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Historic genetic diversity of the endangered white abalone (Haliotis sorenseni)

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HISTORIC GENETIC DIVERSITY OF THE ENDANGERED WHITE ABALONE (*HALIOTIS SORENSENI*)

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ABSTRACT

HISTORIC GENETIC DIVERSITY OF THE ENDANGERED WHITE ABALONE (*HALIOTIS SORENSENI*)

by
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In the 1970's, white abalone populations in California suffered catastrophic declines due to over-fishing, and the species has been listed under the Endangered Species Act since 2001. Genetic diversity of a modern population of white abalone was estimated to be significantly lower than similar *Haliotis* species, but the effect of the recent fishery crash on the species throughout its range was unknown. In this investigation, DNA was extracted from 39 historic and 27 recent dry abalone shells from California, and 18 historic dry shells from Baja California, Mexico. The DNA from the shells was of sufficient quality for the reproducible amplification of 580 bp of the mitochondrial COI gene and 219 bp of the nuclear Histone H3 gene. Two COI haplotypes were distinguished, and no difference ($p>0.05$) was found between the nucleotide or haplotype diversity of California's recent ($\pi=0.0010$; Hd=0.501) and historic baseline ($\pi=0.0010$; Hd=0.505) wild populations of *H. sorenseni*. However, COI diversity in the historic Baja population ($\pi=0.0004$; Hd=0.209) was significantly lower ($p<0.05$) than in California. This study demonstrates the importance of appropriate historic reference groups for threatened or commercially important species, and can aid captive breeding programs manage broodstock and re-introduction design. Stringent controls and treatments to eliminate surface DNA advocate an endogenous source for DNA extracted from shells, but the hypothesis that abalone DNA in shells was remnant of trapped epithelial cells during shell formation could not be verified by histological and fluorescent staining of decalcified abalone shell.

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INTRODUCTION

The white abalone, *Haliotis sorenseni,* is the deepest-living of eight northeastern Pacific *Haliotis* species, occurring between five and 60 meters, with highest densities below 40 meters (Cox 1960; Butler et al. 2006). They can live up to 40 years, reaching maximum lengths of 25 cm (Cox 1960; Tutschulte 1976). However, there is little to no information on the genetic variability of contemporary and historical populations of this species (Gruenthal and Burton 2005). Based on landing data during the peak of the fishery, Rogers-Bennett (2002) estimated a pre-exploitation population size of 360,476 adult white abalone; another estimate that used the amount of suitable habitat and population densities during the peak of the abalone fishery was up to 2.12 million animals in California (Hobday et al. 2001).

Their massive body sizes made white abalone a prized food item along with other similar species, including *H. rufescens,* and *H. cracherodii.* While the species had been collected since the 1850's, commercial and recreational efforts in the late 1960's and 1970's utilized more advanced technologies, making the fishery a valuable resource, especially when populations of shallower species began to decline (Lundy 1997). Commercial landings reached a peak of up to 144,000 lbs. in 1972-1974, then dropped to less than 1,000 lbs. by 1978 (Hobday and Tegner 2000). Population densities failed to recover when the California Department of Fish and Game (CDFG) attempted various commercial and recreational take restrictions, so in March 1996 the fishery was closed in California (Hobday and Tegner, 2000).

In California, unsustainable fishing practices may have driven the population densities so low that successful reproduction in the wild became unlikely. White abalone reproduce by external fertilization and releasing gametes during winter (Leighton 2000). Field and laboratory studies on *H. laevigata* have shown that fertilization is most likely to occur only among animals less than 2 meters distant (1 abalone m^{-2}) (Babcock and Keesing 1999). Diving and submersible surveys confirmed that current populations were orders of magnitude lower than this requirement, even after the closure of the fishery. During the most productive years of the abalone fishery, densities of 857 abalone ha^{-1} of

habitat (0.09 abalone m^{-2}) were reported around Santa Catalina Island (Tutschulte and Connell 1988), and surveys in 1996-1997 and 1999 recorded densities of 1.17 abalone ha^{-1} ($1.17x10^{-4}$ abalone m^{-2}) and 2.73 abalone ha^{-1} ($2.73x10^{-4}$ abalone m^{-2}) (Davis et al. 1998; Hobday et al. 2001). These surveys report few or no juveniles or individuals smaller than 15 cm, indicating that no significant recruitment has occurred since before the 1970's; it was estimated that by 2010 most of the remaining individuals would have died or become senescent (Hobday et al. 2001). Due to critically low densities, apparent recruitment failure, and continued destruction of habitat from human activity, it is evident that this species will not survive or recover without human intervention. Therefore, in 2001 *Haliotis sorenseni* was listed under the Endangered Species Act throughout its range, from Point Conception, California, USA, to Punta Abreojos, Baja California, Mexico (66 FR 29046).

Low population densities and subsequent recruitment failure likely led to reduced genetic variability of the white abalone species in California. A great loss in genetic diversity can potentially affect the reproductive potential and mortality of current, and future white abalone populations on a large geographic scope. The NMFS White Abalone Recovery Plan (2008) established several goals that would ultimately ensure self-sustaining *H. sorenseni* populations in the wild in California. These are to assess, monitor, and protect wild white abalone and their habitat, and to breed, rear, and outplant captive brood stock. Among the recovery actions are those that will alleviate the threat of reduced genetic diversity for wild and captive populations (NMFS 2008). Assessing the current and historical genetic differentiation among wild populations can help design the best captive propagation, field planting and translocation efforts that will maintain or rebuild the genetic structure of wild abalone in California (NMFS 2008: Actions 1.4 and 4.2).

In Baja California, green and pink abalone (*H. fulgens* and *H. corrugata)* comprise 99% of the commercial landings, but white abalone remain legally harvested as a secondary species (CONAPESCA 2004). This 1% reported landing value (*H. sorenseni* and *H. cracherodii* combined) translated to 75,000 lbs. live weight annually caught

during the 1960's fishery peak, and an average of 1,800 lbs. live weight annually since the 1990's (CONAPESCA 2004). The US Endangered Species Act of 2001 does not have jurisdiction in Mexico, but fishery restrictions on size, number, season, and location have been in place for all Mexican abalone species since 1994 (CONAPESCA 1994). The white abalone is considered rare, yet populations are not as closely monitored as the green and pink species. Therefore, molecular techniques offer valuable data for this alternatively fished species, as populations of other species continue to decline.

Assessing the genetic variation of species over time and space has helped conservationists predict the recovered populations of other abalone species. Miller et al. (2009) compared the genetic diversity of *Haliotis rubra* in Tasmania, Australia, among populations in highly productive areas and those that experienced severe fishery depletion in the 1980's. Using seven microsatellite loci, they found that the recovering populations actually had much greater genetic diversity than the healthy areas, likely due to migrant recruits making up the largest proportion of individuals. While few wild white abalone recruits have been documented, knowing what variation may exist or existed historically will shed light on the potential for the recovery of the genetic variability in this species.

To date, the only study of the current genetic variability in white abalone used the mitochondrial DNA genes cytochrome c oxidase subunit I (COI) and cytochrome b (CytB), along with one nuclear gene and five microsatellite loci, to verify species identity and to measure the genetic diversity in 19 adult wild-caught abalone from Farnsworth Bank near Santa Catalina Island, California, USA (Gruenthal and Burton 2005). Only two haplotypes of COI, one haplotype of Cyt B were observed, and over both mitochondrial genes (663 bp) there were four nucleotide sites that were phylogenetically informative to distinguish *H. sorenseni* from the most similar species, *H. kamtschatkana* and *H. k. assimilis.*

Mitochondrial DNA markers are commonly used to analyze population structure and phylogenetics and to design breeding programs due to its high per-cell copy number, high mutation rate, and high stability over time relative to proteins and nuclear DNA

(Maynard et al. 2005). Jiang et al. (1995) found fixed differences in mtDNA haplotypes between neighboring populations of Taiwan abalone (*H. diversicolor*) and relatively low genetic variation in cultured stocks; Maynard et al. (2005) has outlined the mitochondrial DNA sequence and gene organization in the *H. rubra* genome. However, *H. sorenseni* has shown much lower variability at the COI gene than other *Haliotis* species (Figure 1). Gruenthal et al. (2007) found 43 haplotypes over 483 bp of the COI gene among 309 red abalone (*H. rufescens*). In 2008, Gruenthal and Burton found 32 haplotypes over 403 bp of the same gene among 238 black abalone (*H. cracherodii*). Both of these species also experienced population declines due to disease and overfishing and the black abalone was listed as endangered in 2009 (74 FR 1937), but their populations did not fall to such low numbers as with white abalone, and recovery actions have been more timely and successful (Micheli et al. 2008). On the other hand, Clark et al. (2009) observed 3 haplotypes over 473 bp of the COI gene among 28 pink abalone (*H. corrugata*) at one location in California.

This evidence leads to the question of whether white abalone populations were as diverse historically as these other California species before exploitation, or if the species has gone through a longer-term decline in variability. Until recently, it seemed unlikely that enough historic samples existed to attempt to answer this question. Very few whole white abalone or tissue samples were kept (and not eaten or exported) before the end of the fishery collapse. Therefore, a different source of mitochondrial DNA must be applied if historic genetic information is to be revealed.

Although the flesh of the abalone was consumed, harvesters often retained the large, pearly shells as keepsakes or for morphological studies (Cox 1960), many of which remain as dried whole shells in museum and municipal collections, such as the Natural History Museum of Los Angeles County, the Santa Barbara Museum of Natural History, the California Academy of Science, the Channel Island National Park Service, and NOAA Fisheries Service. However, the mollusk shell is not cellular tissue and is not generally thought of as a source of DNA. In abalones, it is made of two calcium carbonate layers secreted by the mantle at the lip, where it is protected by the

periostracum (Dauphin et al. 1989). The outer colored layer is made of prismatic calcite crystals and is crack- and puncture-resistant, while the pearly white nacreous layer is accreted on the inside of the shell as layered aragonite tiles that are ductile and fracture-resistant (Su et al. 2002; Zhao et al. 2004)

Geller and Hawk (*in prep*) hypothesized that during the process of biomineralization, mantle epithelial cells could become trapped among calcium carbonate crystals where DNA might remain intact, albeit in very low concentrations, preserved for many years. They developed a novel method to extract, amplify via PCR and sequence this "entombed" DNA from the shells of various gastropods and bivalves, including several *Haliotis rufescense* and one *Haliotis sorenseni*, supporting the hypothesis of endogenous DNA within mollusk shells.

Because the DNA found in abalone shell is degraded, present in low concentrations, and is from an "unusual source", it bears similarity to "ancient" DNA. Therefore, it is necessary to follow certain preparation criteria used in ancient DNA analyses to maximize DNA extraction success and control against contamination (Pääbo et al. 2004). In the last two decades, the analysis of ancient DNA has greatly contributed to the fields of paleontology, anthropology, molecular evolution, and conservation genetics because it allows the inclusion of long-deceased individuals and extinct populations in order to directly examine spatial and temporal patterns of genetic variation. Sequences have been obtained from Pleistocene mammoths, ground sloths and cave bears to subfossil and recent rodents and birds from materials such as dried tissue, bones, teeth, feathers, and egg shells (reviewed in Pääbo et al. 2004; Oskam et al. 2010); in one noteworthy study, subfossil remains were used to examine the genetic structure and the loss of genetic variation in the endangered kiwi species of New Zealand since the arrival of humans (Shepherd and Lambert 2008).

Although amplifiable DNA has been extracted from such varied sources, no previous studies to date have attempted to do the same with mollusk shells, probably because such skeletal material, which has neither cells nor DNA-enclosing organelles, would be regarded as an unlikely source of genetic material. Numerous studies have sought to

characterize the ultrastructure and chemical composition of mollusk shell with the most common purpose of understanding the processes of protein-mediated biomineralization. The first biochemical analyses of shell nacre showed ultrastructure, mineralogy, and chemical composition variation among bivalve, nautiloid, and gastropod shells (Mutvei 1978)—even differences among *Haliotis* species (Dauphin et al. 1989). Since then, most studies have used electron microscopy (Watabe, 1963; Zhao et al. 2004), amino acid and protein sequence analysis (reviewed in Evans 2008; Zhang and Zhang 2006), and epithelial tissue histology (Jolly et al. 2004) to answer questions about shell structure, composition and production.

