Studying the Genetic Structure of Sponge Populations

by

Evelyn Lise Jensen

A REPORT SUBMITTED IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR BIOL 499

Supervisor: Dr. Sally Leys

Co-supervisor: Dr. Corey Davis

UNIVERSITY OF ALBERTA

2011

Abstract

The sponge reefs, found only off the coast of British Columbia, Canada, are a unique and alien deep sea ecosystem that is not well understood and is threatened by human activities. Here I developed a genetic barcode to differentiate between the two most common sponges on the reefs, *Aphrocallistes vastus* and *Heterochone calyx*. I also developed microsatellite and mitochondrial marker systems to determine the population genetics of *A. vastus* to see if there is genetic structure at the reef level in the Strait of Georgia. Both the microsatellite and mitochondrial markers indicate that the reefs in the Strait of Georgia are all part of a single genetically homogeneous population. It does not appear that asexual reproduction in *A. vastus* is a significant process in the growth of reefs. To determine the mechanisms by which gene flow is occurring between reefs, further investigations into larval behaviour are needed.

Introduction

Sponge taxonomy is currently undergoing a "splitting" phase. Researchers are finding that even very small morphological differences, such as colour, can actually be an indication of species-level differentiation (Boury-Esnault and Solé-Cava 2004). Based on this finding, there is increased interest in the population genetics of sponges. It is believed that sponge populations are highly structured as a result of limited dispersal ability and strong exclusion from habitats which do not meet specific microclimate requirements (Boury-Esnault and Solé-Cava 2004). Structured and reproductively isolated populations of sponges are likely to be, or evolve into distinct species. If we know how populations are genetically structured, we can begin to make inferences about systematics and evolution, and make informed conservation decisions.

The reproductive biology of sponges has been studied extensively in a few species; however there is great variability in reproductive process among species, so generalizations are not easy to make (Bergquist 1978). Regular sampling over long periods of time are required to determine the reproductive cycle of a species, which is difficult to achieve, particularly in subtidal or deep sea sponges (Bergquist 1978). All known sponges are capable of sexual reproduction, and in general are hermaphroditic. Most sponges are also capable of asexual reproduction through gemmule formation, budding and dripping tissue (Bergquist 1978).

One of the questions of interest in sponge population genetics is how common is asexual reproduction? If it is a common process, is it an important factor is structuring populations? The sessile nature of sponges means that dispersal only occurs during reproductive processes, and knowing the relative occurrence of sexual and asexual reproduction would provide a lot of insight into sponge biology. For example, if populations are established through asexual reproduction, the population may not have the genetic diversity to be able to withstand change in their environment. Also, if the dispersal ability of sexually produced larvae is limited, then local populations may be genetically isolated and be likely to diverge from other isolated populations. On the other hand, sponge populations may be genetically homogeneous over large spatial scales, as was found in the species *Chondrosia reniformis* (Lazoski et al. 2001), despite sponges being generally thought to have limited dispersal abilities. This could have implications for the way that sponge reproduction is studied, as well as inform recommendations for sponge conservation.

The purpose of the experiments I completed as part of my honours project was to determine the extent to which asexual reproduction was contributing to the dynamics of populations. I did this by looking at two different species of sponge using two totally different approaches. The focus of this paper is the population genetics of reef forming glass sponges (class Hexactinellida), but a short description of the experiments performed on the other species, *Suberites* sp., can be found in Appendix 1.

In 1986 in the Queen Charlotte Sound, a regional geographic survey found an acoustic anomaly on the ocean floor (Conway 1991). Subsequent investigations in the following years discovered massive reefs made of glass sponges, several kilometers long, at depths between 150m and 250m (Conway 1991). The reefs in the Queen Charlotte Sound cover more than 700 km^2 of discontinuous ocean floor (Conway 1991). Since then, at least 7 major reefs have been found in the Strait of Georgia and the Hecate Strait, each covering between 2 and 10 km^2 (Leys et al. 2004). Glass sponge reefs were common during the Mesozoic, but until the discovery of these reefs, it had been thought that the reef forming habit had gone extinct during the Cenozoic Conway 1991). The west coast of B.C. is the only place in the world that sponge reefs are found in the modern (Conway 1991). For this reason, the reefs have been referred to as “living fossils”, and may provide insight into the ecology of ancient reef analogues (Conway 2001).

In the Strait of Georgia there are two species of glass sponge which are the reef builders: *Aphrocallistes vastus* and *Heterochone calyx* (Leys et al. 2004). They belong to closely related genera within the family Aphrocallistidae (Dohrmann et al. 2008). There are several other species of sponge which live on the reefs, but as their skeletons disarticulate after death, they are not an important part of the reef building process (Leys et al. 2004). Both *A. vastus* and *H. calyx* have dictyonine skeletons, where the spicules fuse to form large ridged skeletons which persist after the death of the sponge and serve to baffle sediment and act as settlement substrate for sponge larvae (Krautter et al. 2006). The sponge reefs are home to many species of crustacean, euphausids and commercially valuable rockfish (Conway 2001).

The west coast of B.C. is a commercially active zone with fishing and trawling occurring around the reefs. It is estimated that half of the reefs in the Strait of Georgia have been damaged by trawling activity (Cook et al. 2008). Observations of damaged sponges indicate that their healing potential is limited, and that sponges cut or otherwise broken do not survive (Austin et al. 2007). Austin et al. (2007) surveyed for baby *A. vastus* in the Saanich Inlet and concluded that they were very rare, implying recruitment is not occurring at a rate that will replace broken individuals. It may take hundreds of years for the reefs to recover from recent trawling damage, if recovery is possible at all (Cook et al. 2008). Reefs in the Hectate Strait have no-trawl zones around them, and it has been recommended that the Strait of Georgia reefs receive the same protection (Cook et al. 2008).

Besides the desire to better understand the reproduction of reef forming sponges, population genetics also has practical, conservation applications in this context. If each reef is a genetically isolated population, then each reef has greater intrinsic conservation value than if there are many reefs which are part of a single genetically homogeneous population. To assess the population structure of *A. vastus* on the reefs in the Strait of Georgia, I used both nuclear and mitochondrial molecular markers.

Microsatellites are regions of typically non-coding nuclear DNA which contain a repeating sequence. The most commonly used repeats are di-nucleotide, such as CT, but tri- and tetra-nucleotide repeats also exist. Because of the nature of DNA replication, mutations occur more frequently over these repeating sequences, resulting in an insertion or deletion of a repeat unit

(See figure 2 in Ellegren 2004). Alleles of microsatellites are different copies of the repeating sequence, which have a different number of repeats. Because the mutations occur with the insertion or deletion of a repeat unit, alleles differ in the length of the microsatellite by multiples of two nucleotides for a di-nucleotide repeat. Instead of sequencing the microsatellite to count the number of repeat units to determine the allele, it is possible to just measure the length of the microsatellite by running PCR product on a gel or through a sequencer set to a fragment analysis program. When this is done, the presence of a microsatellite of a particular length can be identified. Microsatellites are co-dominant, so if only one length appears on the gel or sequencer, then that individual is a homozygote for that allele of the microsatellite. Microsatellites are inherited the same way that other nuclear genes are, and therefore they can be used to trace parentage and determine relatedness (Ellegren 2004). Microsatellites are useful for population level studies because they are super-variable, and many alleles may exist for each locus. For a discussion of why microsatellites are preferred over other types of molecular techniques, see Jarne and Logoda (1996). In this study, I also used sequence data from three mitochondrial genes to assess population structure.

