A STUDY OF PENCIL URCHIN POPULATION GENETICS AT HANNIBAL BANK

Hannah Lee

ABSTRACT

Hannibal Bank is a seamount-like feature known for its high productivity and biodiversity that is located in an UNESCO World Heritage Site off the Pacific coast of Panama. The biodiversity and biogeography of two species of pencil urchins at Hannibal Bank were studied using genetic analysis and geographical imaging systems. DNA barcoding of the COI mitochondrial gene identified gene sequences for 80 samples of two species of pencil urchins (Genus *Hesperocidaris*). Analyses of the geography and bathymetry of the bank indicated that both pencil urchin species demonstrated high gene flow and no significant genetic population structure. Samples of species 1 were distributed primarily on the northern and southern flanks of the bank with some near the peaks with recent population expansion indicated by haplotypes. Samples of species 2 were distributed intermittently along the flank of the bank's perimeter and the haplotype network indicated that it is an evolutionary stable population.

INTRODUCTION

Seamounts are deep-sea structures that allow for high levels of endemic fauna to form habitats within and on the hard substrate (Waller, Scanlon, & Robinson, 2011). Often isolated structures, seamounts are found all throughout the ocean and are defined as underwater mountains that rise above an elevation 1000 m but do not break the surface of the water column (Cho & Shank, 2010). These features are often formed by mantle plumes associated with large geologic events such as plate tectonic shifts. Due to their unique geological structure, seamounts are often studied as a region for dispersal, genetic isolation, and speciation to occur in deep-sea populations (Cho & Shank, 2010).

Hannibal Bank, the site of our study, is a seamount-like feature located off of the Pacific coast of Panama. It is included as part of an UNESCO World Heritage Site and lies within Panama's Coiba National Park (UNESCO.org 2018). It is a marine protected area that is known for its sports fishing and local fishing communities (Cunningham, Guzman, & Bates, 2013). Although an area known for high levels of biodiversity, Hannibal Bank did not have any detailed bathymetry or ecological studies performed until recently. The bank rises to approximately 416

m above the seafloor and therefore does not meet the general definition for elevation of a seamount, but Hannibal Bank shares many of the same physical characteristics of a seamount despite its smaller size (Cunningham et al., 2013).

The biological and geological structure of Hannibal Bank has been studied over the past few years for patterns of local biodiversity since seamounts often contain physical and biological factors that result in biodiversity hotspots (Pineda et al., 2016). The physical structure of the seamount can potentially influence several different biological processes that affect the local communities. In 2013, the first bathymetric study of Hannibal Bank was conducted (Cunningham et al., 2013). The study found that the steeply sided features of the seamount as well as the upright pinnacles on the top may be acting with the surrounding Panama current, Columbia current, and the western equatorial undercurrent to produce upwelling, a process that brings nutrient rich water from deeper depths towards the surface which promotes an area of high productivity around the seamount compared to the surrounding area. It is possible that seamounts in oceanographically stable regions may serve as refuge sites for biota from the effects of climate change since seamounts are generally found at depths near 1000 m below the surface and may not experience as strong of an effect from changes in climate (Du Preez, Curtis, & Clarke, 2016). Current and future changes in the proximity and level of human activity near the bank may also have a strong effect on the overall faunal composition and diversity of the bank (Cunningham et al., 2013).

The need for conservation efforts has increased as the marine environment becomes more vulnerable to human threats (Clark et al., 2010). While fishing is limited to sports fishing and artisanal fishing at Hannibal Bank, benthic communities may still be threatened by the existing practices near the seamount. While small scale fishing does not appear to have as huge of an impact on the local underwater communities as commercial fishing does, it may still be influencing the biodiversity of the seamount. Fishing may be threatening the survival of endemic species located at the seamount due to their limited habitat range (Rogers, 2004).

Diverse communities of organisms are known to inhabit seamounts all over the world and the knowledge about what species settle around a particular seamount can lead to insights on the reasoning for the geographical location and biological interaction of various species (Rogers, 2004). Species interaction can be assessed by focusing on the biological and spatial interactions between or within a specific species or group of organisms. Sea urchins are an invertebrate

marine species within the phylum Echinodermata and the class Echinoidea. There are over 700 species of sea urchins that are found throughout all oceans (Skerry, 2009). As omnivorous grazers, sea urchins feed with a beak-like structure called Aristotle's lantern (Ziegler, Schroder, Ogurreck, Faber, & Stach, 2012). They are able to potentially destroy all of the available algae in an area, especially in kelp forest communities, if unregulated by predation (Tegner & Dayton, 2000). Sea urchins reproduce by external fertilization via broadcast spawning, or releasing gametes into the water column, and fertilization is influenced by many environmental cues such as water flow direction and the timing of gamete release (Rothschild & Swann, 1951). Geographical isolation can limit the ability of different sea urchin populations to reproduce with other populations and increase the available gene pool.

The target organism of this study is the sea urchin, specifically two species of pencil urchins found at Hannibal Bank preliminarily identified as part of the genus *Hesperocidaris*. Pencil urchins belong to a primitive order known as Cidaroida, which includes 123 extant species in 33 genera, and they are characterized by their distinct thick, blunt spines compared to the thin spines of other orders of urchins (Brosseau et al., 2012). There is little phylogenetic data based on molecular characters available on the order Cidaroida although there is a lot of speculation on the ability of molecular data to help resolve interspecies relationships of echinoids.

DNA barcoding involves the use of sequencing a specific region of the genome as a way to genetically discriminate animal species. Mitochondrial genes are often used in species identification because they are passed down to offspring through maternal inheritance. These genes have a high number of copies within the mitochondria, lack introns, have high substitution rates, and have no recombination, which allows scientists to easily identify changes within the nucleotide sequence (Raupach et al., 2015). The cytochrome oxidase I (COI) mitochondrial gene is often used as a molecular marker in evolutionary studies because it evolves in a relatively short time frame. The COI gene has been proven to be effective in discriminating species for all classes of Echinodermata (Ward, Holmes, & O'Hara, 2008). The use of DNA barcoding can potentially identify all of the species involved in a particular food web as well as identify cryptic species (Smith et al., 2011). DNA barcoding is useful in determining species identification of both the pencil urchin species used in this study. The COI gene in echinoderms has over 18,000

entries on GenBank with over 100 entries within the order Cidaroida. However, there are no known entries for the COI mitochondrial gene for the genus *Hesperocidaris*.

Watson conducted a study of the population of one species of pencil urchin as well as one species of polychaete found at Hannibal Bank (Watson, 2016). Her samples included 49 of the pencil urchins collected from Hannibal Bank. The COI mitochondrial gene was used as a molecular marker to study the evolutionary relationships between samples due to the fast-evolving nature of the gene. It was predicted that the collection sites comprised a single population present at the seamount. Watson identified two species of pencil urchins but only studied 40 of the 49 samples due to limitations by the lower sample size of the second species (2016). The results of her research determined that there is a high level of gene flow but no significant differences in molecular variance between populations found in any geographic or depth comparison. The study concluded that there was a lack of isolation by distance that contributed to the lack of genetic structure that may be a result of the limited geographic range of the Hannibal Bank study sites. However, a study by Abdala assessing the biodiversity of communities at Hannibal Bank found that substrate type, seamount geography, and depth had a significant effect on the formation of community structure (2018).