Histochemical investigations into the macromolecular organization of mollusk nacre (Crenshaw and Ristedt 1976; Nudelman et al. 2006) have applied diverse color and fluorescent stains to identify calcium binding sites, carbohydrates, cellulose, chitin, glycoprotein, insoluble proteins, and polysaccharide groups in decalcified mollusk shells, including *Haliotis sorenseni* (Bezares et al. 2008). However, there have been no attempts, to date, to use similar histological methods to detect DNA in shell. The histological labels hematoxylin and eosin (H&E) are commonly used to stain cell structures and genetic material (Lillie 1965), and the fluorochrome 4', 6-diamidino-2-phenylindole (DAPI) is highly sensitive to double-stranded DNA (Invitrogen 2010). If amplifiable amounts of DNA can be extracted from shell material (Geller and Hawk *in prep*), then it should be detectable using these DNA-specific stains.

OBJECTIVES:

The goal of this investigation was to apply a novel method of DNA extraction on dried shell specimens collected from the 1930's to 2000 in order to measure the historic genetic diversity of wild populations of the endangered and data-deficient *Haliotis sorenseni,* at the mitochondrial DNA gene COI, and compare it to estimates of contemporary genetic diversity.

Rarefaction curves of haplotype richness in two other California abalone species (Gruenthal et al. 2007; Gruenthal and Burton 2008) provide a basis for comparison to large commercially exploited *Haliotis* populations (Gruenthal and Burton 2005). Rejection of the null hypothesis (no change in diversity over time) would indicate that the fishery collapse is likely to have been the primary cause of a major genetic bottleneck. Failure to reject the null hypothesis—that genetic diversity among historic samples is not significantly greater than observed in contemporary specimens—would be evidence of a longer-term population decline or an undocumented bottleneck that occurred before the fishery ramped up.

A secondary goal was to investigate the phenomenon of endogenous DNA in mollusk shell material, which makes the primary aspects of the present study possible. Three assays were used to verify the source and origin of the shell DNA. First, external sources of DNA were eliminated by chemical cleaning of shell pieces, with the hypothesis that *H. sorenseni* sequences could be obtained from shells after treatment, indicating that the DNA extracted from shells had been entombed within the shell layers. Second, non-tissue samples (water and mucus) were collected from a living *H. rufescens,* with the hypothesis that abalone DNA is present at and near biomineralization sites. Third, methods for H&E histological staining and DAPI fluorescent staining were modified to visualize DNA in abalone shell.

METHODS

1. DNA Purification from Shell

1.1. Samples

Dry shell specimens with known collection dates and locations (N=94) were acquired from California archive collections: 55 from Natural History Museum of Los Angeles County (NHMLAC), 27 from Channel Islands National Park Service (CHIS),

and 12 from Santa Barbara Museum of Natural History (SBMNH). Shells were the remnants of adult individuals, 10 – 24 cm maximum length. Museum shells had been collected live between 1940 and 2008 by commercial, recreational, and scientific diving expeditions. Collection sites of the museum specimens were distributed throughout the species range (Figure 2). The shells fell into three distinct temporal groups: 16 specimens were collected before the commercial fishing industry ("Pre-Fishery" 1940-1959), 42 shells were collected during the fishery ("Fishery" 1960-1979), and 31 specimens were collected after the fishery collapse ("Recent" 1993-2008). Table 1 summarizes the distribution of samples through time over the species range. Shells obtained from CHIS were collected in 2008, at depth, around Santa Catalina Island (Farnsworth Bank and Eagle Rock); these shells were dead, heavily bio-fouled and eroded. The known growth rates of *Crassadoma giganteum* (Leighton and Phleger 1977) and *Balanophyllia elegans* (Fadlallah 1983) found on the inside of the shells were used to estimate that the mortality dates for these abalone were between 2003 and 2008.

1.2. Shell Preparation

Whole shells were first examined to identify the cleanest and most inconspicuous sampling location along the shell margin in order to minimize processing and preserve the features of the specimen. An area of about 2 cm^2 was cleaned of visible encrusting organisms and cut using a handheld rotary device (Dremel). All cleaning and cutting took place either inside a clean fume hood or in a clean, well-ventilated outdoor location. To minimize contamination between specimens, all shells were handled inside clean Ziploc bags and cut with clean disposable slicing wheels. Disposable respirator, eye shield and gloves were worn for protection from fragments and hazardous shell dust. Shells were rinsed thoroughly with deionized water to remove remaining dust; museum shells were cut and rinsed on-site then transported to MLML in clean plastic vials.

1.3. Decalcification

Shell samples were decalcified in 0.5 M EDTA chelating solution (pH 8.0) in sealed vials. This step took between 5 to 20 days depending on the fragment thickness and the degree of bio-erosion. To decrease decalcification time, vials were constantly agitated on a rotating plate at 80 r/min, and EDTA was replaced as necessary. Decalcification was complete when shell fragments became flexible and wholly translucent. To reduce material loss, samples were not fixed, but instead stored in EDTA solution.

Alternative methods for shell processing were compared to this method, including pulverizing and grinding shell nacre, with and without decalcification steps. However, these experiments resulted in lower PCR success rates, and were not used for analysis (Table 2). Similarly, PCR success rate also increased when fragment size was increased to 2 cm^2. These phenomena may be explained by the extremely high temperatures, due to friction from the Dremel tool, which can damage or denature DNA.

1.4. Contamination Controls

Contamination controls were applied where appropriate, following Pääbo et al. (2004). Both DNA purification and PCR were performed on separate bleach-cleaned lab benches where abalone templates had never been processed, and samples were processed in small batches that included blank extractions as negative controls. Independent redundant DNA extraction and PCR were performed to verify results. In addition, shells for templates for which PCR initially failed were independently re-sampled, extracted, and tested with optimized PCR conditions to confirm the results.

1.5. DNA Extraction

Several methods of DNA extraction were employed and compared (Geller and Hawk *in prep*), and the following DNeasy Blood and Tissue protocol (© 2011 QIAGEN,

Valencia, CA) yielded the greatest PCR success and was used throughout the study. The manufacturer's protocol for animal tissue was followed, with the following specifications. DNA was purified from 15-25 mg of decalcified shell material (corresponding to 150-200 mg of raw dry shell); samples were homogenized with sterilized pestles in 180 *ul* of Buffer ATL and 20 µl proteinase-K. After an incubation of 3 hours at 50° C, samples were centrifuged for one minute at 9.200 x g to pellet remaining solid matter, and the supernatant was transferred to spin columns. No change was observed in amount of solid matter after longer or overnight incubation, so 3 hours was adopted as the standard time for proteinase-K treatment. Finally, two 100 µl eluates of purified DNA solution were combined for maximum yield. Nucleic acid concentration was measured for each template using the NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.).

2. PCR & Sequencing

2.1. COI

Abalone-specific PCR primers from Metz et al. (1998) were used to amplify a 580 base pair (bp) fragment of the mitochondrial cyctochrome oxidase subunit one (COI) gene (AB-COIF Forward 5'-TGATCCGGCTTAGTCGGACTGC; and AB-COIR Reverse 5'-GATGTCTTGAAATTACGGTCGGT). Reactions were carried out in 50 µl volumes using 1.2 µl DNA template, 25 µl 2x GoTaq® Green Master Mix (© 2011 Promega Corporation, Madison, WI) augmented to 2.5 mM $MgCl_2$ final concentration and 0.04 mg/ml BSA, and 0.14 *uM* of each primer. Reactions were incubated at 94° C for 3 min prior to a cycling program of 1 min at 94° C, 1 min at 56° C, and 1.5 min at 72° C for 36-40 cycles. Positive PCR products were sequenced by Elim Biopharmaceuticals, Inc. (Hayward, CA). Low quality sequences or those with ambiguities were rerun with independent PCR and sequencing reactions.

Sequences were aligned and edited to 539 bp with CodonCode Aligner (CodonCode Corporation, Dedham, MA). BLAST searches compared sequences to

existing GenBank data and confirmed sequences as COI from *Haliotis*. Sequences were aligned to the complete COI coding sequences of *H. rubra* and *H. tuberculata tuberculata* (NCBI GenBank Gene IDs 2846698 and 8690458) and translated using the invertebrate (*Drosophila melanogaster*) mtDNA code to verify lack of stop codons or missense mutations indicative of pseudogenes. Haplotype inference was performed in Arlequin 3.5 (Excoffier et al. 2005) and diversity indices—including nucleotide diversity, π, and haplotype diversity, Hd—were calculated in DnaSP v5 (Librado and Rozas 2009). A neighbor-joining phylogenetic tree (Tajima-Nei method) of representative COI haplotypes from 27 *Haliotis* species was created in MEGA 4 (Tamura et al. 2007) to confirm species identities. To compare observed haplotype richness among four *Haliotis* congeners, rarefaction curves were generated in EcoSim (Gotelli and Entsminger 2010).

For temporal and spatial comparisons, *Haliotis sorenseni* sequences were grouped by location (along the latitudinal species range) and by time (1940-1959 "pre-fishery", 1960-1979 "fishery", and 2000-2008 "recent"). Spatial and temporal variation in haplotype frequencies were analyzed in PASW® Statistics 18 (IBM Corporation). Differences among diversity estimates were measured by comparing the intervals of two standard errors from each diversity index.

2.2. Histone H3

Haliotis-specific primers for the nuclear Histone H3 PCR gene F1 (GGCTCGTACCAAGCAGACGGC), R1 (TCCTGCAGAGCCATGACGGC) and R2 (GGTGACACGCTTGGCGTGGA) were designed based on alignments of seven species (GenBank accessions AY923952 - AY923958) using Primer3 (Rozen and Skaletsky 2000) and IDT SciTools Oligo Analyzer 3.1 (Owczarzy et al. 2008). They correspond to nucleotide positions 76-96, 338-357, and 411-430 in the complete Histone H3 coding sequence of *H. discus discus* (GenBank accession EF103400).

Three shell-extracted DNA templates and two of each *H. rufescens, H. walallensis,* and *H. assimilis* tissue-extracted DNA templates were amplified using combinations of

the Histone H3 PCR primers. Two of the shell templates (MYC93 & SCA00) were selected because the mtDNA COI sequences suggested that they were not white abalone; the third shell template was selected as an exemplar of *H. sorenseni* because of the high quality of the COI sequence it yielded. Reactions were in 50 µl volumes, using 1.2 µl DNA template, 25 µl 2x GoTaq® Green Master Mix (© 2011 Promega Corporation, Madison, WI) augmented to 2.5 mM $MgCl_2$ final concentration, 0.04 mg/ml BSA, and 0.14 *uM* of each primer. Reactions were incubated at 94° C for 3 min prior to thermal cycling of 1 min at 94° C, 1 min at 56° C, and 1.5 min at 72° C for 36-38 cycles. Amplified products were sequenced, edited to 256 bp with CodonCode Aligner and aligned with eight congeneric Histone H3 sequences from GenBank in MEGA 4. Species identification was performed by including the sequences with all other published *Haliotis* Histone H3 data on GenBank on a neighbor-joining (Jukes-Cantor method) tree in MEGA 4.

3. DNA Source Experiments

To test the hypothesis that abalone DNA extracted from shell material is endogenous, external contamination and surface DNA sources were eliminated using three cleaning treatments to shell fragments. Three 2 cm^2 fragments were cut from one shell which had been previously sequenced using the standard method described above. Fragments were rinsed and sonicated for one minute in deionized water to remove particles from the surface. Each sample was then treated with one of the following at room temperature: A) 2 h soak in 10% formalin (museum grade); B) 2 h soak in 20% bleach (Pääbo et al. 2004; Kemp and Smith 2005); and C) 5 min soak in 20% HCl, resulting in 55% shell dissolution by mass. Samples were then removed from the chemical baths and rinsed under running deionized water until neutral pH was achieved. The three cleaned shell pieces were decalcified in 0.5 M EDTA (pH 8.0), then DNA was isolated using the aforementioned modified DNeasy protocol. A 580 bp segment of COI was amplified from the three DNA templates and product length and quality was verified

using agarose gel electrophoresis. Successful products were sequenced then edited and aligned with *H. sorenseni* and *H. rufescens* reference sequences in MEGA 4.