Traditionally, sponge taxonomy has been largely based on spicule chemistry and morphology (Bergquist 1978). Because the skills of a sponge taxonomy expert are often required to be able to identify specimens to the species level, phylum Porifera is a good candidate for the practical application of DNA barcoding. In 2002, Hebert put forth the cytochrome *c* oxidase subunit 1 (COI) mitochondrial gene as a universal marker for animal species identification. Currently in the Barcode of Life Data systems (BOLD) database (Ratnasingham and Hebert 2007), there are only 150 species of sponge with a barcode, and in the whole class Hexactinellida only one specimen has been barcoded. Barcoding has many practical applications and could be used more widely in the study of sponge biology. For example, on the reefs, individuals of *A. vastus* and *H. calyx* are intermixed and cannot be distinguished based on macro-scale morphology. The main species of interest in this study was *A. vastus* however, because the two species were indistinguishable at the time of collection, some individuals of *H. calyx* were inadvertently sampled. This led to the desire to find a molecular way of distinguishing between the two, and the study was expanded to include *H. calyx* for the purpose of barcoding. *Staurocalyptus* sp. is another Hexactinellid found on the reefs in the Strait of Georgia, and was used as an outgroup for the barcode analysis. As part of my honours thesis, I sequenced the COI

gene for each of these species, and characterized their barcodes, providing a molecular tool which can definitively identify them in the absence of morphological data.

Methods

Collections

For all the experiments described below, the tissue samples used were collected during a cruise in the Strait of Georgia in 2007 at depths between 69m and 175m. A legend of the reef names and the code used to identify tissue samples collected from each site can be found in Table 1. A map showing the locations of reefs can be seen in Figure 1. Tissue samples were collected by ROV, and the precise depth and GIS coordinates for each sample were recorded. Groups of branches which appeared separate from other branches were considered an individual. Samples were collected from separate individuals between 0.3 and 5m apart which were part of a mound, and several mounds were sampled on each reef. The tissue samples were stored in 95% ethanol at -20˚C. The DNA was extracted using a Qiagen DNeasy blood and tissue kit from a piece of tissue approximately 0.75cm x 0.5cm x 0.75cm and was diluted to 20ng/µl.

Microsatellites

Developing the Library

The microsatellite library was developed from DNA from a single individual of *A. vastus* using the SNX linker system (Hamilton et al. 1999). The only deviation made from Hamilton et al.'s protocol was that the DNA was not enzymatically digested as a first step, because it was already of appropriate lengths. The library was enriched for CT repeats using magnetic beads with CT probes. The plasmids from positive colonies were sequenced on an ABI3730 sequencer. Five candidate loci were chosen, and primers were designed for the region flanking the microsatellite and were ordered from ABI (Table 2).

Testing the Primers

The five sets of direct labelled primers were tested on a panel of 7 individuals of *A. vastus*. The PCR mix contained approximately 50ng of template DNA in a 15 µl reaction with: 1X PCR buffer (50 mM Tris-Cl, pH 9.2, 1.8 mM MgCl2, 10 mM (NH4)2(SO4)), 0.16 mM dNTPs, 0.03 µM of each of the forward and reverse primers, and 0.1 µl of Taq DNA polymerase

(made by the department).The PCR conditions were as follows: 94˚C for 2 minutes, (94˚C for 30 sec, 52˚C for 20 sec, 72˚C for 10 sec)x 33, 72˚C for 10 minutes.

Two of the primer sets (GS10 and GS169) amplified more than two fragment lengths in several of the individuals. The presence of more than two fragment lengths was an indication that non-specific amplification was occurring. A gradient PCR was performed to try an increase the specificity of the primers; the variables tested were combinations of Mg from 1.5 to 2.5mMol and annealing temperature from 48˚Cto 58˚C. The gradient PCR did not improve the amplification, and more than 2 fragments were still present. For this reason, those two primer sets were rejected for use on the larger panel.

Typing Individuals

The remaining three primer sets (GS3, GS21 and GS119) were used on all 83 individuals of *A. vastus* collected in 2007, and fragments were visualized on an ABI 3730 sequencer. On this larger panel of individuals it was found that these three primer sets also produce more than two fragment lengths in some individuals; as many as four fragment lengths were found in an individual (Figure 2).

Analysing the Fragment Data

The traditional analyses used to determine population substructure are Wright's F-statistics (Wright 1951). These tests require the calculation of estimates of gene frequencies, which cannot be done for the microsatellite data here, because there is no way to determine the number of copies of an allele an individual has. When only two fragments are detected, it is possible that that individual has three copies of one allele, and one copy of the other, but there is no way to know for sure.

To analyse the data, the genotype for each individual was coded as the presence or absence of each of the 35 possible fragment lengths. A matrix of presence or absence of each of the 35 possible fragments for each individual was made (Appendix 2). From this, a pairwise matrix was created using the Excel add-in GenAlEx (Peakall and Smouse 2006), which described the number of instances where the presence or absence of a fragment is not the same between two individuals (i.e. present in one individual but absent in the other). This number was divided by 35 to represent the proportion of fragments where the character state was different

between two individuals. Using GenAlEx (Peakall and Smouse 2006), the mean proportion of difference of individuals within a reef was calculated, as well as the mean proportion of differences between individuals from different reefs (Table 3).

Barcoding

Developing the Primers

Primers to amplify the COI gene (Table 4) were developed specifically for *A. vastus* using the mitochondrial genome accessed through Genbank (Accession number: EU000309.1).

Amplification and Sequencing

The COI gene was sequenced in the same 83 individuals of *A. vastus* used in the mircrosatellite experiment, as well as in 11 individuals of *H. calyx* and a single individual of *Staurocalyptus* sp. The PCR amplification contained: approximately 50ng of template DNA in a 10 µl reaction with 1X PCR buffer (50 mM Tris-Cl, pH 9.2, 1.8 mM MgCl2, 10 mM (NH4)2(SO4)), 0.2 mM dNTPs, 0.5 µM of each of the forward and reverse primers, and 1U of Taq DNA polymerase (made by the department). The PCR conditions were as follows: 94˚C for 4 minutes, (94 ˚C for 15 sec, 50 ˚C for 30 sec, 72 ˚C for 45 sec)x 30, 72 ˚C for 5 minutes.

The forward and reverse sequences were assembled in SeqMan (DNASTAR) and aligned in MegAlign (DNASTAR). A pairwise matrix of sequence distances was made. From this, the mean percent sequence difference within and between species was calculated in Excel (2010).

Mitochondrial Markers

Developing the Primers

The ATPase 6 (ATP6) and Cytochrome oxidase *c* subunit 2 (COII) genes were suggested by Rua et al. (2011) as mitochondrial markers which would be variable enough within species to be suitable for population genetics and phylogeography studies. Primers to amplify the ATP6 and COII genes (Table 4) were developed specifically for *A. vastus* using the mitochondrial genome accessed through Genbank (Accession number: EU000309.1).

