Studies at Hannibal Bank, Panama: A Study of Evolutionary History of Mollusks and Developing a Program to Review Dive Track Data

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Abstract

This study focuses on the phylum Mollusca from Hannibal Bank in Panama. Through DNA sequencing and phylogenetic analyses, the biodiversity and evolutionary history of mollusks were evaluated. The results of this study were found to support molluscan relationships reported in previous studies. Seventeen distinct species were collected. Bayesian and Maximum Likelihood analyses revealed that the class Polyplacophora was basal to the sister clades Bivalvia and Gastropoda. Gastropoda was also found to have two distinct clades in both analyses: Vetigastropoda and Caenogastropoda. In addition to the phylogenetic analysis, a user-friendly program was written in R to help review dive track data and to better visualize where samples were found. This streamlines removing errors from dive track data received from research cruises and allows the points to be mapped and visualized.

Introduction

The phylum Mollusca is a very diverse group. Of the benthic marine invertebrates, it is one of the largest phyla with an estimated 100,000 species. Mollusks are defined by certain characteristics. They are bilaterally symmetrical, triploblastic, protostomes, and have a complete gut with an anus and circulatory, digestive, and nervous system. Mollusks also have cephalization and a radula (except for bivalves), a shell, muscular foot, mantle tissue, and mantle cavity. There are seven different classes in the Mollusca phylum: Polyplacophora, Gastropoda,

Bivalvia, Aplacophora, Monoplacophora, Scaphopoda, and Cephalopoda (Levinton, 2014). This study focuses on three of the classes: Polyplacophora, Gastropoda, and Bivalvia.

Polyplacophora are generally known as chitons and have a radula, flattened foot, and eight dorsal plates. They are herbivorous grazers. Gastropoda are generally snails and have a radula, flattened foot, shell, and an operculum. They are distinguished by torsion, which is when the body of the gastropod twists during development, and the anus and mouth both connect to the shell's one opening. Bivalvia are generally clams and have a mantle that secretes a shell and gills that help the bivalve breathe and collect phytoplankton. They are typically distinguished by two symmetrical valves that open on a hinge region (Levinton, 2014).

Mollusca samples were collected at Hannibal Bank. Hannibal Bank is located off the Pacific coast of Panama and is an area of high productivity and biodiversity. It is a top sports fishing area and supports local fishing communities but is a marine protected area where commercial fishing is prohibited (Cunningham, M Guzman, & Bates, 2013). Hannibal Bank is part of Panama's Coiba National Park and is a UNESCO World Heritage site (UNESCO.org, 2016). Hannibal Bank itself is technically considered to be a sea hill by some due to its size as it is less than 500m tall. However, Hannibal Bank has certain features that share characteristics with seamounts and is often referred to as a seamount (Yesson, Clark, Taylor, & Rogers, 2011). Seamounts are an important habitat for benthic organisms because they provide substrate for organisms to live on and may increase the water column's productivity by affecting currents (Cho & Shank, 2010).

In this study, two genes were used to study the evolutionary history of mollusks at Hannibal Bank: COI and 18S. COI is a mitochondrial gene that is often used in barcoding for identifying species. Mitochondrial genes are maternally inherited, have many copies, no introns,

high substitution rates, and no recombination (Raupach et al., 2015). COI is therefore good at resolving shallow nodes of a phylogenetic tree. The non-protein coding nuclear rRNA 18S gene is slow-evolving. This conservative gene is good at resolving deep nodes of a phylogenetic tree and providing discrimination above the taxa of family (Wilke et al., 2001). Two different phylogenetic analyses were also used in this study: Bayesian and Maximum Likelihood. Bayesian analyses are based on the idea of posterior probabilities. These are probabilities that are based on a model after learning something from the data. It searches for the best set of trees representing relationships between the samples. After many generations, the analysis narrows down to a set of a few trees where the likelihoods are very similar and accepting or rejecting a change becomes a random choice; this is called a stable likelihood value (Hall, 2001). Maximum Likelihood analyses infer a tree by finding a tree that maximizes the probability of seeing the data. They look for a tree with the largest log likelihood. It's often impossible to look at all possible trees, so a search method is used to find the most likely tree (Hall, 2001).

