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DIVERSITY AND BIOGEOGRAPHY OF THE UNIQUE, TROPICAL PHYLUM PLACOZOA

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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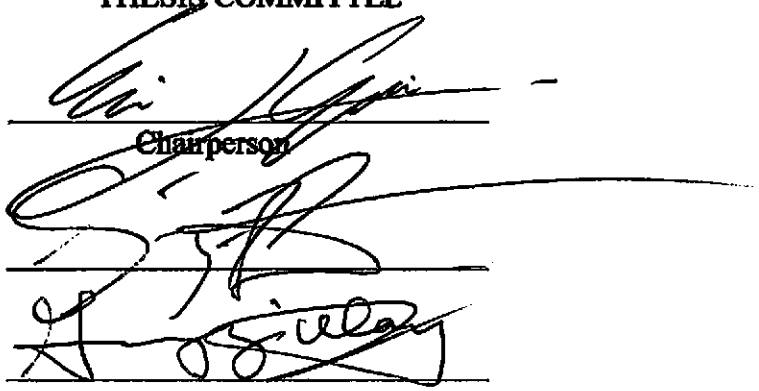
We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Oceanography.

THESIS COMMITTEE



Chairperson

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Abstract

Phylum Placozoa consists of only a single described species, *Trichoplax adhaerens*. Although organisms within the phylum are morphologically indistinct, recent molecular evidence indicates cryptic diversity. To date there are 8 described placozoan haplotypes found in tropical regions world wide. Presented below is a community analysis of 30 placozoan isolates obtained from Hawai'i, as well as 14 samples collected from Puerto Rico. It was found that at least 5 of the 11 described haplotypes are found in Hawai'i, while only 2 different haplotypes were obtained from the Puerto Rican samples. Two separate genetic loci were analyzed using DNA from the Hawai'ian isolates, the mitochondrial 16S rDNA and a more variable location on the mitochondrial genome that encompasses the *cox*2 gene as well as an intergenic spacer region. It was found that both loci exhibited low levels of intra-haplotype variation and moderate levels of inter-haplotype variation, however the 16S locus was better able to capture diversity because the average single nucleotide polymorphism per base pair rate (SNP/bp) is 0.188 compared to the *cox* SNP/bp rate of 0.095 on average. Preliminary work was also done to explore the placozoan life cycle. Samples of near-shore water (between five and 11 liters) were taken during each of two field collections from 2 locations on Oahu and filtered. DNA was extracted from the filter and placozoan 16S rDNA primers were used to amplify product from the extract. Possible product was detected by gel electrophoresis, but more work must be done to conclude whether or not placozoans inhabit the water column and at what concentration. Placozoans are tractable, easily cultured organisms making them ideal for ecological, speciation, and evolution studies.

Table of Contents

List of Tables

List of Figures

Preface

The following project employed molecular techniques, along with supplemental bioinformatics to: 1) Test whether placozoans are occupying the water column 2) Describe the diversity of placozoans found within sea tables at Kewalo Marine Lab as well as samples from an open water site off Isla Magueyes, Puerto Rico and test whether there is haplotype/clade overlap and 3) Determine if it is possible to capture more of the diversity than previous work by analyzing a more variable region on the mitochondrion. Thirty samples from Kewalo Marine Lab, Hawai'i and 14 samples from Puerto Rico were analyzed across two different genetic loci, namely 700 base pairs (bp) of the conserved mitochondrial 16S and 1300 bp of a more variable region spanning mitochondrial genes of the cytochrome oxidase subunit (*cox*) 2 and *cox*1.1a.

The placozoan life cycle is largely unknown. It has been suggested that placozoans can spend ~10 days in the water column before settling on the benthos (Pearse & Voigt, 2007). An experiment was designed to determine whether placozoans could be identified in DNA extractions of filtered seawater. Amplification of the extracts was performed using placozoan-specific primers. Presence of a band on an electrophoresis gel indicated successful amplification of placozoans from the water column. A quantitative polymerase chain reaction was also performed to quantify the amount of placozoan DNA in seawater. Information from the water column work could provide new insight into the placozoan life cycle, namely whether they have a pelagic phase as well as a benthic phase and by comparing community structure of the pelagic

phase could result in the identification of new haplotypes. The data provided in this study is only preliminary, but it includes protocols that could be used in future work.

Recent research using molecular markers has shown significant diversity within the phylum, with organisms taken from various locations worldwide exhibiting differences consistent with genus- or family-level diversity in other taxa (Voigt et al., 2004). To date, 6 clades comprising 11 haplotypes have been described based on comparison of 16S rDNA sequences (Pearse & Voigt, 2007). The most extensive sampling reported in the literature was performed in Twin Cays, Belize by Signorovitch et al., (2006) in which 20 individuals belonging to 5 haplotypes and 3 clades were obtained.

Are these surveys fully capturing diversity within Phylum Placozoa? A seminal study by Voigt et al. (2004) used amplified fragments from two loci, the 16S rDNA (mitochondrial) and the ITS/5.8S region (nuclear) and uncovered 8 different haplotypes. The 16S rRNA molecule is extremely conserved (Woese et al., 1975) and unsuitable for high-resolution molecular systematics. While the Voigt study on the nuclear ITS demonstrated molecular diversity, nuclear genes are subject to recombination/allele sharing. If the gene studied is prone to recombining, it collapses any phylogenetic tree based on it, resulting in an under-representation of diversity. Another potential problem with the Voigt study is that their samples were amplified and sequenced only once, introducing possible errors through the Polymerase Chain Reaction (PCR) process. This project amplified a less conserved mitochondrial region in an effort to detect more of the variability between isolates and also amplified and sequenced each loci twice to mitigate PCR error. Surprisingly, however, it was found that the 16S locus depicted more between-haplotype variation than the second locus, the *cox*2.

Placozoans have been found in tropical oceans worldwide. Some haplotypes have been found to be widespread, with representatives found in the Atlantic Ocean, Pacific Ocean, Indian Ocean, and the Mediterranean Sea. This suggests that placozoans have a cosmopolitan distribution. Placozoans were collected from field sites on the islands of Oahu and Puerto Rico and community structure was described for the samples obtained. To determine whether placozoans have a biogeography or whether they are simply found everywhere, it is imperative to correctly describe the true diversity within the phylum. This project provided information on 30 placozoan samples obtained from Hawai'i, making Hawai'i the most sampled location for placozoans. It also provided placozoan haplotype information from a previously unstudied site, Puerto Rico.

Chapter 1. Introduction to phylum Placozoa

Cellular organization

Placozoans are marine animals. Placozoa comes from the Greek word *Plakos* meaning flat and *Zoon* for animal. Morphologically, these organisms resemble hairy discs but are also able to continuously change their form (Fig. 1). They can reach 3 mm in diameter, though most placozoans collected in Hawai'i are ≤1 mm, and are composed of a few thousand cells. Although they do not have bilateral symmetry, they do have a dorsal and ventral side. There are flagella covering the entire outer surface, which allow the animal to move in the water column and grab substrates. When in contact with a substrate, two types of movement are possible: gliding by means of the ventral flagella and changes of shape resembling amoeboid movement (Grell & Ruthmann, 1991).

Three cell layers can be distinguished, a dorsal (or upper) layer, a ventral (or lower) layer that faces the substrate, and a loosely connected interstitial layer (Fig 2). The dorsal and ventral epithelia lack a basal lamina and the cell junctions are connected by microfilaments. Placozoans contain only four different somatic cell types, compared to sponges which contain ~15, or flies which have ~90 (Collins, 2000). The four placozoan cell types are cover cells, cylinder cells, gland cells, and fiber cells. Column-shaped cylinder cells, which contain flagella and gland cells without flagella, make up the ventral surface. The dorsal surface consists of a layer of cover cells, which are ciliated and flattened toward the outside of the animal. The fourth type of somatic cell is the fiber cells, which are star-shaped and occupy the space between the upper and lower layers.

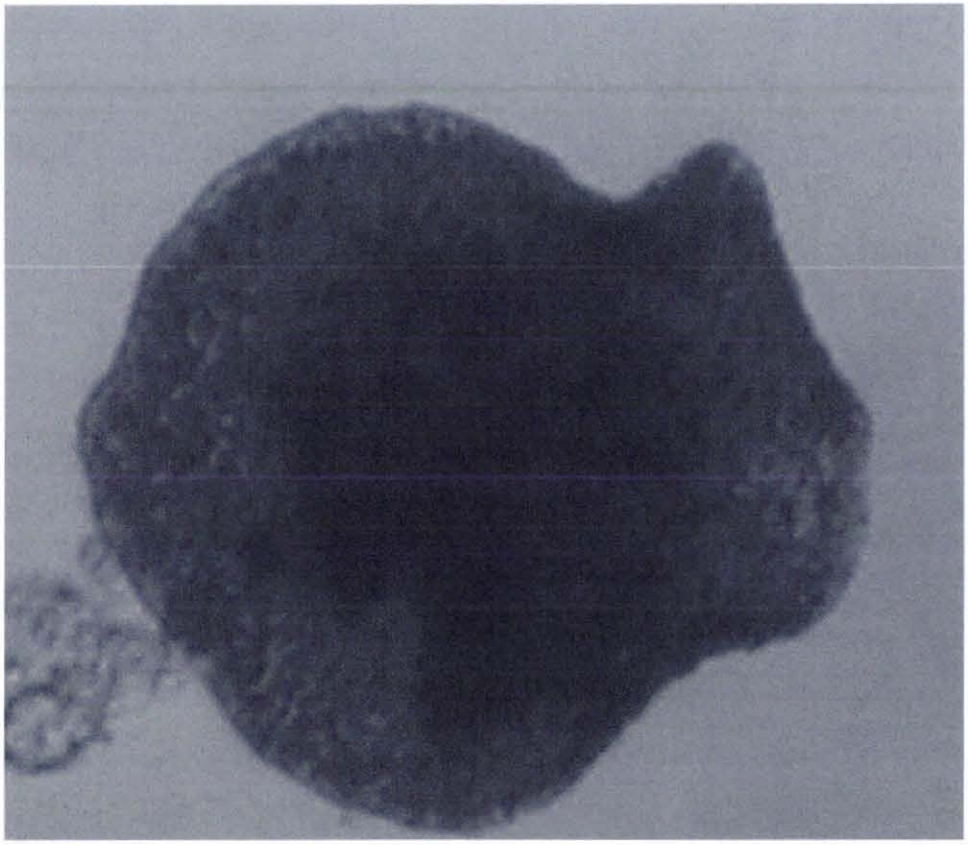


Figure 1. Picture of placozoan from Hawai'i, ~0.5 mm in diameter

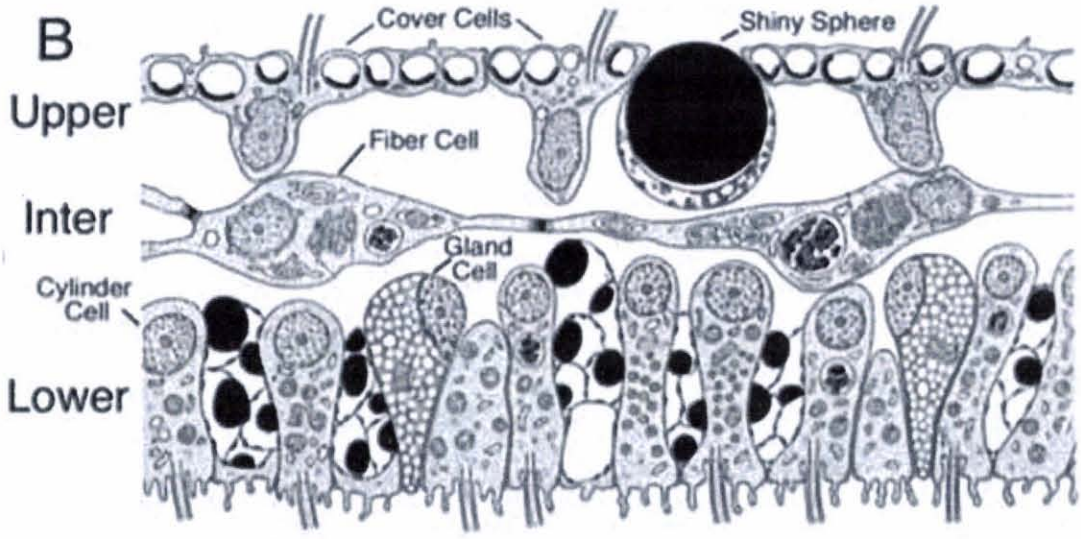


Figure 2. Cross-section of a placozoan, indicating cell types and layers from Grell and Ruthmann, 1991

Thin extensions of the fiber cells connect each other in a network, giving them the star shape. Cellular material such as microtubules and microfilaments criss-cross the fiber cell junctions and it is hypothesized that this system of connected cells is important in coordinating the movement of placozoans (Grell & Ruthmann, 1991). The other conspicuous components of the placozoan body plan are the "shiny spheres" which regularly dots the dorsal side of the organism. These are lipid inclusions within the cover cells whose purpose is uncertain. It has been suggested that the shiny spheres may contain toxins and act as a defense mechanism for the animal, as some grazing animals (snails, seastars, etc.) pull back upon encountering placozoans (V. Pearse, pers. comm.).

Placozoans are nearly transparent but can take up pigments from their food sources. Placozoans feed extracellularly. It has been suggested that the gland cells and possibly the cylinder cells on the ventral side produce exoenzymes for the extrasomatic predigestion of the food (Grell & Ruthmann, 1991). Absorption of digested nutrients is then performed by the ventral cylinder cells. Commonly, the animal elevates from the substrate by contracting filament bundles and forms a digestive bag, aiding in predigestion (Grell & Ruthmann, 1991).

Sex/life cycle

In the laboratory, placozoans are observed undergoing asexual reproduction through binary fission and, less often, budding. During fission, the organism begins to cleave in the center as the two daughter organisms pull apart. A thin, multicellular strand connects the two daughters for 15 min to a few hours before finally splitting, at which time the strand becomes integrated into roughly equal-sized daughter animals

(Schierwater, 2005). Budding, on the other hand, begins by pinching off a small portion on the dorsal side of the mother. The cleaved portion is called a "swarmer" and is a hollow sphere with a single layer of cells from both the dorsal and ventral epithelia (Thiemann & Ruthmann, 1989). This is a dispersal stage and can persist for up to one week (Schierwater, 2005), after which time, the concave ventral epithelium flattens and forms new flagella to adhere to the substrate and the cell types differentiate the dorsal and ventral epithelia.

In laboratory cultures, when the placozoan population density becomes high, placozoans start to degenerate. Usually a single, large (~100μm) oocyte develops and is released as the mother organism disintegrates (Schierwater, 2005). This can also happen under conditions of starvation. Small, non-flagellated round cells are also formed during the break up and are inferred to be sperm cells (Miller & Ball, 2005). Fertilization has never been observed in the laboratory. At some point cleavage begins. Development has only been documented to the 64-cell stage, after which point separation of the cells ceases, but the nuclear DNA continues to multiply, this results in the bursting of the nucleus causing the developing organism to die (Schierwater, 2005).

