A Taxonomic Study of *Lingula reevii* and Survey of Abundance in Relation to Varying Environmental Parameters in South Kāneʻohe Bay

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Abstract

Lingula reevii is an inarticulated brachiopod that is listed as a species of concern within Kāneʻohe Bay, Hawaiʻi. The abundance of *L. reevii* throughout Kāneʻohe Bay has been decreasing since it was first studied in 1969, causing NOAA to add it to the Species of Concern list in 2004. In this study, *L. reevii* was genetically sequenced and compared to similar species in a phylogenetic tree. The data clearly showed that *L. reevii* was genetically distinct from the other species included in the tree. Field observations were conducted to determine if the abundance of *L. reevii* was decreasing and if a decrease could be caused by environmental factors such as salinity, pH, temperature, and sediment grain size. Transect surveys were performed at 11 sites throughout the bay. A general linear model was used to see if these factors were statistically significant. The effects of salinity, pH and the two factors combined were all determined to be significant. This study was unable to compare the abundance of *L. reevii* found in past years' studies because the study methods were not analogous. Future management efforts should include standardizing survey methods and using genetic analysis to compare populations from Hawaiʻi to those in Japan and Indonesia. Also, to assess variability within the Hawaiian population as well as increasing the sample size to improve the length equation.

Introduction

Lingula is one of the oldest living genera, having existed since the Cretaceous period, and is considered to be a living fossil due to its exceptionally slow rate of evolution (Emig 2008). The species *Lingula reevii* is an inarticulate brachiopod that burrows in firm substrates such as sand and mixed sediment in intertidal areas (Hunter *et al.* 2008). This species feeds by filtering detritus, bacteria and microscopic organisms from the water and can be readily located in the sand by the characteristic shape of its siphons, which form three small holes positioned in a straight line. *Lingula reevii* is gonochoristic, meaning each individual is either male or female, and reproduces by releasing gametes into the surrounding water. *Lingula reevii* produces lecithotrophic larvae, meaning that after release, the eggs and sperm unite to form free-swimming, non-feeding larvae that remain in the plankton from two to four weeks (Paine 1963).

The National Oceanic and Atmospheric Administration (NOAA) deemed *Lingula reevii* a Species of Concern (SOC) because it has shown signs of decreasing abundance; however, it cannot be classified under the Endangered Species Act (ESA) due to insufficient information (NOAA National Marine Fisheries Service 2007). *Lingula reevii* has been found throughout

Kāneʻohe Bay in Hawaiʻi, but studies have shown a noticeable drop in abundance (Worcester 1969). This species can also be found in southern Japan and Ambon, Indonesia (Emig 1997).

In this study, *Lingula reevii* DNA will be analyzed and compared to data from studies conducted in Japan in an attempt to further establish *L. reevii* as a Species of Concern. DNA from the mitochondrial coxI gene will be analyzed and compared to known samples in GenBank (Endo *et al.* 2001). Finally, microscopic images and morphological measurements will add to the taxonomic and morphologic descriptions of the species, which may be used to supplement future research, such as studies investigating potential size variations over time. Overall, this data is expected to show that *L. reevii* has enough genetic and morphological variation from its Japanese counterparts to determine that it is, in fact, endemic in Hawaiʻi and should be listed as endangered under the ESA. This distinction could make it easier to protect *L. reevii* from a management standpoint.

The abundance of *L. reevii* as well as specific environmental parameters will be measured where *L. reevii* has been found previously by Dr. Hunter and her students. In addition, *L. reevii* studies will be conducted in new sites to establish a baseline. The study will contain sites that have *L. reevii* and sites that do not, in order to determine if significant environmental differences occur. The environmental parameters measured in this study include temperature, salinity, pH variations, and sediment grain size at each site. This study will provide insight into what environmental factors may be affecting the abundance and density of *L. reevii* throughout Kāneʻohe Bay. The density of *L. reevii* in Kāneʻohe Bay will be compared to previous years' data, while the abundance of *L. reevii* will be compared between different sites in this study.

It is hypothesized that the density of *L. reevii* is decreasing and that this decrease is due to various environmental parameters such as temperature, sediment grain size, salinity and pH. The

second hypothesis is that genetic data from *L. reevii* in Kāneʻohe Bay will be significantly different from genetic data of other *Lingula* species.

Materials and Methods

Geographic Coordinates

Coordinates using latitude and longitude were randomly selected using Google Earth™ for each reef location surveyed. A 25 m line was drawn using the ruler application to delineate distance between transects. Six GPS coordinates were selected for every site with the exception of Reef 20, due to small area constraints. GPS points were entered into a Garmin Venture HC GPS. Data projections were based on WGS 84 datum and were displayed in ArcGIS. The reefs surveyed were Sandbar (SBM1 & SB1), Reef 15, Reef 16, Reef 20, Reef 28, Fringing Reef (FRJ, FRB2, and FRB3), Goby Bay and Lilipuna Pier (Table 1 and Fig. 1).

Table 1. GPS locations of the eleven sites at Kāneʻohe Bay where *Lingula reevii* were surveyed.

Site name	GPS location
SBM1	21.46160, -157.80839
SB1	21.46930, -157.81850
Reef 15	21.45355, -157.80324
Reef 16	21.45447, -157.80470
Reef 20	21.46063, -157.80790
Reef 28	21.46900, -157.82000
FRJ	21.42940, -157.79961
FRB2	21.42660, -157.76982
FRB3	21.42490, -157.76913
Goby Bay	21.43355, -157.79109
Lilipuna Pier	21.42935, -157.79258

Figure 1. Map of Kāneʻohe Bay from Google Earth™ consisting of eleven sites where *Lingula reevii* were surveyed.

Abundance

Abundance of *L. reevii* was measured using 25 m transects that were placed at a randomly assigned start point within each location and laid out parallel to shore. From the end of the transects, groups measured 6 m towards shore and deployed another 25 m transect to create a 25 m by 6 m (150 m^2) rectangular survey area. Surveyors searched for *L. reevii* for 20 minutes and flagged the location of each individual. The total number of *L. reevii* per m^2 was determined for comparison with past studies. On larger reefs, six replicates were conducted, while size restrictions on smaller reefs allowed for fewer replicates.

Temperature

The temperature of each reef was recorded using an Onset HOBO Pendant™ Data Logger UA-001-08 attached to a lead weight and float for stable positioning. One temperature logger was deployed at each location to collect the temperature of the surrounding environment every ten minutes. Loggers recorded temperatures between 20 and 70 °C with an accuracy of $\pm$ 0.53 °C from 0 to 50 °C. Loggers were calibrated in 0°C and 33-35°C water baths.

pH

The National Institute of Science and Technology (NIST) traceable standards buffer calibration kit was used to calibrate the PCTestr 35 Multi-Parameter pH meter. For accurate pH readings, buffer solutions of pH 4.00, 7.00, and 10.00 were used for calibration, with a deionized water rinse between buffer solutions to ensure the readings were not contaminated. Water samples collected in Falcon tubes were tested using the pH meter in the field and in the lab to determine differences between samples.

Salinity

A standard refractometer was used to determine salinity differences between sites. The salinity was measured both in the field and in the lab to determine if there were any differences between the samples. Between each sample test the refractometer was rinsed with deionized water to ensure there was no contamination between sites.

Grain Size

Sediment samples were taken from every two transects at each location. Samples were then combined and dried overnight in a drying oven. Subsamples were then taken and dry sieved to determine six size fractions: 5000μm, 2000μm, 500μm, 250μm, 125μm, 63μm (USA Standard Testing Sieve: A.S.T.M.E.-11 specifications). Seven size fractions were determined: gravel

(>5000µm), granule (2000-5000µm), coarse and very coarse sand (500-2000µm), medium sand (250-500µm), fine sand (125-250µm), very fine sand (63-125µm), and silt/clay (<63µm) in accordance with the Wentworth scale (Folk 1974). Each size fraction was collected in pre-weighed filters and weighed to determine the proportion of each size fraction. Extremely large pieces were removed prior to sorting to reduce variability and eliminate overweighting of some samples by a single piece of material.