Due to the high diversity of the molluscan phylum, there is no consensus for the evolutionary relationships of mollusks. Rather, there are three competing hypotheses of molluscan relationships that are generally discussed. The first is Aculifera that has Polyplacophora sister to Aplacophora and basal to all remaining Mollusca. The second is Testaria that has Aplacophora basal to Polyplacophora and all remaining Mollusca. The third is Serialia that has a clade of Polyplacophora and Monoplacophora separate from all remaining Mollusca (Sigwart & Lindberg, 2015). The results of this study will be compared with the results of a review by Sigwart & Lindberg (2015) that evaluated the molecular and morphological phylogenies of Mollusca. They identified eight different supertrees that served as hypotheses for

different current molluscan evolutionary relationships. We will use our samples to test these hypotheses.

The purpose of this study was to review the diversity and the evolutionary history of mollusks found at Hannibal Bank, Panama. This data will provide information for the biodiversity and number of species found at Hannibal Bank. This information can be useful to legislators trying to preserve and protect the bank. This data will also contribute to the general knowledge of the phylum Mollusca and will hopefully provide some support to clarify the phylum's relationships.

Along with reviewing the evolutionary history of mollusks, the purpose of this study was to develop a program to review dive track data. This program will help researchers to visualize and remove outliers and errors in the dive track data. Dive track data is collected on research cruises from the ship and submersibles and is typically a log of the date, time, latitude, and longitude of the vehicle, as well as additional metadata (depth, altitude, heading, etc.). The program was created using the R language (R. C. Team, 2018) through RStudio (R. Team, 2015) and uploaded to R Shiny so that the program could be accessed from any computer at any time. This will streamline the process of "cleaning up" dive track data.

Materials and Methods

Sample Collection:

Samples in this study were collected on a research cruise. In April of 2015, the M/V Alucia collected samples on cruise AL150302 at Hannibal Bank. The bank was explored with two manned submersibles - Deep Rover 2 and Nadir. They each completed 15 dives and over 500 discrete samples were collected. This study focused on 19 mollusk samples collected by the

submersibles (Figure 1 and Table 1). Mollusk DNA was extracted at sea using a modified Chelex extraction protocol (Sean Walsh, A Metzger, & Higushi, 2013). Bits of tissue were cut off the sample, minced, and added to a 5% Chelex solution, incubated in a 65°C water bath for 3 hours, vortexed, and incubated at 95°C for 10 minutes. After this, samples were kept at -20°C or -80°C until they were needed for further processing in the lab. In order to be processed and used, samples were completely thawed and centrifuged at 13,000 rpm for 5 minutes.
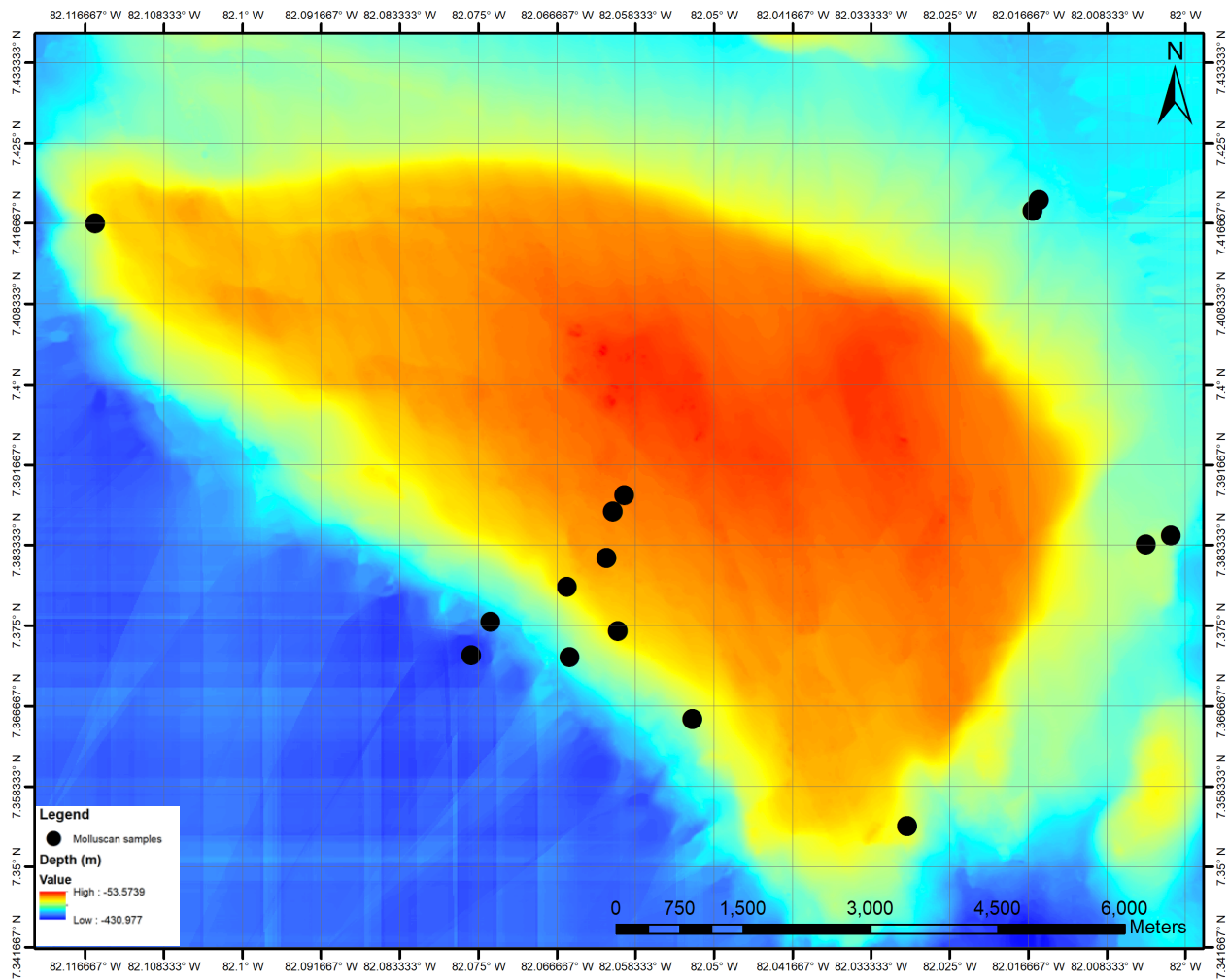