There is recent evidence for sex among individuals in Phylum Placozoa (Signorovitch et al., 2005). In a sexually reproducing population, the overall population and the within-individual (between copy) level of variation are expected to be equal. In the Signorovitch et al. study (2005), seven nuclear loci were analyzed and the observed values of within-individual and within-population variation levels were not only much lower than what would be found in asexual species but the values were also generally similar to each other, suggesting the existence of a sexual lifecycle. The absence of

nucleotide sites that were heterozygous for the same allele pair in all individuals provides evidence against ancient asexuality (i.e. they cannot be recently sexual) (Signorovitch et al., 2005). Homozygosity is a consequence of populations that are inbreeding or allow the fusion of nuclei or cells from the same parent. The data obtained from Signorovitch et al. (2005) also did not contain any locus that was homozygous among all genotypes, therefore, it is unlikely that the isolates obtained by Signorovitch et al. (2005) were asexual as the allelic recombination between homozygous and heterozygous individuals can only occur through sex.

Ecology

Placozoans have been collected on glass slides suspended in the water column (usually for 2-3 weeks), from pieces of algae and coral, and on the glass slides of aquaria. Placozoans are found in tropical, littoral regions around the world (Hawai'i, Pacific Islands, Japan, Australia, Red Sea, Mediterranean, Caribbean, and North/South America) (Pearse & Voigt, 2007). In Hawai'i, placozoans have been captured between the months of June and September, inclusive. Pearse & Voigt (2007) also mention collection only during these months in Japan and the Pacific Islands.

Being nearly microscopic benthic meiofauna, placozoans have not been directly observed in the natural environment, but they are thought to scavenge detritus, protozoa, and algae (Grell & Ruthmann, 1991). In the lab, placozoans have been cultivated to feed on the flagellated alga *Cryptomonas* (Grell & Benwitz, 1974) as well as other filter feeder food including heat-killed yeast (Wenderoth, 1986) and *Artemia* nauplii (Grell, 1983). When given a constant food source, placozoans double approximately once every 1-3

days (Pearse, 1989). It is unknown which organisms, if any, feed upon placozoans. As mentioned above, it is possible the shiny spheres are a defense mechanism, making them unpalatable to possible predators.

Placozoans have been shown to be affected by changes in salinity, where contact with seawater diluted to 75% salinity for 1 h proves 100% fatal; this is also evident in field collections, as placozoans are rarely recovered after rains (Pearse & Voigt, 2007). Placozoans also react to low temperatures (placement at 4°C for an hour has resulted in detachment from the substrate) and ultraviolet light (exposure causes the placozoans to detach and move violently for 15-20 seconds or until light is removed) (Pearse & Voigt, 2007). Placozoans also respond to Ca^{2+} concentration in the seawater. Exposure to calcium-free seawater results in complete disaggregation of cells and seawater with 2X Ca^{2+} results in intense placozoan movement (Grell & Ruthmann, 1991, also observed in Gaidos Laboratory, E. Gaidos, pers. comm.).

Phylogeny

Placozoans are basal metazoans, meaning they group at, or close to, the base of the animal tree of life. Understanding the phylogenetic position of these organisms could aid in discerning the evolution of metazoans. The phylogenetic position of the phylum is highly controversial: *Trichoplax* has been grouped as sister to the Bilateria, Porifera, or Ctenophora, or at the base of all animals depending on the gene(s) and taxa used and reconstruction methodology (Wallberg et al., 2004; Cavelier-Smith & Chao, 2003; Schierwater, 2005; Dellaporta et al., 2006).

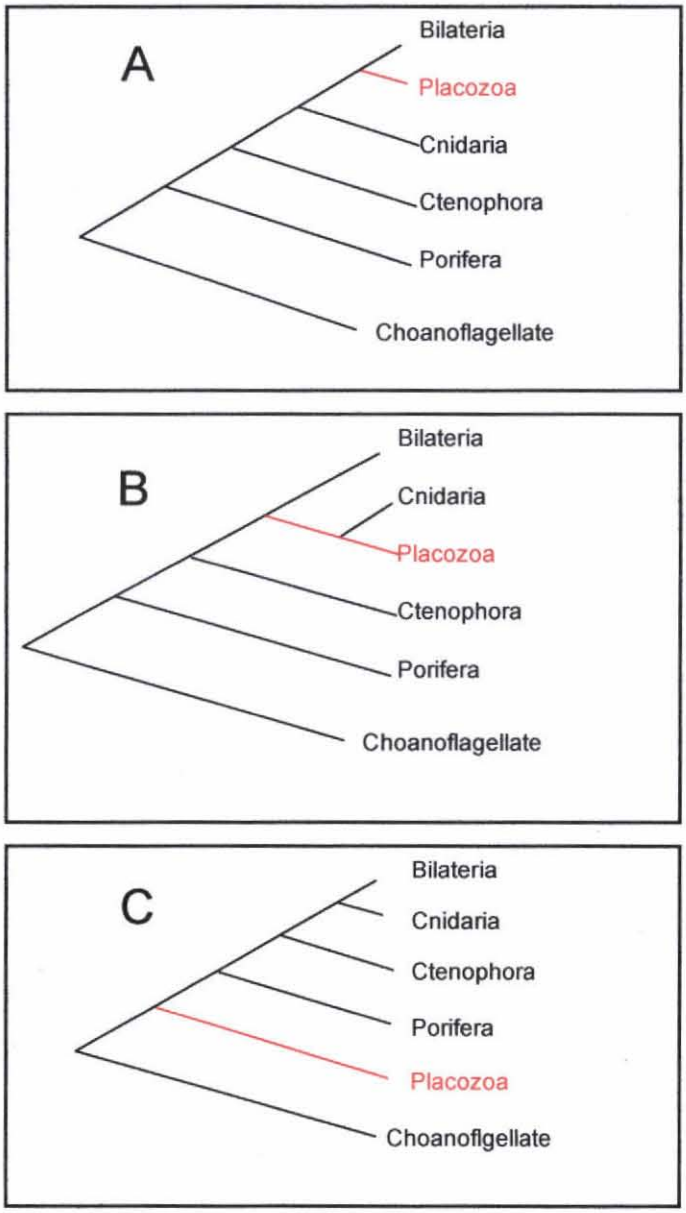


Figure 3. Conflicting placement of placozoans among the basal metazoans

There are three competing hypotheses for the origin of the basal metazoan lineages (Fig. 3). The first hypothesis (Fig. 3, A) suggests that placozoans are sister to bilaterians, or an extremely derived cnidarian itself (Wallberg et al., 2004, Collins, 1998). Supporting this is the fact that Placozoans positively stain for RFamide (a bilaterian neurotransmitter) in antibody tests (Schuchert, 1993). Martinelli & Spring (2003, 2004) have also amplified and shown expression of orthologs to T-box genes *tbx*2/3 and *brachyury* in placozoa. Both genes are involved in the formation of the notochord in chordates. It can be argued that because placozoans contain the remnants of neurotransmission, they may have secondarily lost a nervous system. However these genes could be nervous system precursors, which would indicate a more basal position. Other work by Collins (1998) supporting the position in Box A is that placozoans contain only one *hox* gene that is highly similar to a bilaterian homolog. Collins (1998) also points out that placozoans are dorso-ventrally differentiated with a mesenchymal syncytium similar to the central syncytium of acoel flatworms. The criticism of this hypothesis is that most secondary simplifications are associated with a parasitic lifestyle and placozoans are free-living organisms. Also, if placozoans are derived, not only would they have had to lose a nervous system, they would have had to lose bilateral symmetry, mesoderm, gut, and mouth (Collins, 1998).

The second hypothesis (Fig. 3, B) shows Placozoans as sister to the Cnidarians. Several molecular reconstructions have supported this order based on 18S and 23S rDNA data sets involving a wide range of taxa and Maximum Parsimony (MP), Maximum Likelihood (ML) and Baysian inference (Wainright et al., 1993, Pawlowski, 1996, Kim et al., 1999, Borchiellini et al., 2001, Cavelier-Smith & Chao, 2003) reconstruction

methods. However, this premise has largely been discounted based on 23S and 16S-like secondary structures (Odorico & Miller, 1997, Ender & Schierwater, 2003), and analysis of *hox* (Jakob et al., 2004), *pax* (Hadrys et al., 2005), EF1-α and actin (Martinelli & Spring, 2003) genes. Trees based on morphology of these molecules have suggested a split of Placozoa earlier and separate from Cnidaria.

The third hypothesis (Fig 3, C) has Phylum Placozoa in the basal position, either before or after the Poriferan divergence (Peterson & Eernisse, 2001, Halanych, 2004, Schierwater, 2005). Although the 18S and 23S data (the most commonly used molecules in molecular phylogeny) consistently place Phylum Placozoa within the Cnidarian clade (except Wallberg et al., 2004), there is little morphological support for this location. Standard dogma assumes that simplicity implies ancestry. If this is the case, then the fact that Placozoans have only 4 cell types and are lacking a mouth, gut, nervous system, symmetry, and basal lamina, indicates an ancestral position for Placozoans (Schierwater, 2005).

The genome of *Trichoplax* is approximately 106 Mbp, among the smallest animal genomes, and has been sequenced by the Joint Genome Institute (data released in 2007, Srivastava et al., in press). With genome data available, a number of phylogenetic, expression, directed development and ecological experiments become feasible. Whole genome evaluations of a choanoflagellate (*Monosiga brevicollis*), cnidarian (*Nematostella, Hydra*), ctenophoran (*Mnemiopsis*) and poriferan (*Reniera*) have also been released recently, providing an excellent opportunity for evolutionary studies of the basal metazoans. If *Trichoplax* is found to be the most basal metazoan, it becomes an ideal organism to study in animal evolution. By reconstructing basal metazoan

phylogeny we can assemble a timeline of major animal-specific evolutionary events such as the beginnings of cell-cell communication, cellular differentiation and the development of a nervous system and gastric system.

Diversity

The Placozoan phylum includes only a single described species, *Trichoplax adhaerens*. Although most studies have focused on a single isolate of this species collected from the Red Sea (Grell, 1971), recent research using molecular markers has shown significant diversity within the phylum, with organisms taken from various locations worldwide exhibiting differences consistent with genus- or family-level diversity in other taxa (Voigt et al., 2004; Wolf et al., 2007). This indicates that there is possible cryptic speciation within this phylum, as all isolates generally look the same (although some haplotypes produce birefringing granules and others do not (E. Gaidos, pers. comm.)) but they are molecularly distinct.

Phylum Placozoa may harbor a number of haplotypes that will differentiate into species, genus, family and possibly higher taxonomic levels, however a lack of morphological or ecological differences and the absence of life-cycle information hinders a more systematic classification (Schierwater, 2005). To date, 6 clades comprising 11 haplotypes have been described based on comparison of 16S rDNA sequences, the most recent phylogram is shown in Fig. 4 (Pearse & Voigt, 2007). The most extensive sampling in the published data was performed in Twin Cays, Belize by Signorovitch et al., (2006) in which 20 individual strains belonging to haplotypes H2, H3, H4, H6, H7, and H8 were obtained.

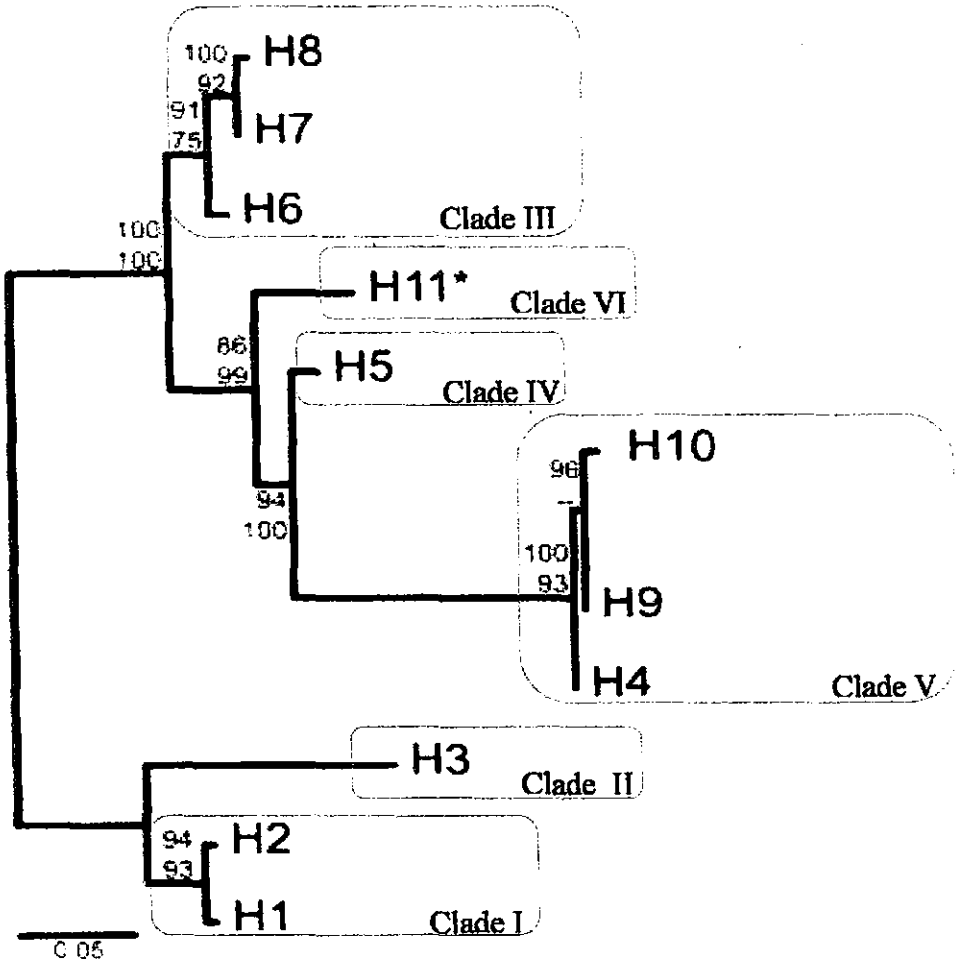


Figure 4. Phylogenetic relationship of placozoan 16S rDNA-gene haplotypes. A maximum likelihood tree, support values >50 are shown at the corresponding nodes; bootstrap values (500 replicates) are given above Bayesian clade credibility values. Length of the bar indicates 0.05 substitutions per site (Pearse & Voigt, 2007).

Biogeography

It has been suggested that organisms such as protozoa and bacteria that are <2mm in diameter have a cosmopolitan distribution due to their small body size and high abundance (Finlay, 2002). Placozoans fall within this range. Placozoans are found worldwide and many identified haplotypes have overlapping distributions, sometimes with two or more haplotypes occurring on the same slide together during collection (Fig. 5) (Pearse & Voigt, 2007). As illustrated, many placozoan haplotypes are represented at each sampling locale and most haplotypes have a broad distribution. It should be noted that this represents a small number of isolates from each location (5-20), and that with greater sampling; it is a possibility that more haplotypes have yet to be uncovered. Voigt et al (2004) uncovered 8 haplotypes with their efforts in sampling the Red Sea, Panama, Venezuela, Italy, and the Guam, while Pearse & Voigt (2007) were able ascertain 11 haplotypes by sampling deeper in those location as well as from sampling new location such as California, the Sargasso Sea, and Australia. Therefore, it should also be expected that more placozoans would be uncovered from locations that have not yet been sampled.