Data Analysis

General linear models were conducted in MINITab17 Student software to determine the significance of the relationships between *L. reevii* abundance and temperature, sediment grain size, pH, and salinity. Next, a *t*-test was conducted to compare the abundance of *L. reevii* at one site and reported abundance of *L. reevii* at the same site during a 2013 study.

Molecular Analysis

DNA was extracted from a single sample of *L. reevii* found dead in Goby Bay in July 2014. An E.Z.N.A. Mollusc DNA Extraction Kit (D3373-01) was used, and provided instructions were followed with the exception of three 50µL elutions performed instead of two. A small sub-sample of tissue was taken from each specimen, crushed with a mortar and pestle, mixed with 25µL of Proteinase K and 350µL of ML1 Buffer, and mixed at 60°C until solubilized. Next, 350µL of 24:1 chloroform:iso-amyl alcohol was added to each tube, and they were centrifuged for 2 minutes at 10,000g. The upper aqueous layer was then transferred to a fresh tube, and one volume of MBL buffer along with 10µL RNase was added. The tubes were vortexed and incubated at 70 °C for 10 minutes. After cooling to room temperature, one volume of 100% ethanol was added, and the tubes were vortexed.

Hi-Bind DNA mini columns were inserted into 2 mL collection tubes, 100μL of 3M NaOH was added, the tubes were centrifuged on maximum speed for 60 seconds, and the filtrate was discarded. Next, 750μL of sample was added, the tubes were centrifuged at 10,000g for 1 minute, the filtrate was discarded and the filter columns were placed into fresh collection tubes. Then, 10μL of HBC buffer diluted with isopropanol was added, the tubes were centrifuged at 10,000g for 30 seconds and the filtrate was discarded. Subsequently, 700μL of DNA wash buffer diluted with ethanol was added, the tubes were centrifuged at 10,000g for 1 minute, the filtrate was discarded and the DNA wash step was repeated. Empty tubes were then centrifuged for 2 minutes at maximum speed to dry the membrane, and the column was transferred to a new 1.5mL microcentrifuge tube. Then, 50μL of elution buffer (previously heated to 70 °C) was added to the tubes, the tubes sat at room temperature for 2 minutes, they were centrifuged at 10,000g for 1 minute, and the filtrate was stored as the DNA sample. The elution step was repeated two more times, and the DNA was stored at -20 °C. Finally, the DNA was run on a 1% agarose gel for 20 minutes at 90 volts.

The samples that had clear bands on the gel were then diluted and used as templates for polymerase chain reactions (PCRs). Each sample had dilutions made to 2:1 (2μL pure sample), 1:1 (1μL pure sample), 1:2 (3μL sample and 3μL Low TE buffer), 1:10 (1μL sample and 9μL Low TE buffer) and 1:50 (1μL sample and 49μL Low TE buffer). Then, 9μL of a master mix composed of 5μL Biomix Red, 0.13μL John Gellar's forward primer, 0.13μL John Gellar's reverse primer and 3.74μL water was added to each tube. Next, the tubes were placed into a thermocycler with the following progression of events: 96 °C for 32 minutes; 96 °C for 45 seconds, 50 °C for 45 seconds, 72 °C for 45 seconds (35 times); 72 °C for 10 minutes; 15 °C

until removed. The PCR products were then run through a 1% agarose gel for 20 minutes at 90 volts.

The samples that had clear bands on the gel were then enzymatically cleaned and sent for sequencing. First, 1.125µL of ExoFAP (exonuclease and shrimp alkaline phosphotase) was added to each tube. Then, 7.5µL of sample was added, and the tubes were vortexed and then placed in the thermocycler for the following progression of events: 37°C for 60 minutes; 85°C for 15 minutes. The tubes were vortexed again, and then 4µL from each tube was placed into a new tube. Next, 1µL of the appropriate John Gellar's primer (one forward and one reverse for each sample in separate tubes) was added, and the tubes were stored in the refrigerator before being sent to the University of Mānoa at Hawai'i Core Sequencing Lab.

Finally, the sequenced data was examined. Each sequence was edited to remove and clarify unknown base pairs using Geneious vR6.1.8. Each edited sequence was then analyzed with blast on GenBank. The DNA sequences were compared to *Lingula* samples previously in the system. After the sequences were aligned and edited, a phylogenetic tree was created with MEGA6 using the maximum likelihood method and with the Tamura-Nei method (Tamura and Nei, 1993).

To visually analyze the specimens, they were photographed using a Macrofire by Optronics camera with an Olympus SZX7 dissection microscope. The photos were captured by using the program, Picture Frame. Each specimen was measured using a caliper; length was measured across the longest part of the shell, width was measured across the widest part of the shell, and height was measured across the deepest part of the shell (Figure 2). The length measurements were compared to an expected length based on the equation: $L=W*2.29+0.15mm$, which was postulated by Worcester (1969). A new equation for length: $L=W*2.23$ was devised

based on specimen measurements. Finally, one specimen was dissected and photographed under the dissection microscope and was subsequently labeled.

Results

Statistical Analysis

A histogram of abundance was created to visualize the distribution of abundances, which was left skewed due to large number of transects where no *L. reevii* were found (Fig. 2). A general linear model was used to compare abundance, salinity, temperature and pH by location. The relationship between pH and abundance were statistically significant (p = 0.007) (Fig. 3). The relationship between salinity and abundance was statistically significant (p = 0.006) (Fig. 4). The relationship between salinity, pH and abundance was statistically significant (p = 0.006). Reefs 16, 20, and 28 were not included in the statistical analysis due to incompatible substrate that does not support *L. reevii* populations.
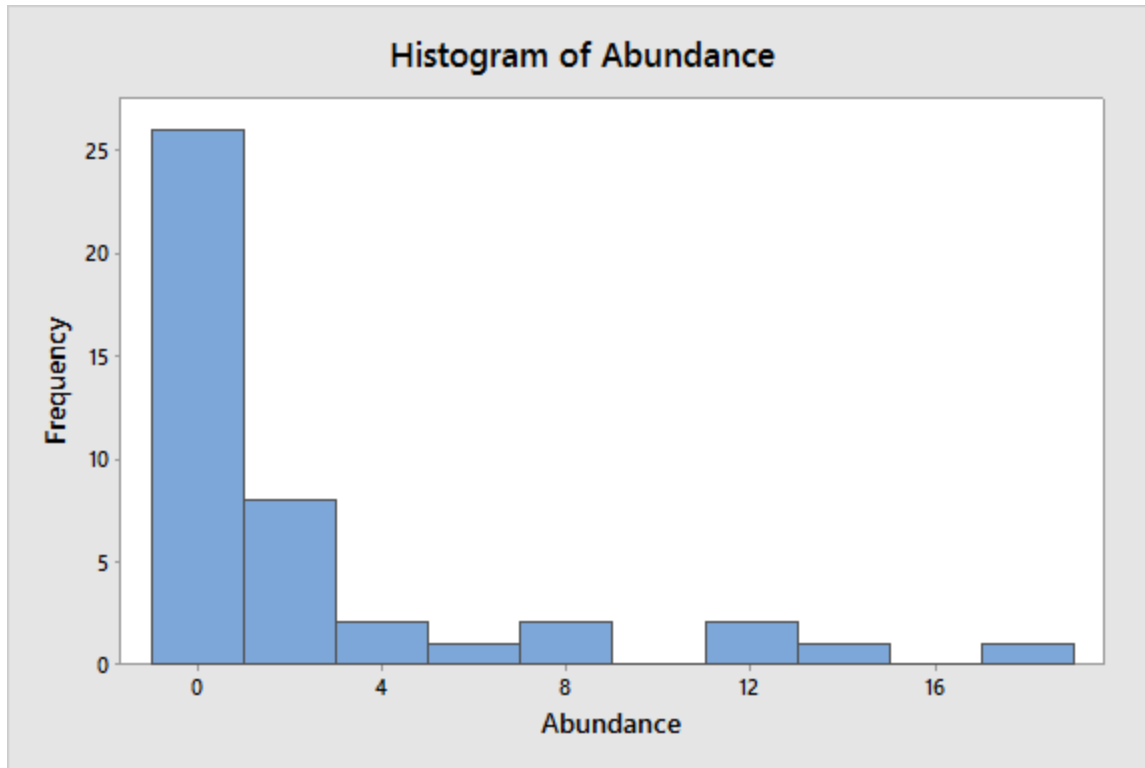
Figure 2. Histogram of abundance of *L. reevii*.