The hypothesis that significant quantities of abalone DNA are available to become entombed in the vicinity of the growing shell margin was investigated using one live *H. rufescens*. First, the inside of the shell was swabbed with filter paper. Approximately 5 µl of fresh mucus was collected from the region of the inner shell and smeared onto a glass slide for microscopy. The same animal was rinsed by pouring 400 ml of filtered seawater over its underside and into a beaker. The rinse water was collected and filtered through a glass microfiber filter. The modified DNeasy protocol was used to purify DNA from 1 cm^2 of each filter paper. DNA from filtered seawater inflow and fresh tentacle tissue from the same animal were also purified as negative and positive controls. The 580 bp fragment of COI was amplified and visible products were sequenced. Sequences were edited in CodonCode Aligner and aligned with *H. sorenseni* and *H. rufescens* reference sequences in MEGA 4.

4. Histology

Rectangular sections about 4 cm^2 were cleaned of encrusting organisms and cut from the outer lip of one *H. sorenseni* shell and one *H. rufescens* shell. The samples were decalcified inside embedding cassettes in 0.5 M EDTA (pH 8.0), constantly agitated at 80 r/min. Paraffin embedding, microtome sectioning, and glass slide mounting steps were performed by the Community Hospital of Monterey Peninsula histology lab (CHOMP, Monterey, CA). Longitudinal slices resulted in stratified serial 4 cm^2 subsamples.

Two slides from each stratum were stained with H&E stains by the CHOMP histology lab, slides were examined with compound microscopy, and images were captured and with Leica DFG FireCam (Leica Microsystems). One slide from each stratum was stained with DAPI according to manufacturer protocols (Gallardo-Escárate et al. 2005; Invitrogen 2010). The slides were examined using an epifluorescent compound microscope fitted with a DAPI filter cube, and images were captured using

Spot Digital Camera (Spot Diagnostic Instruments). Photomicrographs were scaled and compared in ImageJ (Rasband 2009), and shell structures and microorganisms were identified (Vogel et al 2000; Nudelman et al. 2006; Gektidis et al. 2007; Bezares et al. 2008).

RESULTS

1 Shell DNA

1.1 DNA and Molecular Markers

The method of purifying mitochondrial DNA from 94 white abalone shell specimens yielded 90 positive PCR products and 86 high quality COI mtDNA sequences (539 bp), a success rate of 91.5% overall. The DNA yields for shell extractions were low ($\bar{x}$=1091.50 ng, SD=802.67) but were still within the expected range of yields for tissue extractions (Figure 3). It may be noted that this value is an overestimate of target molecule concentration because DNA extracted from shell can include non-target molecules, including those from microendolithic bacteria, fungi, and sponges. Homozygous sequences of 282 bp of the nuclear Histone H3 gene were obtained for three *H. sorenseni* shell templates using the F1/R1 primers; and a 355 bp homozygous sequence was obtained for the *H. rufescens* tissue template using the F1/R2 primers.

1.2 Control Experiments

After treatment, the bleached shell appeared to have only slightly less color on the outer prismatic layer, and no change in mass. After 5 minutes in 20% HCl, 55% dissolution by mass was observed, and the outer layers of the shell were visibly diminished and bleached in color. No change was detected in either the color or mass of the formalin-soaked shell. Positive COI PCR products were obtained from all three treated shell fragments, and the sequences matched with the untreated, independently processed *H. sorenseni* shell (FB20). This experiment supports the hypothesis that DNA

obtained from shell samples originates from within the layers of the shell and not from surface sources.

Verified COI PCR products were obtained from DNA purifications of *H. rufescens* tissue, shell swab, and seawater rinse; no product was observed from the inflow water extraction. The three successful products had the same sequence, which was one *H. rufescens* haplotype not previously observed in our laboratory. In addition, the unstained mucus slide revealed several cell types under 20X magnification. While the cells were not qualified as abalone or otherwise, this experiment showed that abalone mucus harbors not only visible cells, but also high concentrations of DNA in the immediate vicinity of shell mineralization sites.

2 COI Analysis

2.1 Identification & Phylogeny

Using BLAST and phylogenetic methods, 84 of the shell COI sequences were identified as *H. sorenseni*, including five shells obtained from a Morro Bay shell shop in 1966. However, one sequence from a shell collected in Monterey County in 1993 and morphologially identified as *H. sorensensi* most closely matched *H. walallensis* (GenBank accession AY817716), which was different from *H. sorenseni* at 6 nucleotide sites (of 539); and one sequence from a shell collected in "southern California" in 2000 matched *H. kamtschatkana* or *H. assimilis* (GenBank accessions AF060845 and AY817709), which were different from *H. sorenseni* at 4 shared positions (Table 3).

The neighbor-joining phylogenetic tree generated using representative COI haplotypes of 27 *Haliotis* species showed the close relationship of eastern Pacific abalone species, and distinguished three clades (Figure 4). The agreement of these relationships with previously published phylogenies using ITS (Coleman and Vacquier 2002), COI and 16S (An et al. 2005), and COII (Degnan et al. 2006), genetic markers and multi-gene Protein Gel Electrophoresis (Brown and Murray 1992) further confirm the white abalone species identity of the sequences produced in this experiment. However, the

phylogenetic relationships among the larger clades and other species worldwide could not be inferred with sufficient power ($p > 0.5$).

2.2 Genetic Diversity

In the full 539 bp sequenced, two haplotypes were observed among the 84 *H. sorenseni* shell samples, distinguished at site 236 by a single synonymous transition. For analysis, a 403 bp section (sites 84 – 486) was extracted in order to standardize sequences to published data of three congeners (Gruenthal et al. 2007; Gruenthal and Burton 2008; Clark et al. 2009).

The null hypothesis of no difference in diversity (Figure 5) among the historic 1940-1959 and 1960-1979 populations [π=0.530 (n=12, S.E.=0.022) and π=0.513 (n=27, S.E.=0.007) respectively] and the 2000-2008 population [π=0.501 (n=27, S.E.=0.008)] of *H. sorenseni* in California could not be rejected (p>>0.05). No recent samples (after 1971) were obtained from Baja California, Mexico, so regional comparisons were conducted for historic (1940-1972) populations only. The haplotype diversity was significantly greater (p<0.05) in historic California populations than in historic Baja California populations [π=0.503 (S.E.=0.006) and π=0.209 (S.E.=0.027) respectively].

The difference in diversity estimates between California and Baja is explained in part by the small sample size from northern Baja and the observation of only one haplotype among samples (n=15) south of 30°N, which represents 38% of the latitudinal range of the species (Figure 6). In a hypergeometric distribution, the probability of collecting 15 white abalone of haplotype C without encountering any other haplotype is 2.72×10^{-6}; therefore, the historic Baja California white abalone population represented a separate stock than the more northern populations.

Among all 84 samples, the two haplotypes exhibited nearly equal frequency (n_1=40, n_2=44) with low and even diversity (π=0.00125; Hd=0.505). Within the 403 bp subsample, the nucleotide, π, and haplotype, Hd, diversity of *H. sorenseni* was significantly lower (p<0.05) than both *H. rufescens* and *H. cracherodii* (Figure 7).

To correct for unequal sample sizes among species, rarefaction curves were generated with 1000 iterations for haplotype richness (SH) as a function of sample size (Figure 8). Data from *H. rufescens* (n=309) and *H. cracherodii* (n=238) (Gruenthal et al. 2007; Gruenthal and Burton 2008) predicted 14.4 and 18.5 haplotypes in a sub-sample of 84 individuals. The curve for 28 *H. corrugata* (Clark et al. 2009) was fitted to a logarithmic model to predict 3.6 haplotypes in a sample of 84 abalone.

This interspecies comparison suggests that larger sample sizes are needed for *H. rufescens* and *H. cracherodii*, as data for neither species approached an asymptote. An asymptote for a rarefaction curve would indicate a representative sampling of a population, when further sampling is unlikely to yield more variation. The population of *H. sorenseni* reached an asymptote of two haplotypes; further sampling of this species is unlikely to reveal additional haplotypes. The small sample of *H. corrugata* suggests an asymptote of 3-4, but this may be because all samples were taken from one location.

3 Histone H3 Analysis

When edited to 256 bp and included with 8 worldwide congeners on a Jukes-Cantor neighbor-joining tree (Figure 9), the eastern Pacific species clustered similarly to the COI phylogeny. The sequence for shell SCA00 was identical to both *H. assimilis* sequences and to that of the published *H. kamtschatkana* data. Therefore, SCA00 was clearly not a white abalone, and the museum specimen may be relabeled as such.

The MYC93 shell sequence was identical to that of the *H. sorenseni* exemplar shell, and they were different from the two identical *H. walallensis* sequences by one transition (Table 4). More data are required to determine which relationship—COI or Histone H3—is more indicative of species identity, or whether the shell truly represents a rare hybrid.

4 Histology

The H&E stain was used to color nuclei blue and other cellular structures red. This method revealed aspects of the shell intralamellar sheath as well as a diversity of microorganisms such as fungi, algae, sponges, bacteria, and Archaea (Figure 10a).

Fluorescent microscopy using DAPI staining proved a more sensitive method to visualize microscopic details in decalcificed shell sections. When excited with ultraviolet light (~360nm), the blue emission (456-460 nm) revealed microorganisms with less obstruction from structural shell proteins (Figure 10b). Despite the high resolution for detecting micro-endolithic life forms, abalone cells and DNA were not specifically identifiable using this approach, as non-specific binding cannot be rejected.

Certain structures, visible using both H&E and DAPI staining methods, remained unidentified (Figure 10c). They consisted of clusters of many 2 - 5 um structures, possibly nucleated cells, surrounded by scale- or plate-like tiles, interpreted as undecalcified remains of nacre. It was beyond the scope of this assay to definitively identify the cells as animal, bacterial, or otherwise. Therefore, neither staining assay was able to support or refute the hypothesis that amplified DNA originates as sloughed-off cells, nuclei or mitochondria entombed within the shell matrix. This assay underlines the need for an abalone-specific fluorescent *in situ* hybridization (FISH) probe, which could be used with a DAPI counterstain to distinguish specific abalone genes from the microorganism "noise" encountered in this study.

DISCUSSION

1. DNA in Shells

Mitochondrial and nuclear DNA was successfully extracted, amplified via PCR, and analyzed from fresh and historic abalone shell specimens—some as old as 70 years, others having decayed on the sea floor for several years. The low spectrophotometer readings and high number of cycles needed for PCR success imply that DNA in abalone

shell material is in very low quantity, yet this study shows that it still exists in sufficient abundance and quality to be useful for genetic analysis. The shell decontamination and chemical cleaning experiment, as well as precise matching of haplotypes in shell and tissue samples, indicate that DNA amplified from shell samples originated from within the shell and not from the shell surface or laboratory contamination. However, the presence of microorganisms within shells prevented the identification of stained DNA in shell sections as originating from abalone. The presence of abalone DNA swabbed from the inner shell of a living abalone, shown here, is a necessary precondition of the entombment hypothesis. Alternative methods using abalone locus-specific fluorescent *in situ* hybridization (FISH) probes may be the most effective means of visualizing the distributions of entombed abalone DNA.

Only four of the 90 positive PCR products resulted in poor quality sequences, and it is suspected that this was caused by co-amplification of damaged or non-target molecules. Among the four additional shells for which PCR product was not obtained, two were collected dead at Eagle Rock near Santa Catalina Island and were among the shells with the highest levels of erosion and infestation of boring organisms; the other two shells were museum specimens with no observable damage. Erosion and endolith load may not be the only factors responsible for extraction or PCR failure. Shell grinding and cutting experiments suggested that exposure to high temperatures could further damage entombed DNA molecules. Similarly, failed attempts at PCR using *H. rufescens* shells used decoratively in landscaping (data not presented) suggests that UV damage from sun exposure may also affect the success rate. Further investigation into variables affecting quality of DNA in mollusk shells is needed to verify these speculations. On the other hand, this study sequenced DNA from several shells that had been in a museum for over 60 years, yet it remains unknown how long genetic material may remain preserved in shells.