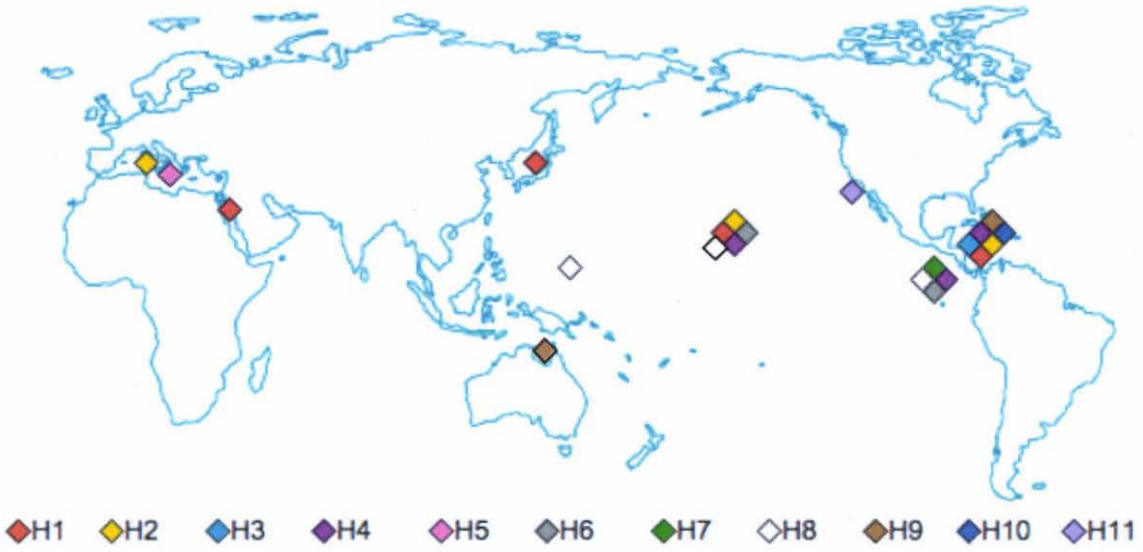


Figure 5. World map illustrating haplotype distribution. Haplotype information collected from this project as well as data from Pearse & Voigt, 2007.

As mentioned above, placozoans have a pelagic phase and can be collected from the water column, as opposed to the benthos. They have not been found in the upper 500mm of surface water, however, possibly due to intense ultraviolet radiation (Pearse & Voigt, 2007). Also, when slides are suspended parallel with the sea floor, placozoans tend to settle on the under surface possibly to escape sedimentation and uv rays (Pearse & Voigt, 2007).

Placozoans prefer tropical and subtropical protected, near-shore environments, especially coral reefs and mangroves (Pearse & Voigt, 2007). They also prefer hard substrates over sandy bottoms. Although whole regions on the map of placozoan distribution are devoid of isolates, this is more likely due to lack of sampling, rather than a lack of placozoans. It is expected that more sampling will uncover more isolates, however Pearse & Voigt (2007) noted that placozoans have yet to be found in the Antarctic.

Chapter 2. A search for placozoans in the water column

Introduction

The full life cycle of placozoans is unknown, including the proportion they spend in a pelagic phase. Many organisms have both a benthic and a pelagic phase, for example three out of four classes within the Phylum Cnidaria (Cubozoa, Hydrozoa, and Scyphozoa) have an asexual, sessile, benthic polyp phase and a medusa phase, which is the pelagic, sexual phase (Galliot & Schmid, 2002). Interestingly, the class Anthozoa, considered the basal class, does not have a pelagic phase. The purpose of the study presented in Chapter 2 was to design an experiment to search for placozoans in the water column, that could be indicative of an abundant, detectable pelagic phase to the placozoan life cycle. Another goal of the experiment was to use molecular methods to compare the placozoan community structure in the water column with the community that settles out in the benthic phase.

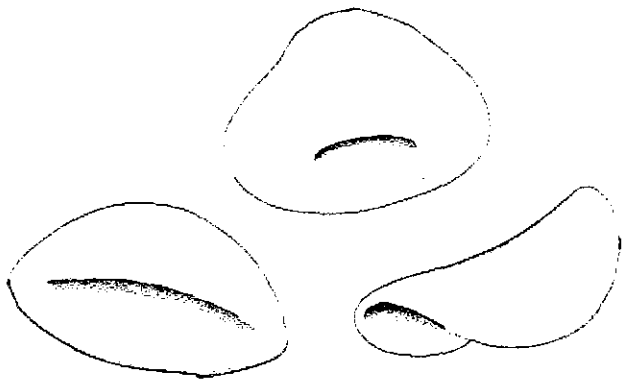


Figure 6. Drawing of pelagic placozoan swarmers. Drawing by J. Keller and C. Patton, based on observations by Pearse & Voigt (2007).

Fig. 6 is an illustration of representative "swarmers" based on observations by Pearse & Voigt (2007) in Petri dishes in the laboratory. The swarmers are estimated to be approximately 200μm in diameter and are hollow, budded off from the dorsal epithelium.

In order to determine whether successful amplification of the DNA extracts occurred using placozoan-specific 16S rDNA primers, the product was run on an electrophoresis gel. Quantitative polymerase chain reaction (qPCR) is a useful way to quantify the amount of DNA in a sample, by comparison of the unknowns to standard wells of known concentration using real-time dye incorporation data. This method was employed to quantify the amount of placozoan DNA in the water column extracts. In order to determine which placozoan haplotypes were present in the water column samples, the 16S rDNA was amplified in three separate reactions and then pooled together. This pooled sample was used to create a clone library and 48 colonies were sequenced.

Water was collected and filtered from two separate locations on the island on Oahu, Hawai'i: a sea table at Kewalo Marine Laboratory and the eastern side of Pearl Harbor. The Kewalo site was chosen because placozoans were found settling on slides on previous occasions at that location. The Pearl Harbor site was chosen an open water field site because the material and seawater that supplies the Kewalo Marine Laboratory sea table is taken from Pearl Harbor.

Materials and Methods

Seawater Collection/Filtration: 5.5L of seawater was collected from a seawater table at Kewalo Marine Laboratory, Hawai'i on 2/27/08 and 10.7L of seawater was collected from a mangrove habitat in Pearl Harbor, Hawai'i on 3/26/08 (Fig. 7). The water was filtered onto a GF/F filter (Whatman, pore size ~4-7 mm). The filter was transferred to a 15 ml Falcon tube.

Figure 7. Map of Hawai'i field sites

DNA Extraction: 5 ml of DNA extraction buffer (2% SDS, 100 mM EDTA, 20 mM Tris, pH 8.0) was added to the Falcon tube containing the filter. One hundred twenty five µl of proteinase K (Roche) was added to the tube and incubated at 50°C overnight with shaking. Afterwards, 5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tube, vortexed, centrifuged at maximum speed for 20 min, and the aqueous phase is

transferred to a new 15 ml Falcon tube. This step was repeated one time. After, 5 ml of chloroform: isoamyl alcohol (24:1) was added to the supernatant and vortexed. The tube was centrifuged at max speed (14,000rpm) for 2 min and the supernatant was transferred to a new tube and quantified. One volume of 95% ethanol was added to the supernatant and the tube was incubated at -80°C for 1.5 hours. Afterward, the tube was centrifuged at max speed for 20 min and the supernatant was removed carefully to avoid disturbing the pellet. The pellet was washed with 1 ml of 75% ethanol and centrifuged at maximum speed for 10 min. The supernatant was removed and the pellet was allowed to air dry for ~45 min. The pellet was then resuspended in 50μL of nanopure water and quantified on a spectrophotometer (Genespec, Hitachi).

PCR: Placozoan-specific primers were designed by Signorovitch et al. (2006) to span a 700bp region of the 16S rDNA: (5'-CGAGAAGACCCCATTGAGCTTTACTA-3' forward, and 5'-TACGCTGTTATCCCCATGGTAACTTT-3' reverse). PCR amplification was performed on a Biorad MyIQ® thermal gradient cycler. PCRs were carried out under the following parameters: 5 min at 94°C; then 38 cycles of 30 sec/94°C, 30 sec/56.5°C, 90 sec, 72°C; 5 min/72°C; hold at 4°C. Two μl of each extraction was used in the PCR reactions. Quantitative PCR analysis was performed to determine the concentration of DNA in the water column filter samples. Eight standard runs were set up and each was run in duplicate to produce a standard curve. The 8 standards contained 0.1 ng, 1 ng, 10 ng, 25 ng, 50 ng, 75 ng, 100 ng, and 150 ng of whole genomic placozoan DNA starting material from previous pure extracts. The unknowns from Kewalo and Pearl Harbor were also assayed in duplicate and quantified by comparison to the standard

curve to determine the concentration of placozoan 16S rDNA in the filtered samples. Analysis of all melt curve data for sample wells and negative wells was done using the MyiQ software.

Cloning: Three separate placozoan 16S rDNA PCR products from each water collection site were combined. The samples were cleaned using a Qiagen Qiaquick PCR cleanup kit. Four μL of the clean 16S rDNA PCR product was used in an Invitrogen TOPO TA cloning kit to generate plasmid vectors containing the 700 bp insert of the placozoan 16S. Transformed colonies were grown on Luria-Bertani (LB) agar plates containing 100 mg/ ml ampicillin. Three colonies were selected from each water column sample plate and grown at 37°C in LB containing ampicillin overnight with shaking. The cell pellets were spun down and a Promega SV miniprep was done on the cell pellets to obtain pure plasmids using the manufacturers recommended protocol. An *Eco*RI restriction digest was performed on the plasmids verify that the insert size was correct. Colonly picking, plasmid preparation and sequencing of additional clones on an ABI 3730XL was performed at the University of Hawai'i Center for Genomics, Proteomics and Bioinformatics. Forty-eight colonies were picked from the plate of the Kewalo Marine Lab water column sample and forty-eight colonies were picked from the Pearl Harbor water column sample. The colonies were prepped in a 96-well plate and sequenced in the forward direction only. A pairwise comparison was performed on the sequences against the publicly available National Center for Biotechnology Information (NCBI) sequence database to align matches and assign haplotype to the placozoan community found in the water column.

Results

After filtration and DNA extraction of the samples, the extracts were analyzed on a spectrophotometer to determine the absorbency ratio of A260 nm/A280 nm, a measure of the quality of the extracted DNA, and concentration. The ratio of the Kewalo sample was 1.515 and the concentration was 247 μg/ml. The ratio for the Pearl Harbor extraction was 1.830 and the concentration was 46.20 μg/ml.

An electrophoresis gel of the PCR products was run at 70 volts for 35 minutes to check amplification of the DNA (Fig. 8).

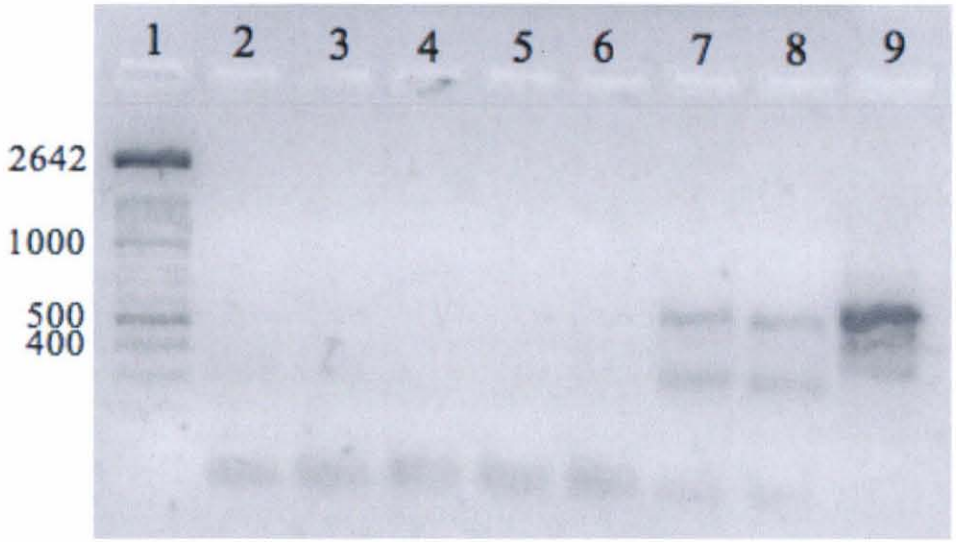


Figure 8. Gel of Kewalo and Pearl Harbor Water Column DNA extractions amplified with placozoan 16S rDNA primers. Lane 1=100bp marker, Lane 2-6= Pearl Harbor 16S 1/100, 1/50, 1/25, 1/10, and 1/5 dilutions of extract from Pearl Harbor, Lane 7= Pearl Harbor 16S amplification at 1X, Lane 8= Kewalo 16S amplification at 1/10 dilution, Lane 9= H1 Placozoan amplification, positive control

As shown, the band sizes for the 16S rDNA gene in the water column were approximately the correct size, ~700 bp, compared to the positive control. Lanes 2-6 showed those amplifications failed when using smaller concentrations of the Pearl Harbor DNA extract (i.e. 1/100 dilution, 1/50, 1/25, 1/10, and 1/5). The working concentration of starting material was 2 μL of a $1/10^{th}$ dilution for the Kewalo Water Column sample and 2 μL of the extraction from Pearl Harbor without dilution. As shown, there are two prominent bands on the gel. The larger band is the predicted size of the targeted 16S rDNA gene fragment; it is undetermined what the second band at 300 bp represents. Negative controls were also run on the Biorad MyiQ thermocycler, but not included in the gel. There was some indication that non-specific amplification was occurring, i.e. smearing on the gel and an exponential curve on the real-time dye incorporation data on the MyiQ screen, in the negative controls. Further analysis was done on the melt curve data to determine if there was contamination amplification of primer-dimers. Upon examination of the melt curves, it was found that negative wells displayed a melt curve peak at 76°C, while wells that contained known product from previously amplified and sequenced extracts peaked at 84°C, therefore, it was determined that false-positives could be identified and disregarded. Only those wells that possessed a product peak on the melt curve were used in subsequent analysis and data collection.

In order to determine approximately how much placozoan DNA was obtained from the water column, a qPCR was performed using a Biorad MyiQ with SYBR green I dye detection. The threshold cycle was calculated for each run. The average of the duplicates was taken for the standards and plotted against the log of the starting quantity. A least squares linear regression trend line was plotted with an R^2 value of 0.9694. By

comparing the unknowns to the trend line, it was determined that the Kewalo water column extraction contained ~1ng of starting placozoan DNA and the Pearl Harbor water column extraction contained ~0.2ng of starting placozoan DNA (Fig. 9).