Table 2. Calculated densities of *L. reevii* at each surveyed site including average density of all sites in 2013 and 2014. Total abundance of *Lingula reevii* found at each site was recorded.

Location	Density (2013)	Density (2014)	Total abundance of *L. reevii* found (2014)
SBM1	N/A	0.0678	43
FRB2	N/A	0.0133	12
FRB3	0.08	0.0044	4
R15	0.06	0.0000	0
SB1	N/A	0.0056	13
FRJ	0	0.0044	4
GBA	N/A	0.0111	10
LPA	N/A	0.0000	0
Average	0.1	0.0149	

Table 3. Average densities of *L. reevii* in Kāneʻohe Bay from 1969 to 2014.

Year	Density
1969	500
1981	100
2004	4
2007	0.94
2008	0.87
2009	0.09
2010	2.93
2011	0.71
2013	0.15
2014	0.015
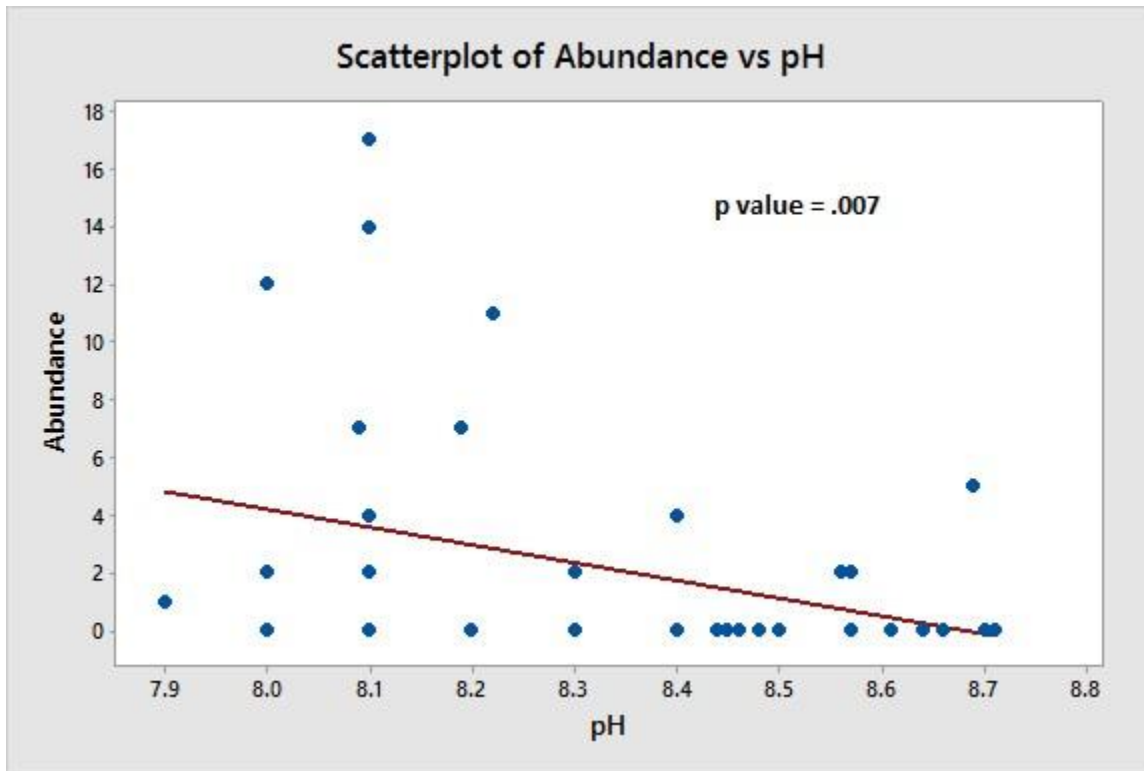
Figure 3. A scatter plot of the pH data in relationship to *L. reevii* abundance, not including data from Reefs 16, 20, and 28.
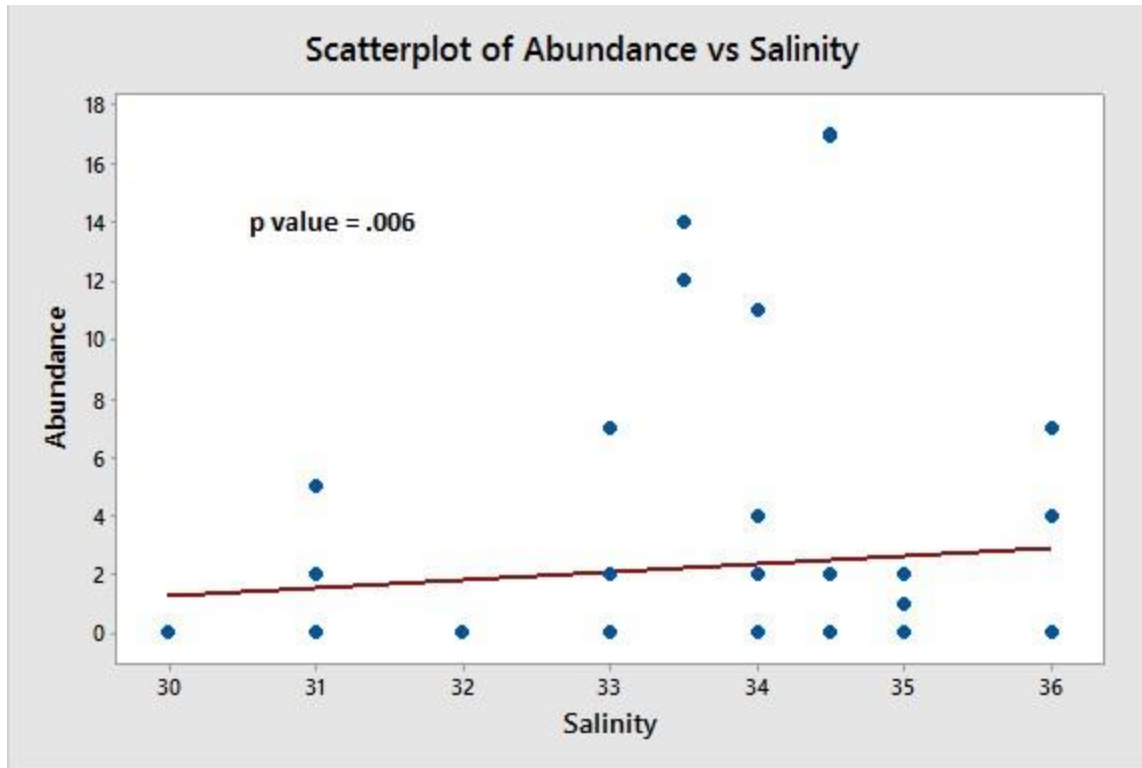
Figure 4. A scatter plot of the salinity data (ppt) in relationship to *L. reevii* abundance, not including data from Reefs 16, 20, and 28.

A *t*-test was performed in MiniTab17 to compare average densities of *L. reevii* at FRB3 to results from 2013 (Table 2 & Table 3). In 2014 the mean density of *L. reevii* found at FRB3 was 0.0044. In 2013 the mean density of *L. reevii* found at FRB3 was 0.08. A two-sample t-test determined that the difference in density between the two years was not statistically significant due to high variability ($p = 0.365$).