Figure 1: Map of the Hannibal Bank with depth indicated by color. Samples used in this study are plotted with black circles.

Data Collection:

One mitochondrial gene, COI, and one nuclear gene, 18S, were used for amplification. Chelex DNA extractions were centrifuged for 5 minutes and then diluted to between 17-23 ng/μL to be used in a polymerase chain reaction (PCR) for amplification. Primers were amplified in 25μL PCR reactions. Each reaction contained 5x PCR Buffer (Promega), 2.5mM of $MgCl_2$, 0.2mM of each dNTP, 0.5μM of each primer, 0.2 U of Taq polymerase (Promega), and 1μL of sample. PCR products were visualized using ethidium bromide on a 1.5% agarose gel. If single bands were visible, they were purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer protocols, with slight modifications. When Buffer PB was added to the PCR sample, it was mixed and allowed to sit for 20 minutes at 4°C. If double bands were visible, they were also gel extracted using the QIAquick PCR purification kit (Qiagen). After purification, the sample concentrations were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and sent to MWG Eurofins Operon for sequencing in the forward and reverse directions.

The Mollusca samples were amplified using COI and 18S primers. The universal COI primers HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') were used to amplify the COI region (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). The PCR program used to amplify the COI gene had an initial denaturation at 94°C for 1 minute, followed by 5 cycles of a 1 minute denaturation step at 94°C, a 1.5 minute annealing step at 45°C, and a 1.5 minute elongation step at 72°C. This was followed by another 35 cycles of a 1 minute denaturation step at 94°C, a 1.5 minute annealing step at 50°C, and a 1 minute elongation step at 72°C. The reaction ended with a final elongation step at 72°C for 5 minutes. Products were then stored at 4°C. The universal 18S primers 18S-5'

(5' - CTGGTTGATYCTGCCAGT - 3') (M.H. Winnepenninckx, G. Reid, & Backeljau, 1998) and 18S1100R (5' - CTTCGAACCTCTGACTTTCG - 3') (Williams, Reid, & Littlewood, 2003) were used to amplify the 18S region. The PCR program used to amplify the 18S gene had an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of a 45 second denaturation step at 94°C, a 45 second annealing step at 54°C, and a 2 minute elongation step at 72°C, followed by a final elongation step at 72°C for 10 minutes. Products were then stored at 4°C.