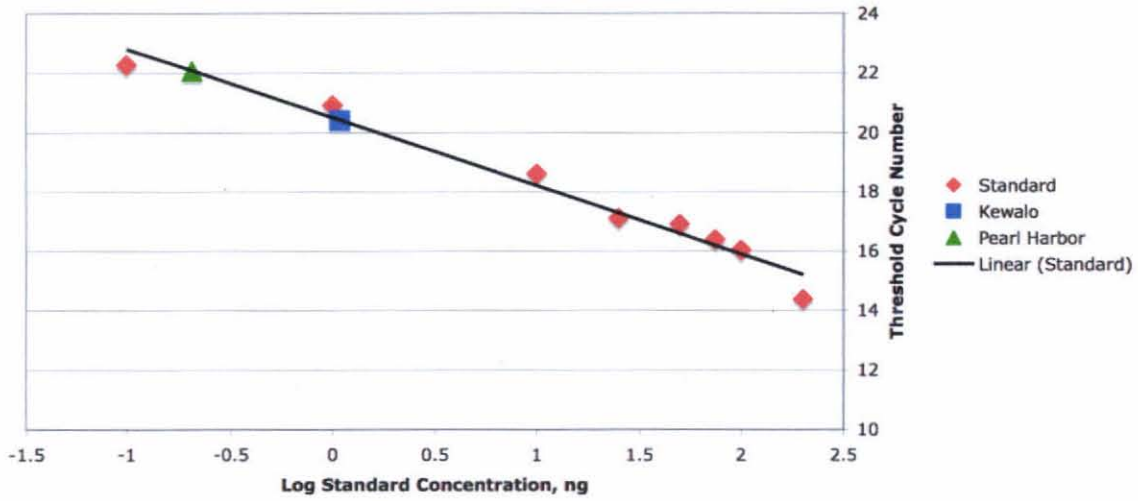


Figure 9. Trend line plot of standards from qPCR analysis of 16S amplifications.

Comparison of Water column unknowns show:

Kewalo	Pearl Harbor
Water Column: 20.41=-2.296x + 20.495	Water column: 22.08=-2.296x+20.495
X=-0.085/-2.296	X=1.585/-2.296
X=0.03702	X=-0.6903
X=10^0.03702=~1.1ng	X=10^-0.6903=~0.2ng

To determine how many organisms this relates to, a simple calculation can be done to determine the amount of DNA per individual:

$$\left(\frac{\text{Size of genome x molecular weight of DNA}}{\text{Avogadro's Number}}\right) \text{ x \# of cells/organism x 2 (diploid ind.)} =$$

ng of DNA/individual, or the following for placozoans:

$$\left(\frac{(105{,}600{,}000\text{bpx}660\text{g/mol})}{6.02\text{x}10^{23}\text{ molecules/mol}}\right) \text{ X } 10^3 \text{ X } 2 = 0.23 \text{ ng DNA/individual}$$

One nanogram of DNA and 0.2 ng for the Kewalo and Pearl Harbor water column extracts, respectively, represents ~1-4 individuals per 5 L of water (or 0.19 ng of placozoan DNA/L at Kewalo and 0.019 ng of placozoan DNA/L at Pearl Harbor, both are less than one organism per Liter). This could be affected by a number of factors including, but not limited to, inefficient extraction from the filter, biased dilution of the starting material in the PCR reactions, and competitive binding of the PCR primers with other templates. Also, the pelagic swarmers that bud from the mother cell are typically much smaller than the ~1mm organism, therefore 0.23 ng would be an overestimation of the amount on DNA found in those individuals. Also, during the time of water collection and filtration, no placozoans were found on slides that were placed on the benthos or in the sea table (see Appendix A). Future experimentation on water column samples should correspond to when placozoans are found on slides placed in the water to demonstrate that placozoans are found in the environment during the course of sampling.

A final experiment was done on the water column samples to determine which haplotypes were found in the water column and if the community structure was comparable to the haplotypes found on the slides from the same water table in Kewalo (see Materials and Methods, Chapter 3). A set of three duplicate PCR reactions was set up for the Kewalo and Pearl Harbor samples using the placozoan 16S rDNA gene primers, the PCR bands appeared as a smear when run on an electrophoresis gel (Fig. 11). Forty-eight colonies were picked from the Kewalo plates and 48 colonies were picked

from the Pearl Harbor plates. Forty-two of the 48 sequences from Kewalo and 44 of the 48 sequences from Pearl Harbor were false positives (i.e. contained no insert), or vector-only sequences. The other combined 10 sequences all aligned to each other and contained ~6 repeats of 40 bps weakly aligning to placozoan and sponge 16S rDNA gene sequences (Fig. 10).

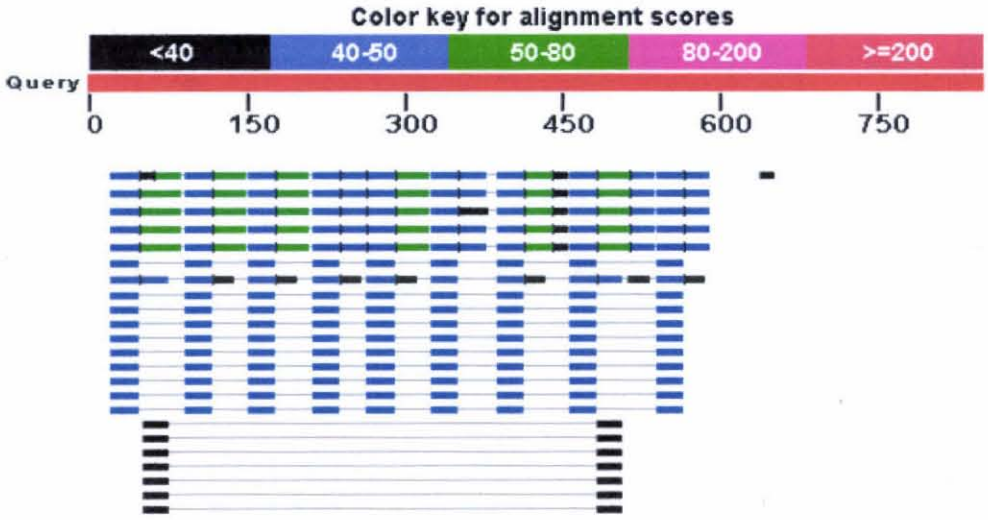


Figure 10. Example blast report of cloned water column sequence, top hit: Placozoa BZ10101, second hit: Topsentia (sponge)

As shown, the scores are very low because the sequences are short. Although the % identity for the green portions, or higher scoring portions, was 100%, when analyzed further, each green portion of the alignment corresponds to the same position on the hit, indicating that each green section in the query sequence is an exact copy of the one before.

Discussion

The failure to obtain high quality 16S rDNA gene sequences from the water column samples appears, at least in part, to be due to technical issues. The original dilutions from the Pearl Harbor and Kewalo samples, which were successfully amplified in earlier trials, were depleted, after which a second set of dilutions was made. Also, a second set of primers had to be purchased during the course of the amplifications. After the switch to the new dilutions and primers, the PCR bands appeared as a smear (Fig. 11) indicating non-specific amplification with the new dilutions and primers. These non-specific products appear to have resulted in inefficient cloning and cloning of concatenated small fragments. More troubleshooting is necessary to clean up the reactions to obtain clear bands for the water column samples as shown in Fig. 8 and produce a higher quality clone library.

The matches of portions of the cloned fragments to both sponge and placozoa also suggests that the primers may not be sufficiently specific. A search of the primers used in the PCR amplifications against the NCBI database showed that, although the primers are placozoan specific, they differ by only two base pairs from the homologous region in sponges. Therefore, the primers may not be as specific as is desirable. Without sequence verification, it is not certain whether the bands amplified earlier (Fig. 8) are actually derived from placozoans.

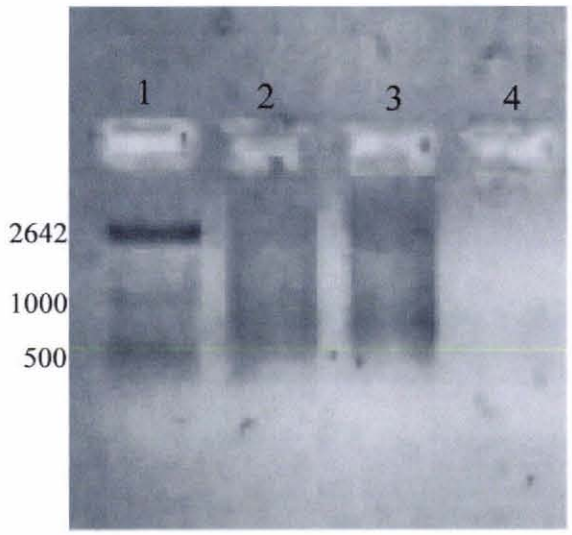


Figure 11 Gel indicating DNA smears of 16S reactions of water column samples after switch in primers and dilutions. Lane 1=100bp Marker, Lane 2=Pearl Harbor water column 16S product, Lane 3=Kewalo water column 16S product, Lane 4= negative control

In conclusion, the amplification of DNA fragments of the expected for placozoans from water column samples is compelling and indicates that placozoans might be found in the water column. It is necessary, however, to develop more specific primers and to sequence the resulting product to make a persuasive argument. Then the community structure of placozoans in the water column might be compared with those acquired from the benthos.

Chapter 3. The diversity of Hawai'ian placozoans

Introduction

The entire placozoan phylum is comprised of only a single described species, *Trichoplax adhaerens*. It has been suggested, however, that there is extensive cryptic diversity between individuals. Based on sequences of the mitochondrial 16S rDNA, eleven haplotypes have been identified and arranged into a phylogenetic tree (Fig. 4). Not all haplotypes have been reported at all locations, but whether this represents a true geographic division or is the result of limited sampling at any given site is uncertain. Successive sampling tends to recover previously reported haplotypes, but it is speculated that with increased sampling, more divergent haplotypes will be discovered.

Placozoans are a challenging subject for evolutionary and population genetics studies. Morphological homoplasies are difficult to infer when dealing with such general features as mouths, mesoderm, and symmetry (Collins, 1998), therefore molecular analysis must be employed for taxonomic classification in this phylum. Because placozoans are invertebrates with morphological simplicity and plasticity, poor taxonomy confounds the resolution of this phylum.

Using molecular tools in taxonomy is hardly a new concept; the process dates back 30 years to when rDNA probes were used to identify eubacteria and archeabacteria (Fox et al., 1980). In fact one of the most prominent projects in oceanography today is the Census of Marine Life, a project that uses molecular similarities in mitochondrial DNA sequences to, in effect, "barcode" the life in the sea. Large-scale projects such as this will revolutionize taxonomy, however the validity of such approaches has been

controversial. A key assumption is that variation in mitochondrial DNA is congruent with variation in nuclear markers and that the variation can adequately divide closely related genotypes into naturally separated groups to define species limits (Vogler & Monaghan, 2006). This assumption allows the analysis of short sequences of the mitochondrial DNA to be used as a method for assigning species. However, most studies choose mitochondrial loci that are highly conserved. Diversity studies based on conserved regions could lead to an underestimation of true diversity, which would be detrimental to evolutionary studies, biodiversity measurements and conservation efforts. In organisms where there are substantial amounts of genetic variability in morphologically similar individuals, such as placozoans, it could be helpful to use another, more variable locus to describe the variation.

Past molecular research on placozoans relied on the sequences of the highly conserved 16S rDNA mitochondrial locus to assign haplotype (Pearse & Voigt, 2007; Signorovitch et al., 2006; Voigt et al., 2004). This project focused on finding a second, variable region on the mitochondrial genome as another mode to describe the diversity among placozoans. Mitochondrial loci, as opposed to nuclear loci, were considered because mitochondria usually evolve at a faster rate, allowing greater resolution between closely related individuals (Moritz et al, 1987). Also, the mitochondrion has a low (or zero) rate of recombination (Piganeau et al, 2004), is usually maternally inherited, and it has a simple (mostly circular, relatively small, and generally conserved) genetic structure. Nuclear loci, on the other hand, can be difficult to study due to heterozygosity, low chromosome copy number, and paralogous loci resulting in multiple copies of a single gene (Schubert et al, 2000). To date there have been four placozoan mitochondrial

genomes sequenced. Typical animal mitochondria are circular, relatively short (~16 kb), and code for 12-13 genes, 2 rRNA genes, and 22 tRNAs. Placozoan mitochondria, however, are much larger (from 32-37 kb). Although they contain the typical 12 respiratory chain genes and 2 rRNA genes, they code for 24 tRNAs, have large portions of intergenic spacer regions, several introns especially within the *cox*1 gene, and large open reading frames of unknown function (Fig. 12).

A region on the placozoan mitochondrion was identified that had conserved gene synteny in the four sequenced placozoan mitochondrial genomes (Signorovitch et al., 2007), namely the *cox*2 - *cox*1.1 region (Fig. 12). Primers were designed to encompass ~400 bp of the protein-coding *cox*2 gene, a highly variable intergenic spacer region, the methionine tRNA, more intergenic spacer region, and ~200 bp of the *cox*1.1 protein-coding gene (Fig. 12). Diversity within phylum Placozoa was assessed based on this locus and the traditional, conserved 16S rDNA gene locus.