This current project focused on the expansion of the Watson (2016) study of the pencil urchins collected at Hannibal Bank. The availability of larger sample numbers made it possible for a more robust study of the population genetic structure of the pencil urchin species that is commonly found near Panama as well as the inclusion of a second species of pencil urchin. This study intended to determine the gene flow and genetic diversity found at Hannibal Bank based on the pencil urchin as a model organism. The expansion of the available data allows further comparisons to be made between and within the identified sea urchin species collected from various points of the bank.

MATERIALS & METHODS

Sample Collection:

Samples were collected in April 2015 on cruise AL150302 to Hannibal Bank in the eastern Pacific Ocean off the coast of Panama on the M/V Alucia. Organisms were collected by the manned submersibles Deep Rover 2 and Nadir at multiple sites at the peak and long the flanks of the Bank (Figure 1). Upon collection, sea urchins were catalogued individually at sea.

DNA was extracted on board using Chelex extractions modified from the procedure described by Walsh et al. (Walsh, Metzger, & Higuchi, 1991). Small pieces of tissue were taken from the sample, minced, and then added to a 5% Chelex solution. The Chelex extractions were incubated in a 65°C water bath for 3 hours, vortexed, incubated at 95°C for 10 minutes, and centrifuged at 13,000 RPM for 5 minutes prior to use. Extractions were stored at -80°C or -20°C until further processing in the lab.

Eighty-four sea urchins were collected at Hannibal Bank (Table 1) and eighty individuals were amplified using Polymerase Chain Reaction (PCR). Eighty COI sequences with a length of 535 base pairs were obtained from these samples. *Eucidaris tribuloides*, with GenBank accession number KC626171.1, is a pencil urchin with 14% divergence from the samples that was used as an outgroup for phylogenetic analysis because the sequence was closely related to, but outside of the genus *Hesperocidaris*.

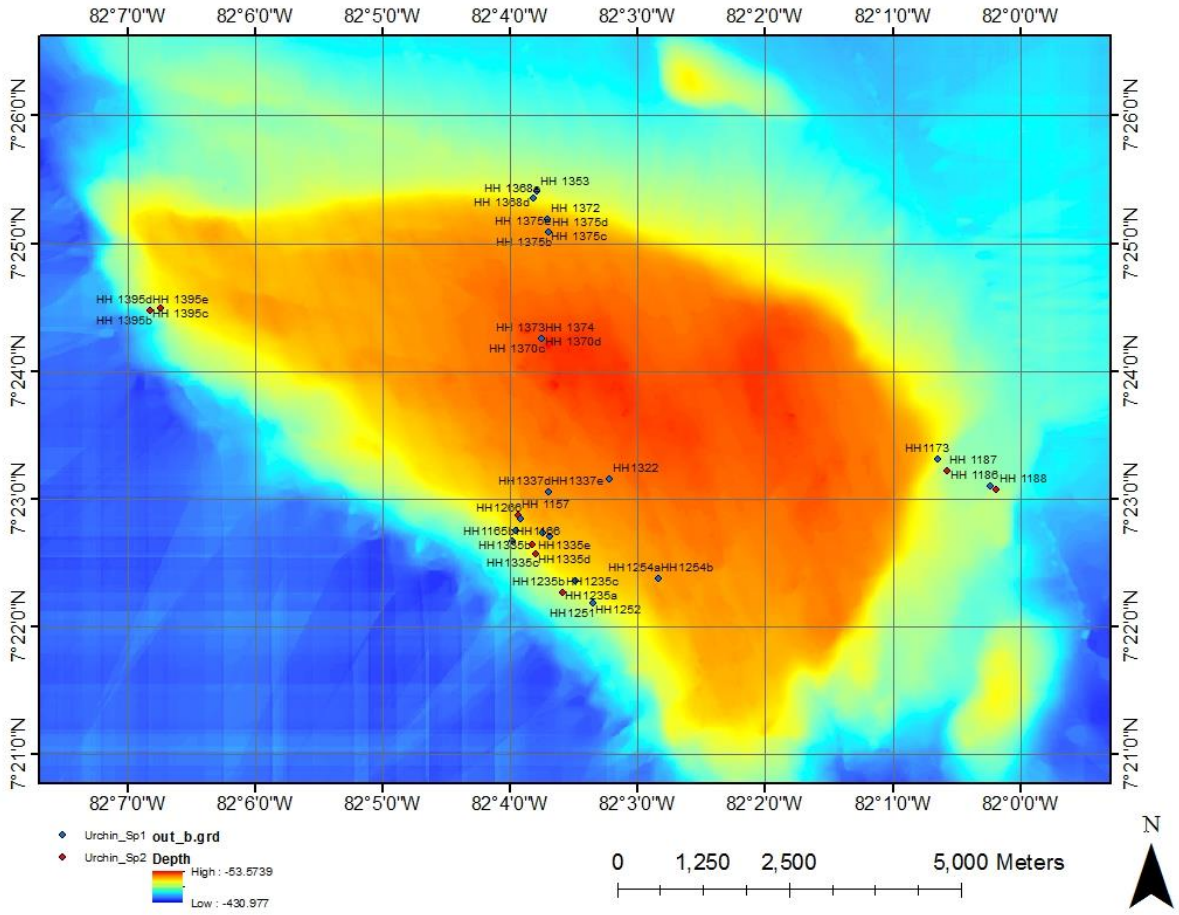

Figure 1: Map of sample sites at Hannibal Bank with depth indicated by color. Samples used in this study are plotted with dot markers: blue represents *Hesperocidaris* Sp.1 sampled populations and red represents *Hesperocidaris* sp. 2 sampled populations. Sample names are labeled with "HH#".

Table 1: Table of metadata for *Hesperocidaris* species samples used in this study based on geographical location. This table includes species, population, coordinates from the center of each population, sample quantity, the dive (indicated by DR# or N#) and sites (indicated by a letter or number after the dive number), and depth included in each population.

Species	Population	Latitude	Longitude	Quantity	Dive and Sites	Depth (m)
1	1	7.421368	-82.062375	18	N299_F	194
					DR320_B	170
					DR320_D	117
					N300_17:13	100

	2	7.404331	-82.062565	5	DR320_C, N300_C	130
	3	7.376063	-82.061043	17	DR316_WP1, DR316_B, N298_G	130
					DR316_Q1	134
					DR311_WP4	164
					N291_D	224
					N294_J	156
					N295_D	192
	4	7.380727	-82.052096	8	N298_H	100
					DR317_C	89
					N295_G	108
	5	7.387009	-82.007172	2	DR312_E, N292_WP3	177
					N292_WP3	224
2	1	7.408249	-82.113121	11	DR322_C	251
					N290_L	Unknown; Sample picked up mid-dive
					DR322_E	193
	2	7.411937	-82.063121	2	DR320_D	117
					N300_C	130
	3	7.376602	-82.064223	15	DR311_WP5	164
					N291_D	224
					N298_E	211
					N298_F	172
					N294_I	209
	4	7.38612	-82.006402	2	N292_WP4	189
					N292_D	224

DNA Processing:

DNA extractions were diluted to between 18-22 ng/μL for use in polymerase chain reaction (PCR) to amplify the DNA. The markers were amplified in 25 μL PCR reactions containing 5 μL 5X PCR Buffer, 2.5 mM of $MgCl_2$, 0.5 μM of each primer, 0.2 mM of dNTP, 1 μL of extracted DNA template and 0.2 U of Taq polymerase (Promega). 0.4 μL of BSA (bovine serum albumin) was used in some PCR reactions as an additive to increase samples with low PCR product yield (Bribiesca-Contreras et al., 2013). Optimal annealing temperatures were determined using a gradient PCR. PCR products were visualized on a 1.5% agarose gel with ethidium bromide and subsequently purified using the QIAquick PCR purification Kit (Qiagen) following manufacturer protocols. The purified PCR products were quantified for DNA concentration using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and sent to MWG Eurofins Operon for Sanger sequencing in both directions of DNA.