Phylogenetic Analyses:

After samples were sequenced and the results sent back, alignments and contigs were created using the program CodonCode Aligner (v. 8.0.1; Codon Code Corporation, Dedham, MA, USA). In CodonCode Aligner, forward and reverse sequences were aligned to form a contig and generate a consensus sequence of a single sample, and then they were checked and edited by eye. Since COI is a protein-coding gene, the COI sequences were also translated to amino acids to ensure the absence of stop codons, which would indicate the presence of pseudogenes. After contigs were cleaned up, they were aligned with an outgroup using Clustal W 1.83 (Larkin et al., 2007). The outgroups were both sipunculids for COI (GenBank Accession number: DQ300165.2) and 18S (GenBank Accession number: X79874.1). Alignments were then checked by eye. The consensus sequences of each sample were also compared to known sequences in GenBank by running them in the NCBI nucleotide BLAST tool online (Altschul et al., 1997), using the Nucleotide collection database and optimized for blastn. Each COI sequence was checked to identify the lowest taxonomic level, according to divergence threshold values reported in the literature (Hebert, Ratnasingham, & deWaard, 2003) (Sun et al., 2016). The alignment was exported to MEGA7 (Kumar, Stecher, & Tamura, 2016) to view the alignment and edit it. Ends were trimmed and gaps or mutations/deletions were confirmed to be real by

checking the original chromatograms. A concatenated alignment was also created by manually copying the COI sequence alignments and appending them to the 18S sequence alignments.

After the alignments were trimmed and checked, phylogenetic trees were constructed and visualized using Bayesian and Maximum-Likelihood analyses on the website phylogeny.fr (Alexis Dereeper, Audic, Claverie, & Blanc, 2010; A. Dereeper et al., 2008). The best-fitting substitution model for each gene was determined using j-Model Test, using the Bosque software V. 2.0.2 (http://bosque.udec.cl/software/bosque.html) (Ramirez-Flandes & Ulloa, 2008). Bayesian analyses were run on a program called MrBayes 3.2.6 with the following settings: 6 substitution types (GTR), default substitution model, invariable+gamma rates variation across sites, 100,000 generations, a tree sampled every 10 generations, and the first 500 trees were discarded. Maximum-Likelihood analyses were run on the Advanced Mode program. Only the PhyML and TreeDyn options were selected and the workflow was run all at once. The bootstrapping procedure was selected with 500 bootstraps, along with the GTR substitution model.

The COI alignment was also used to create a pairwise distance table and group mean distances to estimate evolutionary divergence between the samples and to count the number of species found using MEGA7 (Kumar et al., 2016) with the settings: no variance estimation method, nucleotide substitution type, Kimura 2-parameter model, uniform rates among sites, pairwise deletion of gaps/missing data, and all codon positions were selected.

User Program for Reviewing Dive Track Data:

The R language was used to create a user program that cleaned up dive track data. First, the formatting of the dive track data had to be standardized and data was formatted with only the following labelled columns: Date, Time, Latitude, Longitude, Depth, Altitude and Heading. The

packages ggplot2 (Wickham, 2009) and shiny (Chang, Cheng, Allaire, Xie, & McPherson, 2018) were then imported. Commands and statements were written to be passed between the user interface and the server in R Shiny. Final code was uploaded and published to R Shiny online to a private account. Instructions on how to use the code can be found in the Appendix. The full code can be found on the website GitHub at this link: https://github.com/sjelsenbroek/Reviewing DiveTrackData/blob/b7114e0f552b58a6989bea023a2c088a944a96f2/HonorsProjectCode#L1-L153. The program can also be found on the online R shiny website at this link: https://cholab.shinyapps.io/DiveTrackData/.

Results

Thirty-four Mollusca samples were collected at Hannibal Bank. However, three of these samples were lost, one of them didn't collect any tissue, two never successfully amplified, and two were incorrectly identified and untrustworthy, leaving twenty-six total samples for study (Table 1). Nineteen of the twenty-six samples were successfully amplified using the COI primers, and all twenty-six of the samples were successfully amplified using the 18S primers. The COI genes were also confirmed to not be pseudogenes. The final Mollusca dataset included nineteen samples of concatenated COI and 18S sequences; covering 1,110 base pairs. There were eight species matches found on GenBank within the 11.1 % divergence threshold of the COI mitochondrial gene reported by Hebert et al. (2003). There were also samples in the dataset that were found to be the same species and the final dataset contained seventeen species (Table 2). HH1144 and HH1204 had a distance of 0.0%, and HH1203 and HH1348 had a distance of 0.2%, confirming that these samples were the same species. HH1282 was also found to be in the same

genus, *Calliostoma*, as HH1144 and HH1204. Samples HH1012 and HH1325 were found to be

in the same family too, *Colloniidae*.