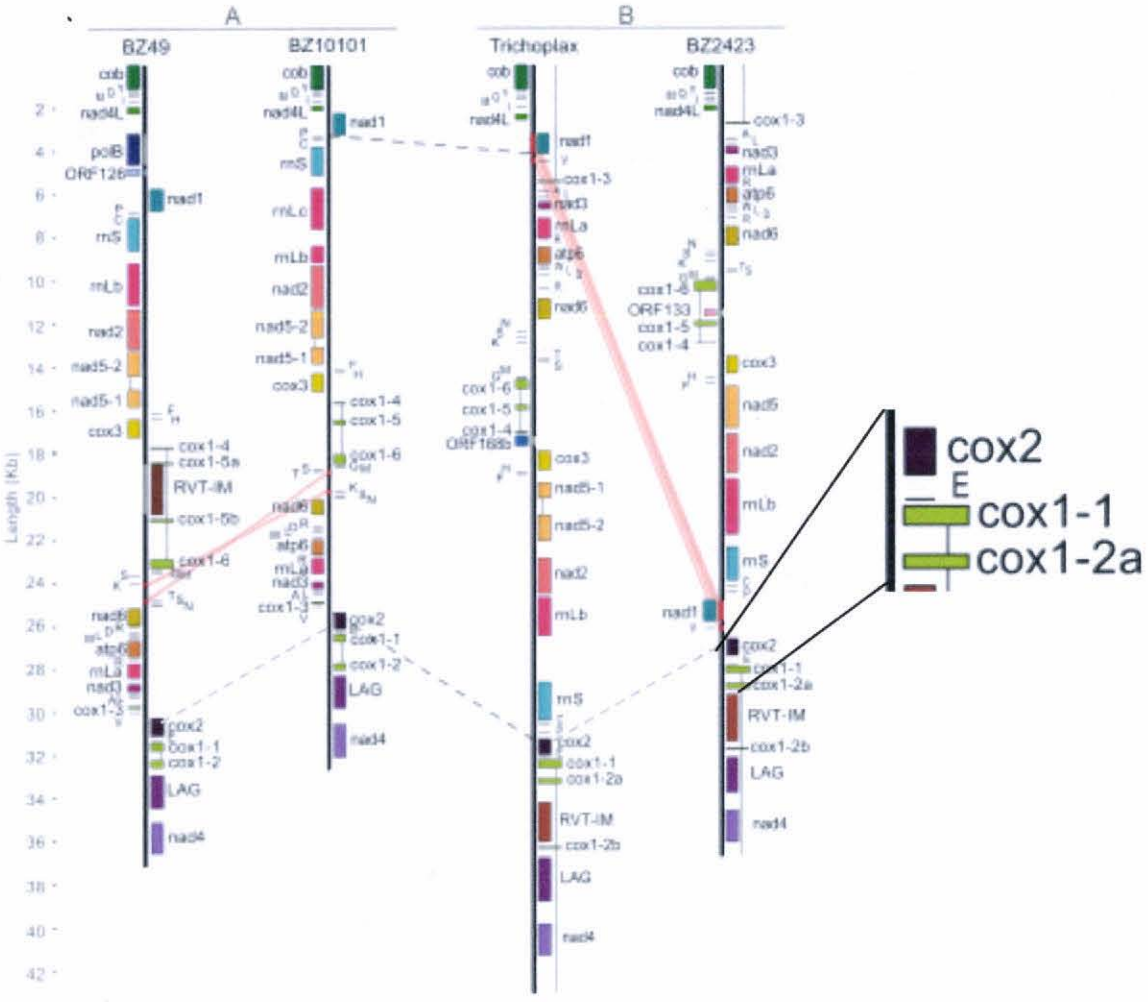


Figure 12. Map of the 4 sequenced placozoan genomes. Indicated is the *cox*2 - *cox*1.1 region. From Signorovitch et al., 2007

Materials and Methods

Isolation/Cultivation: One-hundred-thirty-five placozoan isolates were collected from slide racks placed on the bottom of a sea table containing corals and sponges collected from Pearl Harbor and maintained at Kewalo Marine Laboratory, Hawai'i. The slides were placed in the water for ~3 weeks. After which time, the slides were brought to the lab and examined for placozoans under a dissecting scope. Single placozoan isolates were transferred to a petri dish containing filtered seawater and filter feeder food (Liquifry Marine, (Interpet) contains dextrin pea flower, egg, yeast, spinach, and preservative). Placozoans were fed every 2-3 days and were kept at 26°C.

DNA Extraction: Of the 135 isolates collected, 30 dishes grew to contain ~ 15 individuals. Once the populations reached this density, the placozoans were transferred to a microfuge tube containing 450 µL of DNA extraction buffer (2% SDS, 100 mM EDTA, 20 mM Tris, pH 8.0). 12.5 µL of proteinase K (Roche) was added to the tube and the extraction was incubated at 50°C overnight with shaking. Afterwards, 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the tube. The tubes were then vortexed, centrifuged at maximum speed for 20 min, and the aqueous phase was transferred to a new tube. This step was repeated once. The supernatants were then extracted with 500 µL of chloroform:isoamyl alcohol (24:1) by vortexing, centrifuging at maximum speed for 2 min and transfer of the aqueous phase to a new tube. One volume of 95% ethanol was added to the extract and the tube was incubated at -80°C for 1.5 hours. Afterward, the tube was centrifuged at maximum speed for 20 min and the supernatant was removed, being careful to avoid disturbing the pellet. The pellet was

washed with 1 ml of 75% ethanol and centrifuged at maximum speed for 10 min. The supernatant was removed and the pellet was allowed to air dry ~40 min. The pellet was then resuspended in 50 µL of ultrapure water (Barnstead NanoPure®) and stored at -20°C. DNA was sequenced from 30 different isolates and the DNA was quantified on a Genespec low-volume spectrophotometer (Hitachi).

Primer Design: The 4 placozoan mitochondrial genomes that have been sequenced and deposited in the NCBI database were aligned for the portion spanning the entire *cox*2 gene to the *cox*1.1 gene using ClustalX. The consensus sequence was used in the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to design placozoan specific primers spanning 1300 bp including portions of both the *cox*2 gene and *cox*1.1 gene, 2 transgenic non-coding regions, and the tRNA coding for methionine.

PCR and Sequencing: The polymerase chain reaction amplifications were performed on a Biorad MyIQ® thermal gradient cycler. Amplification of a 700 bp fragment of the mitochondrial 16S rDNA region was done using primers designed by Signorovitch et al. (2006) as detailed in Chapter 2. PCRs of the *cox* region were carried out under the following parameters: 5 min at 94°C; then 38 cycles of 30 sec/94°C, 30 sec/58°C, 90 sec, 72°C; a final 5 min/72°C; and a hold at 4°C. Two µl of each extraction was used in the PCR reactions. To eliminate PCR and sequencing errors, two independent PCR reaction products were generated and sequenced in the forward direction. Only informative sites (Single Nucleotide Polymorphisms, SNPs) that appeared in both sequences were used in subsequent analysis. Sequencing was done by the University of Hawai'i Center for

Advanced Studies in Genomics, Proteomics and Bioinformatics on an ABI 3730XL. The BLAST program in GenBank was used to determine haplotype matches (Altshul et al., 1990). The sequences were aligned in ClustalX. The polymorphisms were counted by hand for each alignment within haplotype and between haplotype. Only *cox* sequences that had agreement with the 16S haplotype were used. Gaps were not analyzed.

Results

The results of the DNA extraction are shown in Appendix B. Although some of the concentrations are low, they all produced a band when 2μL of template was amplified with the placozoan-specific primers by Signorovitch et al. (2006).

After the DNA was extracted from each isolate, PCR amplifications were performed in duplicate for each isolate using the 16S rDNA gene primers and the *cox*2-*cox*1.1 primers that were designed using Primer3. The products were checked on a gel and the products that failed to produce a band were re-run until all samples produced a band or showed a product peak on the Biorad qPCR melt curve analysis software. The products were then cleaned using a Qiagen Qiaquick clean-up kit and directly sequenced. The resulting 16S rDNA gene fragment sequences varied in length from 116-552 bp and the *cox* sequences varied from 122-676 bp. The predicted length for the 16S rDNA gene fragment and *cox* fragment was 700 bp and 1350 bp, respectively. Each product was sequenced only in the forward direction (within the conserved *cox*2 gene product).

Table 1 shows the classification of each isolate and the haplotypes that were matched for each locus. As shown, sequences were not obtained for isolates 13, 17, 21, and 30 for the 16S rDNA gene, or for isolates 1, 3, 4, 5, 8, 10, 11, 12, 14, 15, 18, 23, and

24 for the *cox* locus. A "no" in the column indicates that the isolate failed to sequence for that loci. The "haplotype matched" is the top hit from a pairwise BLAST comparison against the NCBI sequence database.

Table 1. Best haplotype matches for each isolate for the 16S and *cox* loci

	Haplotype matched	
Isolate	16S	*cox*
1	1	No
2	1	1
3	1	No
4	6	No
5	6	No
6	6	1
7	8	8
8	8	No
9	4	1
10	8	No
11	8	No
12	8	No
13	No	8
14	1	No
15	1	No
16	4	4
17	No	4
18	2	No
19	2	1
20	6	1
21	No	1
22	2	1
23	2	No
24	1	No
25	2	1
26	1	1
27	2	1
28	1	1
29	1	1
30	No	4

As shown, there are a number of *cox* sequences that match to the H1 haplotype, this is due to the fact that only four placozoan mitochondrial genomes have been sequenced, the H1, H3, H4, and H8. It is possible that the isolates that match to H1 could in fact be H2 isolates because, as seen in the phylogenetic tree (Fig. 4) these two haplotypes are very similar, but because there is not a mitochondrial H2 genome, the match defers to the H1. For this reason, when the 16S rDNA and *cox* sequences match to different haplotypes within the same clade, it is not considered a discrepancy. Table 1 shows there are three discrepancies between the closest haplotype matches for the 16S rDNA and *cox* loci for isolates 6, 9, and 20. For isolate 6, the 16S rDNA gene sequence length was 417 bp with an e-value of 0.0 and an identity of 411/411 matching to H6 while the *cox* sequence length was 166 bp, an e-value of e^{-55} and an identity of 130/138. For this isolate the H6 haplotype is a better designation. For isolate 9, the 16S rDNA gene sequence length was 163 bp, an e-value of e^{-42} and an identity of 99/99 and 35/40 while the *cox* sequence length was 167 bp, an e-value of e^{-45} and an identity of 107/110. Because the quality of the sequence is the same for both loci, it is ambiguous which haplotype the isolate is and will be removed from further analysis. For isolate 20, the 16S rDNA gene sequence was 255 bp long, had an e-value of e^{-115} and an identity of 245/255 while the *cox* sequence was 212 bp long, an e-value of e^{-40} and an identity of 151/195, it also had two insertions and one deletion in the alignment. For those reasons, isolate 20 is most likely H6 haplotype. The number of Hawaiian placozoan haplotypes that could be inferred among these samples therefore differed between the 16s rDNA and the *cox* loci (Fig. 13 & Fig. 14).

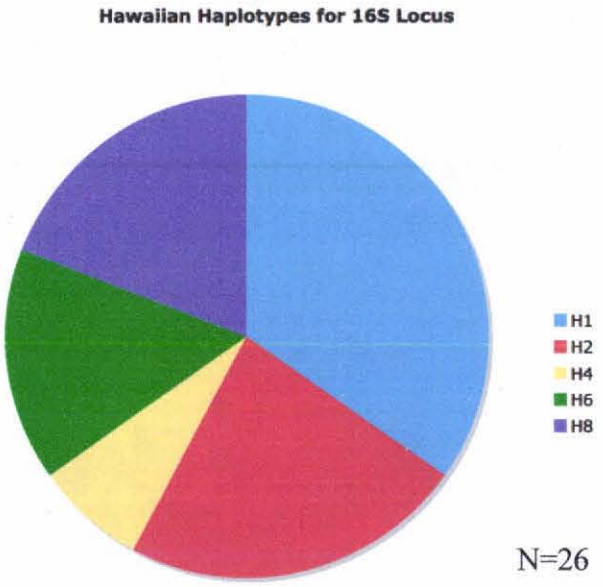


Figure 13. Pie chart of the community structure of Hawai'ian placozoans according to the 16S rDNA gene locus

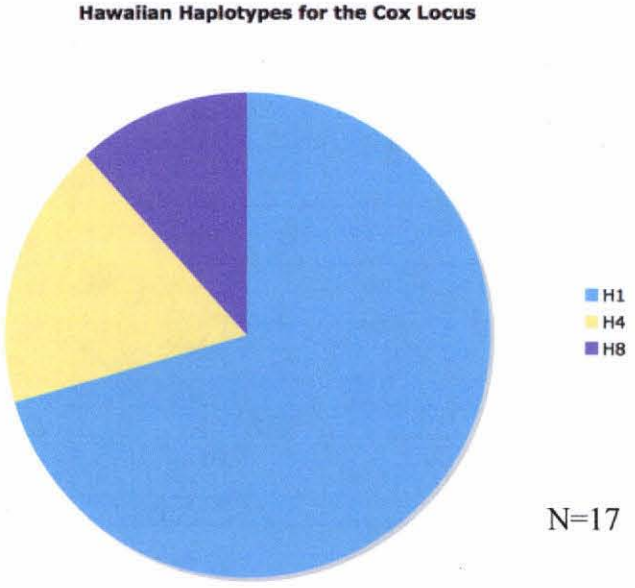


Figure 14. Pie chartsof the community structure of Hawai'ian placozoans according to the *cox* locus

In order to determine whether the 16S rDNA region or the *cox* region captured more of the variability between isolates, single nucleotide polymorphisms (SNPs) were calculated per length of sequence for each locus within each haplotype and between each haplotype (Table 2). The 16S rDNA dataset consisted of 20 sequences, 9 were H1, 2 were H2, 4 were H6, and 5 were H8. Isolates 9, 13, 16, 17, 18, 19, 21, 22, 27, and 30 were not used in the 16S rDNA dataset because of failed or bad sequence. The *cox* dataset consisted of only 8 sequences, 3 were H1, 3 were H4, and 2 were H8. Isolates 1, 3, 4, 5, 8, 10, 11, 12, 14, 15, 18, 20, 22, 23, 24, 27, and 29 were not used because of failed or bad sequence, while isolates 2, 6, 9, 19, and 21 were not used because the BLAST report indicated that it aligned with the *cox*1.1 gene instead of the *cox*2 gene and could therefore not be compared.

Table 2. Amount of single nucleotide polymorphisms per base pair within haplotype and between haplotype for each locus

	16S		***cox***
	Within haplotype SNP		Within haplotype SNP
H1	0.008	H1	0
H2	0.008	H4	0.0023
H6	0.005	H8	0
H8	0.003		
	Between haplotype SNP		Between haplotype SNP
H1 with H2	0	H1 with H4	0.112
H1 with H6	0.213	H1 with H8	0.126
H1 with H8	0.323	H4 with H8	0.047
H2 with H6	0.213		
H2 with H8	0.312		
H6 with H8	0.07		

The average within-haplotype variation for the 16S rDNA gene was 0.006 ± 0.0021, while the average within-haplotype variation for the *cox* locus was 0.0014 ± 0.00097. The average between-haplotype variation for the 16S locus was 0.1885±0.014, while the average between-haplotype variation for the *cox* locus was 0.095±0.0079. Surprisingly, the 16S rDNA locus contained more polymorphisms per base pair and was therefore better able to detect diversity. This can be explained by the fact that the *cox* sequences used in this dataset only spanned the coding region of the *cox*2 and were therefore conserved. The values indicate cryptic speciation is not occurring within haplotypes because the within-haplotype SNP average is very small. The between-haplotype variation, on the other hand, is consistent with genera-level taxonomic rankings in Cnidaria and Porifera which have average genera SNP per base pair values of 0.2243 and 0.1804, respectively, for the 16S rDNA locus based on sequences obtained from the NCBI database and aligned in ClustalX.

Maximum parsimony phylogenetic trees were constructed using the program PAUP 4.0 to illustrate the relationship between the haplotypes in Hawai'i according to the 16S rDNA (Fig. 15) and *cox* (Fig. 16) sequences obtained. The cnidarian *Metridium senile* was used as an outgroup in both trees (accession number for the 16S rDNA gene was AY345876, accession number for the *cox*2 gene was NC000933). The numbers on the internal nodes depict bootstrap values across 100 replicates. As shown, the 16S rDNA phylogram depicts the H6 and H8 as closely related and the H1 and H2 as closely related, this is consistent with other published results (Voigt et al., 2004; Pearse & Voigt, 2007). The *cox* phylogram shows H8 as more closely related to H4 than H1, this is also consistent with published results.