The sea urchin samples were amplified using echinoderm specific universal COI primers. The primers COIceF (5'- ACTGCCCACGCCCTAGTAATGATATTTTTATGGTNATGCC-3') and COIceR (5'- TCGTGTGTCTACGTCCATTCCTACTGTRAACATRTG-3') were used to amplify a portion of the COI region in 72 samples (Hoareau & Boissin, 2010). Eight sea urchin samples were alternatively amplified by the primers OphiF (5'- ATAATGATAGGAGG-ATTTGGAAA-3') and COIceR (5'-TCGTGTGTCTACGTCCATTCCTACTGTRAACATRTG-3') for the same portion of the COI region (Bribiesca-Contreras et al., 2013). The PCR program used had an initial denaturation at 95°C for 3 to 7 minutes, followed by 40 cycles of a 45 second denaturation step at 94-95°C, a 45-70 second annealing step at 48-50°C, a 60-80 second elongation step at 72°C, followed by a final elongation step for 3-5 minutes at 72°C.

Population Genetic Analysis:

DNA sequences were edited using GeneStudio Pro 2.2.0.0 (GeneStudio, Inc.) and CodonCode Aligner (CodonCode Corporation). Contigs and alignments were made and checked by eye in GeneStudio Pro and CodonCode Aligner. Alignment files were transferred into MEGA version 7 to check for pseudogenes, form phylogenetic trees, and calculate mean genetic distances (Tamura et al., 2013). COI sequences were translated to amino acids in MEGA to ensure that pseudogenes, indicated by a stop codon, were not present. This section of the COI mitochondrial gene was confirmed to not be a pseudogene. Samples were compared to sequences

in GenBank using the NCBI nucleotide BLAST tool online. Species identity was uncertain for the urchins so phylogenetic analysis was conducted to determine species identity before population genetic analyses were performed. Phylogenetic trees were formed using a Kimura 2-P model with 500 bootstraps (Kimura, 1980). Different species were divided into multiple datasets based on phylogenetic analysis.

Analyses of molecular variance (AMOVA) were conducted by using ARLEQUIN v3.1 on the species datasets constructed from phylogenetic analysis (Excoffier, Laval, & Schneider, 2007). The AMOVA tested for population structure of each sea urchin species at the Hannibal Bank. Geographic isolation was tested by AMOVA for among groups, among populations within groups, and within populations. A separate set of AMOVA tests were run for each sea urchin species. For sea urchin species 1 (sp. 1), tests were run using three different configurations of populations: 1) each individual population versus each of the other populations, 2) northern populations versus southern populations, and 3) northern populations versus southern populations versus eastern populations. Two different configurations of populations were performed for sea urchin species 2 (sp. 2): 1) each individual population versus each of the other populations, and 2) northern populations versus southern populations.

A Mantel test was performed for both sea urchin species to test for isolation by distance using ARLEQUIN v3.6.2.2 (Mantel, 1967). A Tajima's D test was performed using ARLEQUIN v3.6.2.2 as a test statistic for neutral selection (Tajima, 1989). This test also indicates if the data produced from each set of samples fits a model of rapid population expansion.

Haplotype networks were created using TCS 1.21 (Clement, Posada, & Crandall, 2000). Network ambiguities were resolved using criteria from coalescent theory. Haplotypes were considered to be more likely connected to other haplotypes that occur frequently, are interior within the cladogram, or are geographically closer (Crandall & Templeton, 1993); (Pfenninger & Posada, 2002). Nested clade analysis was performed using ANeCA (Panchal, 2006); (Panchal & Beaumont, 2007). This was done in congruence with other analyses as a way to identify evolutionary processes and geographic patterns within the dataset. Haplotype distribution maps were created using the haplotype data for each species to plot the frequency of each haplotype in each population around Hannibal Bank.

AMOVA were performed to test population structure based on depth for both species. This analysis was limited by the relatively shallow depth of Hannibal Bank. Depth analyses were

performed using three different sets of depth bin structures for both species. *Hesperocidaris* sp. 1 urchins were collected from depths of 89 m to 224 m and *Hesperocidaris* sp. 2 urchins were collected from depths of 117 m to 251 m. Sample HH1109 was not included in the depth analyses for *Hesperocidaris* sp. 2 due to unknown depth of sample collection. Bin structures used previously by Watson (2016) were divided into depths of 89-115 m, 116-145 m, 146-193 m, 194-225 m, and 226-255 m. A second set of bin categories developed by Abdala (Abdala, 2018) were divided into depths of 75-150 m and 150-275 m. The third set of bin categories (HL) were divided into depths of 0-175 m and 176-275 m in attempt to equalize the distribution of samples in each category. These AMOVA were performed according to three hierarchical levels: within the depth bin, among the bins but within the depth interval, and among depth intervals. Mantel tests and Tajima's D neutrality tests were also run for all depth analyses.

Spatial analyses of molecular variance (SAMOVA) were run for each species using SAMOVA 2.0 (Dupanloup, Schneider, & Escoffier, 2002). This test is used to indicate the best population groupings that are geographically homogenous for each species dataset without constraint of the sample's geographic composition. Samples are randomly assigned into *K* groups. The simulated annealing process was repeated 100 times from a different initial partition of samples into *K* groups in order to ensure that the final configuration of *K* groups is not affected by a given initial configuration (Dupanloup et al., 2002). SAMOVA was run using values of $K = 2$ to 5 for *Hesperocidaris* sp. 1 and values of $K = 2$ to 4 for *Hesperocidaris* sp. 2 in order to compare the population structures generated by the SAMOVA to the population structures estimated *a priori*.

RESULTS

Phylogenetic analysis revealed two clades depicting *Hesperocidaris* sp. 1 and sp. 2 urchins (Figure 2). Pencil urchin data showed a 4.8% sequence divergence between sp. 1 and sp. 2 and a mean difference of 15% between the two species and the outgroup, *Eucidaris tribuoides*. According to Palumbi et al., echinoderms have a 3% sequence divergence of species (Palumbi, Grabowsky, Duda, Geyer, & Tachino, 1997). Intraspecific genetic variation is 0.40% for *Hesperocidaris* sp. 1 and 0.60% for *Hesperocidaris* sp. 2.