Lingula reevii **distribution**

Six sites throughout Kāneʻohe Bay that had conditions in which *Lingula reevii* were thought to reside (Fig. 6). The largest concentrations of *L. reevii* were found at Sandbar M1 (n=43), followed by Sandbar 1 (n=13), followed by Fringing Reef B2 (n=12), followed by Goby Bay (n=10), with Fringing Reef J and Fringing Reef B3 having the lowest abundances (n=4) (Fig. 7- Fig.12).

Figure 6. Locations where *Lingula reevii* were surveyed. *L. reevii* survey sites selected based on past observations.
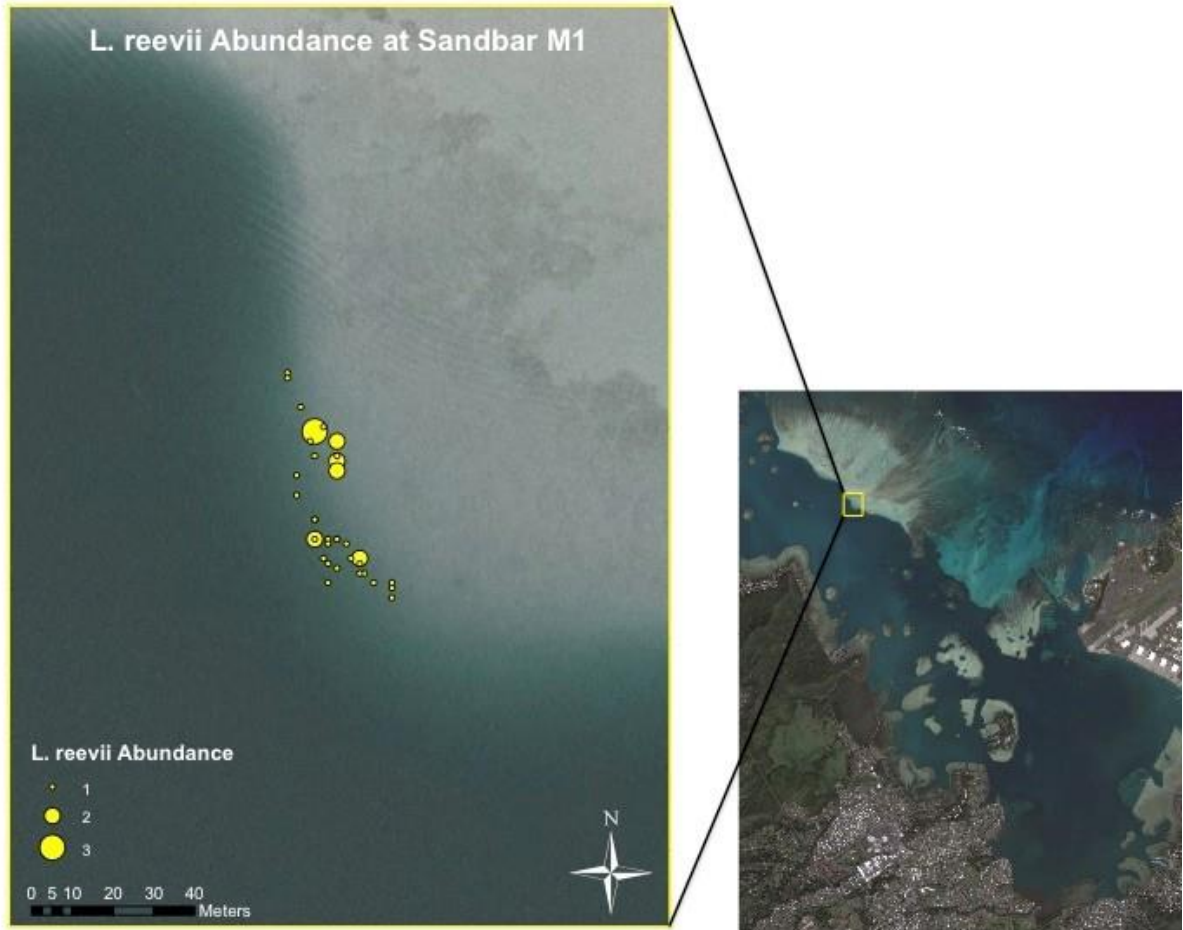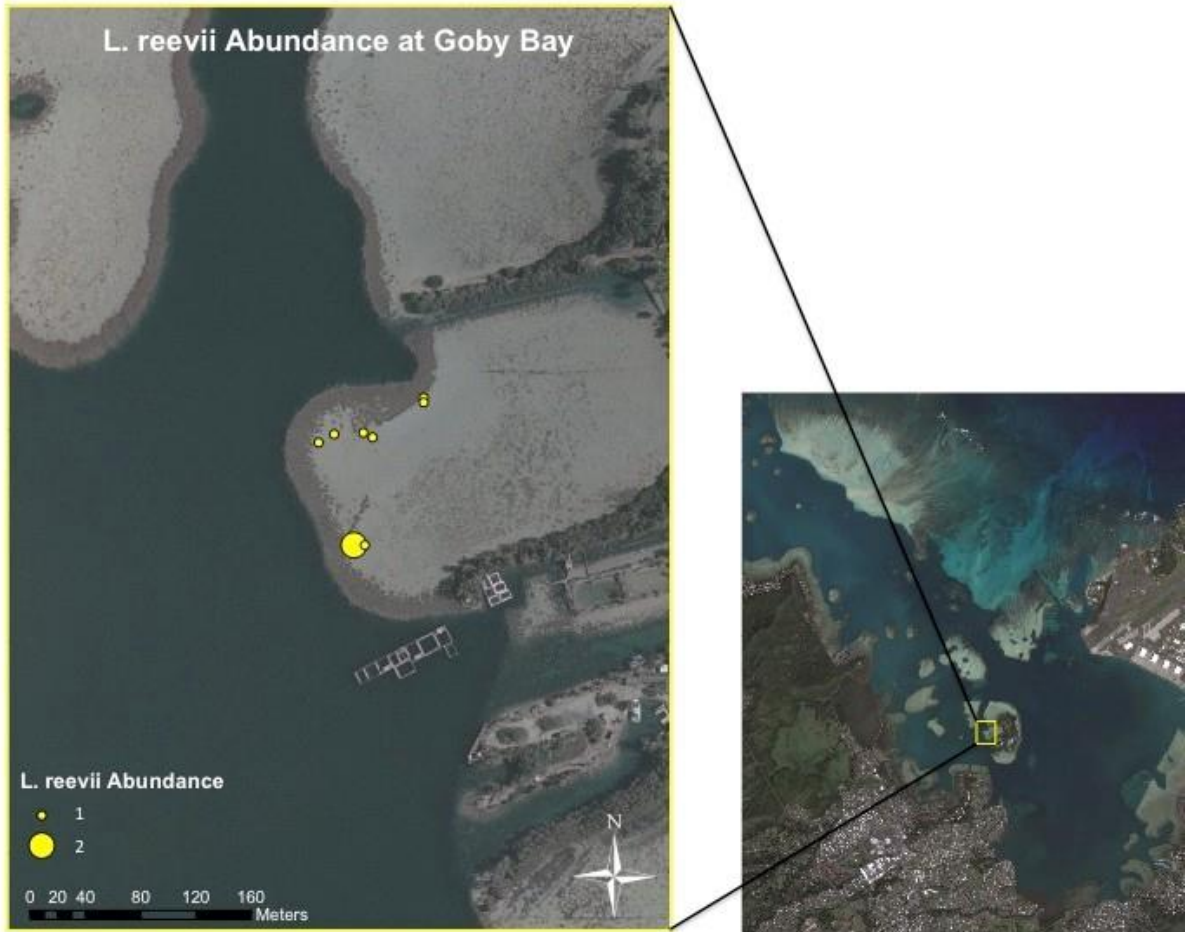
Figure 7. The abundance of *Lingula reevii* observed at Sandbar M1. Graduated bubbles increase with increased abundance.