Amplification and Sequencing

The ATP6 and COII genes were sequenced in 83 individuals of *A. vastus* collected during the 2007 cruise. The sequences were run on an ABI3730 sequencer.

Analysis

The forward and reverse sequences of COII and ATP6, as well as COI were assembled in SeqMan (DNASTAR) and aligned in MegAlign (DNASTAR). The mean percent sequence differences for individuals within a reef, as well as between reefs were calculated in Excel (2010).

The sequences from the COI, COII and ATP6 genes were concatenated for each individual where a full length read of each gene was produced (n=34). These concatenated sequences were aligned in MegAlign (DNASTAR) and exported to PAUP (Swofford 2003, version 4.0).

In MegAlign (DNASTAR), a pairwise matrix of sequence distances was created from the concatenated sequences and haplotypes were identified by hand.

Results

Microsatellites

If two individual sponges have the same trait for each of the 35 characters (fragments), then the proportion of difference between them is 0.000, if they have a different trait for each of the characters, the proportion of difference between them is 1.000. Within reefs, the proportion of differences ranged from 0.185 to 0.215. Between reefs, the proportion differences ranged from 0.187 to 0.221.

There is no correlation between the geographic distance between two sponges and how different they are genetically (Figure 3). The genetic difference between pairs of sponges which are located within 150m of each other is normally distributed (Figure 4), and has roughly the same mean and spread as the genetic difference between all pairs of sponges, up to 70km apart (Figure 4)

Barcoding

The sequence difference of the COI gene within *A. vastus* (n=83) was 0.121%. The sequence difference of the COI gene within *H.calyx* (n=11) was 0.148%. The sequence difference between *A. vastus* (n=83) and *H. calyx* (n=11) is 11.69% (SD=0.1672) in the COI gene. *Staurocalyptus* sp was used as an outgroup (n=1), the sequence difference between it and

A. vastus and *H. calyx* was 24.13% (SD=0.2637) and 23.55% (SD=0.0934) respectively. A distance based phylogeny to graphically display these differences was created in MegAlign (DNASTAR) (Figure 6).

Mitochondrial Markers

For the COI, COII and ATP6 genes individually, the mean percent sequence differences within reefs was less than the between reef differences in some instances (Tables 5, 6 and 7).

The concatenated data set contained 1771 characters, 1761 of which were constant, of the variable characters 7 were parsimony uninformative and three were parsimony informative. Maximum parsimony trees were created from a heuristic search in PAUP (Swofford 2003, version 4.0). There were 5 best trees, each with a tree length of 11.The maximum parsimony trees of the concatenated sequences did not show clear grouping of individuals from the same population (Figure 7).

There were 11 haplotypes among the 34 individuals, two which were shared by more than one individual. The two shared haplotypes were named red and blue. There were 9 unique haplotypes which were only found in a single individual. The haplotype frequencies found in each reef can be seen in Figure 8.

Discussion

Microsatellites

Based on the microsatellite data, there is no indication of within reef population structure for *A. vastus* in the Strait of Georgia region, because there are instances where the between reef differentiation is less than the within reef differentiation. The isolation by distance plot (Figure 3) shows no trend that individuals of *A. vastus* which are geographically close are more similar genetically based on the microsatellite markers. In genetically structured populations, there would have been a positive relationship between genetic differences and increasing geographic distance. Based on the microsatellite data, there is no indication that neighbours are more likely to be related than individuals tens of kilometers apart. The distribution of genetic differences between pairs of *A. vastus* individuals is almost identical at two extreme geographic scales (150 m apart, up to 70 km apart). This further indicates that there is no reef level population structure.

Barcoding

The amount of sequence difference within a species is usually less than 1% (Avise 2000). In this study, the within species sequence difference at the COI gene was much less than 1%, which indicates that *A. vastus* and *H. calyx* are "good" species. The standard amount of sequence difference required to delineate separate species in the COI gene is 2% (Hebert 2003). The sequence difference between *A. vastus* and *H. calyx* is almost 12%, so the COI gene is very capable of differentiation these two species. The traditional method of using spicule morphology to identify sponges requires a trained eye, if not a taxonomy expert to correctly identify species. Before the samples were sequenced for the COI gene, I identified each as *A. vastus*, *H. calyx* or *Staurocalyptus* sp. based on spicule morphology. These identifications were independently corroborated by Dr. Henry Reiswig, a Hexactinellida taxonomy expert. The species identification based on sequence data and morphology of the spicules agreed in every instance. This result gives support to the morphological differences between species being representative of true genetic differences.

Whenever genetic work is done on *H. calyx* there is always some question as to whether the DNA is contaminated with DNA from its hydrozoan symbiont *Brinckmannia hexactinellidophilia* (Schuchert and Reiswig 2006). I am confident that the DNA used in this study was not contaminated by *B. hexactinellidophilia* DNA. *A. vastus* and *H. calyx* had the same common ancestor at the time when they diverged from the ancestor of *Staurocalyptus* (Dohrmann et al. 2008), and one would expect *A. vastus* and *H. calyx* to have very similar sequence differences to *Staurocalyptus* sp., which was the result found here. If the *H. calyx* DNA were contaminated by *B. hexactinellidophilia* DNA then *H. calyx* and *Staurocalyptus* sp. would have had a much greater sequence difference than *A. vastus* and *Staurocalyptus* sp.

Mitochondrial Markers

Concurrent with the results from the microsatellite analyses, some of the between reef sequence differences are less than the within reef sequence differences for each of the three mitochondrial genes. There is also no reef level trend in the occurrence of haplotypes (Figure 8), or in the grouping of haplotypes (Figure 7). These analyses further support the idea that there is no reef level population structure within the Strait of Georgia region. However, conclusions drawn based on the analyses performed with the concatenated sequences are limited by the

extremely small sample size, particularly in reefs 3 and 4. It is likely that with greater sampling at those reefs, unique haplotypes would be found, and with even greater sampling at all reefs, individuals which share the "unique" haplotypes would be found. There is no reason to expect that with increased resolution, a geographic pattern to the occurrence of haplotypes would emerge.

Conclusions

Based on both the nuclear and mitochondrial the marker systems developed during this study, there is no indication of reef level population structure in *A. vastus* in the Strait of Georgia Region. This indicates that there is gene flow or exchange of individuals among reefs. It is likely that it is not gametes which are being exchanged, but that larvae are dispersing from one reef to another. *A. vastus* are hermaphroditic and are thought to release sperm into the water. Although developing embryos have only been found once, it is believed that they brood eggs, which become fertilized within the adult sponge (Leys et al. 2007). It is not known how long the larvae live in the water column until they settle, but in other Hexactinellida it can be up to 7 days (Leys et al. 2007). One week may be sufficient time for larvae to move with the currents from one reef to another, but further information about deep water currents in the Strait of Georgia would be needed to determine this.