2. COI Phylogeny

The relationship of the white abalone with closely related red and northern species is apparent in the COI cladogram, and other works using ITS (Coleman and Vacquier 2002) and combined COI and CytB (Gruenthal and Burton 2005), agree that *H. sorenseni* and *H. kamtschatkana* are the most similar to each other. However, this COI phylogeny incorporates newly published COI sequences and offers several new perspectives of the Haliotidae of the eastern Pacific. Three main eastern Pacific clades were distinguished, although hierarchical relationships were weakly supported. Green and black abalone grouped as one distinct clade (C), while pink abalone were found to be most closely related to four western Pacific species (B), *H. gigantea, H. madaka, H. discus,* and *H. discus hannai.*

The close relationship between the two New Zealand species, *H. iris* and *H. virginea,* and the Australian species *H. laevigata* and *H. rubra* paralleled findings from latest COII analysis of southern hemisphere species (Degnan et al. 2006). Outside of North America, the COI tree grouped most species with less than 50% bootstrap support, but the Degnan et al. (2006) Bayesian MCMC COII tree was better capable of defining the phylogenetic structure of many other *Haliotis* species. The COI sequence used in this study is only 3% of the length of the entire *Haliotis* mitochondrial genome, and longer sequence fragments or additional markers would add to the resolution of these relationships.

3. Species Barcoding with COI

COI is a commonly used "barcoding" marker because it can distinguish most invertebrate species (Hebert et al. 2003; Schander and Willassen 2005); in this study, forty shells were observed to have one *H. sorenseni* haplotype, and 44 shells had a second *H. sorenseni* haplotype. Both had been previously published, and no other COI haplotypes are known for this species. Alignment with all known abalone COI sequences showed that the two haplotypes were unique to the species, which is a premise of the

barcoding method. Mollusk shells could provide an important new source for such barcoding markers in situations where tissue cannot be sampled or when morphological characteristics are insufficient, such as poached shells, historic and museum specimens, and predator stomach contents. Shell sampling may also prove a less invasive sampling method for large scale sampling of historic and live populations of other threatened, endangered—such as California's black abalone (NMFS 2009)—and recently extinct mollusk species, (Régnier et al. 2009, IUCN 2010).

4. COI Diversity

New techniques for extracting and amplifying DNA from shell material were successfully applied to provide a historic baseline to which genetic diversity of recent endangered populations could be compared. This study also underlines the importance of using the most appropriate reference group when dealing with depleted and endangered species (Matocq and Villablanca 2001). Using only current populations of the two well-studied congeners (*H. rufescens* and *H. cracherodii*) as references would have lead to the assumption that the recent fishery crash was the direct cause of the lack of genetic diversity in the white abalone. However, comparison of historic and recent populations of *H. sorenseni* indicates that low genetic diversity is historic and not associated with the most recent population decline. The red and black abalone were affected by the most recent anthropogenic overharvesting on an even larger scale than the white abalone, but both species occur at shallower depths and therefore have different histories of exploitation by humans and animals. Similarly, the historic lack of genetic diversity of the white abalone cannot be used to calibrate changes in genetic diversity of other species. Current diversity estimates among abalone species is variable, so comparable historic studies are needed in order to reach solid conclusions for each.

A historic lack of diversity means that population declines will not be reflected in changed genetic diversity. Overharvesting caused a demographic crash in populations of *H. sorenseni* to the extent that successful reproduction may not have occurred since the

1970's (Hobday and Tegner 2001), but in the absence of mtDNA diversity to erode, no genetic decline was observed. It is not known why or how long this species has been genetically depauperate, relative to similar species, and it may be indicative of a long term decline in population size and viability. Although Withering Syndrome has plagued other abalone species in recent times, it has not been found at significant frequency in white abalone; other undocumented events or cryptic bottlenecks may have governed modern genetic diversity (Luikart et al. 1998). *Haliotis sorenseni* relies on drift algae imported from shallower kelp forests, so populations of this sedentary species may have been susceptible to starvation events in response to climate-driven fluctuations in kelp forest resources known to have occurred over the last 20,000 years (Graham et al. 2010).

Plausibly, prehistoric population bottlenecks may have diminished diversity without sufficient recovery time prior to recent over-exploitation. The historic viewing frame of this investigation spanned 70 years, but the species has been around for up to 2 million years (Metz et al. 1998). It is possible that the populations considered "historic" in this study are the descendents of survivors of previous or ancient bottleneck events. In addition to bottlenecks, the same mechanisms that drive the habitat limits (Tutschulte 1976; Leighton 1972; Davis et al. 1998) may have suppressed genetic diversity throughout this species' history by curbing population densities. Diploid nuclear markers might provide a better signature of the historic decline in population size. This long term perspective may offer one reason for optimism: if pre-fishery abundance estimates of white abalone are correct, then ancient populations apparently recovered to large size despite limited genetic diversity.

The addition of data from nuclear gene or microsatellite markers would supplement this analysis, yet the low historical mtDNA diversity remains a significant pattern. Low genetic diversity in declining populations is associated with inbreeding and introgression, which can reduce a population's long-term survival (Avise 1994). The pre-fishery baseline estimate of genetic diversity for this species underscores the importance of management programs that include genetic monitoring of threatened species and those of ecological and commercial importance. Today's monitoring data will be tomorrow's

baseline reference set, and the information could prevent, or in any case record, genetic changes in populations. In particular, understanding humans' effects on the genetic diversity of commercially exploited species will help conservation and resource management programs make informed decisions about restoration, protection, or sustainable use. Although amplification of microsatellites was not attempted using shell DNA, such markers have been studied using other non-traditional DNA sources and may provide potential markers to detect species bottlenecks (Menotti-Raymond and O'Brien 1993) and aid in monitoring programs (Luikart et al. 1998).

This study is one of many examples of the invaluable resources that museum collections supply for conservation genetics. However, the drawback of relying solely on museum specimens lies in uneven sampling due to sample availability; collections house representative specimens, but do not have the capacity to hold samples without limit from all collection dates and locations. The museum specimens used in this study were collected by scientific expeditions or recreational and commercial divers, and may have been biased toward adults within size ranges defined by landing requirements. However, white abalone habitat is not as cryptic as similar species (Lafferty et al. 2004) so ease-of-capture bias is not considered likely. Growth rates based on bomb radiocarbon (ΔC-14) analysis (Andrews et al. *in prep*) indicate that individuals between 10 and 24 cm were between 10 to 40 years old, which allows for the inclusion of multiple generations within the any sample population. The groups of shells collected dead around Santa Catalina Island were no doubt biased toward larger specimens that were most readily detected by ROV surveys. The amount and source of sample bias in this study may be considerable, but the information gained from the shells remains significant.

Regardless of the small sample size, the observation of only one mtDNA haplotype in the southern one-third of the species' range is a significant finding, since elsewhere the haplotypes occur at nearly equal frequencies. More information on the natural history and connectivity of white abalone in Baja California is necessary to understand this phenomenon; natural populations of white abalone may exhibit cryptic

differences at southern latitudes, and this should be taken into account for recovery and re-introduction programs in both countries.

5. Histone H3

Two museum shells which had been morphologically identified as *H. sorenseni* grouped with the sympatric species *H. kamtschatkana/H. assimilis* and *H. walallensis* at the COI gene. Because the mitochondrial COI gene is known to be maternally inherited in white abalone (Gruenthal and Burton 2005), the marker could not be used alone to determine if the individuals were misidentified or if they were interspecific hybrids. Morphological features are known to vary within abalone species (Leighton 2000; Owen 2005a,b) and many of the eastern Pacific abalones have been hybridized in the lab (Owen et al. 1970). As a nuclear gene, Histone H3 is expected to be biparentally inherited, and thus capable of distinguishing an F1 hybrid individual with parents of different species. Both COI and Histone H3 sequences confirmed that the specimen SCA00 belonged to H. assimilis/H. kamtschatkana. The museum shell MYC93 grouped with *H. walallensis* at the COI but its Histone H3 sequence was identical to *H. sorenseni*.

With the help of additional morphologic examination, the two molecular markers succeeded in recognizing two museum collection oddities. The misidentification of mollusk species and hybrids poses threats to conservation efforts (Supernault et al. 2010), and the data collected from shell DNA may greatly increase the accuracy of future estimates of species abundance and diversity, poaching litigation, and museum studies.

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TABLES & FIGURES

Table 1. Distribution of shell samples through time and over the species range.

Samples	Pre-Fishery 1940-1959	Fishery 1960-1979	Recent 2000-2008	Total
California – N	3	7	4	14
California – S	10	16	27	53
Baja – N	1	3	-	4
Baja – S	2	16	-	18
Unknown	-	-	-	5
Total	16	42	31	94

Table 2. Comparison of success rates among alternative methods of processing abalone shells.

Shell ID	*Haliotis* Species	Qt.	Method	Amtount Shell (mg)	EDTA?	Amount Extracted (mg)	Number Positive PCR	Number Sequences	Success Rate (%)
Nelda	Hruf	1	Ground	15mg	No	15	0	0	0
Nelda	Hruf	1	Ground	25mg	No	25	0	0	0
Nelda	Hruf	1	Ground	50mg	Yes[a]	50	1	0	0
Nelda	Hruf	1	Cut	284 (0.2m^2)	Yes[a]	<25[b]	0	0	0
SWC1, 2	Hruf	2	Cut	150 (0.2m^2)	Yes[a]	<25[b]	2	2	100
ER01-06	Hsor	6	Cut	150-200 (0.2m^2)	Yes[a]	<25[b]	2	2	33
EFB18-20	Hsor	3	Cut	150-200 (0.2m^2)	Yes[a]	<25[b]	1	1	33
Nelda	Hruf	1	Cut	1,200 (2cm^2)	Yes[a]	15-25	1	1	100
ER01-06	Hsor	6	Cut	~730 (2cm^2)	Yes[a]	15-25	5	5	83
FB12-16	Hsor	5	Cut	~730 (2cm^2)	Yes[a]	15-25	5	5	100
Museum & CHIS Shells	Hsor	94	Cut	580-800 (2cm^2)	Yes[a]	15-25	90	86	91

a. Decalcification in 0.5 M EDTA reduces raw shell material to about 1/10th of the original mass.
b. All of the decalcified shell was used for DNA extraction, but amounts varied because the fragile material fell apart easily and significant material was lost in some cases.

Table 3. Mitochondrial DNA COI sequence variation among four California *Haliotis* species.

Species	ID	GenBank Accession	COI site (of 539 bp)													
			34	40	52	115	124	130	148	151	223	236	265	271	436	437
H.sor	SBC40a		G	A	G	T	A	T	A	G	G	C	T	G	C	C
H.sor	BMX47		.	.	.	.	.	.	.	.	.	T	.	.	.	.
H.wal	Hap1	*AF060846*	.	G	A	C	G	.	.	A	.	.	G	A	.	.
H.wal	Hap2	*AY817716*						.	.	A	.	.	G	.	.	.
*	MYC93*		.	G	A	C	G	.	G	A	.	.	G	.	.	.
H.assim	Hap1	*AY817708*						.	.	.	.	.	.	.	T	.
H.assim	Hap2	*AY817713*						C	.	.	A	.	.	.	T	T
H.assim	Hap3	*AY817709*						C	.	.	A	.	.	.	T	.
H.kam	Hap1	*AY923920*	.	.	.	.	.	.	.	.	.	.	.	.	T	.
H.kam	Hap2	*AF060845*	A	.	.	.	.	C	.	.	A	.	.	.	T	.
*	SCA00*		A	.	.	.	.	C	.	.	A	.	.	.	T	.

Table 4. Nuclear Histone H3 sequence variation among six California *Haliotis* species. The COI grouped MYC93* with H. walallensis, while its Histone H3 sequence is identical to *H. sorenseni*. The SCA00* shell matches *H. assimilis/H. kamtschatkana* at both genes. *H. rufescens, H. corrugata*, and *H. virginea* are used as outgroup species to illustrate the low level of variation at this locus.