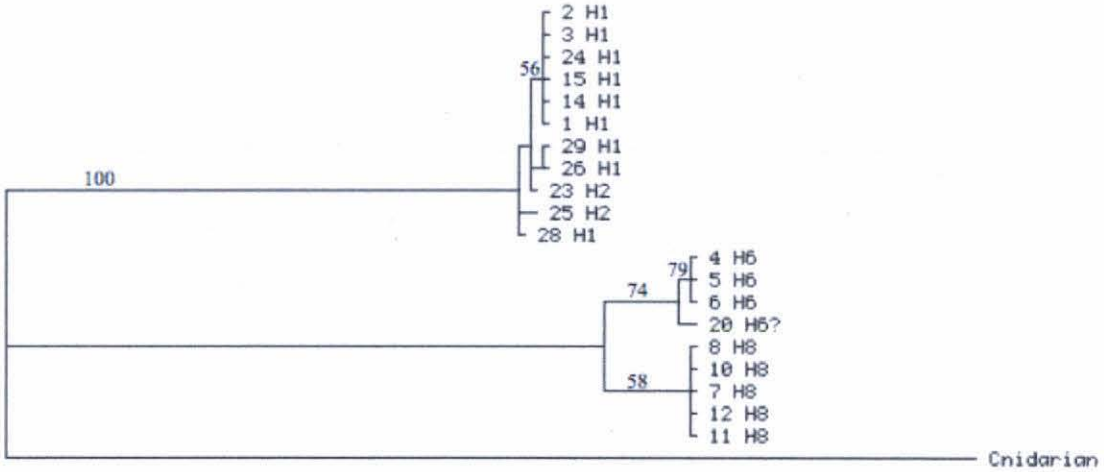


Figure 15. Maximum parsimony phylogenetic tree of haplotypes for 16S rDNA locus. Bootstrap values are indicated at internal nodes.

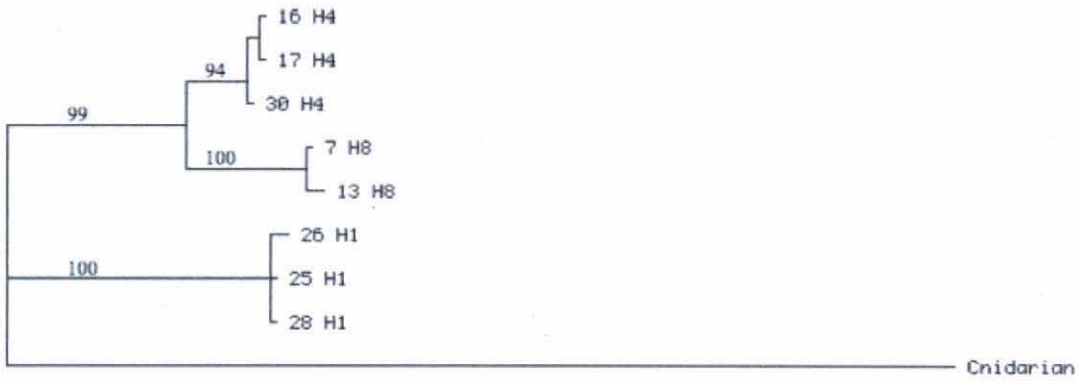


Figure 16. Maximum parsimony phylogenetic tree of haplotypes for *cox* locus. Bootstrap values are indicated at internal nodes.

As shown, there is diversity at the molecular level in the placozoan samples taken from Hawai'i. The extraction techniques developed resulted in good quality DNA and the Signorovitch 16S rDNA primers were used to successfully amplify the 16S rDNA region in the Hawai'ian placozoan mitochondria. The *cox* sequences were highly

variable in length, ranging from 100 – 650 bp. Generally, the *cox* sequences degenerated when the end of the *cox*2 gene was reached before going into the intergenic spacer regions, while 5 of the sequences gathered amplified the *cox*1.1a gene and could not be compared to the others.

A final study was done to compare divergence within placozoan haplotypes and diversity within the Poriferan and Cnidarian phyla. Sequences were obtained from the NCBI database corresponding to the *cox*1-3 and *nad*1-6 mitochondrial genes as well as nuclear genes alpha tubulin and heat shock protein 90 (HSP90) for a number of species within the Cnidarian and Poriferan phyla. Mitochondrial gene sequences were also obtained from the four placozoan mitochondrial genomes that have been sequenced. Primers were designed to amplify placozoan nuclear genes alpha-tubulin and HSP90. Hawai'ian placozoan isolates corresponding to H1 and H6 were then amplified using the alpha-tubulin and HSP90 primers and sequenced. Sequence alignments were created using ClustalX. A pairwise p-distance analysis was performed using the Kimura model. Mean values were calculated between placozoan haplotypes and between families within orders of the Poriferan and Cnidarian phyla. This was done for species and genera for these phyla as well. Although there is no clear relationship between taxonomic rank (esp. between genera and families) and p-distance values (Fig. 17), it is apparent that placozoan haplotypes have p-distance values well in excess of species-level rankings in other basal metazoans. This would suggest that a true delineation should be made between the placozoan haplotypes.

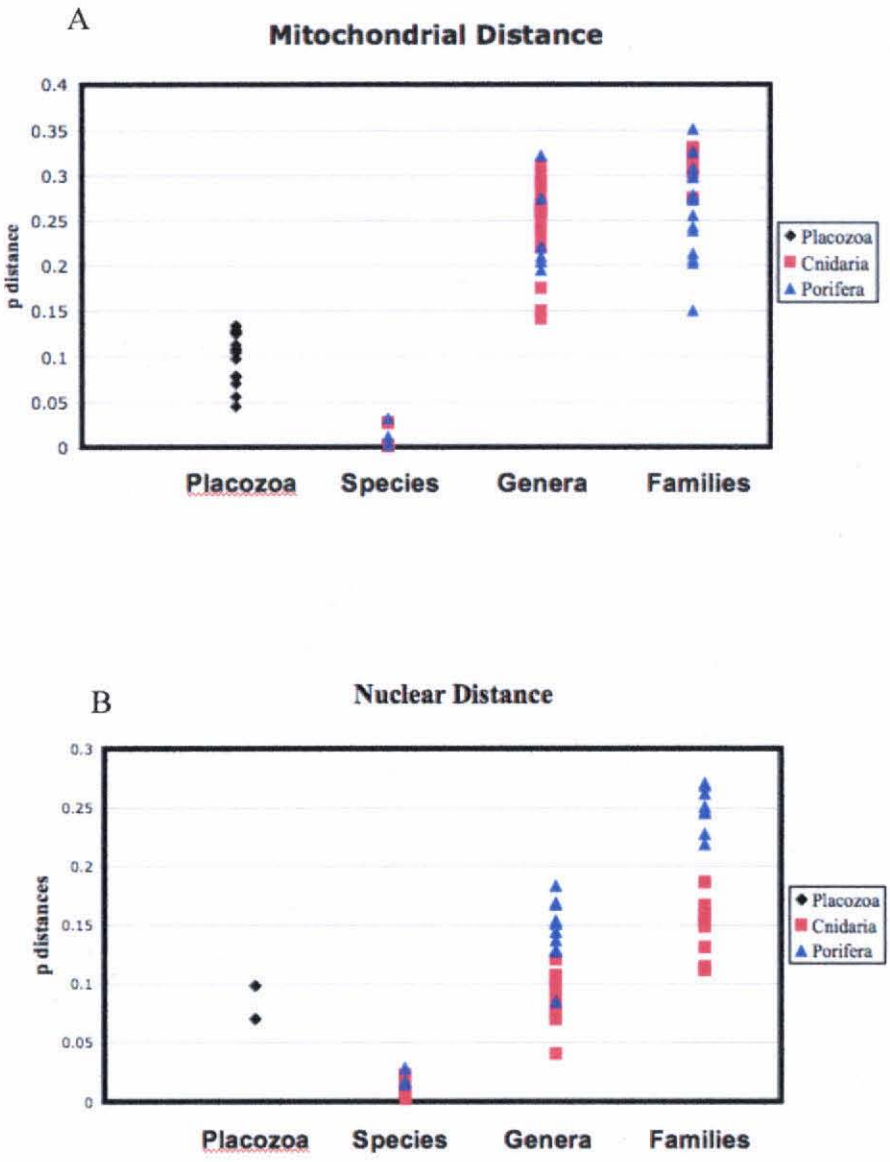


Figure 17. Average P-distance based on Kimura model for (A) mitochondrial genes *cox*1-3 and *nad*1-6 and (B) alpha tubulin and heat shock protein 90 between placozoan haplotypes and between species, genera, and families for Cnidaria and Porifera. P-distances among placozoan haplotypes are higher (thus more divergent than among species for Cnidaria and Porifera.

Discussion

Although placozoans are morphologically very similar, there is underlying cryptic diversity, resulting in the recognition of 11 distinct haplotypes. Molecular techniques must be utilized in order to determine the degree of variation between the different haplotypes. It is possible that sequencing a less conserved genetic region will lead to the discovery of even more variation within the phylum and more than the known 11 haplotypes will arise. More research is needed to identify a suitable locus for a less-conserved region appropriate for recognizing greater variability.

The Hawai'ian placozoan dataset illustrates that there are diverse placozoans found in sea tables at Kewalo Marine Laboratory. As indicated in this study, the 16S rDNA locus was able to capture more of the diversity within the placozoan phylum than the *cox* locus. This is due to the fact that the *cox* sequences were short in length (average of ~300 bp) and did not sequence into the more variable intergenic spacer regions. In effect, all of the *cox* sequences in this study only spanned the highly conserved protein-coding portion and would be expected to show less variability. In order to determine if sequencing the 16S rDNA locus is adequate to uncover all of the diversity of placozoans in the environment, development of new primers that span a separate variable region is necessary. There are two other possible mitochondrial targets that were identified in this study, a region spanning the placozoan open reading frame 2 into the *nad*4 gene and a region extending from the *cob* gene to the *nad*4L gene. These two loci should be targeted first because they have conserved gene synteny across the four placozoan mitochondrial genomes that have been sequenced by Signorovitch et al. (2006). Also in the future,

better sequencing reaction conditions must be identified to ensure that the sequencer will be able to span difficult regions, such as increasing the sequencing temperature.

The work in this study included 30 isolates collected from a single location in a seawater table at one time of the year. Future work should include sampling at different times within the year and across a number of years to determine if the haplotype structure is maintained or if it is variable. Environmental parameters should also be assessed to see if life cycle changes are brought on by specific environmental cues. A concerted effort to find placozoans both in the water column and on the benthos in different locations other than in an artificial environment (seawater table) should also be done, such as deployment in mangrove forests around the island of Oahu, Hawai'i.

Chapter 4. Placozoan biogeography

Introduction

Geographic isolation followed by positive selection and adaptive radiation has been demonstrated to be a strong force in speciation for terrestrial (esp. island) organisms (MacArthur & Wilson, 1967). However, speciation events in the marine realm, where there are few barriers to widespread dispersal and a comparatively high level of gene flow possible, are less understood (Palumbi, 1994). In these cases of marine population sympatry, it is possible that assortative mating and/or habitat preference becomes important in speciation (Bierne, 2003). Marine organisms that have a planktonic life stage, are relatively small (≤2mm), and have larger population sizes are generally considered to be cosmopolitan (Finlay, 2002).

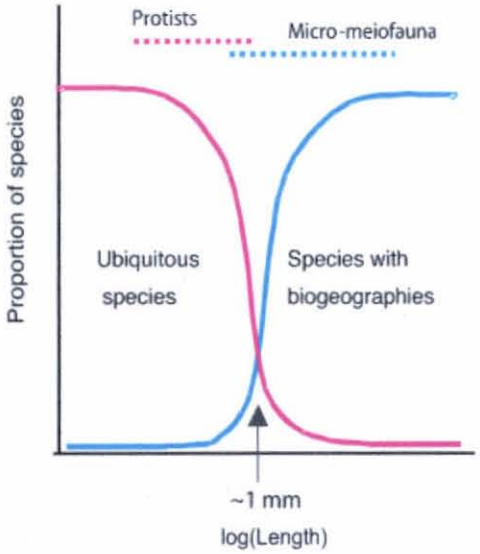


Figure 18. A hypothetical model of the ubiquity-biogeography transition as a function of organism size from Finlay, 2002

It is well known that large animals have restricted geographic ranges, or a biogeography. Long-term isolation, in many cases, led to speciation. Microbes, on the other hand, are found in nearly every aquatic niche worldwide and are subjected to numerous mechanisms of dispersal such as hurricanes, ocean circulation and ballast

water release. Therefore not only are microbes found globally, but often the same species are found again and again. For example, it is quite common to find a ciliate in New Zealand that is nearly identical to a European counterpart (Finlay, 2002). In 1934, this observation led a Dutch microbiologist named Lourens Bass-Becking to exclaim "everything is everywhere" and the proposition that organisms smaller than 1-2mm have no biogeography (Finlay, 2002; Fig. 18). It is not clear whether this is the case with placozoans. Figure 5 (reproduced below) shows the geographic distribution of placozoan haplotypes to date. As shown, not all haplotypes have been reported at all locations, but whether this represents actual biogeography or is the result of limited sampling (often only 5-20 isolates are recovered per location) at any given site is uncertain. Successive sampling does however tend to recover previously reported haplotypes and many haplotypes have a very broad distribution.

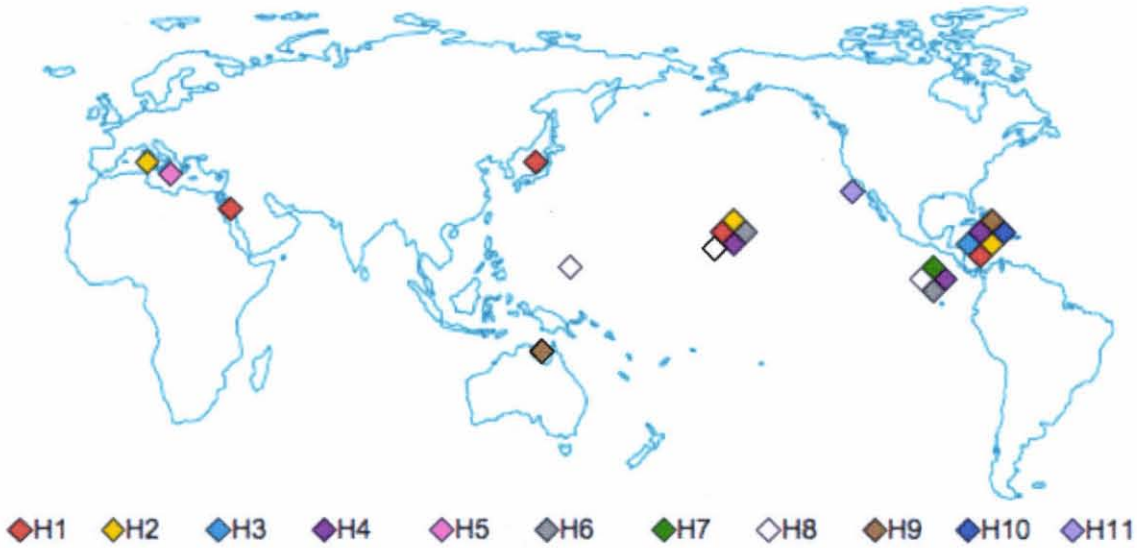


Figure 5. World map illustrating haplotype distribution. Haplotype information collected from this project as well as data from Pearse & Voigt (2007).