Asexual reproduction in *A. vastus* does not appear to be a factor in the development of reefs. Although observations have indicated that budding by "dripping" tissue may be occurring (Austin 2003), no genetically identical pairs of individuals were found in this study. As an internal control to this study, at the time of sampling, pieces of tissue were divided into two collection tubes and labelled as separate samples. Only these double sampled individuals were found to have identical genotypes for the microsatellites. If asexual reproduction by budding was occurring, clones would likely be in close proximity to each other. The sampling method of selecting individuals within the same mound should have picked up at least one instance of clones, if asexual reproduction was an ecologically significant process.

Microsatellites have been used to assess the population structure of sponge species in other parts of the world. For example, seven microsatellite loci were identified in *Scopalina lophyropoda*, a demosponge found in the Mediterranean, which allowed researchers to conclude

that asexual reproduction was not an important factor in that species (Blanquer and Uriz 2010). The sequence of COI has also been used to assess the population structure of *Rhopaloeides odorabile* on the Great Barrier Reef (Whalan et al. 2008). Three haplotypes were identified in *R. odorabile*, one of which was present in all populations at frequencies between 0.55 and 1.00 (Whalan et al. 2008), much like the red haplotype identified in this study.

Further molecular analyses are needed to provide more evidence as to the population genetics of *A. vastus* in the Strait of Georgia. Efforts should be made to isolate microsatellites which are not duplicated in the nuclear genome. To do this, I would recommend creating a library which is enriched for repeats other than CT. In molluscs, difficulties in developing microsatellite markers have been due to the microsatellite locus being located within a transposon, with as many as 19 copies of the transposon found within 14.7 kilobases of genome (McInerney et al. 2011). It is possible that a similar mechanism is responsible for the duplication of the microsatellite loci developed in this study. If that is the case, then it should be possible to identify other non-duplicated microsatellites. If unduplicated microsatellite loci are not found, than a marker system based on SNPs (single nucleotide polymorphism) could be developed as an alternative. Once a good set of markers is developed, they could be applied to reefs outside the Strait of Georgia, such as the northern reefs in the Queen Charlotte Sound. Currently ongoing attempts to find larvae of *A. vastus* should continue. It would be of great advantage to know how long they stay in the water column before they settle, so that it can be determined if it is possible for them to travel with the currents from one reef to another in that timeframe.

Even though *A. vastus* on all reefs are part of a genetically homogeneous population, the results of this study do not reflect on the genetic structure of other organisms living on the reefs. Therefore, this study does not conclude that each reef is equal to the others in overall genetic diversity. There could be genetic structure at the reef level for *H. calyx* populations, or for any of the other species of sponge or other animals. Further study into the population genetics of the other reef-dwelling organisms should be performed before recommendations can be made as to the conservation of particular reefs in the Strait of Georgia based on the premise of preserving genetic diversity.

Acknowledgments

I would like to thank my excellent supervisors for their advice in troubleshooting, positive attitudes and patience; the staff and students at BMSC, particularly Siobhan Gray, for all their help during the fall semester; Dr. Henry Reiswig for identifying *Suberites* sp. as well as the glass sponge specimens; Dr. Dave Coltman and his lab for hosting and advising me; and Jackson Chu for helping with GIS.

Note: See appendix 3 for the list of procedures which were carried out by Dr. Corey Davis.

References

Austin et al., *In prep*. Some New and Old Hadromerida (Porifera: Demospongiae) from British Columbia and Adjacent Waters. *Zootaxa*.

Austin, W. C. 2003. Sponge Gardens: A hidden treasure in British Columbia http://mareco.org/khoyatan/spongegardens

Austin, W. C., Conway, K. W., Barrie, J. V., Krautter, M. 2007. Growth and morphology of a reef-forming glass sponge, *Aphrocallistes vastus* (Hexactinellida), and implications for recovery from widespread trawl damage. *Porifera Research: Biodiversity, Innovation and Sustainability*. 139-145

Avise, J. C. 2000 *Phylogeography. The history and formation of species*. Cambridge, MA: Harvard University Press

Bergquist, P. R. 1978. *Sponges*. Berkley, CA: University of California Press

Blanquer, A., Uriz, M. J. 2010. Population genetics at three spatial scales of a rare sponge living in fragmented habitats. *BMC Evolutionary Biology*. 10:13

Boury-Esnault, N., Solé-Cava, A. M., 2004. Recent contribution of genetics to the study of sponge systematics and biology. *Boll. Mus. Ist. Biol*. Univ. Genova. 68:3-18

Conway, K. W., Barrie, J. V., Austin, W. C., Luternauer, J. L. 1991. Holocene sponge bioherms on the western Candaian continental shelf. *Continental Shelf Research*. 11(8-10): 771-790

Conway, K. W., Kruatter, M., Barrie, J. V., Neuweiler, M. Hexactinellid sponge reefs on the Canadian continental shelf: A unique "living fossil". *Geoscience Canada*. 28(2): 71-78

Cook, S. E., Conway, K. W., Burd, B. 2008. Status of the glass sponge reefs in the Georgia Basin. *Marine Environmental Research*. 66:S80-S86

Dohrmann, M., Janussen, D., Reitner, J., Collins, A. G., Worheide, G. 2008.Phylogeny and Evolution of Glass Sponges (Porifera, Hexactinellida).*Systematic Biology*. 57(3): 388-405

Ellegren, H. 2004. Microsatellites: Simple sequences with complex evolution. *Nature Reviews Genetics*. 5: 435-445

Gaino, E., Bavestrello, G., Magnino, G., 1999. Self/non-self recognition in sponges. *Italalian Journal of Zoolology*. 66:299-315

Hamilton, M. B., Pincus, E. L., Di Fiore, A., Fleischer, R. C. 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*. 27:500-507

Hebert, P. D. N., Cywinska, A., Ball, S. L., deWaard, J. R. 2002. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London*. DOI 10.1098/rspb.2002.2218

Herbert, P. D. N., Ratnasingham, S., deWaard, J. R. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London*, Biological Letters Supplement

Jarne, P., Lagoda, P. J. L. 1996. Microsatellites, from molecules to populations and back. *Tree*. 11(10): 424-429

Kaye, H., Oritz, T., 1981. Strain specificity in a tropical marine sponge. *Marine Biology*. 63: 165-173

Krautter, M., Conway, K. W., Barrie, J. V. 2006.Recent Hexactinosidan sponge reefs (silicate mounds) off British Coloumbia, Canada: Frame building processes. *Journal of Paleontology*. 80(1): 38-48

Lazoski, C., Solé-Cava, A. M., Boury-Esnault, N., Klautau, M., Russo, C. A. M., 2001. Cryptic speciation in a high gene flow scenario in the oviparous marine sponge *Chondrosia reniformis*. *Marine Biology*. 139:421-429

Leys, S. P., Mackie, G. O., Reiswig, H. M. 2007. The biology of glass sponges. *Advances in Marine Biology*. 52: 1-145

Leys, S. P., Wilson, K., Holeton, C. Reiswig, H. M., Austin, W. C., Tunnicliffe, V. 2004. Patterns of glass sponge (Porifera, Hexactinellida) distribution in coastal waters of British Columbia, Canada. *Marine Ecology Progress Series*. 283: 133-149