Histone H3 (256 sites)					
Species	ID	62	158	209	251
H. sor	SBC40a	T	T	C	T
*	MYC93*	.	.	.	.
H. wal	Hwa1 & 2	.	.	.	C
H. kam	AY923920	C	.	.	C
H. assim	Has1 & 2	C	.	.	C
*	SCA00*	C	.	.	C
H. ruf	HrufTent	C	.	T	C
H. corr	FJ977736	C	C	.	C

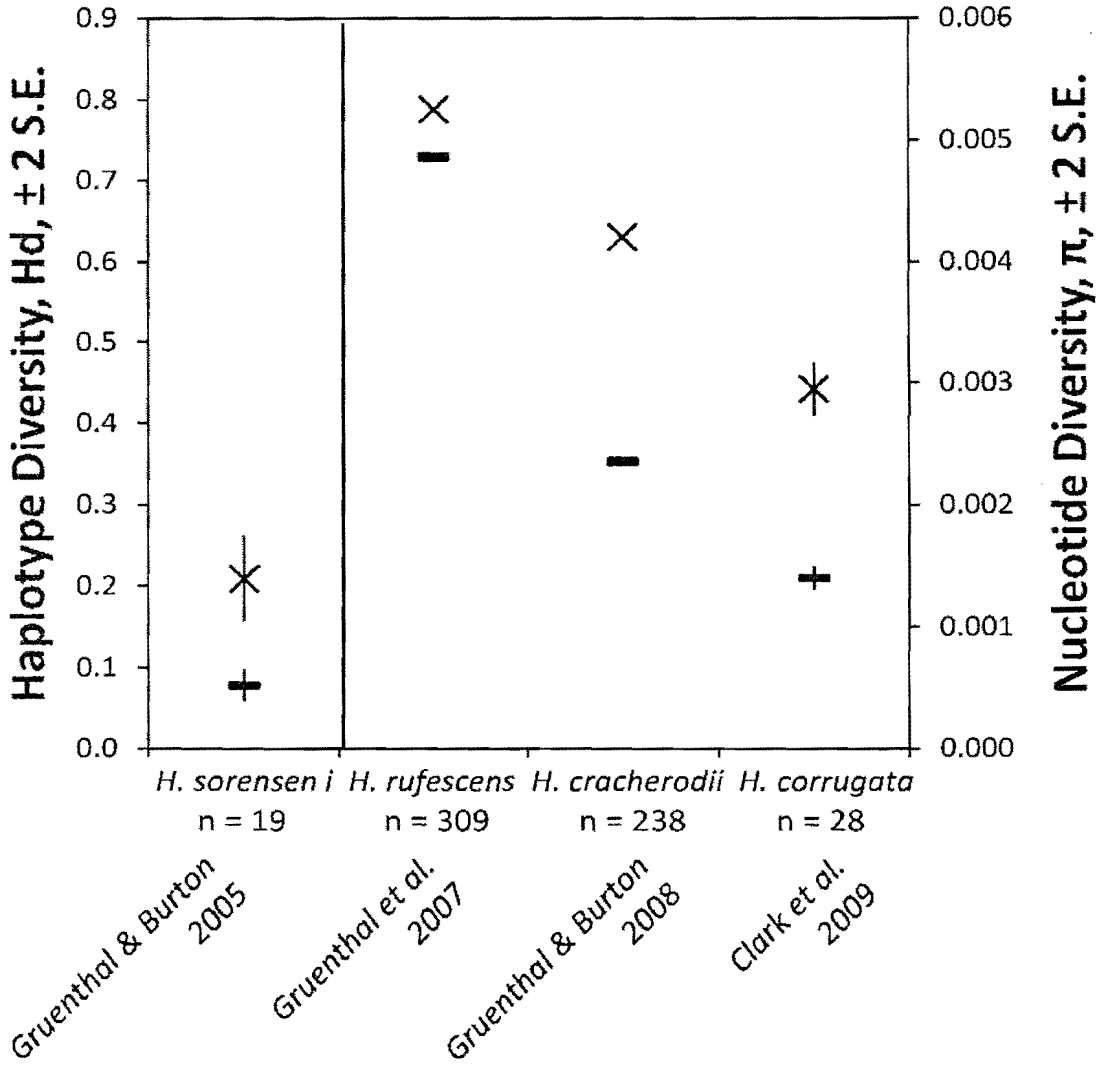

Figure 1. Comparison of nucleotide diversity estimates among recently published California abalone populations at COI. Vertical bars indicate two standard errors of the means. **X**'s correspond to the left-hand Hd axis, and red —'s correspond to the π axis on the right. Significantly lower diversity was observed at COI mitochondrial gene in the 2005 *H. sorenseni* population than in other species. *Note: estimates are *not* standardized to the same length, but all cover approximately the same gene region.

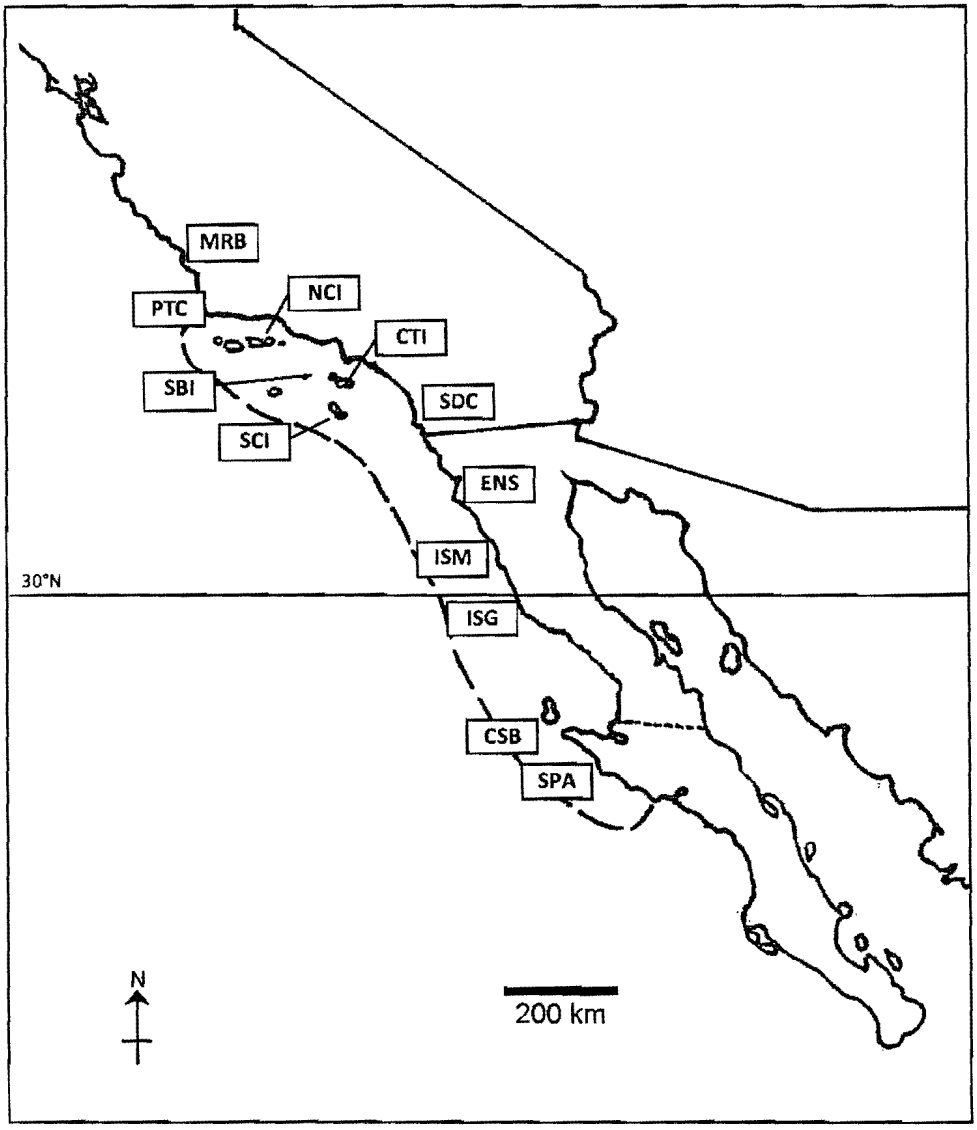

Figure 2. Range map of *H. sorenseni* along California and Baja California, Mexico. Light line indicates the range edges, including presumed locations based on depth and habitat availability. San Pablo & Isla La Asuncion (**SPA**) n=6; Isla Carlos to Bahia San Bartolome (**CSB**) n=12; Isla San Geronimo (**ISG**) n=1; Islas San Martin (**ISM**) n=2; South of Ensenada (**ENS**) n=1; San Diego (**SDC**) n=2; San Clemente Island & outer banks (**SCI**) n=8; Santa Catalina Island (**CTI**) n=36; Santa Barbara Island (**SBI**) n=9; Northern Channel Islands & Mainland (**NCI**) n=9; Point Conception (**PTC**) n=3; Morro Bay Shell Shop (**MRB**) n=5.

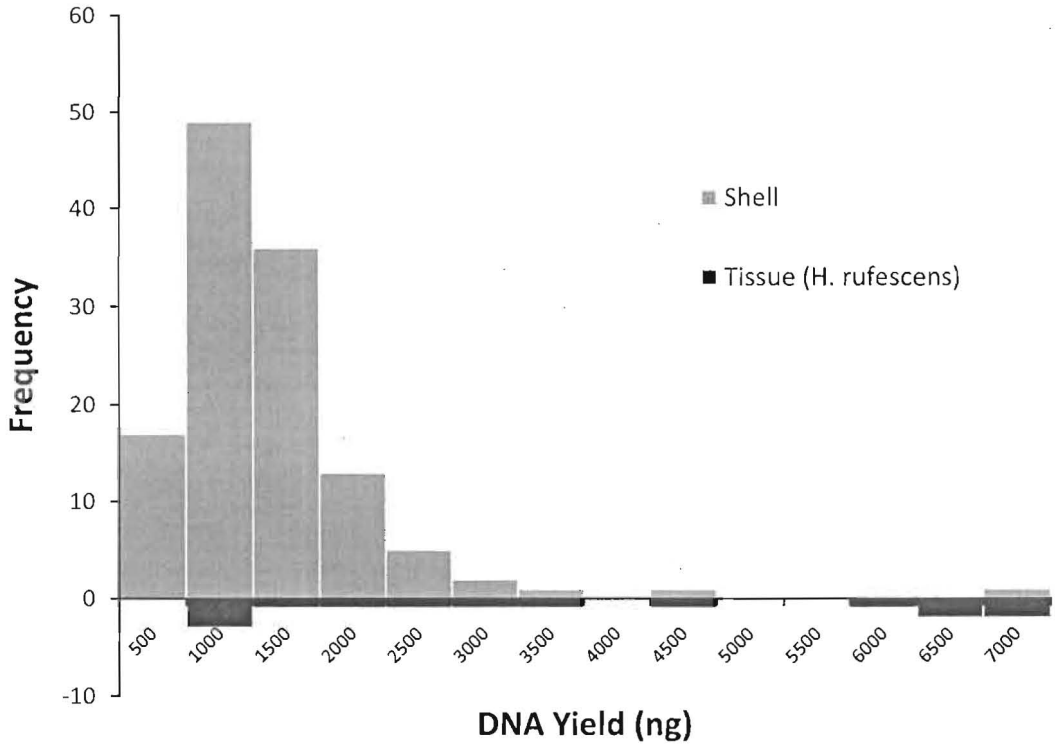

Figure 3. Frequency histogram of DNA extraction yield (ng) from abalone shells (*H. sorenseni, H. rufescens*; n=125) and abalone tissue (*H. rufescens, H. walallensis, H. assimilis*; n=14*)*. Frequency values for tissue samples are expressed on the negative axis. No *H. sorenseni* tissue samples were processed.