Figure 8. The abundance of *Lingula reevii* observed at Sandbar 1. Graduated bubbles
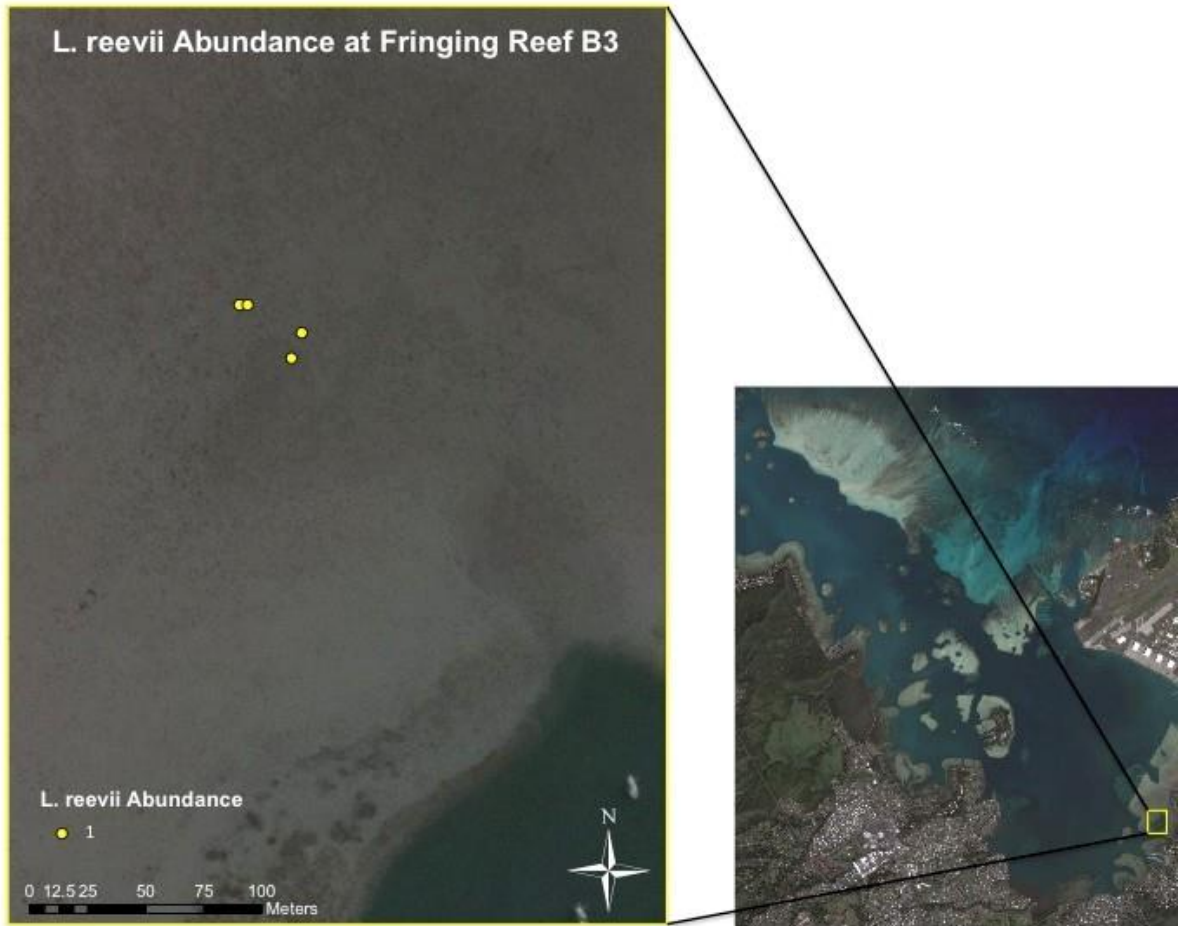increase with increased abundance.

Figure 9. The abundance of *Lingula reevii* observed at Goby Bay. Graduated bubbles increase with increased abundance.

Figure 10. The abundance of *Lingula reevii* observed at Fringing Reef J. Each yellow dot represents a *L. reevii*.

Figure 11. The abundance of *Lingula reevii* observed at Fringing Reef B3. Each yellow dot represents a *L. reevii*.

Figure 12. The abundance of *Lingula reevii* observed at Fringing Reef B2. Graduated
bubbles increase with increased abundance.

Sediments

The proportions of sediment grain sizes were calculated to create pie charts to depict composition at each site. Larger grain sizes are shown with red, orange, and yellow, while smaller grain sizes are shown as green, blue, and purple. The white color represents the smallest grain size of less than 63 microns (Fig. 13).
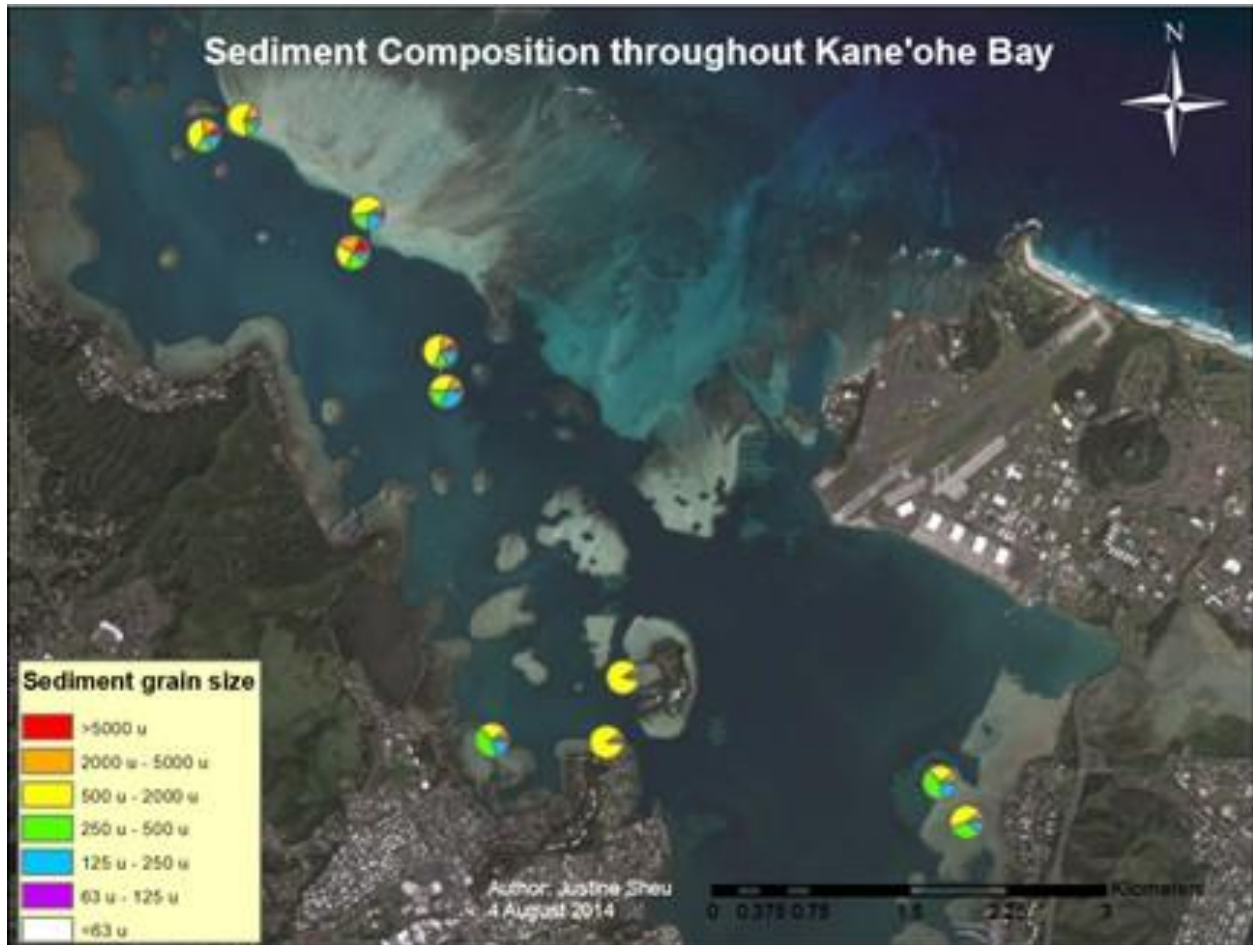
Figure 13. Sediment composition at 11 sites throughout Kāneʻohe Bay. Seven size fractions were determined: gravel (>5000 µm), granule (2000-5000 µm), coarse and very coarse sand (500-2000 µm), medium sand (250-500 µm), fine sand (125-250 µm), very fine sand (63-125 µm), and silt/clay (<63 µm) in accordance with the Wentworth scale (Folk 1974).