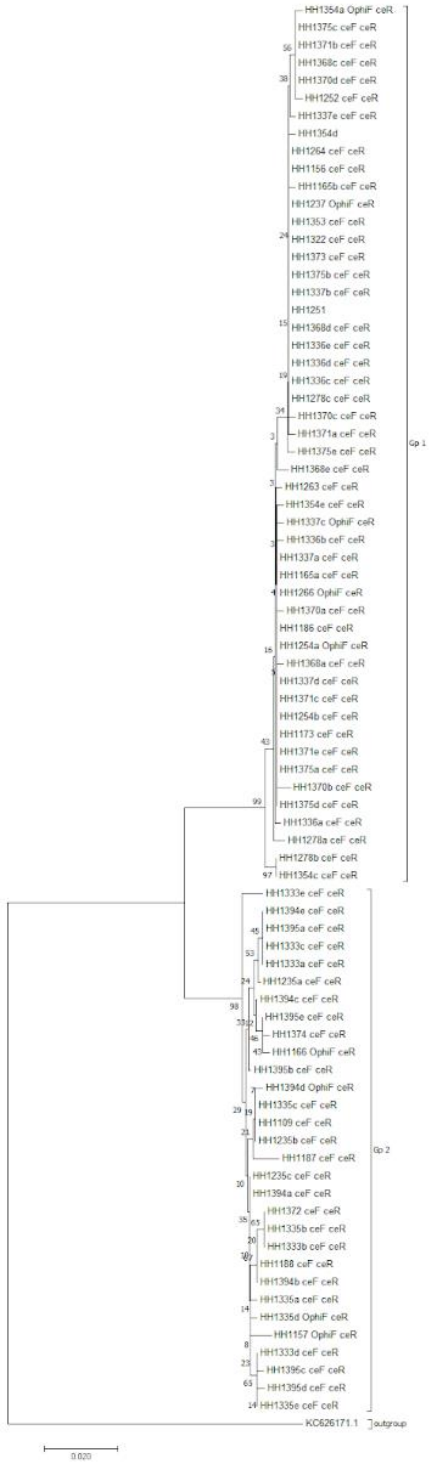

Figure 2: Phylogenetic tree of the COI gene for collected *Hesperocidaris* sp. 1 and sp. 2 samples using the Kimura-2 parameter for genetic distance and a bootstrap of 500. The outgroup is a sample of a *Eucidaris tribuloides* pencil urchin which was obtained from GenBank (KC626171.1).

Analyses of molecular variance (AMOVA) comparing geographic regions were not significant for any population structure for *Hesperocidaris* sp. 1 or sp. 2 (Tables 2 and 3). P-values for each set of populations tested resulted in p-values greater than 0.05 for among groups, among populations within groups, and within groups. Nested clade analysis resulted in no significant population structure within pencil urchin populations. The Mantel test for isolation-by-distance and the Tajima's D neutrality tests were also not significant as they all resulted in p-values of greater than 0.05 for each set of populations tested for both species.

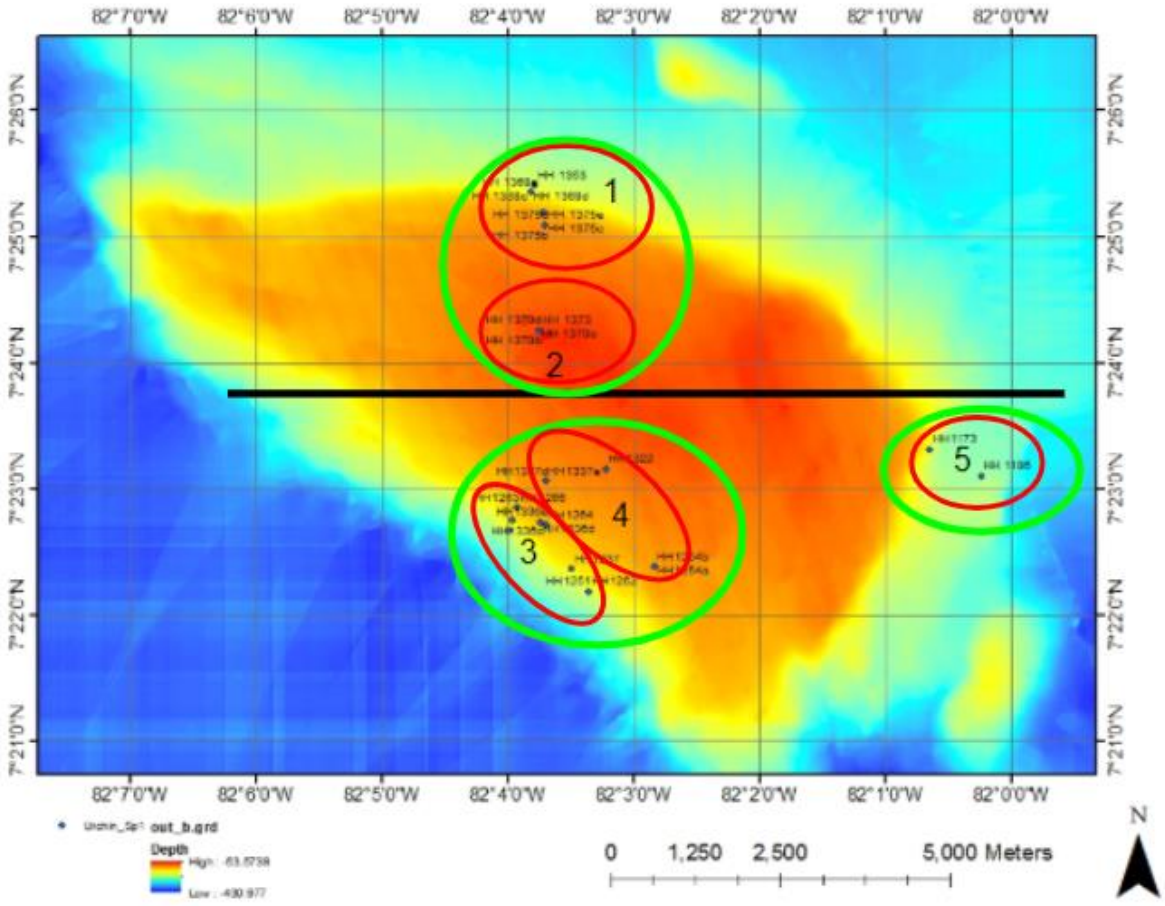

Figure 3: Map of sampled *Hesperocidaris* sp. 1 populations. Geographic populations are denoted by numbers and circled in red. Northern versus southern populations are separated by a black line. Northern versus southern verses eastern populations are denoted by green circles. Sample names are labeled with "HH#".

Table 2: Results for AMOVA, Mantel, and Tajima's D analyses for geographic populations for *Hesperocidaris* sp. 1. P-values under 0.05 are significant and reject the null hypothesis.

Populations Tested	Among Groups	Among Populations Within Groups	Within Groups	Mantel Test	Tajima's D
Sites 1 vs 2 vs 3 vs 4 vs 5	0.39883	0.98240	0.99707	0.59700	0.87442
Sites 1 & 2 vs 3, 4, & 5 (N vs S)	0.25806	0.99218	0.99902	0.56000	0.87189
Sites 1 & 2 vs 3 & 4 vs 5 (N vs S vs E)	0.45357	0.98338	0.99609	0.56800	0.87537