Table 1: Table of metadata for samples used in this study. This table includes sample ID, latitude/longitude coordinates of collection sites, depth, class, and lowest taxonomic level able to be identified based on COI divergence threshold values.

Sample ID	Latitude (°)	Longitude (°)	Depth (m)	Class	Lowest Taxonomic Level Achieved
HH1005	7.3542	-82.0295	181	Gastropoda	family - *Fissurellidae*
HH1012	7.3542	-82.0295	181	Gastropoda	genus - *Collonia*
HH1027	7.3542	-82.0295	181	Gastropoda	species - *Pollia assimilis*
HH1044	7.3542	-82.0295	181	Gastropoda	genus - *Monogamus*
HH1067	7.4191	-82.0155	N/A	Bivalvia	family - *Pectinidae*
HH1068	7.4191	-82.0155	N/A	Bivalvia	N/A
HH1079	7.4180	-82.0162	261	Gastropoda	genus - *Krebsia*
HH1080	7.4180	-82.0162	261	Bivalvia	N/A
HH1144	7.4167	-82.1156	184	Gastropoda	species - *Calliostoma variegatum*
HH1164	7.3790	-82.0656	147	Gastropoda	species - *Fusinus salisburyi*
HH1168B	7.3653	-82.0523	403	Bivalvia	N/A
HH1168C	7.3653	-82.0523	403	Bivalvia	N/A
HH1202	7.3843	-82.0015	234	Bivalvia	N/A
HH1203	7.3834	-82.0042	272	Bivalvia	family - *Yoldiidae*
HH1204	N/A	N/A	N/A	Gastropoda	species - *Calliostoma variegatum*
HH1216	7.3754	-82.0737	325	Polyplacophora	genus - *Callistochiton*
HH1231	7.3719	-82.0757	414	Bivalvia	N/A

HH1232	N/A	N/A	N/A	Gastropoda	species - *Pterotrachea coronata*
HH1267	7.3868	-82.0607	96	Gastropoda	species - *Antillophos sp.*
HH1282	7.3744	-82.0602	81	Gastropoda	species - *Calliostoma ligatum*
HH1298B	7.3820	-82.0614	66	Gastropoda	genus - *Coralliophila*
HH1309	7.3820	-82.0614	66	Gastropoda	genus - *Mitromorpha*
HH1319	7.3885	-82.0595	86	Bivalvia	genus - *Pharaonella*
HH1325	N/A	N/A	N/A	Gastropoda	species - *Homalopoma luridum*
HH1348	7.3717	-82.0653	260	Bivalvia	family - *Yoldiidae*
HH1393B	N/A	N/A	N/A	Bivalvia	N/A