McInerney, C. E., ALlock, A. L., Johnson, M. P., Bailie, D. A., Prodohl, P. A. 2011. Comparative gonomic analysis reveals species-dependent complexities that explain difficulties with microsatellite marker development in moluscs. *Heredity*. 106: 78-87

Müller, W. E. G., Steffen, R., Lorenz, B., Batel, R., Kruse, M., Krasko, A., Müller, I. M., Schröder, H. C., 2001. Suppression of allograft rejection in the sponge *Suberites domuncula* by FK506 and expression of genes encoding FK506-binding proteins in allografts. *Journal of Experimental Biology*. 204:2197-2207

Peakall, R., Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*. 6, 288-295

Ratnasingham, S., Hebert, P. D. N. 2007. BOLD : The Barcode of Life Data System (www.barcodinglife.org).Molecular Ecology Notes 7, 355–364. DOI: 10.1111/j.1471-8286.2006.01678.x

Rua, C. P. J., Zilberberg, C., Sole-Cava, A. M. New polymorphic mitochondrial markers for sponge phylogeography. *Journal of the Marine Biological Association of the United Kingdom*. DOI: 10.1017/S0025315410002122

Schuchert, P., Reiswig, H. M. 2006. *Brinckmannia hexactinellidophila*, n. gen., n. sp.: a hydroid living in tissues of glass sponges of the reefs, fjords, and seamounts of Pacific Canada and Alaska. *Canadian Journal of Zoology*. 84: 564-572

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts

Wilson, H. V., 1907. On some phenomena of coalescence and regeneration in sponges. *J. Exp. Zool*. 5:245-258

Whalan, S., de Nys, R., Smith-Keune, C., Evans, B. S., Battershill, C., Jerry, D. R. 2008. Low genetic variability within and among populations of the brooding sponge *Rhopaloeides odorabile* on the central Great Barrier Reef. *Aquatic Biology*. 3: 11-119

Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics*. 15: 323-354

Tables

Table 1. Legend of Reef numbers and Sample names

Number	**Location Name**	**Number of Samples**	**Sample IDs**
Reef 1	Fraser Reef	27	R10, R11, R17, R18
Reef 2	Galiano Reef	34	R12, R13, R14
Reef 3	McCall Bank	14	R15
Reef 4	Howe Sound	8	R16

Table 2. Microsatellites markers isolated from *Aphrocallistes vastus*, characteristics are based on 83 individuals.

Locus	Forward Primer	Reverse Primer	Repeat motif	Allele size range	No. of alleles
GS3	GCATAATCCTAATCGGTCCT	ACCGTCTTCCAGGTACTAGC	CT	265-322	10
GS10	GACTTCTTCATCCCGATTTC	TTTTGCAGTTGGATTGTCTT	CT	NA	NA
GS21	ATTTTCAGGATGCAACAAAG	ATTTTTGTCATCGCCTTACA	CT	133-159	11
GS119	GGGAACCTGATCGCTTATGA	GAGGGATAGAATCTCAGCAACTG	CT	208-239	14
GS169	GCACGAAATCGGAACTTCA	CAACATAAACGCGGCTGATA	CT	NA	NA

Table 3.The mean proportion difference of fragment occurrence within and between reefs of *Aphrocallistes vastus*.

	Reef 1	**Reef 2**	**Reef 3**	**Reef 4**
Reef 1	0.19367 (SD 0.070489)			
Reef 2	0.205495 (SD 0.064078)	0.215432 (SD 0.062426)		
Reef 3	0.187127 (SD 0.062267)	0.206363 (SD 0.058398)	0.185243 (SD 0.059531)	
Reef 4	0.196978 (SD 0.067217)	0.221218 (SD 0.059972)	0.196429 (SD 0.064532)	0.187755 (SD 0.057894)

Table 4. Mitochondrial primers used for barcoding *Aphrocallistes vastus*, *Heterochone calyx* and *Staurocalyptus* sp., and determining population structure in *A. vastus*. All primers were developed using the published mitochondrial sequence of *A. vastus*.

Gene	Primer Sequence	Product length
COI	F: ATTCAACAAAACCACAAAGATATAGG R:TATACTTCTGGATGTCCAAAGAATCA	569
COII	F:CCTGCCTCTCCTACAATGGA R:CGCCGCATAATTCTGAACAT	577
ATP6	F:CTATTCTCAGTTTCAGAAATCTCTCC R:AGTAATGTGAATACATAGGCTTGGA	625

Table 5. The mean percent sequence difference within and between reefs of *A. vastus* in the COI gene.

	Reef 1	**Reef 2**	**Reef 3**	**Reef 4**
Reef 1	0.119088 (SD 0.106665)			
Reef 2	0.12342 (SD 0.109457)	0.114973 (SD 0.108515)		
Reef 3	0.112169 (SD 0.107096)	0.128782 (SD 0.107954)	0.112088 (SD 0.106286)	
Reef 4	0.122685 (SD 0.116516)	0.134926 (SD 0.107611)	0.116071 (SD 0.11897)	0.139286 (SD 0.119689)

Table 6. The mean percent sequence difference within and between reefs of *A. vastus* in the COII gene.

	Reef 1	**Reef 2**	**Reef 3**	**Reef 4**
Reef 1	0.074462 (SD 0.106546)			
Reef 2	0.056824 (SD 0.097149)	0.038793 (SD 0.082375)		
Reef 3	0.038462 (SD 0.079204)	0.019355 (SD 0.05937)	0 (SD 0)	
Reef 4	0.095604 (SD 0.120232)	0.076498 (SD 0.108229)	0.057143 (SD 0.092009)	0.114286 (SD 0.119523)

Table 7. The mean percent sequence difference within and between reefs of *A. vastus* in the ATP6 gene.

	Reef 1	**Reef** 2	**Reef** 3	**Reef** 4
Reef 1	0.098095 (SD 0.1074)			
Reef 2	0.064167 (SD 0.0962)	0.024793 (SD 0.066183)		
Reef 3	0.078095 (SD 0.103757)	0.040952 (SD 0.082852)	0.05714 (SD 0.092582)	
Reef 4	0.053333 (SD 0.089443)	0.0125 (SD 0.048925)	0.028571 (SD 0.071714)	0 (SD 0)

Figures

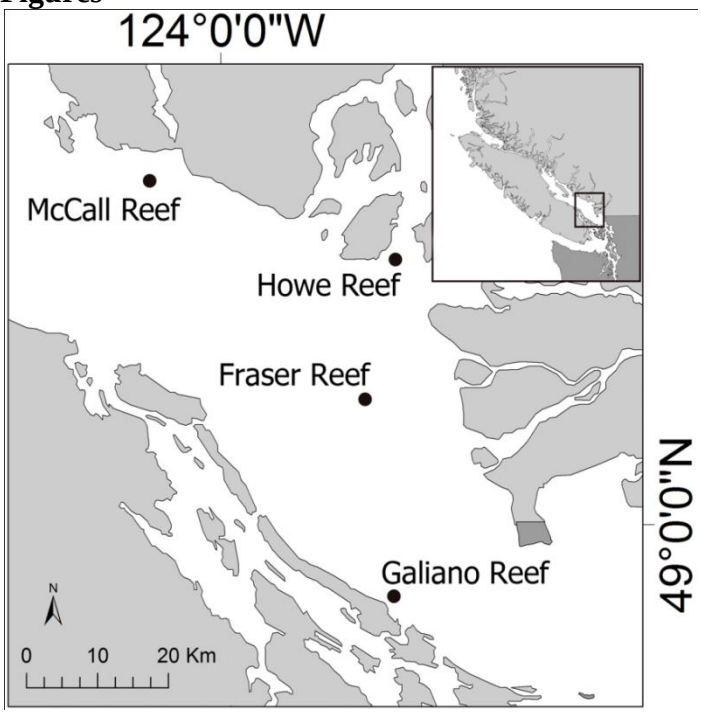

Figure 1. Map of the reefs where tissue samples were collected during the 2007 cruise in the Strait of Georgia.