As a result of research presented in this thesis, Hawai'i is currently the most sampled site of any location for placozoans and diverse haplotypes (H1, H2, H4, H6 and H8) have been obtained from seawater tables at Kewalo Marine Laboratory, Hawai'i. To explore placozoan biogeography, a second, geographically distant, and previously unsampled location was chosen as another field site. Seventy-eight samples were obtained from traps placed in 7 locations on the islands of Puerto Rico and Isla Magueyes, 14 of those samples amplified with the 16S rDNA primers. Given that placozoans are ~2mm in diameter and have been found in many locations around the world, this would suggest that placozoans have a ubiquitous distribution. This section sought to describe the community structure of placozoans in these two distant regions and to determine if the same species that are also present in Puerto Rico are found in Hawai'i. If placozoans are ubiquitous, then it would be assumed that the haplotypes found in Hawai'i would also be found in Puerto Rico.

One challenge to describing the true environmental diversity of any location is limited sample sizes. Small sample sizes can lead to an underestimate of diversity by not capturing all haplotypes present. Without large sample sizes, it is impossible to know whether a specific placozoan haplotype is simply not found at a location, or whether that haplotype was there and missed due to insufficient sampling. In this section, a mathematical probability graph was used to illustrate the effects of sample size on the acquisition of haplotypes between two geographic locations.

Materials and Methods

Isolation: Slide racks were suspended 150mm off the bottom of the seafloor. The field sites included 3 piers on Isla Magueyes, Puerto Rico and 1 pier on the main Puerto Rican island (Figure 19, detailed sampling protocol in Appendix A). The slides were placed in the water for ~3 weeks. After which time, the slides were brought to the lab and examined for placozoans under a dissecting scope. Single placozoan isolates were transferred to a microfuge tube containing 50 μL of DNAzol (Invitrogen). The tubes were placed on a rack and transported back to the Hawai'i laboratory at room temperature.

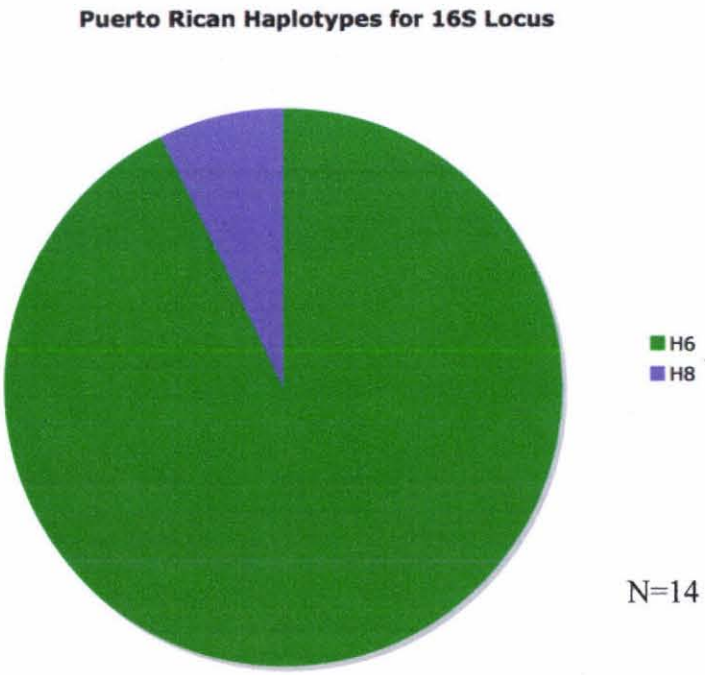
Figure 19. Picture of the slide traps used to collect placozoans in the field and a map of the Puerto Rican field site, Isla Magueyes

PCR and Sequencing: The polymerase chain reaction amplifications were performed on a Biorad MyIQ® thermal gradient cycler. Amplification of a 700 bp fragment of the 16S rDNA region was done using primers designed by Signorovitch et al. (2006) as described in Chapter 2 and the *cox* primers designed with Primer3 as described in Chapter 3. To eliminate PCR and sequencing errors, two independent PCR reaction products were generated and sequenced in the forward direction. Electrophoresis gels were run of the

PCR products; only products that displayed a band were sent for sequencing. Gels were run at 75 volts for 1 hour and products were run with a 100 bp DNA marker. Each gel was stained with SYBR green. Sequencing was done by the University of Hawai'i Center for Genomics, Proteomics and Bioinformatics on an ABI 3730XL. Sequences were compared to the NCBI BLAST database to determine the best haplotype match. Only informative sites (SNPs) that appeared in both sequences being compared were used in subsequent analysis.

Results

A protocol was developed to amplify directly from a single placozoan isolate in a 50 μL sample of DNAzol. The working template volume was 2 μL of a 1/20 dilution into water of the 50 μL starting sample. This was performed on all 78 samples brought back from Puerto Rico, however, the 16S fragment successfully amplified from only 14 of those samples. Although, the bands were checked for amplification using the *cox* primers, the bands appeared as a smear on the gel and resulted in ambiguous sequences. Therefore, only the 16S rRNA gene sequences, which ranged in length from 120-413 bp, are considered here.



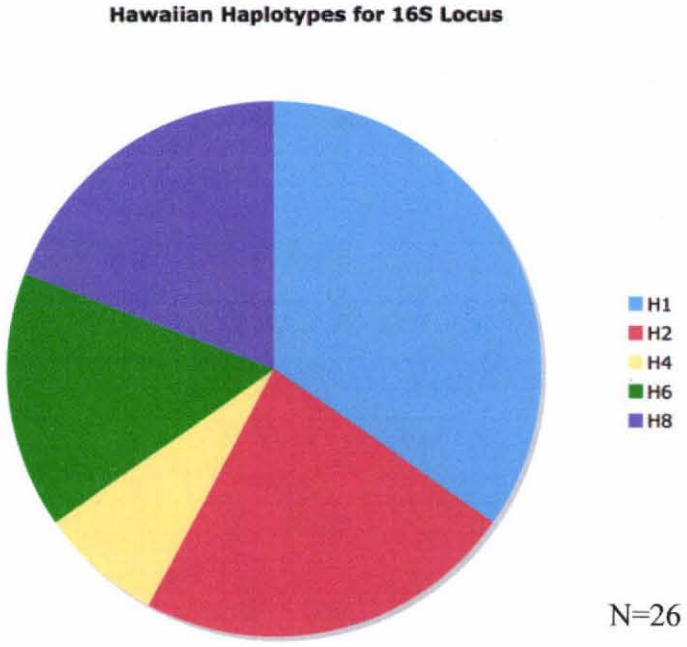


Figure 20. Pie chart of the community structure of the Puerto Rican samples based on sequences of the 16S locus and a reproduction of Fig. 13 from Chapter 3 of the community structure of Hawai'ian placozoans according to the 16S locus sequences

All of the placozoans that were successfully amplified from the Puerto Rico site belonged to clade III according to the 16S rDNA sequence analysis (Fig. 20). This is in contrast to the high diversity recovered in Hawaii. This is surprising because previous research (Signorovitch, et al., 2006) showed that 20 placozoan strains taken from various locations in the Caribbean (i.e. Panama and Venezuela) included representatives from 4 of the placozoan clades.

To determine if this apparent lack of diversity in the samples taken from the Puerto Rican waters was significant, a mathematical probability test was performed. The following equation was used to determine the probability that there will be at least one haplotype found in region 1 that will not be found in region 2 as a function of the number of samples and the number of haplotypes:

Equation 1: $$1-\left[(1-\frac{1}{H})^{N} x(1-\frac{1}{H})^{M}+(1-(1-\frac{1}{H})^{N})x(1-(1-\frac{1}{H})^{M})\right]^{H},$$

where H= the number of possible haplotypes, M= the number of samples taken from region 1, and N= the number of samples taken from region 2 (Equation 1, derivation in Appendix C). This calculation assumes that all haplotypes present at a given location are present in equal abundance according to the neutral theory of biogeography. The actual total number of haplotypes (H) is not known, so probabilities were calculated for a range of possible values of H. To date, there are 6 placozoan clades, 11 known haplotypes, and there are possibly even more haplotypes that haven't been discovered yet due to lack of sampling. By focusing on the hypothetical red line in the graph (haplotype #=8), it is possible to see that there is a high probability (~0.8) that there will be at least one

haplotype found in region 1 (Hawai'i) that will not be found in region 2 (Puerto Rico) or vice versa for sample sizes of 30 isolates from region 1 and 15 isolates from region 2.

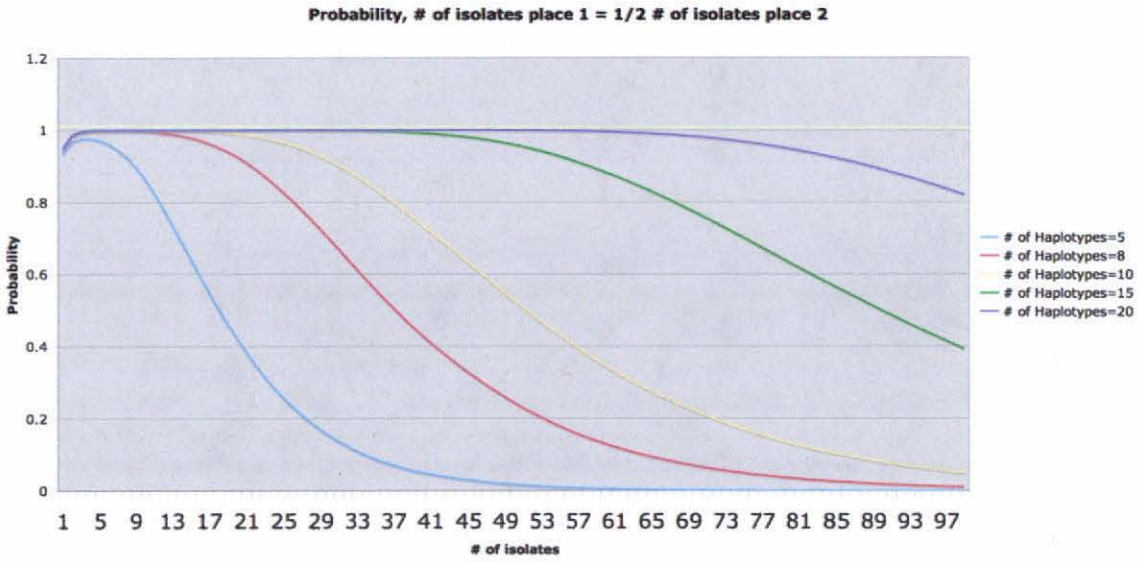


Figure 21. Graph derived from equation 1 depicting the number of samples taken from two different locations and the probability of finding the same haplotype at each place as a function of the number of haplotypes assuming an equal number of haplotypes are present.

As shown in the graph, it would take many samples (>73) from region 1 and >37 samples from region 2 to have a <10% probability of finding a new haplotype in one sample but not the other if there are only 10 haplotypes. Because the sample size was of insufficient size in both Hawai'i and Puerto Rico, it is not surprising that Haplotypes H1, H2, H3, and H4 were obtained from Hawai'i but not from Puerto Rico. Also, if more than 11 haplotypes exist, the minimum required number of samples from each geographic region increases as well.

Discussion

This is the first report of placozoans in Puerto Rico. In total, 78 isolates were collected on glass slides during the month of July. Attempts were made to grow the placozoans in Petri dishes. In Puerto Rico, the placozoans were fed the Liquifry Marine (Interpet) filter feeder food and grown in the air-conditioned laboratory, on the sidewalk in the sun, and outside in a shaded shelter. None of these attempts resulted in the placozoans dividing. Therefore, single isolates had to be manipulated in further experiments. Of the 78 samples collected, only 14 amplified.

There are a number of reasons for the paucity of successful amplifications from the Puerto Rican samples. One possibility is that the DNA template concentration was too low. Trying to amplify from a single individual is difficult. Each PCR reaction was only used 2 μl of a 50 μl sample volume diluted 1/20 (i.e. only 0.002 μl of the original sample/organism per PCR reaction). Another possible cause of failed reactions is inhibition of the PCR enzymes by the DNAzol. The Invitrogen DNAzol product guide indicates that dilution of DNAzol is necessary to prevent inhibition, however the dilution maximum for the Puerto Rican samples was limited because of the low concentration of the template. Another possibility is that the Puerto Rican placozoan ribosomes contained secondary structure that confounded sequencing efforts (a review of the difficulties with secondary structure can be found in Kozak, 1991).

The reason that the placozoans isolated in Puerto Rico failed to divide is unknown. It is possible that the filter feeder food had spoiled during travel, making the food unsuitable and resulting in placozoan degeneration. The DNAzol method was the best available for transporting isolated cultures, however it meant that amplification was

required from very little template. In the future, it would be helpful to grow the organisms on location to at least a density of 10 individuals before performing a DNA extraction. This will allow for a greater amount of template to be used in the PCR amplification process and hopefully result in brighter bands. Having pure DNA extracts will allow for more opportunities to troubleshoot the amplification process and rule out issues such as inhibition. Although amplification from single isolates adhered to Gene Guard filter paper (FTA®) has been documented before (Signorovitch et al., 2006), the materials were not available at the time of field collection from Puerto Rico. In this study, DNAzol was used to preserve the single Puerto Rican isolates, resulting in very dilute samples.

Community structure analyses are difficult to interpret with low sample numbers (Fig. 22), therefore future work should include more than 14 isolates to reliably compare the community structures between two geographic regions. Ideally, equal numbers of samples from the two sites would be evaluated. Still, this project provided information from a previously unstudied location, Puerto Rico, and found an overlapping distribution of haplotypes H6 and H8 in Hawai'i and Puerto Rico.

Chapter 5. Discussion and future work

Overall Discussion

In most of the metazoan phyla, Linnean taxonomic ranking results in an abundance of classes, subclasses, orders, families, genera, and species. The Placozoa phylum, on the other hand, contains only a single described species. Morphologically these organisms are indistinct, however molecular diversity relative to the genus-level in other basal phyla have been demonstrated across a number of genetic loci resulting in the delineation of 11 haplotypes. This project sought to describe the diversity found in placozoan isolates found in Hawai'i and Puerto Rico as well as to compare levels of diversity identified from sequencing two genetic loci.

It has been demonstrated that placozoans exhibit a high level of diversity within the phylum when comparing mitochondrial sequences. Based on the sampling in this project, more diversity was found within the samples taken from Hawai'i than those obtained from Puerto Rico. Haplotypes H1, H2, H4, H6, and H8 were recovered from Hawai'i, while only haplotypes H6 and H8 were recovered from Puerto Rico. It was also found that the 16S rDNA and *cox* loci performed equally well for assessing the diversity within haplotypes, while analysis of the *cox* locus was better for distinguishing the H1 haplotype from H6 and H8 and the H6 haplotype from the H8.