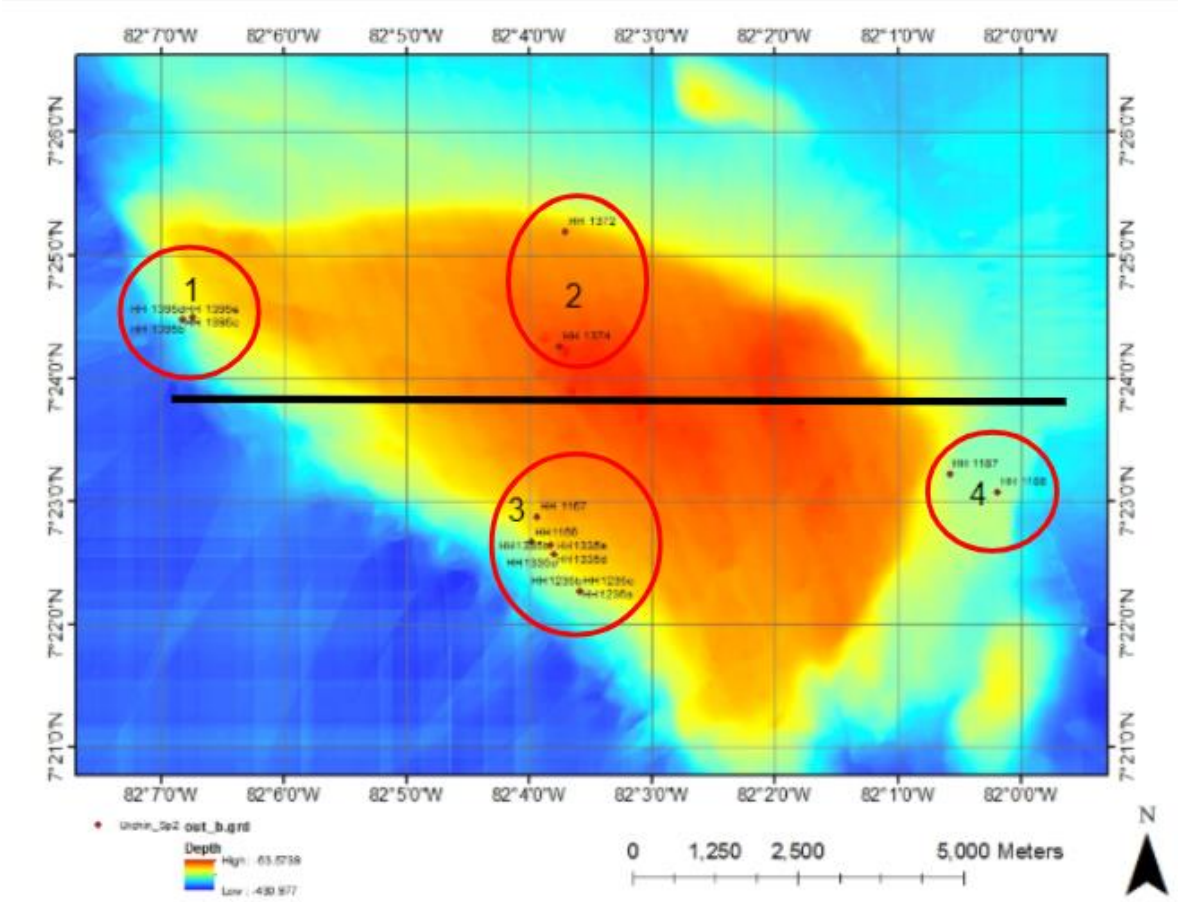

Figure 4: Map of sampled *Hesperocidaris* sp. 2 populations. Geographic populations are denoted by numbers and circled in red. Northern versus southern populations are separated by a black line. Sample names are labeled with "HH#".

Table 3: Results for AMOVA, Mantel, and Tajima's D analyses for geographic populations for *Hesperocidaris* sp. 2. P-values under 0.05 are significant and reject the null hypothesis.

Populations Tested	Among Groups	Among Populations Within Groups	Within Groups	Mantel Test	Tajima's D
Sites 1 vs 2 vs 3 vs 4	0.16227	0.43597	0.29228	0.247000	0.77342
Sites 1 & 2 vs 3 & 4 (N vs S)	0.90420	0.12610	0.14076	0.244000	0.77858

The haplotype network and distribution map for *Hesperocidaris* sp. 1 shows 22 haplotypes within populations of *Hesperocidaris* sp. 1 (Figures 5 and 6). The haplotype network for *Hesperocidaris* sp. 2 shows 20 haplotypes within populations of *Hesperocidaris* sp. 2 (Figures 7 and 8).

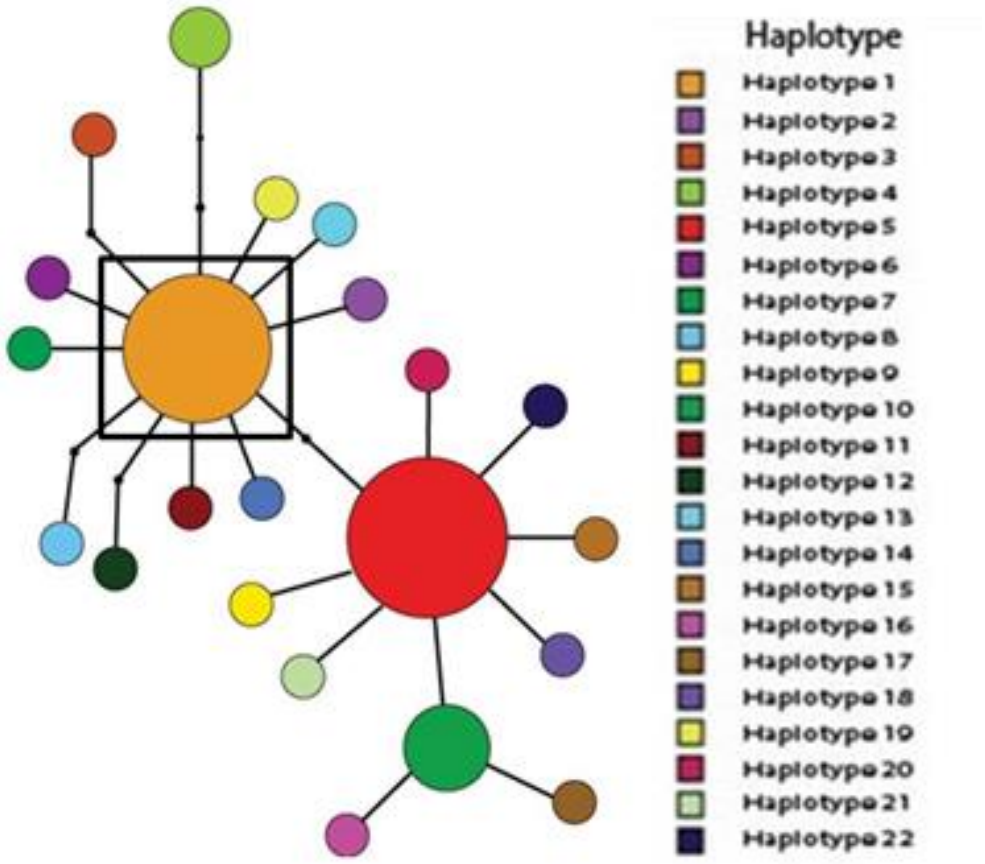

Figure 5: Haplotype network of *Hesperocidaris* sp. 1 urchins. Each color corresponds to the haplotype legend. Each node represents a nucleotide change in the genetic sequence and the size of each haplotype corresponds to its relative abundance. A black square denotes the ancestral node.

