UPPSALA
UNIVERSITET

A phylogeographic study of the deep-sea sponge *Geodia barretti* using Exon-Primed Intron-Crossing (EPIC) markers

Isabel Ordaz Németh

Degree project in biology, Bachelor of science, 2014
Examensarbete i biologi 15 hp till kandidatexamen, 2014
Biology Education Centre and Department of Organismal Biology, Uppsala University
Supervisors: Mikael Thollesson and Paco Cárdenas

Table of Contents

Abstract	**3**
Introduction	**4**
The Poriferan Bauplan	4
Ecology and importance	4
Phylogeny and evolution	5
Geodia barretti	5
Genetic markers: Exon-Primed Intron-Crossing	8
Aims of the project	9
Materials and Methods	**10**
Finding introns	10
Sample dataset	10
PCR program	11
Electrophoresis	12
Purification and sequencing	12
Software used	12
Designing and testing new primers	13
Analysis of the data	14
Results	**15**
Evaluation of the universal primers	15
Pairwise distances between the sponges	15
Phylogenetic trees	17
Discussion	**19**
PCR troubleshooting	19
Pairwise distance matrix and phylogenetic trees	19
Conclusion	**22**
Acknowledgements	22
References	23
Appendix	25

Abstract

Background: There is a limited number of genetic markers suitable for phylogeographic and population genetic studies in non-model organisms, particularly various marine invertebrates, such as sponges. The possibility of using Exon-Primed Intron-Crossing (EPIC) markers in the deep-sea sponge species *Geodia barretti* is explored in this project. The sponge specimens that were tested came from different localities of the North Atlantic. **Results:** An EPIC marker was found to show genetic variability among the sponge specimens. Based on the resulting DNA sequences of the sponges, a phylogenetic analysis was made, which showed the genetic connectivity among them. **Conclusions:** EPIC markers are effective genetic markers variable enough to apply them to poorly studied marine species such as *G. barretti*.

Introduction

The Poriferan Bauplan

Porifera, commonly known as **sponges**, are sessile benthic invertebrates with simple structures. They essentially function as water canal systems that need to generate the right pressure differences for optimum water flow (Leys & Hill, 2012).

They lack true tissues, but three distinct layers can be identified. On the outer surface of the sponge, cells called pinacocytes form the **pinacoderm**. The inner layer, known as the **choanoderm**, is made up by unique flagellated cells called choanocytes, which continuosuly circulate water throughout the body, enabling filter-feeding and gas exchange. Water comes in through small pores on the **pinacoderm**, and is expelled through openings called osculae. The **mesohyl** is the middle layer of the sponge, which makes up most of the sponge's body and contains different types of cells, including totipotent archaeocytes (Brusca & Brusca, 1990).

The skeleton of a sponge can contain organic and inorganic elements; in most sponges it is primarily made up of a fibrillar collagen framework which is secreted by different types of cells. Sclerocyte cells are in charge of making mineral skeletal elements called **spicules**. Spicule morphology and their location within the sponge provide useful information for the systematics of this phylum as well as for species identification (Brusca & Brusca, 1990).

Ecology and Importance

Sponges play an important role in marine ecosystems for their filtering capacity; for example, in coral reefs cavities, encrusting sponges are known to remove significant amounts of dissolved organic carbon (Goeji, 2009).They also represent a source of food to other animals, such as nudibranchs, sea turtles and some tropical fishes, and offer protection and refuge to smaller organisms (Brusca & Brusca, 1990). Additionally, they host exceptionally diverse microbial communities (Thacker & Freeman, 2012).

When it comes to the economical value of sponges, there is great interest from natural product chemists and pharmaceutical companies to discover new drugs from the oceans (Genta-Jouve & Thomas, 2012). Substances with anti-inflammatory, antibiotic and other

healing properties have been found in many sponge species. For example, one species native to New Zealand, *Halichondria moorei,* has long been used by Maori people to heal wounds; it turns out that this species has a high concentration of potassium fluorosilicate, an anti-inflammatory agent (Brusca & Brusca, 1990).