Slides were placed in 7 locations on Oahu (Appendix A), however, only the slides placed in sea tables containing sponges and cnidarians at Kewalo Marine Laboratory collected specimens. The sea table sponges and cnidarians at Kewalo were transported there from Pearl Harbor (M. Hadfield, pers. comm.). A study by Coles et al. (1999) was

done to assess the overall organismal diversity in Pearl Harbor, Hawai'i. They found a number of introduced species with natural habitats in the Indo-West Pacific, as well as several species indigenous to the Red Sea (belonging to Phyla Mollusca and Arthropoda) and the Caribbean Sea (belonging to Phyla Porifera and Cnidaria). Many placozoan haplotypes that have been collected from the Kewalo sea tables are also found in the Red Sea (H2) and the Caribbean (H1, H2 and H4). It is possible that the placozoans were introduced to Hawai'i by accompanying other organisms such as mollusks, sponges, and cnidarians.

Hawai'i is now the most extensively sampled location for placozoans. Another accomplishment of this thesis work was that it provided information on a previously unsampled location (Puerto Rico). This project also provided preliminary data suggesting that placozoans can be found in the water column, providing insight into placozoan life cycle. Along with the data, a number of important protocols, both for cultivation and molecular work, were developed to aid in future research on placozoans.

Future Work

After analyzing the placozoan-specific primers used to amplify the 16S rDNA, it was found that the primers differ by two base pairs from sponge rDNA. Designing new primers that are more specific to a less conserved region will be a better tool for determining if amplification of placozoan DNA is occurring from the water column DNA extracts. The presence or absence of a bright band on a gel will be a preliminary positive result, indicative of placozoans in the water column before sequencing the product. Continuing the water column work using quantitative methods such as qPCR or

fluorescent in situ hybridization would be of value to determine whether placozoans spend at least a portion of their life in the water column. It would also be interesting to sequence the resulting amplicons to determine whether the community structure in the water column is similar to that found settling on the benthos.

Another worthy project would be to design new primers like those of the *cox* region and troubleshoot the sequencing process in order to sequence over the intergenic spacer region. This would result in highly variable sequences across many haplotypes that could be analyzed for diversity, possibly uncovering new haplotypes, or lending additional support for the current 11 haplotypes that are already defined. Simply increasing the temperature in the sequencing oven may help to sequence across a highly variable region with possible secondary structure and should be attempted in the future. None of the *cox* sequences were longer than ~500 bp (never reaching the methionine tRNA). The product was supposed to be 1350 bp. Also, DMSO can be added to the reaction, which reduces secondary structure folding and could aid in obtaining quality sequence (Shen & Hohn, 1992).

Another future project to carry out would be a rigorous sampling program across a number of years. In this project, the slides were deployed when convenient and collected anywhere from 3 weeks to 2 months later. It was found that placozoans were present on slides from July-September, but not every month of the year was sampled and alternate locations were not sampled until March, 2008. For a more complete sampling, I would suggest deployment at Kewalo Marine Laboratory, Pearl Harbor, He'eia, and the mangroves around Kahuku. I would suggest deployment once a month and collection exactly one month later while recording temperature, salinity, and turbidity. This project

should run for a minimum of 2 years and could be combined with water column extractions as well. This would provide information on whether Hawai'ian placozoans are found only at certain times of the year, whether community structure changes throughout the year and whether temperature and salinity differences affect the presence of placozoans.

A final suggestion for future work would be to more extensively sample a second location, as well as obtain more samples from the Hawai'i location. By having a thorough sampling from one location, such as 100 isolates, it would be possible to make some conclusions about whether or not some haplotypes are being overlooked or undersampled and to start comparing relative abundances of the different haplotypes. In order to reliably compare community structure between two geographic locations, more samples must be obtained to get a low probability of finding a haplotype in Hawai'i that is not at a second location. Therefore, more extensive sampling must be done at the Puerto Rico location and more efficient methods of amplifying from single isolates must be established.

The work presented here has provided a glimpse of placozoan diversity in both Hawai'i and Puerto Rico. Many protocols were generated to grow and maintain placozoan cultures in the laboratory, to perform DNA extractions on a limited number of placozoans from cultures and from GF/F filters, to perform RNA extractions, to create cDNA libraries, to achieve PCR amplification from DNA extracts as well as single isolates, and to make clone libraries. Using these tools, a number of new projects can begin. Even now, few laboratories are working on these enigmatic creatures and little is known. Each new project undertaken leads to new discoveries for phylum Placozoa.

Appendix A. Detailed sampling protocol

Detailed Collection of Placozoans: Hawai'i

Placozoans were collected from Kewalo Marine Laboratory, Station 1 (Fig. 22), on four separate occasions: 7/2004 (slides deployed for weeks, found many placozoans, 5 extracted), 7/5/06 (slides deployed 5/5/06, found many placozoans, 3 extracted), 7/29/07 (slides deployed 6/3/07, found 3 placozoans, none extracted), 9/18/07 (slides deployed for several weeks, found 135 placozoans, 21 extracted). Also slides were collected on 10/17/07, 1/18/08, 2/27/08, and 3/26/08. No placozoans were found on these occasions. Slides were deployed at Station 2, He'eia Fishpond, on 8/24/07 and collected 9/13/07 and again deployed 9/15/07 and collected 10/24/07. No placozoans were found on these occasions either. Slides were deployed at Station 3 on 2/29/08 and collected 3/26/08. No placozoans were found. Slides were deployed at stations 4-7 on 3/15/08 and collected 4/28/08. No placozoans were found on this occasion either. To date, Kewalo Marine Laboratory is the only location where placozoans have been collected in Hawai'i.

The field work in Puerto Rico and other published studies (esp. Pearse & Voigt, 2007) suggest that placozoans grow well in areas dominated by mangroves and coral and less well in sandy bottom areas. The slides around Oahu were deployed only where mangroves were common and left for 3 weeks to 2 months. Past collections also suggested that placozoan presence could be seasonal. My own sampling on Oahu indicated that placozoans are found most often in the summer months and less often in the winter/spring months. Deployment of slides in Pearl Harbor (Station 3) and Stations 4-7 were done in the spring and could be one reason why placozoans were never

recovered there. A more rigorous sampling schedule is needed to determine whether placozoans can be found in the open environment in Hawai'i, or whether placozoans are an artifact of the seawater table system found at Kewalo Marine Laboratory.

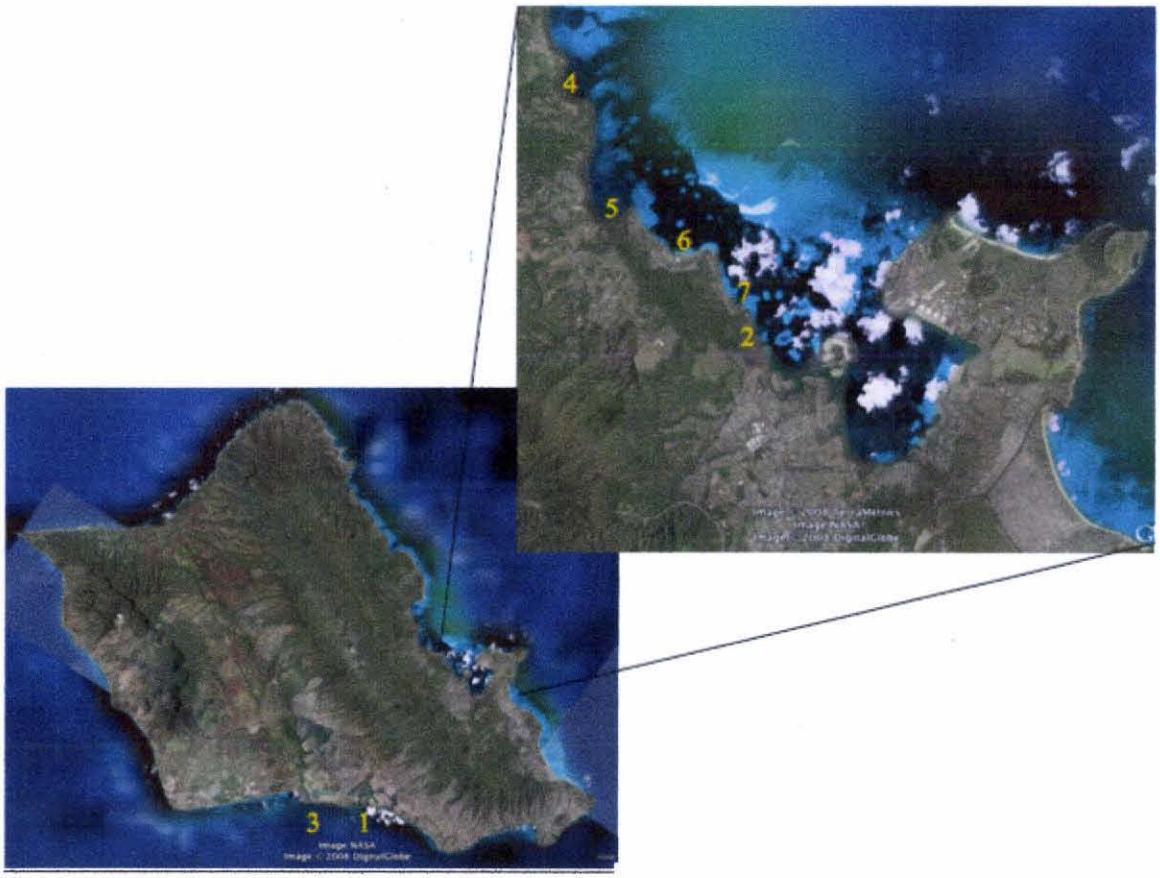


Figure 22. Map of field stations in Hawai'i

Detailed Collection of Placozoans: Puerto Rico

Slides were deployed twice in Puerto Rico, once on 6/15/07 and collected on 7/12/07 and deployed again on 7/13/07 and collected on 7/21/07. Seven traps were set out in four separate field stations and one aquarium containing fire coral. Every slide deployment resulted in the acquisition of placozoans. The field stations are shown in Fig. 23. Traps 1 and 2 were deployed at Station 1, traps 3 and 4 were deployed at Station 2,

trap 5 was deployed at Station 3, trap 6 was deployed at station 4, and trap 7 was deployed in the aquarium in the host lab. Table 3 details the placozoans that were collected and isolated in DNAzol (Invitrogen), the date of collection, the station number and trap number and whether amplification of the 16S rDNA was successful.

Figure 23 Map of field stations in Puerto Rico

Table 3. Puerto Rican samples collected from each trap and each station

Collection Date	Station #	Trap #	Isolate #	Amplification?
7/12/07	1	1	1	yes
7/12/07	1	1	2	yes
7/12/07	1	1	3	yes
7/12/07	1	1	4	yes
7/12/07	1	1	5	yes
7/12/07	1	1	6	yes
7/12/07	1	1	7	yes
7/12/07	1	1	8	yes
7/12/07	1	1	9	no
7/12/07	1	1	10	no
7/12/07	1	2	1	no
7/12/07	1	2	2	no
7/12/07	1	2	3	no
7/12/07	1	2	4	no
7/12/07	1	2	5	no
7/12/07	1	2	6	no
7/12/07	1	2	7	no
7/12/07	1	2	8	no
7/12/07	1	2	9	no
7/12/07	1	2	10	no
7/12/07	1	2	11	no
7/12/07	1	2	12	no
7/12/07	1	2	13	no
7/12/07	2	3	1	no
7/12/07	2	3	2	no
7/12/07	Aquarium	7	1	no
7/12/07	Aquarium	7	2	yes
7/12/07	Aquarium	7	3	yes
7/12/07	Aquarium	7	4	yes
7/21/07	1	1	1	no
7/21/07	1	1	2	no
7/21/07	1	1	3	no
7/21/07	1	1	4	no
7/21/07	1	1	5	no
7/21/07	1	1	6	no
7/21/07	1	1	7	no
7/21/07	1	1	8	no
7/21/07	1	1	9	no
7/21/07	1	1	10	no
7/21/07	1	2	1	no
7/21/07	1	2	2	yes
7/21/07	1	2	3	yes

Table 3. (Continued) Puerto Rican samples collected from each trap and each station

7/21/07	1	2	4	no
7/21/07	1	2	5	no
7/21/07	1	2	6	no
7/21/07	1	2	7	no
7/21/07	1	2	8	no
7/21/07	1	2	9	no
7/21/07	2	3	1	no
7/21/07	2	3	2	no
7/21/07	2	3	3	no
7/21/07	2	3	4	no
7/21/07	2	3	5	no
7/21/07	2	3	6	no
7/21/07	2	3	7	no
7/21/07	2	3	8	no
7/21/07	2	4	1	no
7/21/07	2	4	2	no
7/21/07	2	4	3	no
7/21/07	2	4	4	no
7/21/07	2	4	5	no
7/21/07	2	4	6	no
7/21/07	2	4	7	no
7/21/07	2	4	8	no
7/21/07	3	5	1	no
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7/21/07	3	5	5	no
7/21/07	3	5	6	no
7/21/07	3	5	7	no
7/21/07	3	5	8	no
7/21/07	4	6	1	no
7/21/07	4	6	2	no
7/21/07	4	6	3	no
7/21/07	4	6	4	no
7/21/07	4	6	5	no
7/21/07	4	6	6	no

Appendix B. DNA extraction concentrations

Table 4. Table of UV absorption ratio and concentration for the 30 placozoan isolates collected from Hawai'i

Isolate	A260/A280 Ratio	Concentration (ug/ ml)
1	1.717	39.5
2	1.204	110.1
3	1.88	66.7
4	1.44	63.1
5	2.043	48
6	1.895	36
7	1.659	25.2
8	1.298	16.35
9	1.267	70.76
10	1.303	43
11	1.271	30.95
12	1.881	34.8
13	1.87	56.85
14	2.206	37.5
15	2.347	57.5
16	1.296	8.1
17	1.39	8.6
18	1.721	10.5
19	1.957	27
20	1.182	13
21	1.645	13.65
22	1.314	7.5
23	1.702	9.7
24	1.885	9.8
25	1.459	7.95
26	1.551	3.8
27	1.357	3.8
28	3.875	2
29	1.071	15
30	5.54	12.2

Appendix C. Derivation of Equation 1

H=# haplotypes

N=# of isolates from region 1

M=# of isolates from region 2

$h=1-\frac{1}{H}$ = probability that a given isolate is NOT of a specific haplotype

h^N = probability that N isolates from region 1 are all not of a specific haplotype

$1-h^M$ = probability that a specific haplotype from region 2 appears at least once

$h^N(1-h^M)$ = probability that a haplotype found at least once in region 2 never appears in region 1

$h^M(1-h^N)$ = probability that a haplotype found at least once in region 1 never appears in region 2

The probability that the sample is not of a given haplotype from either region 1 or region 2 is: $1-\left[h^N(1-h^M)+h^M(1-h^N)\right]$. By combining the terms, it follows: $=h^Nh^M+(1-h^N)(1-h^M)$. The probability that at least one of each of H haplotypes is found in the sample taken is determined by raising the equation to the H power: $=\left[h^Nh^M+(1-h^N)(1-h^M)\right]^H$. The probability that at least one haplotype from region 1 will not be found in region 2 is: $=1-\left[h^Nh^M+(1-h^N)(1-h^M)\right]^H$. By substituting the terms back in, the final equation is:

$$1-\left[(1-\frac{1}{H})^N x(1-\frac{1}{H})^M+(1-(1-\frac{1}{H})^N)x(1-(1-\frac{1}{H})^M)\right]^H.$$

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