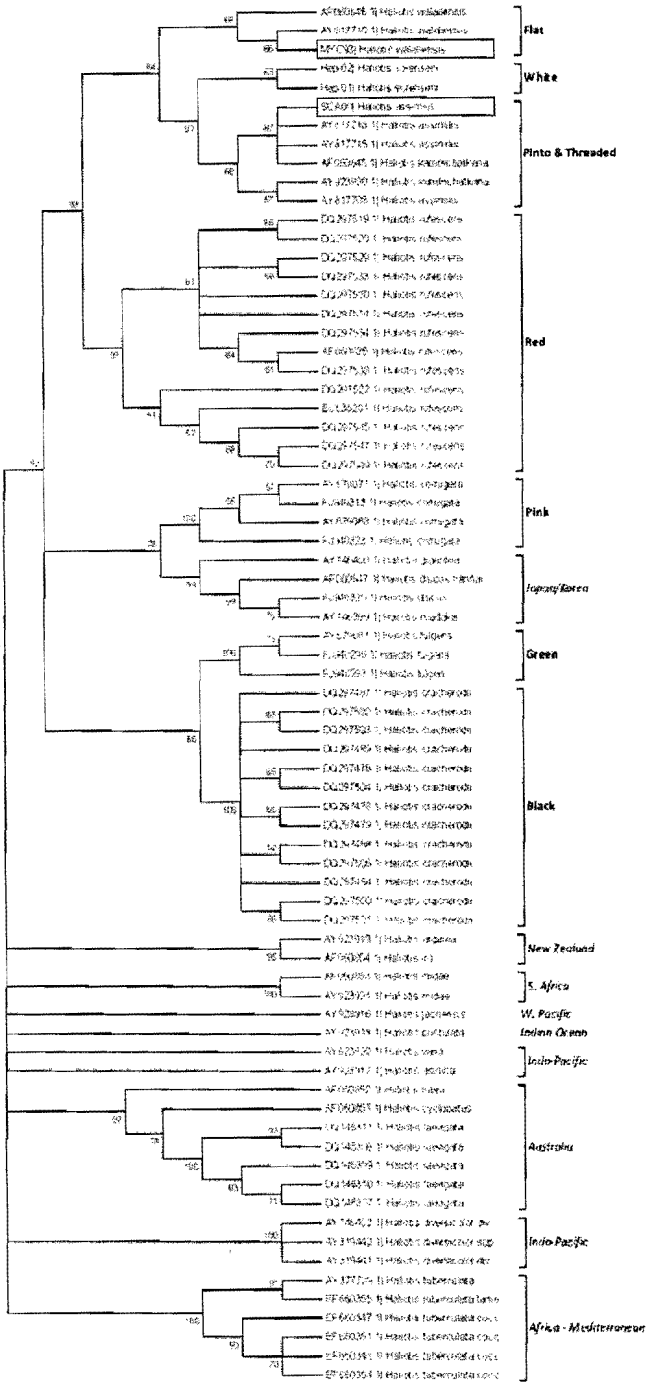

Figure 4. Mitochondrial COI cladogram of 27 *Haliotis* species. Tajima-Nei neighbor-joining consensus tree from 5,000 bootsrap replicates and 354 positions. Branches with agreement <50% are collapsed. Brackets indicate the common names of eastern Pacific species, and geographic range for all others. The boxes indicate the two shells of uncertain identity. Two haplotypes were observed in 84 *H. sorenseni shells* and two shells (MYC93 and SCA00) grouped most closely with flat or pinto and threaded species, respectively.

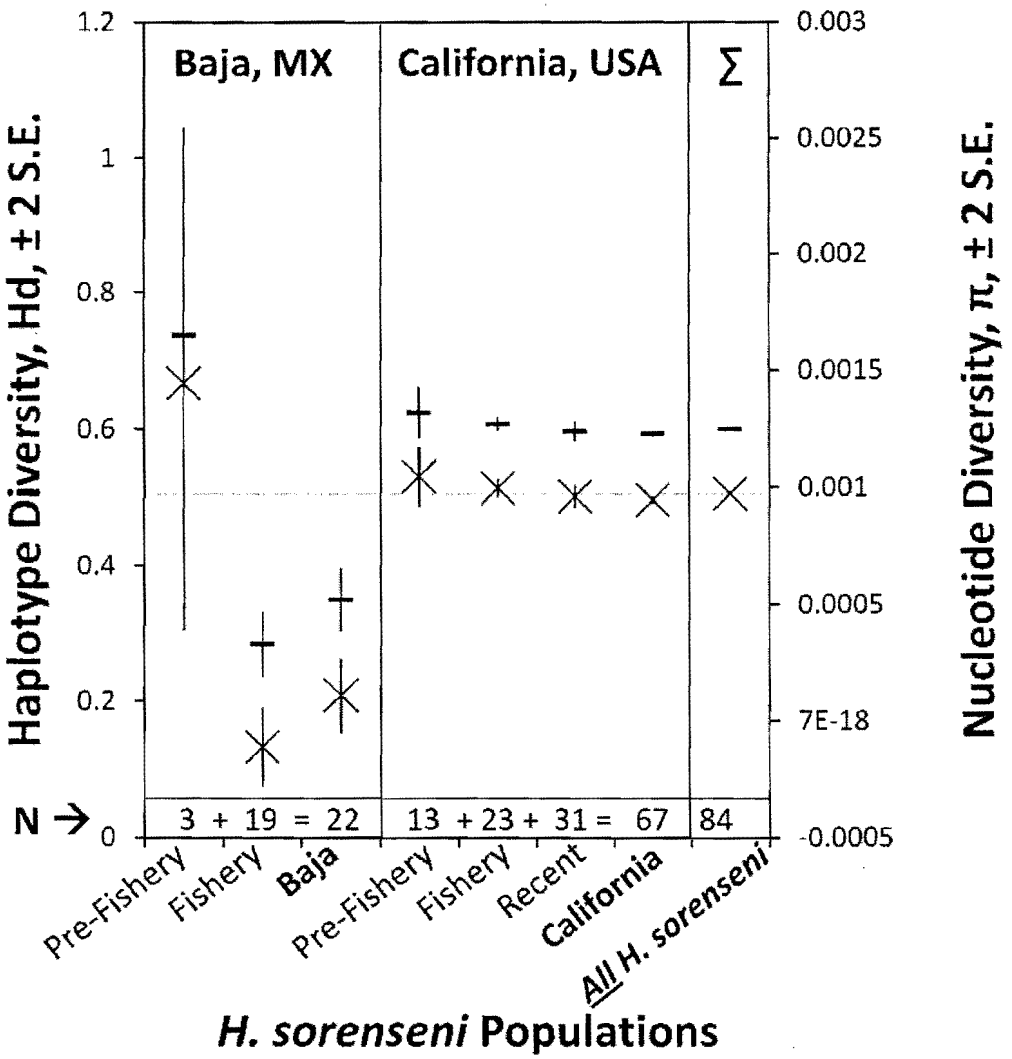

Figure 5. Nucleotide diversity (π) and Haplotype diversity (Hd) of *H. sorenseni* across time in Baja, MX, and California, USA using 403 bp of COI mitochondrial gene. Vertical bars indicate two standard errors of the means. **X**'s correspond to the left-hand Hd axis, and red —'s correspond to the π axis on the right. Overall, diversity in Baja remains significantly lower than in California, and no change in diversity was observed over time in California.

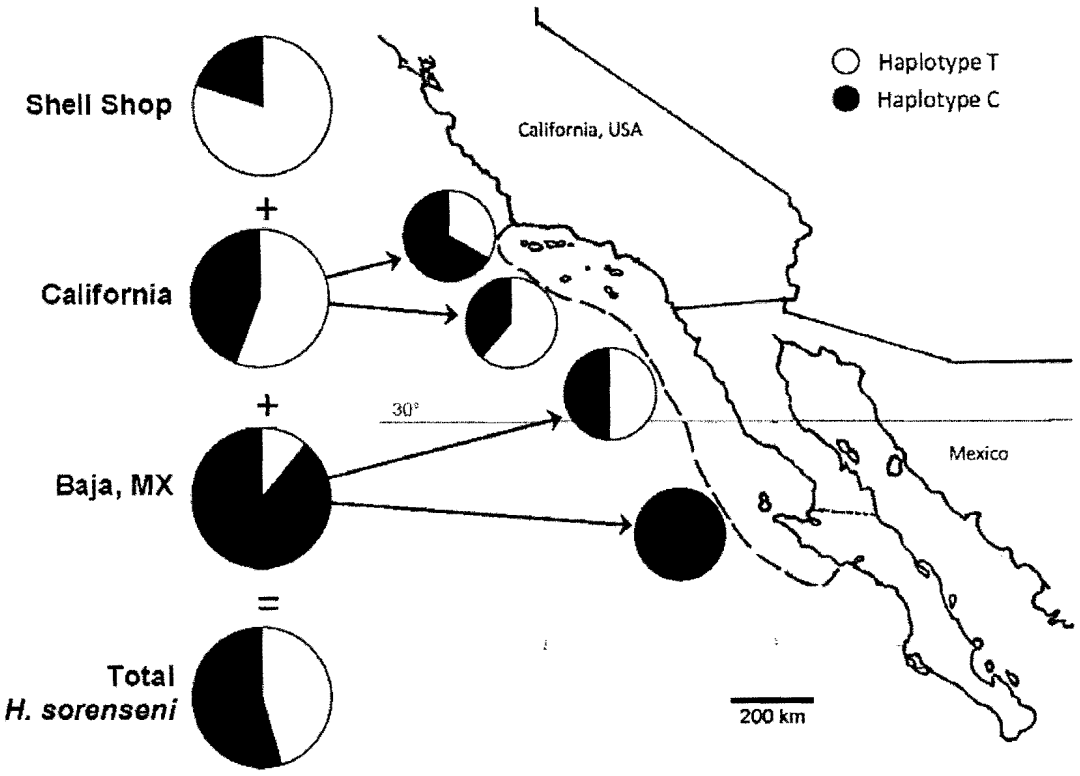

Figure 6. Haplotype Distribution Map. Proportions of two *H. sorenseni* COI haplotypes distributed along species range. Total of 84 shell samples was divided by country, and then by northern and southern regions of each. The five samples from a Morro Bay shell shop, located in San Luis Obispo County in California, were excluded from spatial comparison because collection locations were unknown, but since they were collected (legally) in 1966—prior to peak landing years—and most of them were haplotype 1 (blue), they were most likely collected in the southern Channel Islands, where this haplotype predominates.

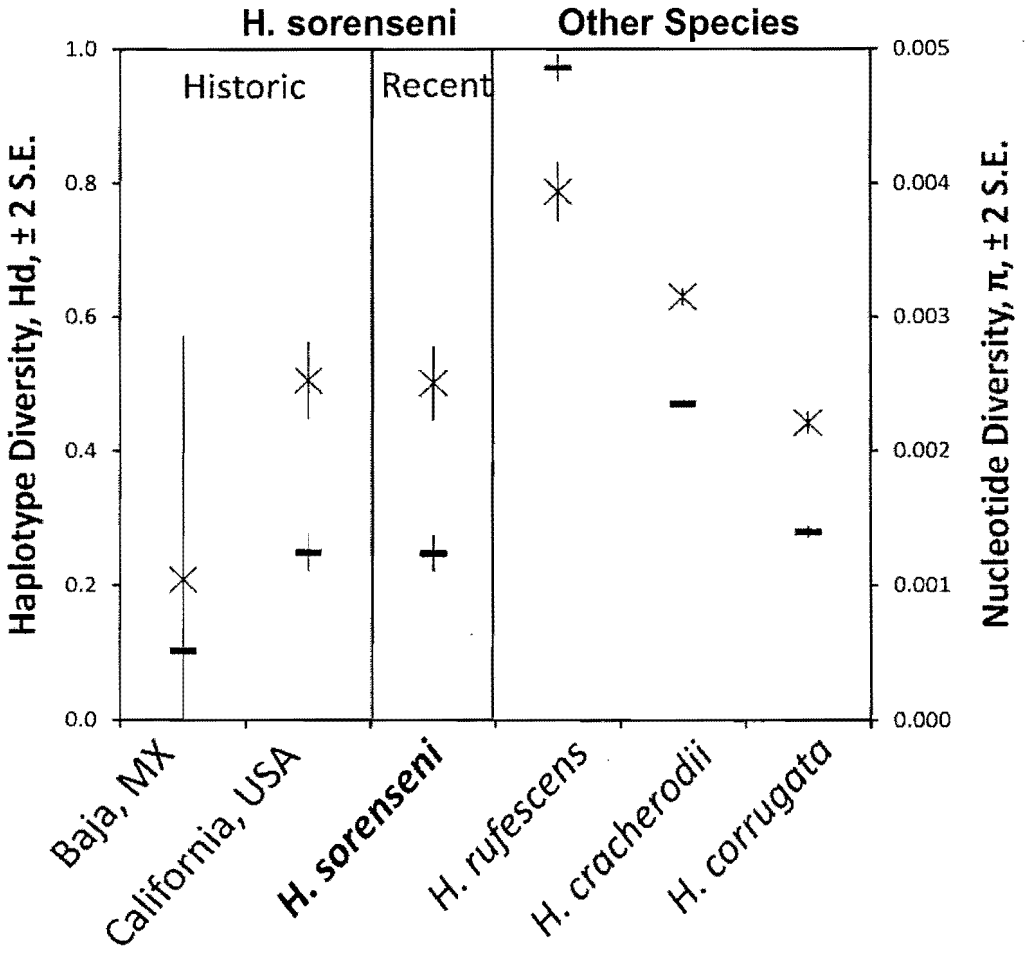

Figure 7. Comparison of nucleotide (π) and haplotype diversity (Hd) of historic and present populations of H. sorenseni and recent populations of other Haliotis species. Vertical bars indicate two standard errors of the means. X's correspond to the left-hand Hd axis, and red —'s correspond to the π axis on the right. Diversity estimates are based on sequences standardized to the same 403 bp COI gene sequence.