Sediments containing high levels of silt/clay and medium levels of gravel are indicative of areas that are sheltered with lower wave energy and are pulled towards the upper right of the figure. In contrast, the three patch reefs, R16, R28, R20, have high levels of gravel and a low levels of silt/clay and are pulled towards the bottom right of the figure (Figure 14).
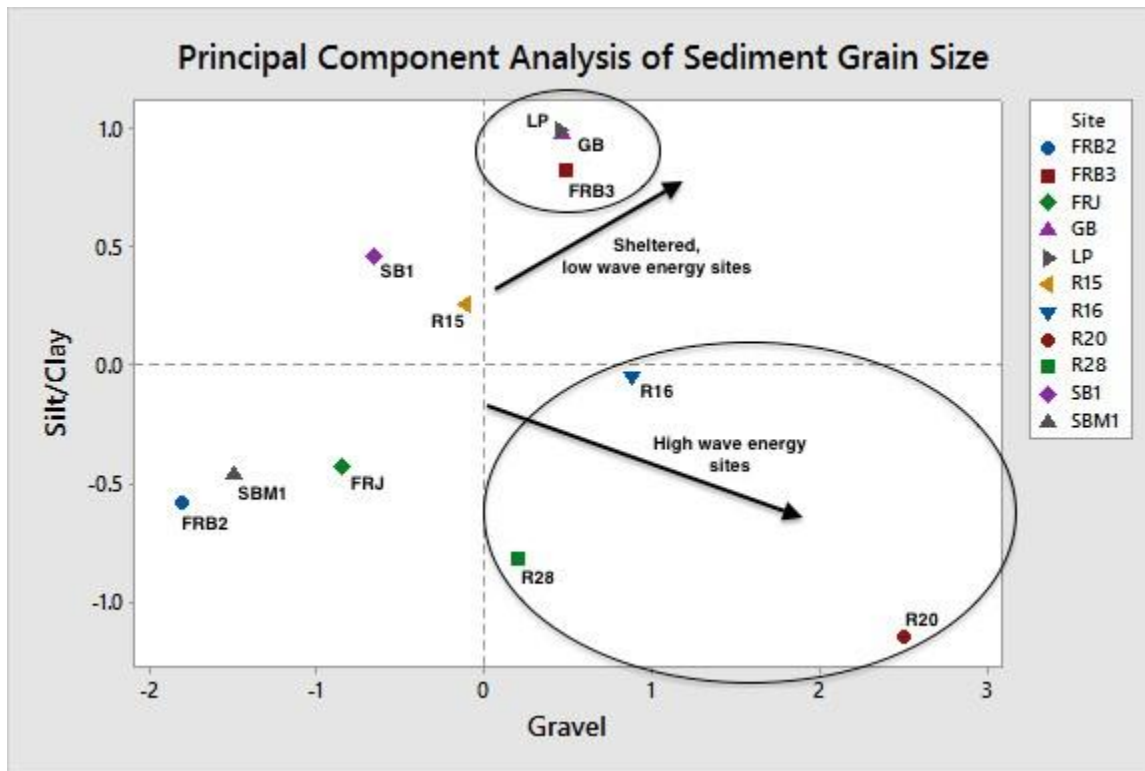

Figure 14. Principal Component Analysis (PCA) of sediment grain size. Two extreme sediment sizes are shown for 11 sites throughout Kāneʻohe Bay.

Molecular Analysis

A phylogenetic tree was constructed to determine the base pair divergence between related species. *Lingula reevii* clearly resides on its own branch within the tree, showing it is genetically distinct from the other species in the tree (Fig. 15). The branch lengths correspond to the legend, which indicates five percent divergence between species (Fig. 15). The length, width and height of the specimens were also measured to further study their morphology in an attempt to distinguish them from *L. reevii* populations in other parts of the world (Fig. 16). These

measurements were recorded and analyzed using two different equations (Table 4). Finally, one

of the specimens was dissected and photographed under a microscope to further analyze internal