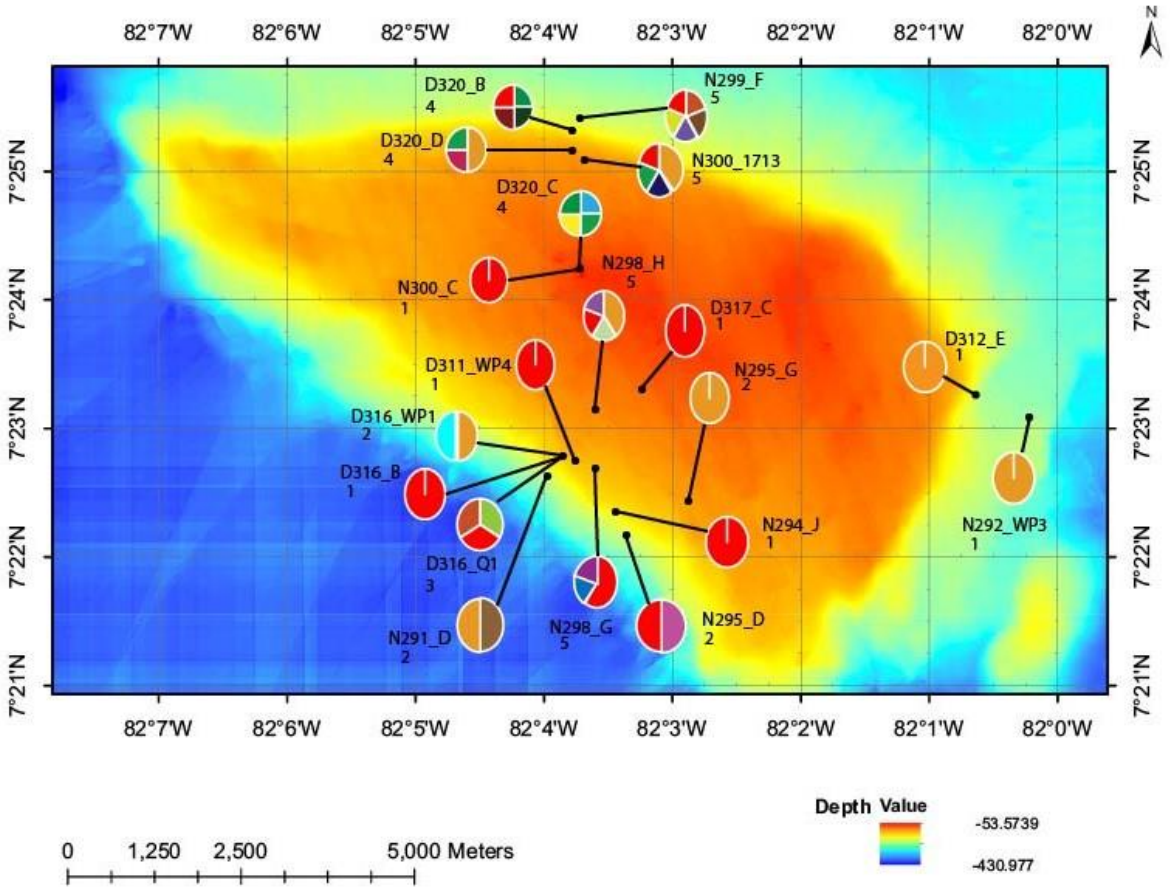

Figure 6: Map of haplotype distribution for *Hesperocidaris* sp. 1 at Hannibal Bank. Each haplotype is shown with its own unique color that corresponds to the legend in Figure 5. Pie charts represent the proportion of each population that share a certain haplotype. Sample sizes are indicated underneath the population number.

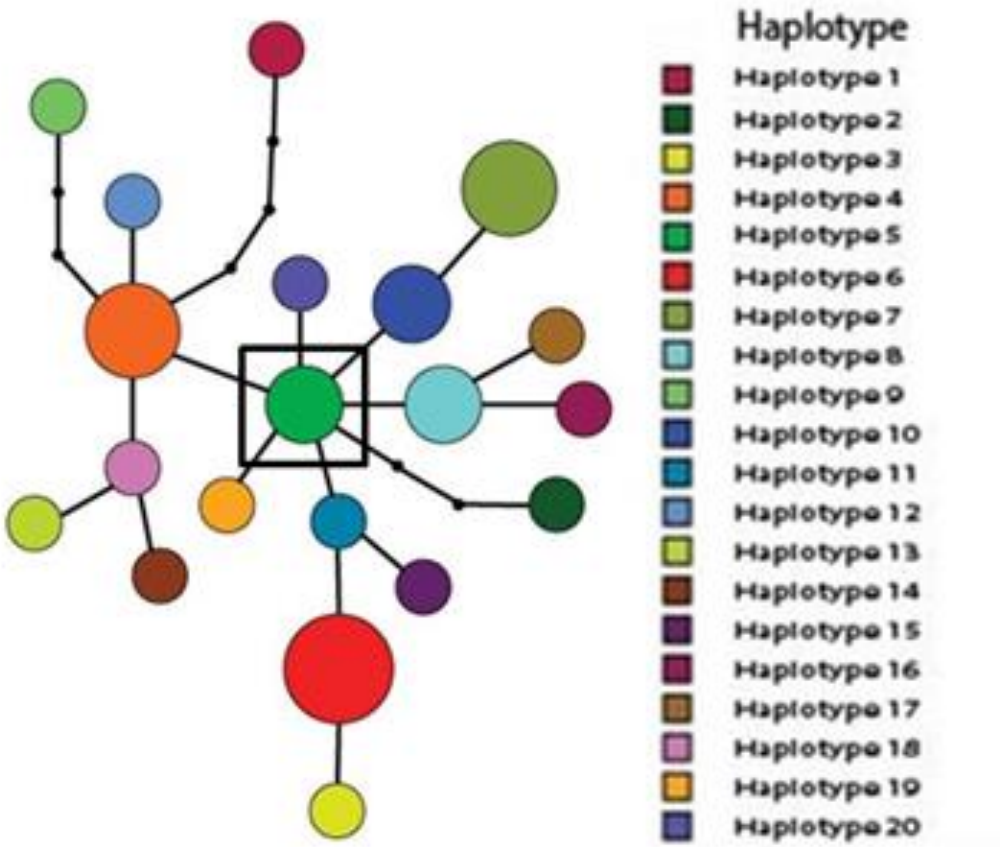

Figure 7: Haplotype network of *Hesperocidaris* sp. 2 urchins. Each color corresponds to the haplotype legend. Each node represents a nucleotide change in the genetic sequence and the size of each haplotype corresponds to its relative abundance. A black square denotes the ancestral node.