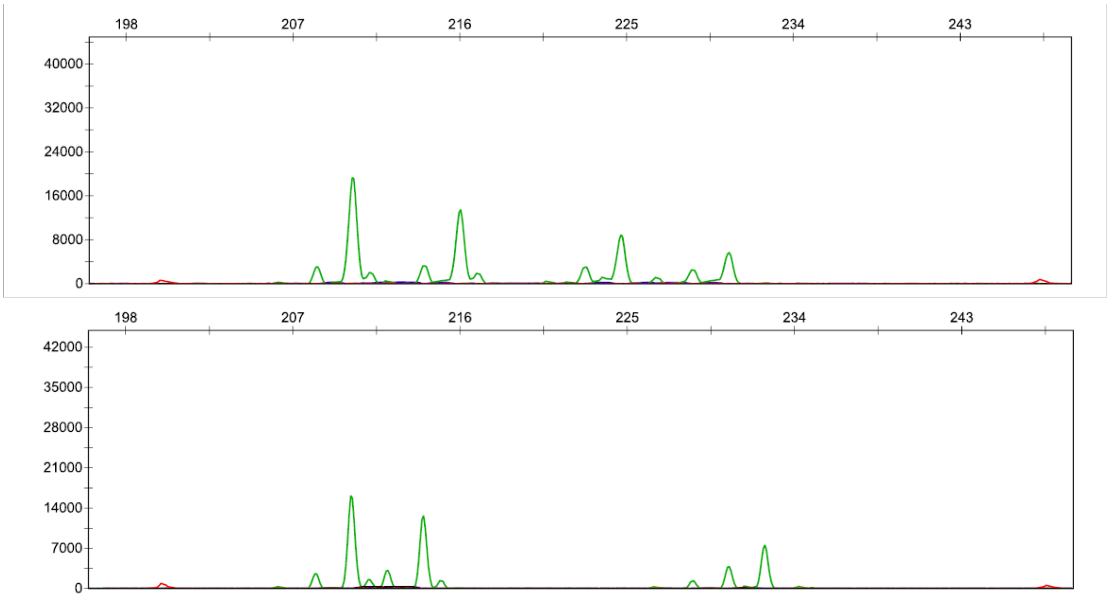

Figure 2. Examples of genotypes at the GS119 locus in *A. vastus*, the top panel individual has four fragments (Alleles: 210, 216, 224 and 230), the bottom panel individual has three fragments (Alleles: 210, 214, and 232). The smaller peaks around the larger peak are stutter bands produced when Taq DNA polymerase slips when replicating the DNA. The allele is the largest peak.

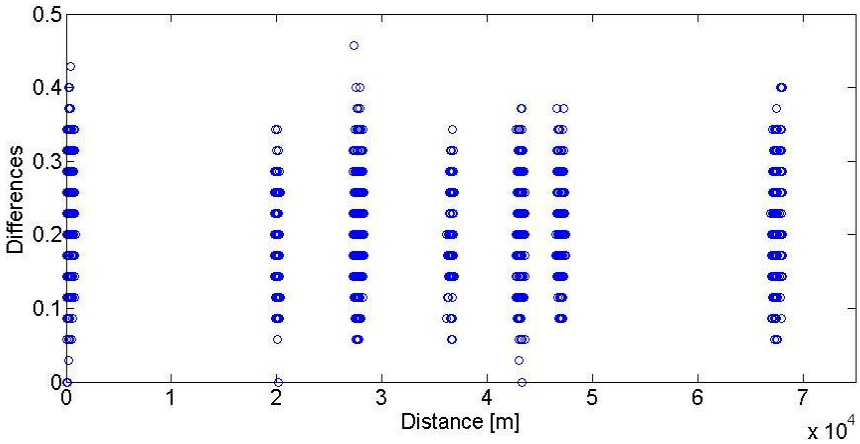

Figure 3. An isolation by distance graph showing the proportional genetic difference between each pair of sponges on the y-axis and the geographic distance between each pair of sponges on the x-axis.

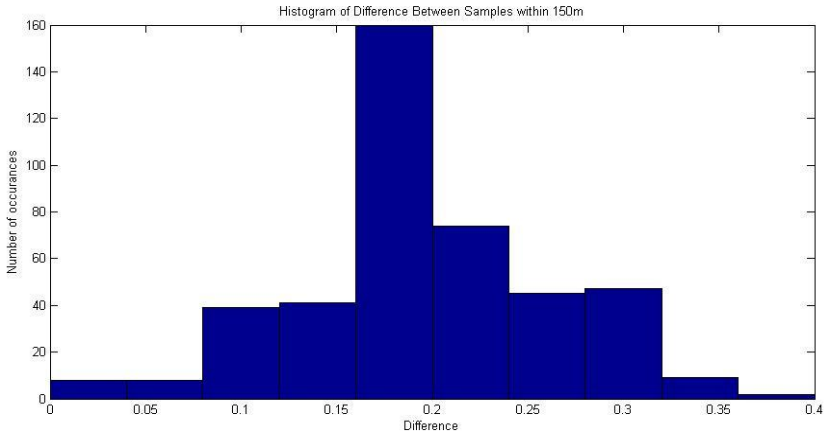

Figure 4.A histogram depicting the frequency of each level of genetic difference from the pairwise comparison of all sponges within 150m of one another.

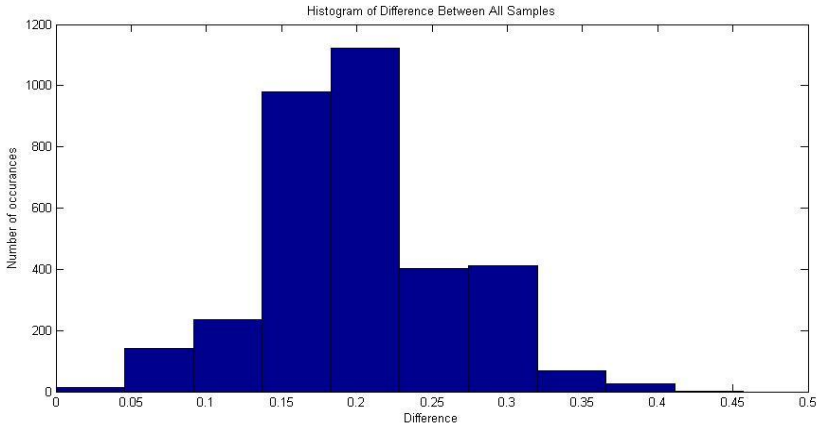

Figure 5.A histogram depicting the frequency of each level of genetic difference from the pairwise comparison of all sponges sampled in 2007.