Bayesian and Maximum Likelihood analyses yielded similar tree topologies (Figure 2). Both analyses found the Polyplacophora to be most basal, with the Bivalvia and Gastropoda clades as sister to each other. In addition, Gastropoda came out with two different clades (Figure 2). One clade was identified to contain only samples of the Gastropoda clade Vetigastropoda, and the other was identified to contain only samples of the clade Caenogastropoda. The taxonomic order of the samples was determined by matching them on GenBank, within the 25.30% order divergence threshold of the COI mitochondrial gene (Sun et al., 2016). These results have high levels of support in both analyses. In the Maximum Likelihood analysis, Gastropoda and Bivalvia were sister to each other 90% of the time, and the two Gastropoda clades came out 100% of the time. Group mean distances were also found between the three classes and between the two gastropod orders. Gastropoda and Bivalvia had a distance of 54.9%, Bivalvia and Polyplacophora had a distance of 56.6%, and Gastropoda and Polyplacophora had a distance of 29.5%. Vetigastropoda and Caenogastropoda had a distance of 32.7%.
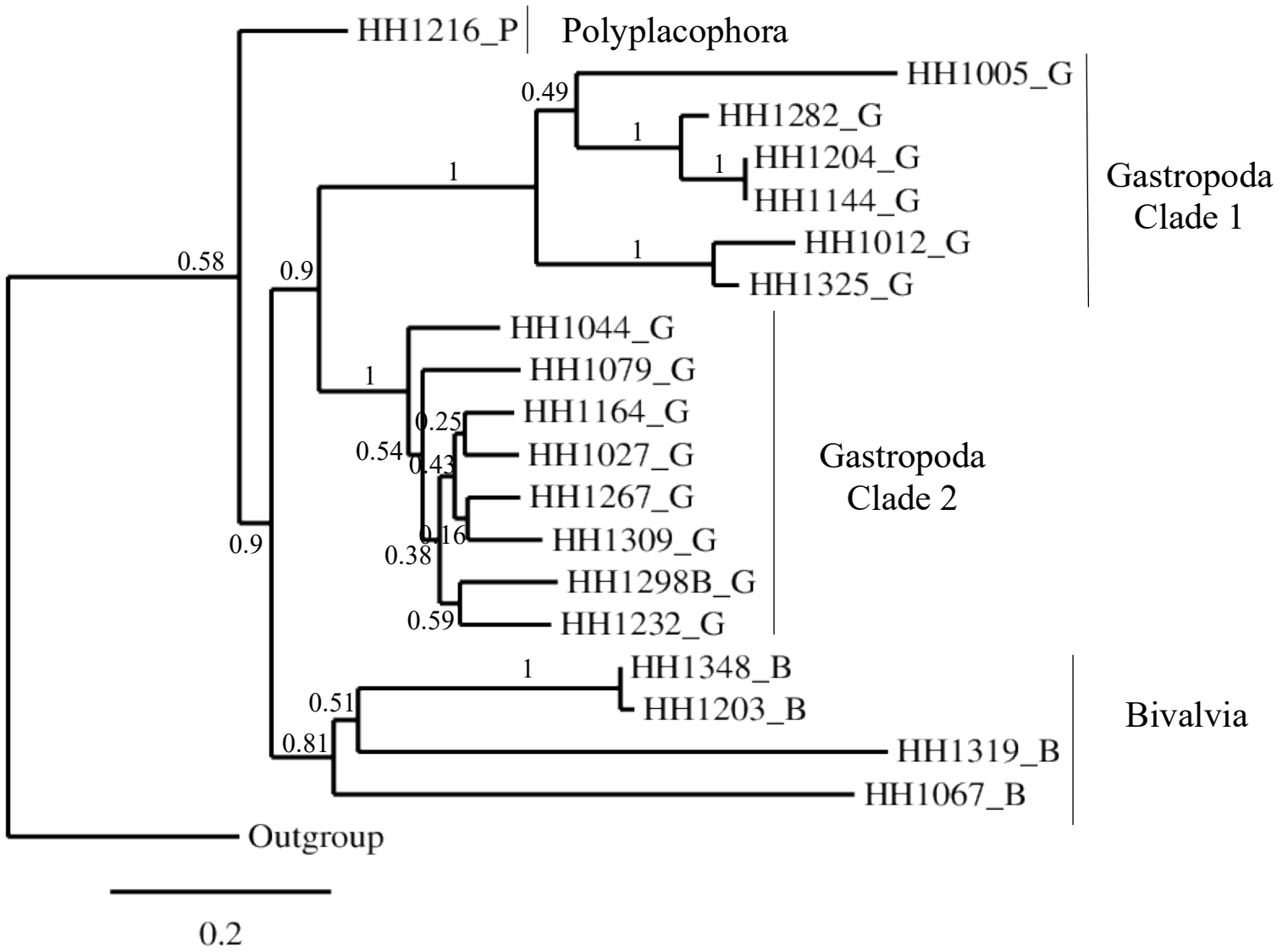
Figure 2: Maximum Likelihood phylogenetic tree of concatenated 18S+COI dataset. Taxa labeled by sample number and class (_P represents Polyplacophora, _G represents Gastropoda, and _B represents Bivalvia). The outgroup is a sipunculid. Gastropoda Clade 1 represents the Vetigastropoda clade. Gastropoda Clade 2 represents the Caenogastropoda clade. Bayesian analysis resulted in the same tree topology.