Panel C shows the variability of levels of diversity among other California abalone, *Haliotis rufescens* (Hruf), *H. cracherodii* (Hcrach), and *H. corrugata* (Hcorr).

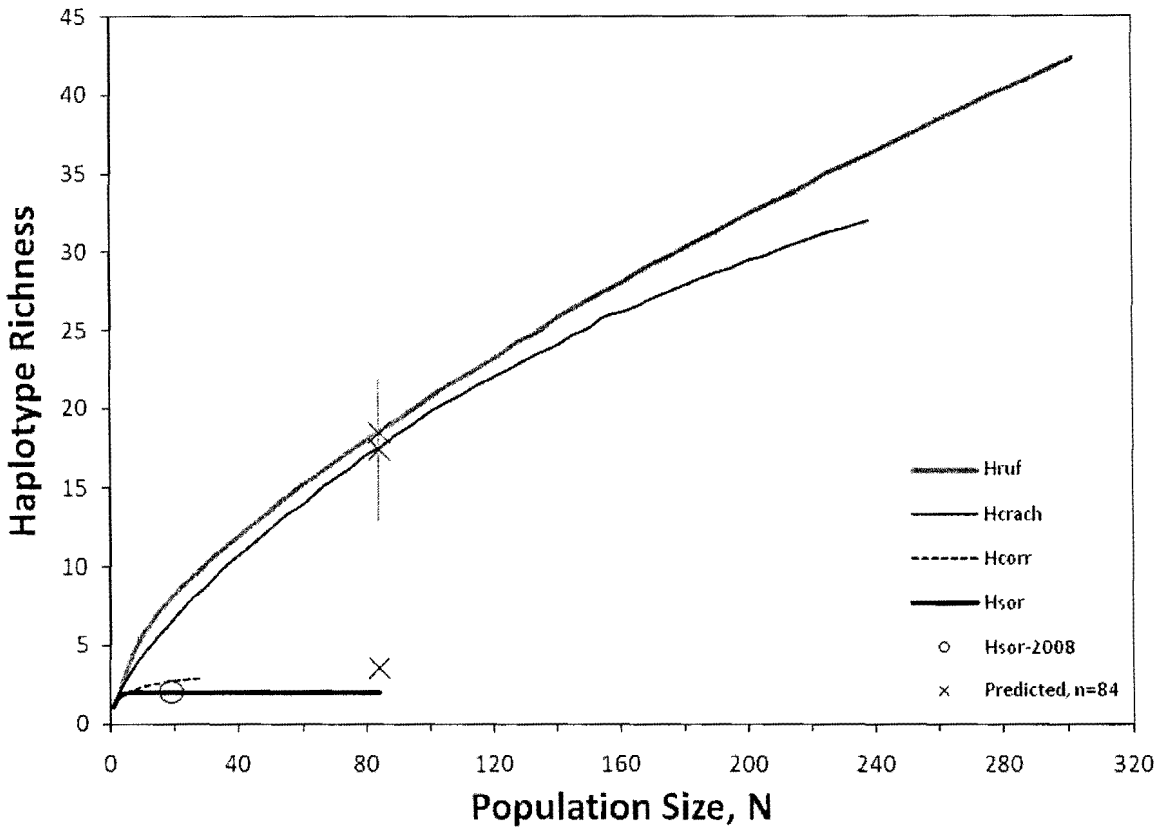

Figure 8. Haplotype richness rarefaction curves of four species of abalone, as a function of population size, n. For a sample size of 84 individuals of *H. rufescens* and *H. cracherodii* (Gruenthal et al. 2007, Gruenthal and Burton 2008), an average of 18 haplotypes was predicted (X), with 95% confidence intervals extending from 13-22 haplotypes. A loglinear model fitted to one population of 28 *H. corrugata* from Point Loma, CA (Clark et al. 2009) predicted 3.6 haplotypes in a sample of 84 pink abalone. The solid Hsor line shows that in a sample size of 84 white abalone, two haplotypes were measured, which is consistent in name and number with the findings of Gruenthal and Burton (2005), in a sample of 19 white abalone (o).

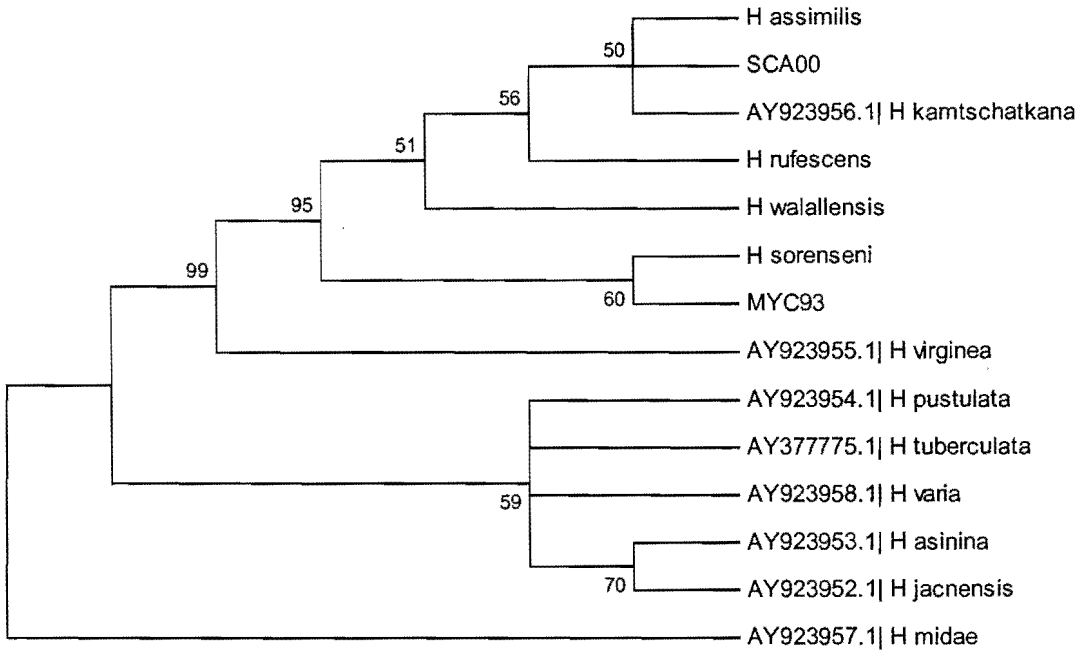

Figure 9. Nuclear Histone H3 Neighbor-joining cladogram of 12 *Haliotis* species over a 256 nucleotide sequence length. Tajima-Nei method, 80,000 bootstrap replicates. Linearized assuming equal evolutionary rates in all lineages, and branch lengths are equal to the number of base substitutions per site. The placement of SCA00 with *H. assimilis/H. kamtschatkana* concurs with the COI relationship, whereas the MYC93 Histone H3 sequence is identical to that of *H. sorenseni* rather than *H. walallensis*. Nodes for *H. assimilis* and *H. walallensis* each represent two identical sequences from separate specimens.

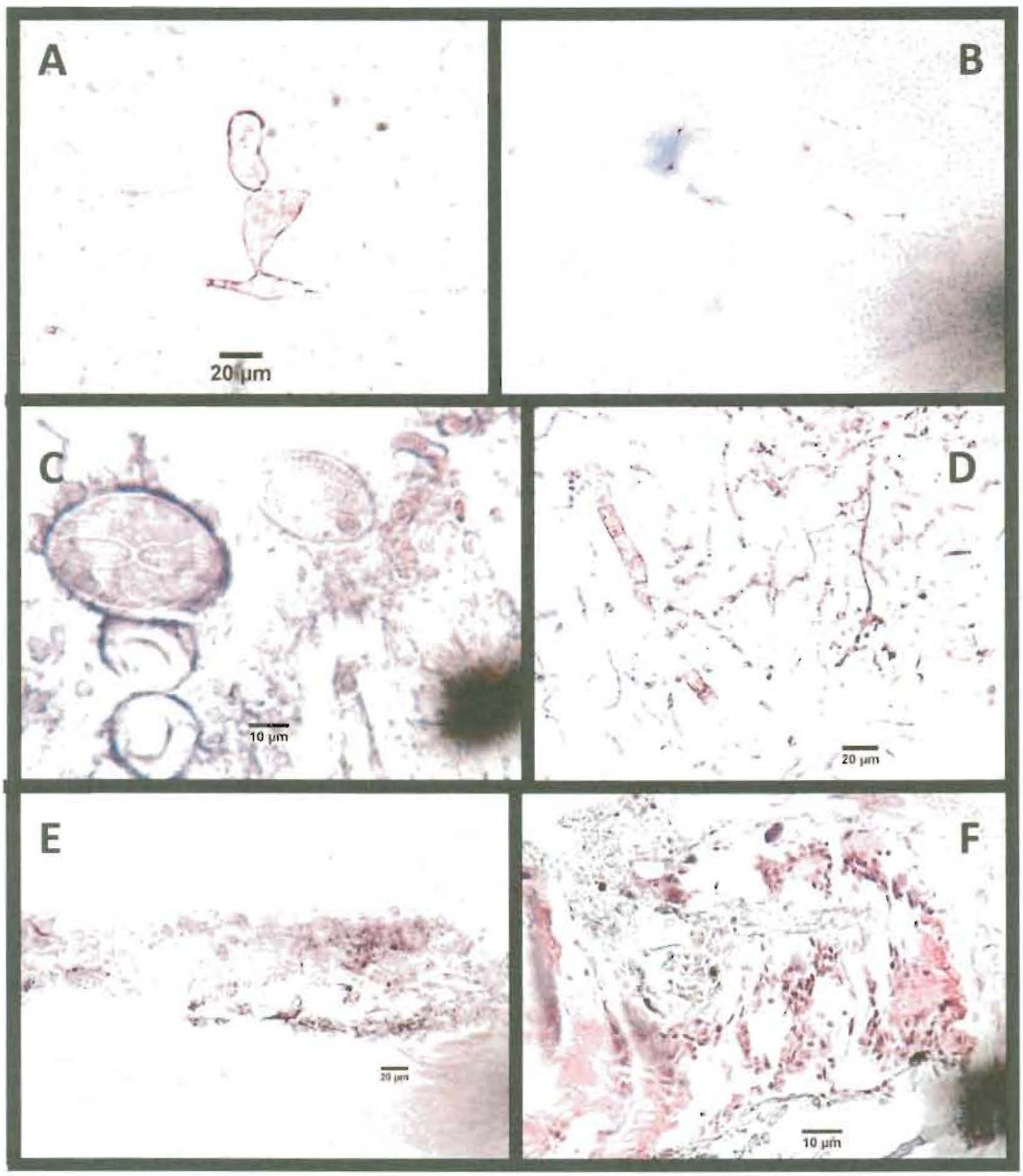

Figure 10a. H&E Stain. A) Red abalone shell, with algal cell and intralamellar membrane structure. B) Irregularities in intralamellar membrane in red abalone shell. C) Diatoms and other organisms amid bio-eroded prismatic layer of white abalone shell. D) Boring algal and fungal cells within middle nacreous layers of white abalone shell E & F) Examples of possible boring sponge exploratory tunnels.