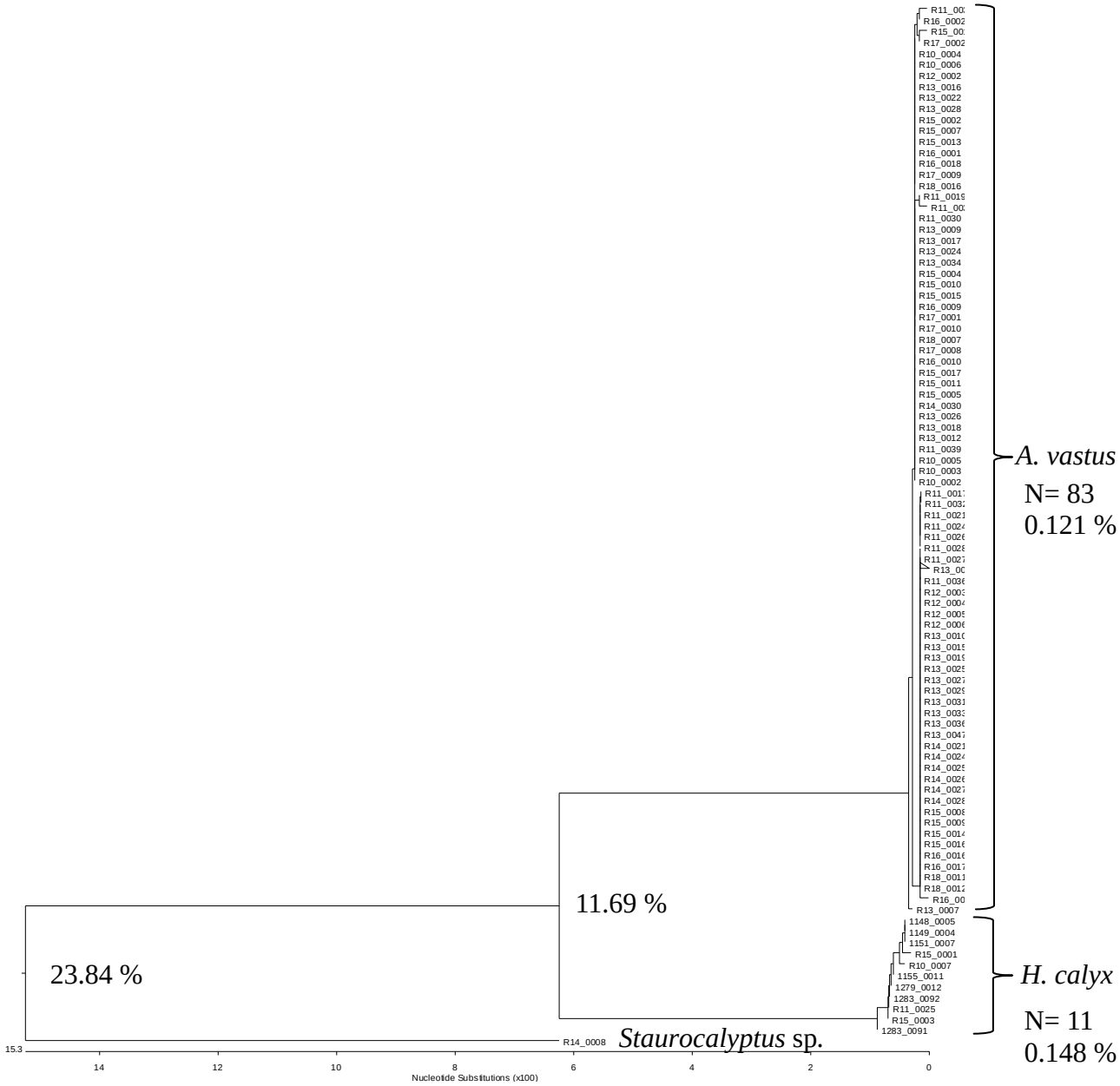

Figure 6. The distance based phylogeny of the COI gene with the percent of the sequence difference that is represented by the branching point indicated at the nodes, and within species sequence difference indicated at the tips.

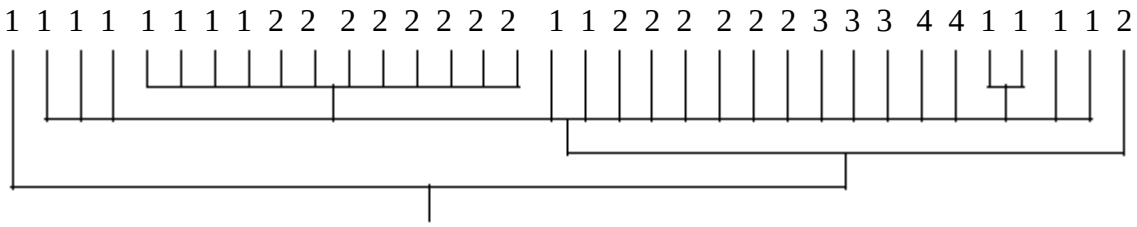

Figure 7. A maximum parsimony tree created from the concatenated sequences (genes COI, COII, and ATP6) of 34 individuals of *A. vastus* with the populations those individuals are from indicated by the numbers at the branch tips (Tree length = 11).