organs (Fig. 17).
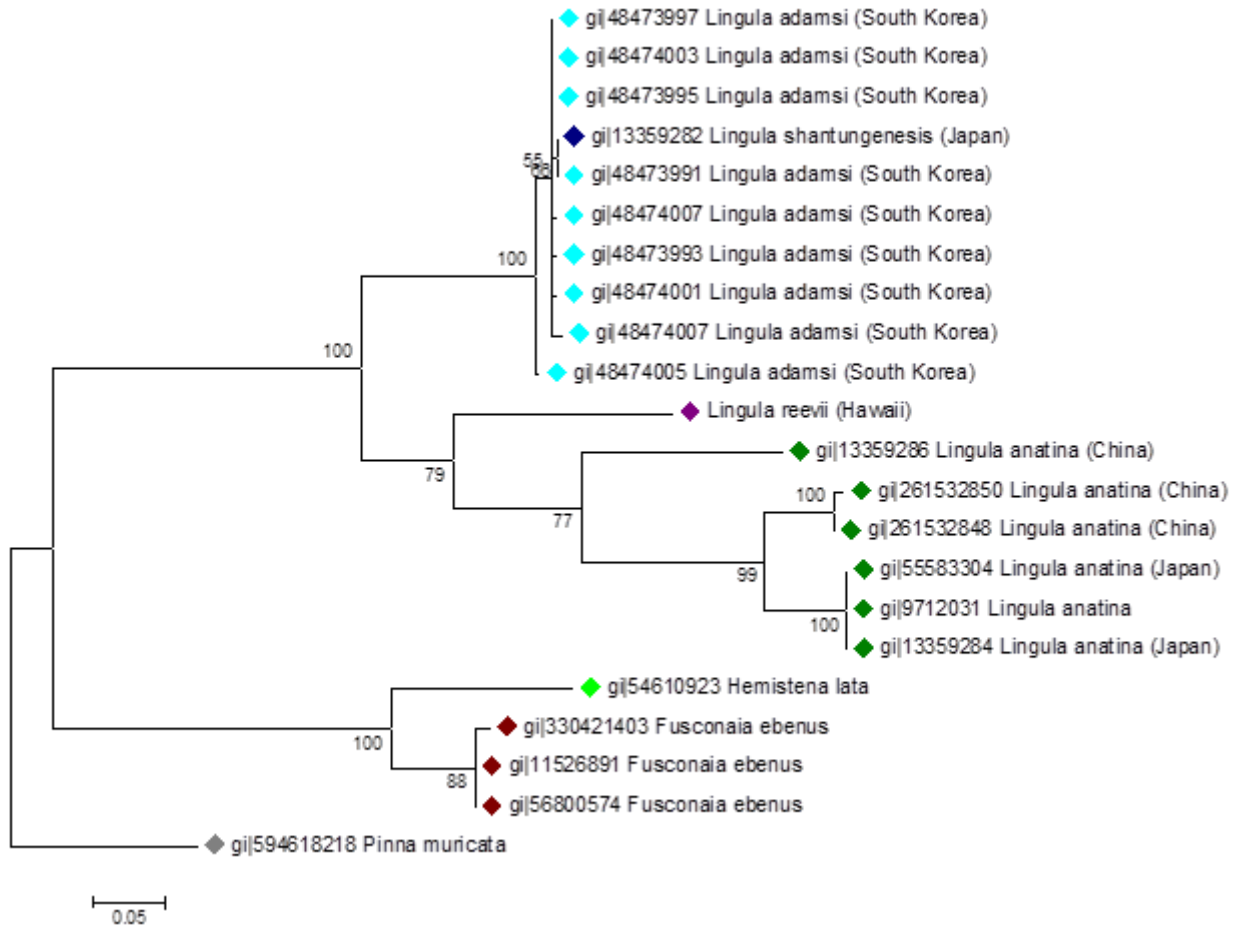
Figure 15. Molecular Phylogenetic analysis by Maximum Likelihood method
Phylogenetic relationships (location given in parentheses). Numeric values represent base pair divergence.
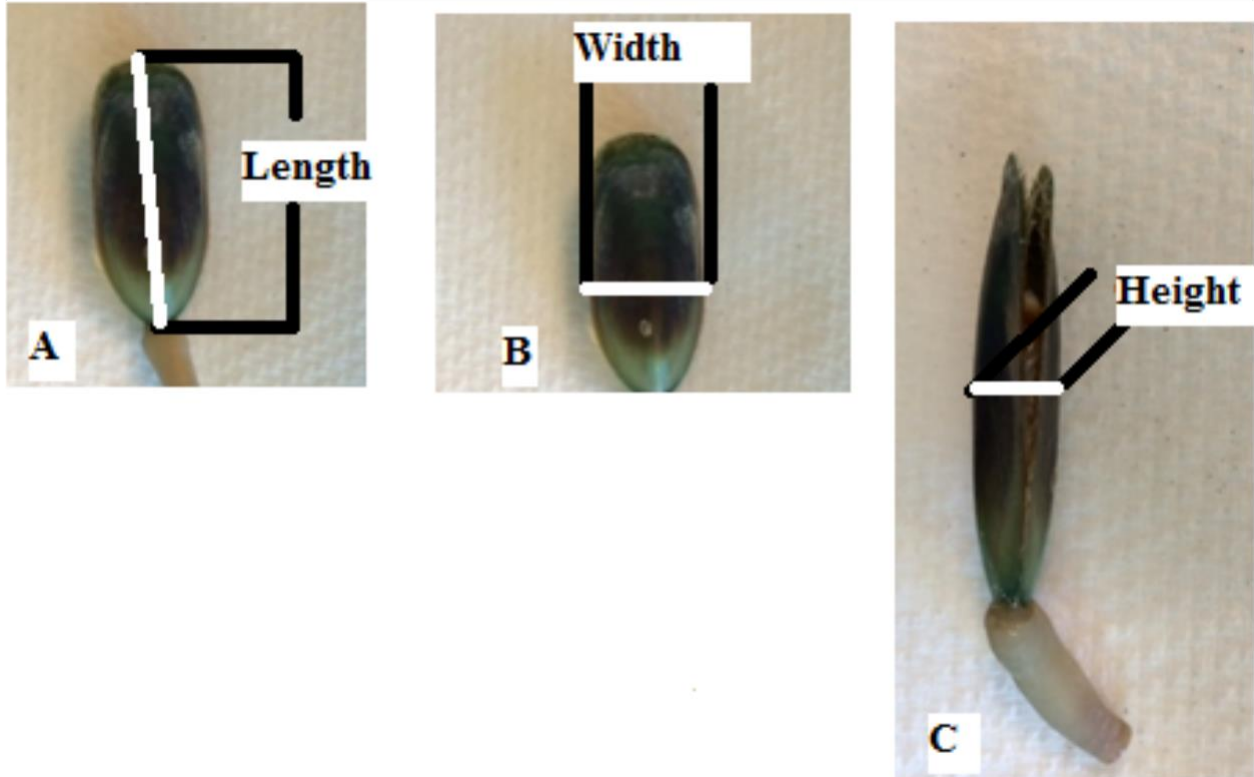
Figure 16. *Lingula reevii* **A**. Method for shell length measurement; **B**. Method for shell width measurement; **C**. Method for shell height measurement.
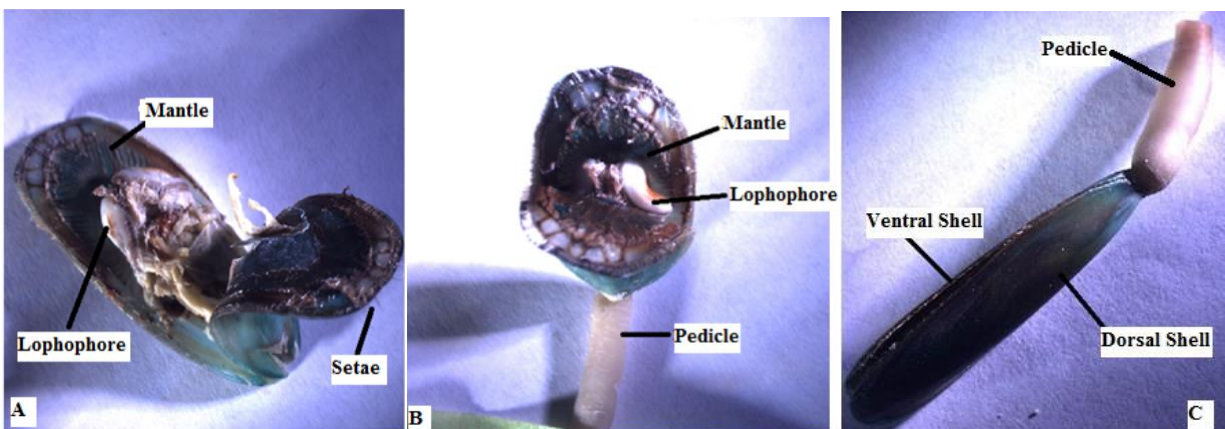
Figure 17. *L. reevii.* **A. & B.** Labeled views of dissected specimen; **C.** Labeled view of complete specimen.

Table 4. Measurements of *L. reevii* using equation from Worcester (1969) as well as a new equation (L=W*2.23).

Specimen	Length (mm)	Width (mm)	Height (mm)	Ratio (L/W)	Worcester equation length estimate (mm)	Difference from Worcester equation (mm)	New equation length estimate (mm)	Difference from new equation (mm)
1	34.35	15.59	6.27	2.2	35.85	1.5	34.77	0.42
2	33.3	14.56	6.24	2.29	33.49	0.19	32.47	0.83
3	35.41	16.64	6.27	2.13	38.26	2.85	37.11	1.7
4	33.32	14.58	6.25	2.29	33.54	0.22	32.51	0.81
5	35.4	15.63	6.28	2.26	35.94	0.54	34.85	0.55
6	36.45	16.65	7.3	2.19	38.28	1.83	37.13	0.68
7	39.56	17.69	8.35	2.24	40.66	1.1	39.45	0.11
Average	35.40	15.91	6.71	2.23	36.57	1.18	35.47	0.73

Discussion

Lingula reevii is only found in southern Japan, Ambon, Indonesia and Kāneʻohe Bay, Hawaiʻi (Hunter et al., 2008). These populations are clearly isolated and may have slightly different genetic make-up. Currently, there is no genomic data available for *L. reevii* for the COI gene from either Japan or Indonesia. There is, however, such data available for other *Lingula* species. Based on the results of DNA sequencing, the *L. reevii* sample from Hawaiʻi was genetically distinct from all the other species included in the tree (Fig. 15). Thus, the hypothesis, *L. reevii* is significantly different genetically from other similar species, could not be rejected. This is important from a management perspective, as *L. reevii* can be considered genetically distinct from other similar species and should therefore be offered a higher level of protection.

It should also be noted that *Lingula shantungenesis* included in this tree may actually be *Lingula adamsi* based on the genetic similarities, so further testing is needed to verify this species (Fig. 15). Furthermore, *L. reevii* samples from the populations in Japan and Indonesia should be genetically analyzed and compared to the Hawaiian samples to determine whether the populations show divergence from one another. If they do, then *L. reevii* in Kāneʻohe Bay would

be considerably rarer than previously thought and should be a focus of conservation. There is already a noted ecological difference between the Japanese and Hawaiian *L. reevii*: the Japanese populations burrow in mud flats, while the Hawaiian populations are found burrowed in the sand (Goto et al., 2014). More individual samples from Kāneʻohe Bay should also be genetically analyzed and compared to one another to determine whether there is genetic variability within the Hawaiian population as well. Samples from two other individuals have been sent out for sequencing, and samples from seven additional individuals have been obtained, but these results will not be available before the culmination of this course. These samples should add to the understanding of the *L. reevii* populations in Kāneʻohe Bay and may be used to aid future research in comparative studies to populations from Japan and Indonesia.