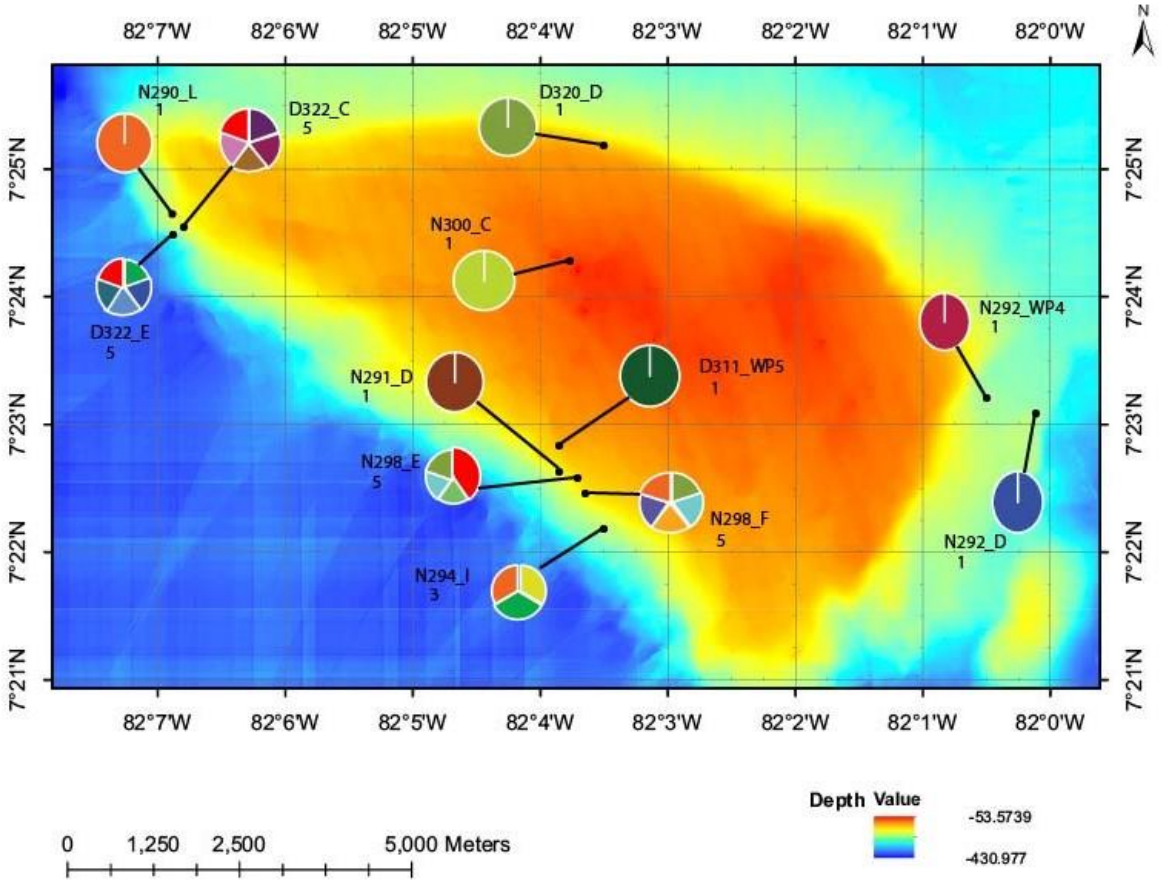

Figure 8: Map of haplotype distribution for *Hesperocidaris* sp. 2 at Hannibal Bank. Each haplotype is shown with its own unique color that corresponds to the legend in Figure 7. Pie charts represent the proportion of each population that shares a certain haplotype. Sample sizes are indicated underneath the population number.

Analyses of molecular variance (AMOVA) comparing bathymetric or depth regions were not significant for any population structure for *Hesperocidaris* sp. 1 or *Hesperocidaris* sp. 2. (Tables 5 and 7). P-values for each set of populations tested resulted in p-values greater than 0.05 for among groups, among populations within groups, and within groups. Nested clade analysis resulted in no significant population structure within pencil urchin populations. The Mantel test for isolation-by-distance and the Tajima's D neutrality tests all resulted in p-values of greater than 0.05 for all depth bin structures for both species.

Table 4: Number of samples in each depth bin for the different depth bin structure in *Hesperocidaris* sp. 1. The depth interval ranges are listed for the depth structures from Watson (2016), Abdala (2018) and the current study.

Depth Bin	SW Bins	JA Bins	HL Bins
1	89-115 m (n=13)	75-150 m (n=33)	0-175 m (n=39)
2	116-145 m (n=20)	150-275 m (n=17)	176-275 (n=11)
3	146-193 m (n=9)		
4	194-225 m (n=8)		
5	226-255 m (n=0)		

Table 5: Results for AMOVA, Mantel, and Tajima's D analyses for bathymetric populations for *Hesperocidaris* sp. 1. P-values under 0.05 are significant and reject the null hypothesis.

	Among Groups	Among Populations Within Groups	Within Groups	Mantel Test	Tajima's D
SW Bins	0.55230	0.99022	0.99707	0.71500	0.87700
JA Bins	0.50635	0.99609	0.99609	0.67800	0.87716
HL Bins	0.68719	0.99609	0.99902	0.66600	0.87389

Table 6: Number of samples in each depth bin for the different depth bin structure in *Hesperocidaris* sp. 2. The depth interval ranges are listed for the depth structures from Watson (2016), Abdala (2018) and the current study.

Depth Bin	SW Bins	JA Bins	HL Bins
1	89-115 m (n=0)	75-150 m (n=2)	0-175 m (n=8)
2	116-145 m (n=2)	150-275 m (n=27)	176-275 (n=21)
3	146-193 m (n=12)		
4	194-225 m (n=10)		
5	226-255 m (n=5)		

Table 7: Results for AMOVA, Mantel, and Tajima's D analyses for bathymetric populations for *Hesperocidaris* sp. 2. P-values under 0.05 are significant and reject the null hypothesis.

	Among Groups	Among Populations Within Groups	Within Groups	Mantel Test	Tajima's D
SW Bins	0.67742	0.08895	0.09971	0.241000	0.75900
JA Bins	0.46041	0.11241	0.09580	0.262000	0.75564
HL Bins	0.16325	0.09384	0.08407	0.356142	0.75591

When divided into two, three, four, or five homogenous groups by the SAMOVA, the AMOVA for *Hesperocidaris* sp. 1 resulted in a significant p-value for among groups (Table 8). When divided into three or four homogenous groups by the SAMOVA, the AMOVA for *Hesperocidaris* sp. 2 resulted in a significant p-value for among groups (Table 9).

Table 8: Results for the AMOVA analyses for *Hesperocidaris* sp. 1. AMOVA analyses were run using the best fit homogenous groups generated by the SAMOVA. P-values under 0.05 are significant.

Number of Groups	Homogeneous Groups	Sample Depth (m)	Among Groups	Among Populations Within Groups	Within Populations
2	D312_E, D316_WP1_Q1, N292_WP3, N295_G	108, 130, 134, 177, 224	0.0098	1.0000	0.99316
	D311_WP4, D316_B, D317_C, D320_B, D320_N300_C, D320_D, N291_D, N294_J, N295_D, N298_G, N298_H, N299_F, N300_1713	89, 100, 117, 130, 156, 164, 170, 192, 194, 224			
3	D311_WP4, D316_B, D317_C, D320_B, D320_N300_C, D320_D, N291_D, N294_J, N295_D, N298_G, N298_H, N299_F, N300_1713	89, 100, 117, 130, 156, 164, 170, 192, 194, 224	0.0000	1.0000	0.99316
	D312_E, N292_WP3, N295_G	108, 177, 224			
	D316_WP1_Q1	130, 134			
4	N295_D	192	0.0000	1.0000	0.99707
	D311_WP4, D316_B, D317_C, D320_B, D320_N300_C,	89, 100, 117, 130,			

	D320_D, N291_D, N294_J, N298_G, N298_H, N299_F, N300_1713	156, 164, 170, 194, 224			
	D312_E, N292_WP3, N295_G	108, 177, 224			
	D316_WP1_Q1	130, 134			
5	D320_B, D320_N300_C, D320_D, N291_D, N298_G, N300_1713	100, 117, 130, 170, 224	0.0000	1.0000	0.99707
	N298_H	100			
	N299_F	194			
	D311_WP4, D316_B, D317_C, N294_J, N295_D	89, 130, 156, 164, 192			
	D312_E, D316_WP1_Q1, N292_WP3, N295_G	108, 130, 134, 177, 224			

Table 9: Results for the AMOVA analyses for *Hesperocidaris* sp. 2. AMOVA analyses were run using the best fit homogeneous groups generated by the SAMOVA. P-values under 0.05 are significant.