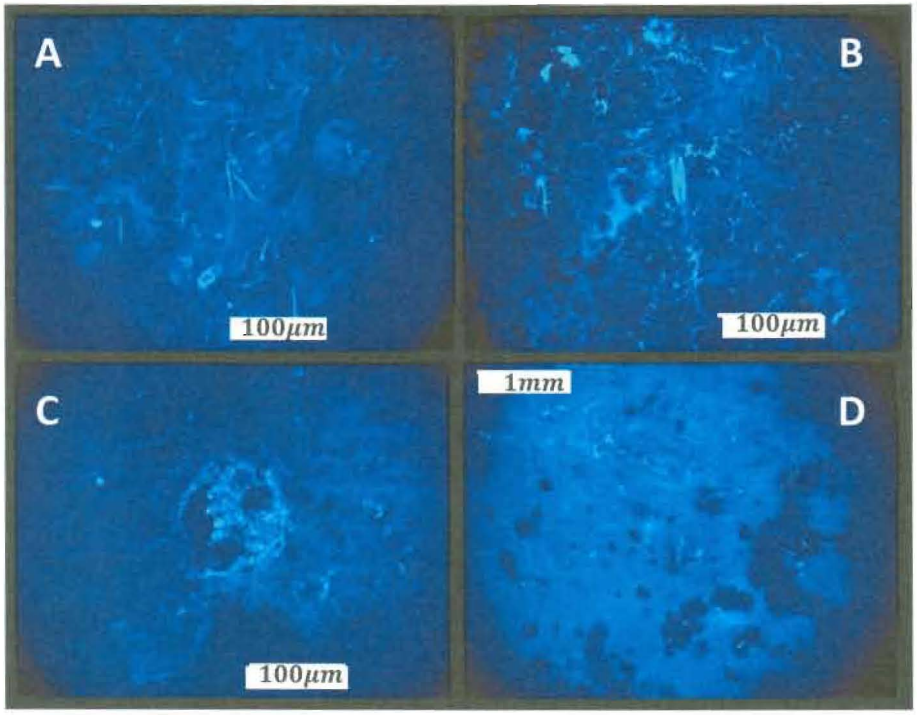

Figure 10b. DAPI Stain. A & B) Examples of boring organisms, including a mix of green algae, cyanobacteria, and fungal cells. C) Cross section of what is believed to be a small tunnel made by a boring sponge. D) Intralamellar membrane with evidence of microorganismal bio-erosion.

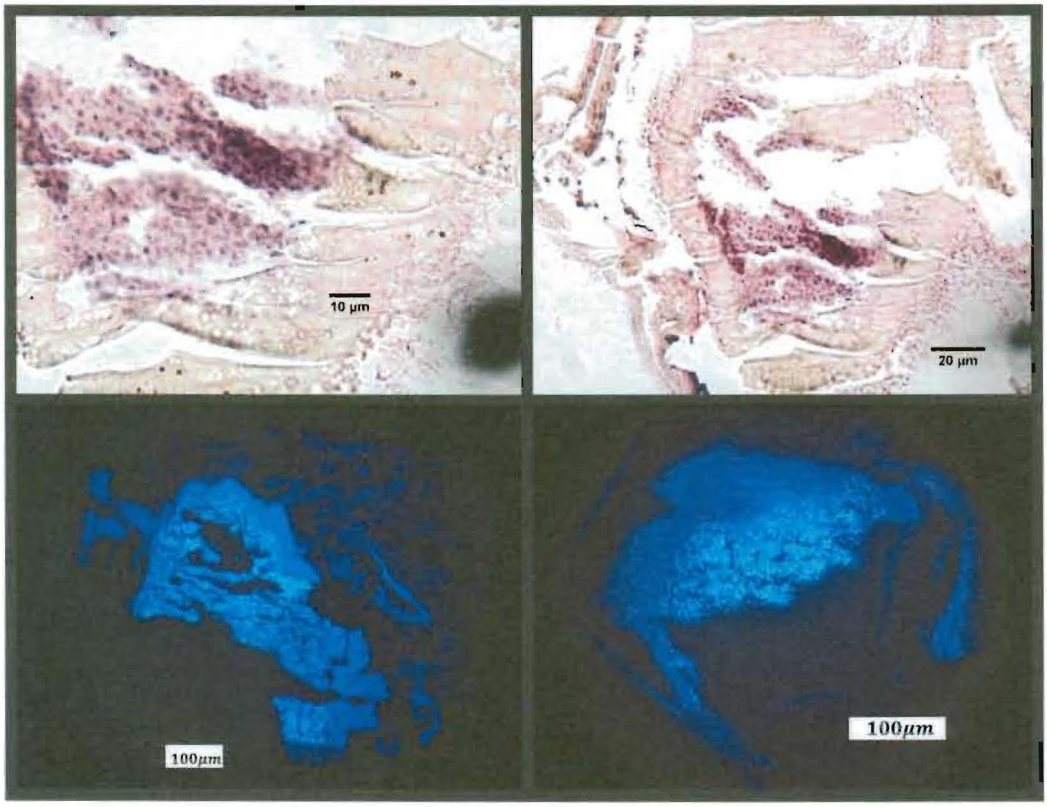

Figure 10c. Other stained structures. Some strucures in the shell were not specifically distinguishable as belonging to any particlular taxon, and it is not known whether these common structures are artifacts of the embedding or staining processes.

APPENDIX

DNA SEQUENCES

Title: *Haliotis sorenseni* COI, haplotype C
Gene: mitochondrial cytochrome c oxidase subunit 1, partial cds
Length: 539 bp
Source: mitochondrion *Haliotis sorenseni*
Organism: *Haliotis sorenseni.* Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
act cag tct ttt aat tcg ggc cga act tgg cca gcc agg agc act ctt ggg gga cga cca act cta taa cgt aat tgt aac agc cca cgc ttt cgt aat aat ctt ctt cct agt tat acc act aat aat tgg agg att tgg aaa ctg act ggt ccc ttt aat att agg ggc acc aga cat agc ctt tcc ccg act aaa taa cat aag att ctg act cct tcc gcc atc ctt aac cct act cct aac atc ggg cgc tgt aga aag tgg agc ggg gac agg ctg aac agt cta tcc tcc cct ctc tag taa cct tgc cca cgc agg agc atc agt aga ctt agc aat ttt ctc cct aca cct agc cgg aat ctc atc aat ttt agg ggc agt aaa ctt tat tac tac agt aat aaa tat acg tgt aaa agc aca gcc cct aga acg aat gcc att att tgt ttg atc agt aaa aat tac cgc cat cct act act cct atc act acc tgt tct agc agg tgc cat tac aat act cct aac cg

Title: *Haliotis sorenseni* COI, haplotype T
Gene: mitochondrial cytochrome c oxidase subunit 1, partial cds
Length: 539 bp
Source: mitochondrion *Haliotis sorenseni*
Organism: *Haliotis sorenseni.* Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
act cag tct ttt aat tcg ggc cga act tgg cca gcc agg agc act ctt ggg gga cga cca act cta taa cgt aat tgt aac agc cca cgc ttt cgt aat aat ctt ctt cct agt tat acc act aat aat tgg agg att tgg aaa ctg act ggt ccc ttt aat att agg ggc acc aga cat agc ctt tcc ccg act aaa taa cat aag att ctg act cct tcc gcc atc ctt aac ctt act cct aac atc ggg cgc tgt aga aag tgg agc ggg gac agg ctg aac agt cta tcc tcc cct ctc tag taa cct tgc cca cgc agg agc atc agt aga ctt agc aat ttt ctc cct aca cct agc cgg aat ctc atc aat ttt agg ggc agt aaa ctt tat tac tac agt aat aaa tat acg tgt aaa agc aca gcc cct aga acg aat gcc att att tgt ttg atc agt aaa aat tac cgc cat cct act act cct atc act acc tgt tct agc agg tgc cat tac aat act cct aac cg

Title: *Haliotis ?sorenseni* COI, MYC93
Gene: mitochondrial cytochrome c oxidase subunit 1, partial cds
Length: 539 bp
Source: mitochondrion *Haliotis ?sorenseni*
Organism: *Haliotis sorenseni.* Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
ttc ttc cta gtc ata cca ctg ata att gga gga ttt gga aac tgg cta gtc cct tta ata tta ggg gca cca gac ata gcc ttt ccc cga cta aat aac ata aga ttc tga ctc ctt ccg cca tcc tta acc cta ctc cta aca tcg ggc gct gta gaa agg gga gcg ggg aca ggc tga aca gtc tat cct ccc ctc tct agt aac ctt gcc cac gca gga gca tca gta gac tta gca att ttc tcc cta cac cta gcc gga atc tca tca att tta ggg gca gta aac ttt att act aca gta ata aat ata cgt gta aaa gca cag ccc cta gaa cga atg cca tta ttt gtt tga tca gta aaa att acc gcc atc cta cta ctc cta tca ct

Title: *Haliotis assimilis/H. kamtschatkana* COI, SCA00
Gene: mitochondrial cytochrome c oxidase subunit 1, partial cds
Length: 539 bp
Source: mitochondrion *Haliotis assimilis/H. kamtschatkana*
Organism: *Haliotis sorenseni.* Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
ttc ttc cta gtt ata cca cta ata atc gga gga ttt gga aac tga ctg gtc cct tta ata tta ggg gca cca gac ata gcc ttt ccc cga cta aat aac ata aga ttc tga ctc ctt cca cca tcc tta acc cta ctc cta aca tcg ggc gct gta gaa agt gga gcg ggg aca ggc tga aca gtc tat cct ccc ctc tct agt aac ctt gcc cac gca gga gca tca gta gac tta gca att ttc tcc cta cac cta gcc gga atc tca tca att tta ggg gca gta aac ttt att act aca gta ata aat ata cgt gta aaa gca cag cct cta gaa cga atg cca tta ttt gtt tga tca gta aaa att acc gcc atc cta cta ctc cta tca ct

Title: *Haliotis sorenseni* Histone H3
Gene: Histone H3 gene, partial cds
Length: 256 bp
Organism: *Haliotis sorenseni.* Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
gta aat cca ccg gag gaa agg ctc ccc gta aac agc tgg cca cca agg ctg ctc gta aga gtg ccc cgg cta cag gag gtg tca aga aac ctc aca gat aca ggc cag gaa cag tcg ccc ttc gtg aga tcc gtc gtt acc aga aga gca ccg agc ttt tga tca gga agc tgc cat tcc agc gtc tgg t?c gtg aaa tcg ccc aag act tca aga ccg atc tcc gat tcc agt ctt cag ccg tca tgg ctc tgc a

Title: *Haliotis rufescens* Histone H3
Gene: Histone H3 gene, partial cds
Length: 256 bp
Organism: *Haliotis rufescens*. Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
gta aat cca ccg gag gaa agg ctc ccc gta aac agc tgg cca cca agg ctg ctc gta aga gcg ccc cgg cta cag gag gtg tca aga aac ctc aca gat aca ggc cag gaa cag tcg ccc ttc gtg aga tcc gtc gtt acc aga aga gca ccg agc ttt tga tca gga agc tgc cat tcc agc gtc tgg tcc gtg aaa tcg ccc aag att tca aga ccg atc tcc gat tcc agt ctt cag ccg tca tgg ccc tgc a

Title: *Haliotis assimilis* Histone H3
Gene: Histone H3 gene, partial cds
Length: 256 bp
Organism: *Haliotis assimilis*. Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
gta aat cca ccg gag gaa agg ctc ccc gta aac agc tgg cca cca agg ctg ctc gta aga gcg ccc cgg cta cag gag gtg tca aga aac ctc aca gat aca ggc cag gaa cag tcg ccc ttc gtg aga tcc gtc gtt acc aga aga gca ccg agc ttt tga tca gga agc tgc cat tcc agc gtc tgg tcc gtg aaa tcg ccc aag act tca aga ccg atc tcc gat tcc agt ctt cag ccg tca tgg ccc tgc a

Title: *Haliotis walallensis* Histone H3
Gene: Histone H3 gene, partial cds
Length: 256 bp
Organism: *Haliotis walallensis*. Eukaryota; Metazoa; Mollusca; Gastropoda; Orthogastropoda; Vetigastropoda; Haliotoidea; Haliotidae; Haliotis.
Sequence:
gta aat cca ccg gag gaa agg ctc ccc gta aac agc tgg cca cca agg ctg ctc gta aga gtg ccc cgg cta cag gag gtg tca aga aac ctc aca gat aca ggc cag gaa cag tcg ccc ttc gtg aga tcc gtc gtt acc aga aga gca ccg agc ttt tga tca gga agc tgc cat tcc agc gtc tgg tcc gtg aaa tcg ccc aag act tca aga ccg atc tcc gat tcc agt ctt cag ccg tca tgg ccc tgc a