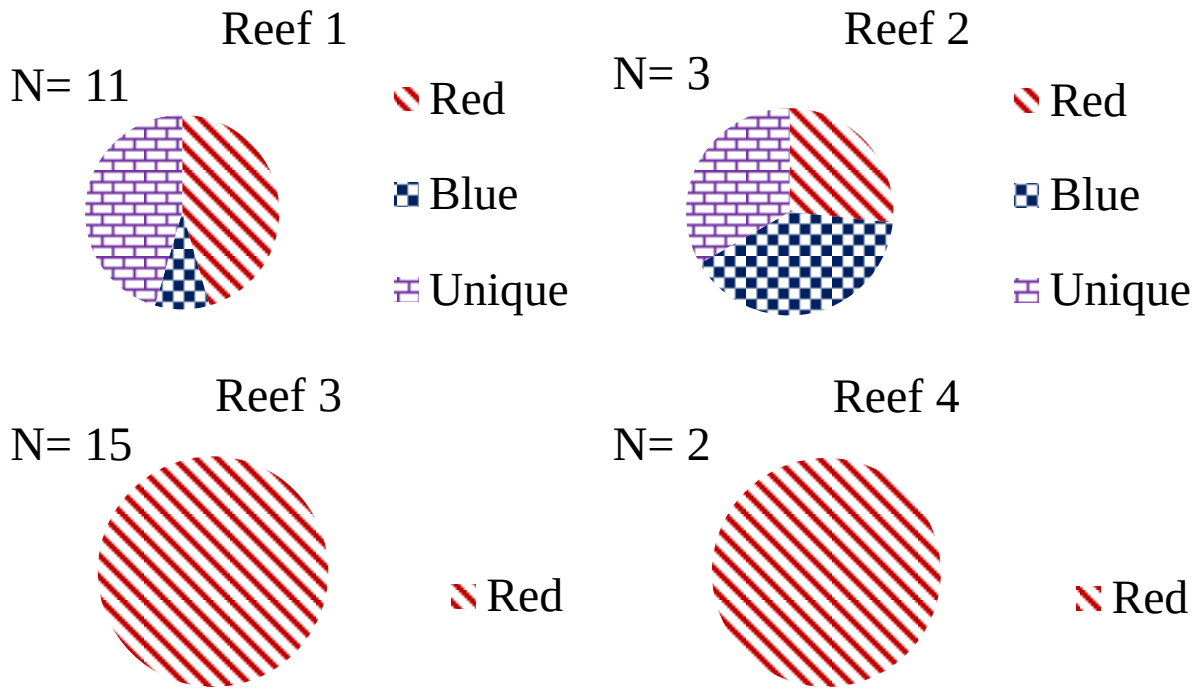

Figure 8.The frequency of haplotypes, named red, blue, and unique. Unique fraction represents haplotypes which were only found in a single individual of *A. vastus*. Haplotypes were determined based on the sequence of the COI, COII and ATP6 genes, in each reef.

Appendix 1

Using tissue grafting as a proxy for genetic identity to understand population structure in a Demosponge, *Suberites* sp.

A simple grafting method has been devised for testing whether two individuals are of the same genetic strain, based on the classic cell dissociation studies of Wilson (1907). By grafting tissue from one sponge onto tissue of another, it is possible to test whether the individuals are from the same genetic strain or are clones (Gaino et al. 1999). If the grafted region fuses and the tissues look as if they will become one functional sponge, then the two individuals involved in the graft are of the same strain. If the graft does not fuse, then the two individuals were not genetically similar. Graft rejection can be characterised by the development of ectosome on each individual at the graft site, or a cytotoxic reaction resulting in necrosis of one or both individuals at the graft site. The rejection is an immune reaction induced in response to the detection of non-self tissue. A graft between two sponges of the same stain is accepted because the tissues recognize each other as self. There is plenty of evidence supporting the idea that it is an immune response, for example, an immunosuppressant, such as FK506, can be used to force graft acceptance between two unrelated conspecific sponges (Müller et al. 2001). This grafting method has been used in sponge population studies both in the field and the lab (e.g. Kay and Oritz 1981)

I set out to determine how many strains (or clonal types) were present in a population of *Suberites* sp. (soon to be described by Austin et al. *in prep*). This species was chosen because it is convenient to access and individuals have discrete margins. I set up grafts in the lab between individuals within a population, and between individuals from different populations. The goal was to find out how common asexual reproduction was within a population, and to see if different populations were the same genetic strain.

The sponges observed for this study are of a soon to be described species in the genus *Suberites* (identification by Henry Reiswig, description in Austin et al. *in press*, Fig.1). They are from class Demospongiae, order Hadromerida, family Suberitidae. *Suberites* sp. is common in the shallow subtidal on rocky substrate between 3 and 10 meters deep in moderately exposed to very exposed areas in Barkley Sound.

The main result of this study is that all individuals of *Suberites* sp. sampled from seven sites were compatible enough with all other individuals for the tissues to apparently fuse. Why the species of *Suberites* studied here did not react to foreign conspecific tissue as expected has two possible explanations. First, all of the individuals sampled, despite being from sites up to almost 9 kilometers apart, could be of the same genetic strain. Alternatively, this particular species of *Suberites* may not express a histoincompatibility reaction with conspecifics, regardless of genotype.

This study on the importance of asexual reproduction in *Suberites* sp. populations relates to the rest of my honours project in the following way. Similar grafting experiments have previously been performed on live tissue of *A. vastus*, to see if asexual reproduction is a major structuring factor in the formation of sponge reefs. The results of that study were opposite to what I found here: all of the allografts failed to fuse. I have taken that study on *A. vastus* to the next step, which is molecular analysis. I was unable to follow up the *Suberites* sp. study in the same way due to time constraints.

Appendix 2

Table of the presence (1) or absence (0) of each of the possible fragment lengths (row 1) for the three microsatellite loci in all individuals of *A. vastus* collected in 2007.

Sample	Reef	133	135	137	141	145	147	149	151	153	157	159	208	210	212	214	216	221	223	227	229	231	233	235	237	239	265	267	269	308	310	312	314	316	320	322
R10_0002	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R10_0003	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R10_0004	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
R10_0005	1	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
R10_0006	1	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
R11_0017	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R11_0019	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
R11_0021	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
R11_0024	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
R11_0026	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
R11_0027	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R11_0028	1	0	1	0	0	0	1	0	1	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
R11_0030	1	0	1	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
R11_0032	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R11_0034	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R11_0036	1	0	1	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R11_0038	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
R11_0039	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
R12_0002	2	0	0	0	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
R12_0003	2	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
R12_0004	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R12_0005	2	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
R12_0006	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R13_0007	2	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R13_0009	2	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0
R13_0010	2	0	0	0	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
R13_0012	2	0	1	0	0	0	1	0	0	0	1	0	0	1	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0
R13_0015	2	0	1	0	0	0	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
R13_0016	2	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
R13_0017	2	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0
R13_0018	2	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
R13_0019	2	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
R13_0020	2	0	1	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0
R13_0022	2	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0
R13_0024	2	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
R13_0025	2	0	0	0	1	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0
R13_0026	2	0	1	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R13_0027	2	0	0	0	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
R13_0028	2	0	1	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R13_0029	2	0	1	0	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
R13_0031	2	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0

R13_0033	2	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0
R13_0034	2	0	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
R13_0036	2	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R13_0047	2	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
R14_0021	2	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	
R14_0024	2	0	0	0	0	0	1	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	
R14_0025	2	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	
R14_0026	2	0	0	0	0	0	1	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	
R14_0027	2	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	
R14_0028	2	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
R14_0030	2	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
R15_0002	3	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
R15_0004	3	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	
R15_0005	3	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	
R15_0007	3	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
R15_0008	3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	
R15_0009	3	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	
R15_0010	3	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	
R15_0011	3	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	
R15_0012	3	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
R15_0013	3	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
R15_0014	3	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
R15_0015	3	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
R15_0016	3	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	
R15_0017	3	0	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	
R16_0001	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
R16_0002	4	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	
R16_0008	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	
R16_0009	4	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
R16_0010	4	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	
R16_0016	4	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	
R16_0017	4	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	
R16_0018	4	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	
R17_0001	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
R17_0002	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
R17_0008	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
R17_0009	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	
R17_0010	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	
R18_0007	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	
R18_0011	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
R18_0012	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
R18_0016	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	

Appendix 3

The parts of this project which were performed by Dr. Corey Davis:

Development of the microsatellite library and primer design

Final typing reactions for microsatellites

Primer design for COI, COII and ATP6

Amplification and sequencing of mitochondrial markers

Contiging for COI