Number of Groups	Homogeneous Groups	Sample Depth (m)	Among Groups	Among Populations Within Groups	Within Populations
2	D311_WP5, D320_D, D322_E, D322_C, N290_L, N291_D, N292_D, N294_I, N298_E, N298_F, N300_C	117, 130, 164, 172, 193, 209, 211, 224, 251	0.07527	0.39394	0.15445
	N292_WP4	189			
3	D320_D, D322_E, D322_C, N290_L, N291_D, N292_D, N294_I, N298_E, N298_F, N300_C	117, 130, 172, 193, 209, 211, 224, 251	0.01075	0.70381	0.13881
	N292_WP4	189			
	D311_WP5	164			
4	D320_D, D322_E, D322_C, N290_L, N291_D, N292_D, N294_I, N298_E, N298_F	117, 172, 193, 209, 211, 224, 251	0.01173	0.79570	0.15347
	N300_C	130			
	N292_WP4	189			
	D311_WP5	164			

DISCUSSION

This study expanded the original assessment of the pencil urchin population structure of Hannibal Bank performed by Watson (2016). Phylogenetic analysis confirmed that there are two distinct species of pencil urchins. Watson used 40 samples of *Hesperocidaris* sp. 1 in the previous study's genetic analyses. This study added an additional 10 samples to the original dataset in order to expand and confirm the results that Watson had previously found. Although Watson had originally obtained 9 samples of *Hesperocidaris* sp. 2, genetic analyses were never performed on those samples. This study added 21 more samples to the original 9 for a total of 30 samples for genetic analysis.

In order to test the population structure of the pencil urchins at Hannibal Bank, a series of genetic analyses were run to test any significant populations based on geography or bathymetry. The analyses of molecular variance (AMOVA) results all had a p-value for among groups, among populations within groups, and within groups greater than 0.05 (Tables 2 and 3) which indicates no significant population structures based on the geography of the bank. The Mantel test showed a lack of isolation-by-distance since the test resulted in p-values that were greater than 0.05, reaffirming the results of the AMOVA that there is no significant geographic population structure. The Tajima's D neutrality test also had an insignificant p-value which indicates that there is no recent population expansion.

The haplotype network for *Hesperocidaris* sp. 1 found 22 different haplotypes from a dataset of 50 samples (Figure 5). The most common haplotypes were haplotype 1 and haplotype 5 with the ancestral node appearing to be haplotype 1. The haplotype network has a star-like formation which indicates that there may have been recent population expansion. The haplotype distribution shows that haplotypes 1 and 5 were widely distributed all over the bank while there are several new haplotypes in the northern populations with a few other singletons in the southern populations (Figure 6). There were only two samples in the eastern population but both samples were members of haplotype 1 indicating that gene flow occurred between the eastern population and the rest of the bank.

The haplotype network for *Hesperocidaris* sp. 2 found 20 different haplotypes from 30 samples (Figure 7). The most common haplotypes were 4, 6, and 7, however haplotype 5 appears to be the ancestral node. Singleton haplotypes were found in all parts of the bank (Figure 8). The northwestern and southern populations were composed of several different singleton haplotypes

(haplotypes 2, 3, 9, 11, 12, 14, 15, 16, 17, 18, 19, & 20) while the northern and eastern populations had only one or two different singletons in each population (haplotypes 1 & 13). The similar number of individuals with each haplotype indicates that *Hesperocidaris* sp. 2 is a more evolutionary stable population in comparison to *Hesperocidaris* sp. 1, which still has many individuals for a few of the same haplotypes. However, the apparent stability of the *Hesperocidaris* sp. 2 haplotype network could also be a result of the low sample size and it is possible that there could be a higher number of individuals for a few of the same haplotypes.

The AMOVA results for populations based on depth for both species resulted in p-values greater than 0.05 for the SW, JA, and HL depth bins for both species (Tables 5 and 7), confirming that there is no significant population structure based on depth at Hannibal Bank. The Mantel test and the Tajima's D p-values were also insignificant for all three depth bin structures for both species. This suggests that depth does not appear to be a determining factor for pencil urchin communities. However, *Hesperocidaris* sp. 2 urchins are found to inhabit deeper depths in comparison to *Hesperocidaris* sp. 1 urchins. *Hesperocidaris* sp. 2 is commonly found at depths of 117 m to 251 m, while *Hesperocidaris* sp. 1 is found at depths of 89 m to 224 m.

The spatial analyses of molecular variance (SAMOVA) identified the maximally differentiated groupings for each species using a simulation rather than identifying groups *a priori*. *Hesperocidaris* sp. 1 urchins were divided into regional groups based off sets of two, three, four, and five homogenous groups (Table 8). The p-value for among groups resulted in a significant difference, which should indicate that there is a genetic difference in populations based on regions. In comparison to the homogenous groups chosen *a priori*, there is no apparent pattern based on geography or depth range for the way homogenous groups were formed via SAMOVA. The number of samples of in each group were also consistently uneven which could have skewed the results.

The SAMOVA results for *Hesperocidaris* sp. 2 urchins divided the samples into regional groups based off sets of two, three, and four homogenous groups (Table 9). The p-values for among groups resulted in a significant p-value for two and three homogenous group structures. The groups were generally divided into regional groups more closely based on depth, with one sample site containing a majority of the samples located at the deepest depths and two to three other sample sites being grouped individually due to being located at slightly shallower depths on the bank. This is different from the groups chosen *a priori* based on geographical coordinates

viewed on the map of the bank. However, there are still inconsistencies in the depth range of each homogenous grouping, therefore any significance could be an artifact of low sample size.

The purpose of this study was to determine the biodiversity found at Hannibal Bank using pencil urchins as the model organism in order to provide information about gene flow on the species level to help guide conservation efforts at Hannibal Bank or in similar study sites. The study confirmed the conclusions that *Hesperocidaris* sp. 1 has high gene flow, but no significant population structure based on geography or depth (Watson, 2016). This study also concluded that *Hesperocidaris* sp. 2 also has high gene flow but no significant population structure based on the geography of the bank or the depth of the samples. The study results were expanded using SAMOVA to generate groups of populations without determining the group structure *a priori*. The groups generated by SAMOVA indicated that there may be a significant difference in the genetic structure among groups for *Hesperocidaris* sp. 2 but not for *Hesperocidaris* sp 1.

There are several ways that gene flow may have spread throughout different parts of the bank. Sea urchins are known to reproduce by broadcast spawning which allows gametes to be released into the water column for fertilization (Rothschild & Swann, 1951). This would allow gametes from different populations to travel through the water column to mix, promoting high gene flow. However the success of broadcast spawning is dependent on the longevity of sperm after release and the amount of turbulent flow that determines the distance gametes can travel for fertilization (Bishop, 1998). The physical structure and depth of the bank can also limit the distance that gametes are able to spread between populations and the areas where individuals are able to survive (Ourens, Freire, Vilar, & Fernandez, 2014). This study of Hannibal Bank suggest that gene flow has not been restricted by the geography or depth of the bank in either species.

Hannibal Bank is one of many seamounts found throughout the world's oceans. While there is still much to be explored, the data that can be obtained from Hannibal Bank can be used to help direct conservation and management of the bank or at other similar deep-sea features. The pencil urchin population at Hannibal Bank appears to act as one genetic population when considering the bank as a whole because there is high gene flow and no population structure found in either species. However, the community structure of both pencil urchin species varies slightly with depth because *Hesperocidaris* sp. 1 is found at a shallower depth range when compared to *Hesperocidaris* sp. 2. If a specific species of pencil urchin is to be conserved, then depth could potentially be a determining factor in protecting a certain depth range from diving or

fishing. Overall, this type of biological data can be correlated with physical, chemical, and geological data to determine other connectivity patterns that may be occurring as a result of other oceanographic processes which can serve to connect or isolate communities (Shank, 2010). The protection of Hannibal Bank as a biodiversity hotspot is important for both preserving endemic species that may inhabit the bank and the seamount's role as a stepping stone for dispersal.

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