In addition to the genetic study, morphological studies were carried out. Seven *L. reevii* samples from Kāneʻohe Bay were measured and analyzed using an equation from Worcester (1969). Worcester provides an equation for calculating the length of *L. reevii* shells if the width is known (L=W*2.29+0.15mm). The estimated length of each sample was calculated using this equation, and the results were compared to the actual lengths of the recent individuals collected (Table 4). Based on the differences, a new length estimation equation was calculated (L=W*2.23). Then, the estimated length of each sample was calculated using the new equation, and the results were compared to the actual lengths of the individuals (Table 4). The average of the difference between Worcester's equation and the actual length was 1.83 mm, and the average of the difference between the new equation and the actual length was 0.73 mm (Table 4). It should be noted that the new equation was calculated based on four samples with samples five through seven used as validation samples to verify the new equation. This new equation should

be tested on a much larger sample size, as there were only seven individuals measured to create it, while Worcester used 406 samples (Worcester, 1969).

Finally, ten *L. reevii* samples were measured and observed in an attempt to improve the existing morphological descriptions of the species for preservation. Two of the samples were from 2012, and one sample was not labeled with a date, so it remains unknown. These three samples had been preserved in ethanol for an extended period of time and were deteriorating. The two halves of the shells were no longer fully connected, the pedicles were missing, and the coloration had faded to a much lighter blue-green than seen in fresh samples. The pedicles on the 2014 samples had clumps at the base that resembled concrete. These began to dissolve within a few days of being placed into the ethanol solution. The material of the pedicle itself began to discolor to a darker brown after it was cut to obtain a sample for DNA extraction, and the samples became rigid in the solution within hours. The 2014 samples retained their darker green and brown coloration for the duration of the study. This information may be important to note for future researchers analyzing specimens preserved in ethanol.

In addition to analyzing molecular data, various field parameters collected throughout Kāneʻohe Bay were also evaluated. There were 11 sites surveyed; however, only eight of those sites were used in the statistical analysis because the data was skewed towards zero (Fig. 2). Reefs 16, 20, and 28 were exempt from statistical analysis because the habitats were unfavorable to support *Lingula reevii*. Out of the eight sites, Lilipuna and Reef 15, did not have any observed *L. reevii*, but were included in statistical analysis because previous studies have found *L. reevii* at these sites. These two sites were not used in mapping the distribution of *L. reevii* (Fig. 6).

Statistical analysis was unable to scientifically reject or support the hypothesis that *L. reevii* is decreasing in Kāneʻohe Bay, because survey sites and methods have not been consistent.

For example, the methods used in 2010 utilized SCUBA technology to survey 23 sites with a total of 68 transects, while the methods in 2011 utilized free diving to survey three sites using quadrats (Table 3). Due to this discrepancy, no comparisons can be made with past *L. reevii* density studies. For future studies, survey sites and methods should be consistent so comparisons can be made to monitor the progress of this SOC. This study can serve as the starting point for future studies in order to create appropriate conservation and management plans for *Lingula reevii*.

However, among the sites that were surveyed in this study, the environmental parameters were tested to determine the effect on *L. reevii* density including salinity, pH, temperature, and sediment grain size. There was a significant relationship between the abundance of *L. reevii* and salinity and pH at the eight sites. The optimal conditions for *L. reevii* were found to be in areas of high salinity and lower pH (Fig. 2 and 3). This was supported by our data because the highest abundance of *L. reevii* was observed at Sandbar M1 (n=43), which had an average pH of 8.1 and an average salinity of 33.9 ‰ (Fig. 7). In contrast, Fringing reef J (total abundance of 4) had an average pH of 8.4 and an average salinity of 31.8 ‰ (Fig. 10).

Sediment data collected throughout Kāneʻohe Bay show a wide range of sediment composition ranging from gravel (>5000 μm) to silt/clay (<63 μm) (Fig. 13). Areas such as Reefs 16, 20, and 28 have a higher proportion of larger sediment grain size because of strong currents and high wave flush that remove fine sediments (Fig. 14). Another contributing factor for larger sediment grain size could be due to those reefs' locations in areas exposed to high boat traffic that disperse fine sediments. In contrast, Fringing Reef B3 (Fig. 11), Goby Bay (Fig. 9), and Lilipuna Pier are indicative of sheltered sites and contain sediments with high levels of smaller grain sizes (Fig. 14). In areas with a presence of *Lingula reevii*, there were lower proportions of

gravel. For example, Fringing Reef B2 and Sandbar M1 had the greatest abundance of *L. reevii* and had the lowest level of gravel and lower levels of silt/clay sediment (Fig. 12). An anomaly at SB1 was found where this site is exposed to high wave energy but, has high levels of silt/clay sediment and a low proportion of gravel, and 13 *L. reevii* were observed (Fig. 8).

In conclusion, we rejected the null hypothesis in support of the alternative hypothesis that *L. reevii* has significant genetic differences from similar species. This should more firmly establish *L. reevii* as an SOC in Kāneʻohe Bay. Additionally, the length estimation equation proposed by Worcester in 1969 may need revision based on the findings of this study. The *L. reevii* found in Kāne'ohe Bay are genetically distinct from similar species in other parts of the world and are decreasing in abundance making it more important to protect this unique species. Furthermore, this study has provided data that pH, salinity, and sediment grain size data can provide a starting point for future studies in determining areas that have favorable habitat conditions where *L. reevii* can be found. To avoid problems in the field, a detailed methodology design should be created so that problems such as inconsistent survey sites and methods can be avoided to provide for appropriate conservation and management plans for this Species of Concern, *Lingula reevii*.

Supplemental Materials

General Linear Model: Abundance versus pH, Salinity, Temp_High, Location

```
The following terms cannot be estimated and were removed:
  Salinity*Location

Method
Factor coding  (-1, 0, +1)

Rows unused    6
Backward Elimination of Terms
α to remove = 0.05
```

```
Analysis of Variance
Source                  DF  Adj SS  Adj MS  F-Value  P-Value
 pH                      1   19.72  19.720  8.40     0.007
 Salinity                1   20.44  20.438  8.70     0.006
 pH*Salinity             1   20.17  20.173  8.59     0.006
Error                   33   77.51   2.349
Total                   36  101.89

Model Summary
     S R-sq  R-sq(adj)  R-sq(pred)
     1.53254 23.93%     17.02%   0.00%
Coefficients
Term                    Coef  SE Coef  T-Value  P-Value   VIF
Constant                -651   224     -2.91    0.006
pH                       77.0  26.6     2.90    0.007   531.91
Salinity                 19.57  6.63    2.95    0.006  2335.87
pH*Salinity             -2.311  0.789 - 2.93    0.006  2208.27
Regression Equation
Abundance = -651 + 77.0 pH + 19.57 Salinity - 2.311 pH*Salinity
Fits and Diagnostics for Unusual Observations

Obs  Abundance Fit  Resid  Resid Std
 7     7.000  2.966  4.034   2.95  R
24     5.000  1.833  3.167   2.35  R
37     4.000  0.740  3.260   2.17  R
R  Large residual
```

T-Test Results

```
Two-Sample T-Test and CI

Sample  N    Mean      StDev   SE Mean
2014    6   0.00444  0.00688   0.0028
2013   12    0.083    0.288    0.083


Difference = μ (1) - μ (2)
Estimate for difference:  -0.0786
95% CI for difference:  (-0.2613, 0.1042)
T-Test of difference = 0 (vs ≠): T-Value = -0.95 P-Value = 0.365  DF = 11
```

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