Phylogeny and Evolution

Sponges are among the oldest metazoans. There are spicule fossil records from the beginning of the Cambrian period (Brusca & Brusca, 1990). For this reason they hold valuable information that could give a better understanding of the early evolution, development and molecular genetics of animals (Wörheide et al., 2012).

Within the phylum Porifera, the latest classification divides sponges into four classes: Calcarea, Hexactinellida, Demospongiae and Homoscleromorpha. At present there are approximately 8,500 poriferan species according to the World Porifera Database (Van Soest et al., 2014), of which the majority belong to the class Demospongiae, making it the most diverse group (Wörheide et al., 2012).

The phylogenetic relationships to other metazoan taxa and the relationships within the phylum are still questionable; nevertheless, morphological and molecular data support the idea that the four sponge classes form a monophyletic group (Wörheide et al., 2012).

Geodia barretti

The model organism of this project is the deep-sea sponge ***Geodia barretti***. It is classified as:

Class: Demospongiae Sollas, 1885
Order: Tetractinellida Marshall, 1876
Sub-order: Astrophorina Sollas, 1888
Family: Geodiidae Gray, 1867
Sub-family: Geodiinae Sollas, 1888
Genus: Geodia Lamarck, 1815
Geodia barretti Bowerbank, 1858

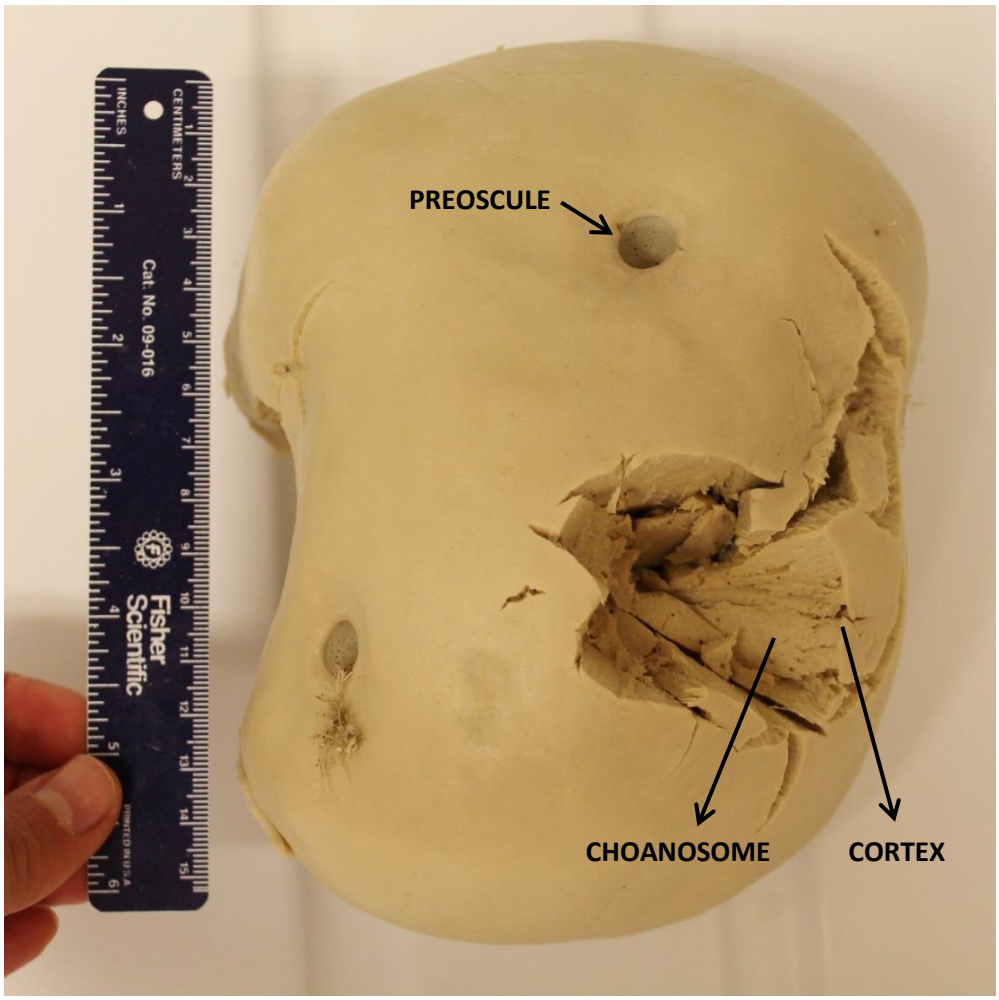
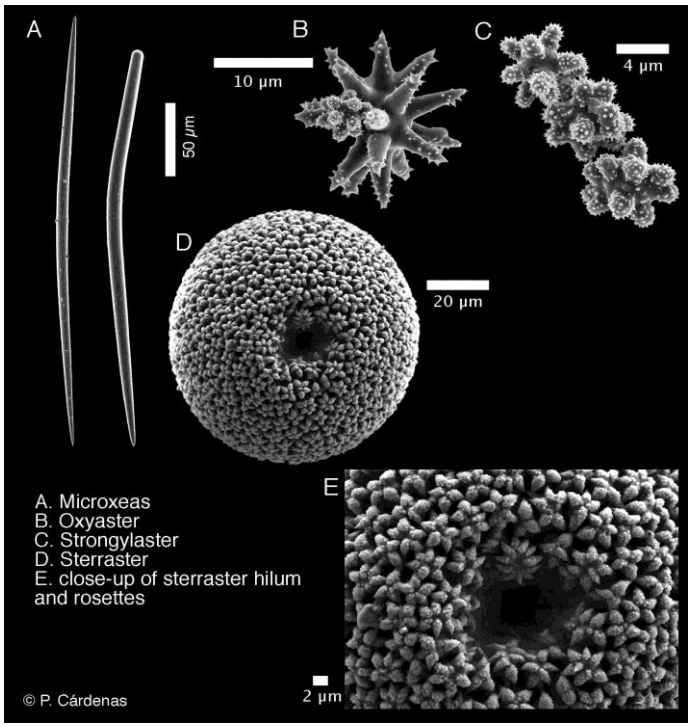