Table 2: Estimates of Evolutionary Divergence between Sequences for COI Dataset. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 450 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Highlighted cells show samples that are below the threshold to be considered the same species (Hebert et al., 2003).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 HH1005_G																				
2 HH1298B_G	0.355																			
3 HH1079_G	0.326	0.283																		
4 HH1203_B	0.524	0.513	0.467																	
5 HH1216_P	0.354	0.293	0.310	0.511																
6 HH1282_G	0.407	0.321	0.345	0.548	0.360															
7 HH1012_G	0.388	0.324	0.331	0.487	0.333	0.339														
8 HH1164_G	0.359	0.230	0.252	0.438	0.224	0.322	0.263													
9 HH1144_G	0.420	0.358	0.344	0.525	0.313	0.176	0.406	0.318												
10 HH1325_G	0.294	0.298	0.287	0.492	0.314	0.349	0.204	0.258	0.372											
11 Outgroup	0.505	0.436	0.459	0.605	0.370	0.380	0.492	0.369	0.375	0.499										
12 HH1309_G	0.381	0.251	0.268	0.479	0.230	0.359	0.274	0.189	0.338	0.278	0.393									
13 HH1027_G	0.409	0.249	0.249	0.402	0.259	0.333	0.328	0.150	0.311	0.307	0.377	0.195								
14 HH1044_G	0.347	0.292	0.298	0.474	0.290	0.357	0.300	0.251	0.353	0.310	0.415	0.273	0.265							
15 HH1204_G	0.420	0.358	0.344	0.525	0.313	0.176	0.406	0.318	0.000	0.372	0.375	0.338	0.311	0.353						
16 HH1067_B	0.560	0.683	0.635	0.639	0.666	0.699	0.692	0.711	0.676	0.598	0.739	0.658	0.719	0.704	0.676					
17 HH1319_B	0.586	0.544	0.539	0.628	0.572	0.614	0.521	0.521	0.624	0.521	0.634	0.495	0.478	0.521	0.624	0.724				
18 HH1267_G	0.331	0.242	0.259	0.430	0.251	0.336	0.285	0.148	0.321	0.294	0.395	0.203	0.170	0.261	0.321	0.672	0.517			
19 HH1348_B	0.520	0.508	0.472	0.002	0.516	0.543	0.482	0.442	0.530	0.497	0.611	0.483	0.406	0.479	0.530	0.639	0.623	0.434		
20 HH1232_G	0.417	0.277	0.287	0.520	0.285	0.300	0.343	0.254	0.300	0.315	0.410	0.282	0.258	0.320	0.300	0.720	0.558	0.241	0.524	

The user program to remove outliers was successful. It achieved its function to visualize the data and to remove outliers. A final copy of the data was able to be downloaded in a csv format without the points selected by the user. Functionality and use are further described in the Appendix.
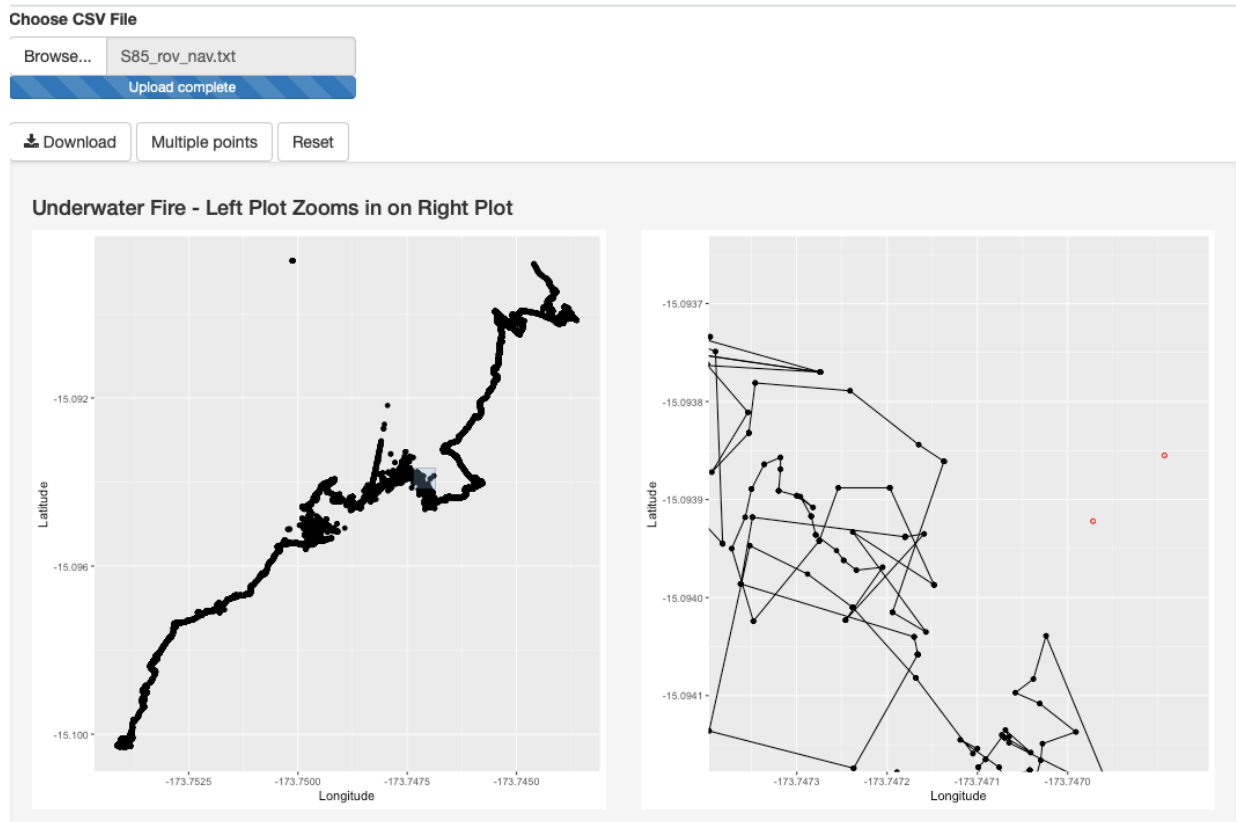

Figure 3: Screenshot of user program interface. The map on the left is used to view the whole dive and to zoom-in on a section of the dive. The map on the right is the zoomed-in region of the dive and is used to remove points and see metadata of the points.

Discussion

The purpose of this study was to review the diversity and the evolutionary history of mollusks found at Hannibal Bank, Panama. We described seventeen distinct species found across Hannibal Bank. We were also able to show that the class Polyplacophora is basal to the sister

clades of Bivalvia and Gastropoda. This study mostly matched two of the eight hypotheses in the Sigwart and Lindberg (2015) study. The Sigwart and Lindberg (2015) study analyzed forty-two published trees on molluscan relationships and created eight super tree hypotheses. These hypotheses were produced using different algorithms and approaches. Our results also seem to support the Testaria hypothesis that has Aplacophora basal to Polyplacophora and all remaining Mollusca (Sigwart & Lindberg, 2015).

Within the Gastropoda class, two main clades were found. In general, there are five proposed clades of Gastropoda: Patellogastropoda, Vetigastropoda, Neritimorpha, Caenogastropoda, and Heterobranchia (Zapata et al., 2014). The results of this study matched the consensus tree of the Zapata et al. (2014) study with Vetigastropoda and Caenogastropoda sister to each other. The Zapata et al. (2014) study tested six hypotheses of Gastropoda internal relationships by analyzing transcriptome data from thirty-four gastropods and six other mollusks. This study also matched three of the six hypothesis trees which were run using a variety of different analyses (Zapata et al., 2014).

This study provides support for certain hypothesized molluscan relationships. High support found in our phylogenetic tree and clear relationships that are described in other studies indicate that we can be fairly certain in our results. However, our study only analyzed a molecular dataset. When other types of data, like paleontological, developmental, and morphological data, are included, other molluscan relationships are better supported (Wanninger & Wollesen, 2018). Our study supports other findings that have used only molecular data, but these results are most likely not true representations of molluscan evolutionary relationships since they fail to account for other types of data. Future studies would preferably include other

types of data, representatives of each class of Mollusca, as well as the orders found in each class. More samples would add to the confidence of these relationships.

The user program created in R is also very useful in making the process of cleaning up dive track data more efficient. The user can visualize the data points from the dive and zoom in and click on points thought to be outliers or errors. Then the user can give the new csv file a name and save it without the points that were clicked on. This program can be applied immediately to help researchers with past, current, and future dives that they need to clean up. There is also plenty of room for improvement or upgrade to the program including packages that allow being able to zoom farther in and out, or the final data could be automatically mapped on a bathymetric map.

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Appendix

   The user program works by first clicking Browse… to select the csv or txt file that you wish to view and analyze (Figure 3). The map will then appear. The user can manipulate the data by highlighting a section of the map on the left that will zoom in on that region and display that new map on the right. By highlighting points on the right map, metadata (longitude, latitude, depth…) of the points are found below the maps. If the user thinks a point is erroneous, he/she can click on the point on the right map to remove it. The point will turn red and will no longer be connected to the rest of the points. If a user highlights multiple points, they can all be deleted at once by clicking the Multiple points button. If a user mistakenly clicks to remove a point, he/she can simply click the point again to save it or they can click the Reset button to bring the file back to how it was originally. Once the user is satisfied with the map and the dive track data, he/she can enter in what name they would like to save the file as and then click on Download to save the new dive track data without the erroneous points. The file is saved to the user's download folder under the entered name.

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