Figure 1. External morphology of a *Geodia barretti* specimen from Tromsø, Norway. The sponge is about 16 cm long and has 2 preoscules, which hold the true oscules.

Geodia barretti specimens have generally round but irregular forms, and can measure up to 80 cm in diameter. The surface is usually smooth and white, although the color can vary to light yellow or light brown (Fig. 1). The texture can also be rather hispid, as it has been observed in some North Western Atlantic (NWA) specimens. Sponges may have one to several preoscules, which contain and protect the true oscules. The cortex, which is 0.5 mm thick, is made up of sterrasters and dichotriaenes, which are types of spicules (Fig. 2). Spicules called microxeas and strongylasters surround the oscules. Each osculum has its own sphincter (Cárdenas et al., 2013).



A study from two populations in Norwegian and Swedish fjords revealed that *G. barretti* is dioecious and oviparous, and that gametes are released once or twice a year, in early summer and in the autumn. Asexual reproduction has not been detected in this species. Furthermore, it has been observed that gamete release coincides with algal blooms for both populations (Spetland et al., 2007).

Figure 2. Spicules of *Geodia barretti* (modified from Cárdenas et al., 2013)

There is a wide diversity of sponge larvae; at least 11 different larval types have been described, but nothing is known about the larval stage of *G. barretti* (Wörheide et al., 2012). Most demosponges release a type of larva called parenchymella, which has a short swimming phase (usually a few days) before settling down (Brusca & Brusca., 1990).

Not much is known about the ecology of this species. In the North Eastern Atlantic (NEA) at least one sponge-feeding chiton species, *Hanleya nagelfar*, has been found living on *G. barretti* (Todt et al. 2009). Other than chitons, different microbial communities have been detected; one analysis discovered the three most abundant microbial groups to be phylum Chloroflexi, phylum Acidobacteria, and probably phylum Poribacteria (Radax et al., 2012).

G. barretti has been found at depths ranging from 30 to 2000 m; in the NEA it is commonly found 200 to 500 m deep. The temperatures at which specimens have been found also vary, from 0.4°C in the Denmark Strait, up to 13°C in the Mediterranean Sea. Specimens have been found in North Eastern and North Western Atlantic locations, and as far south as the Mediterranean Sea (Fig. 3) (Cárdenas et al., 2013).

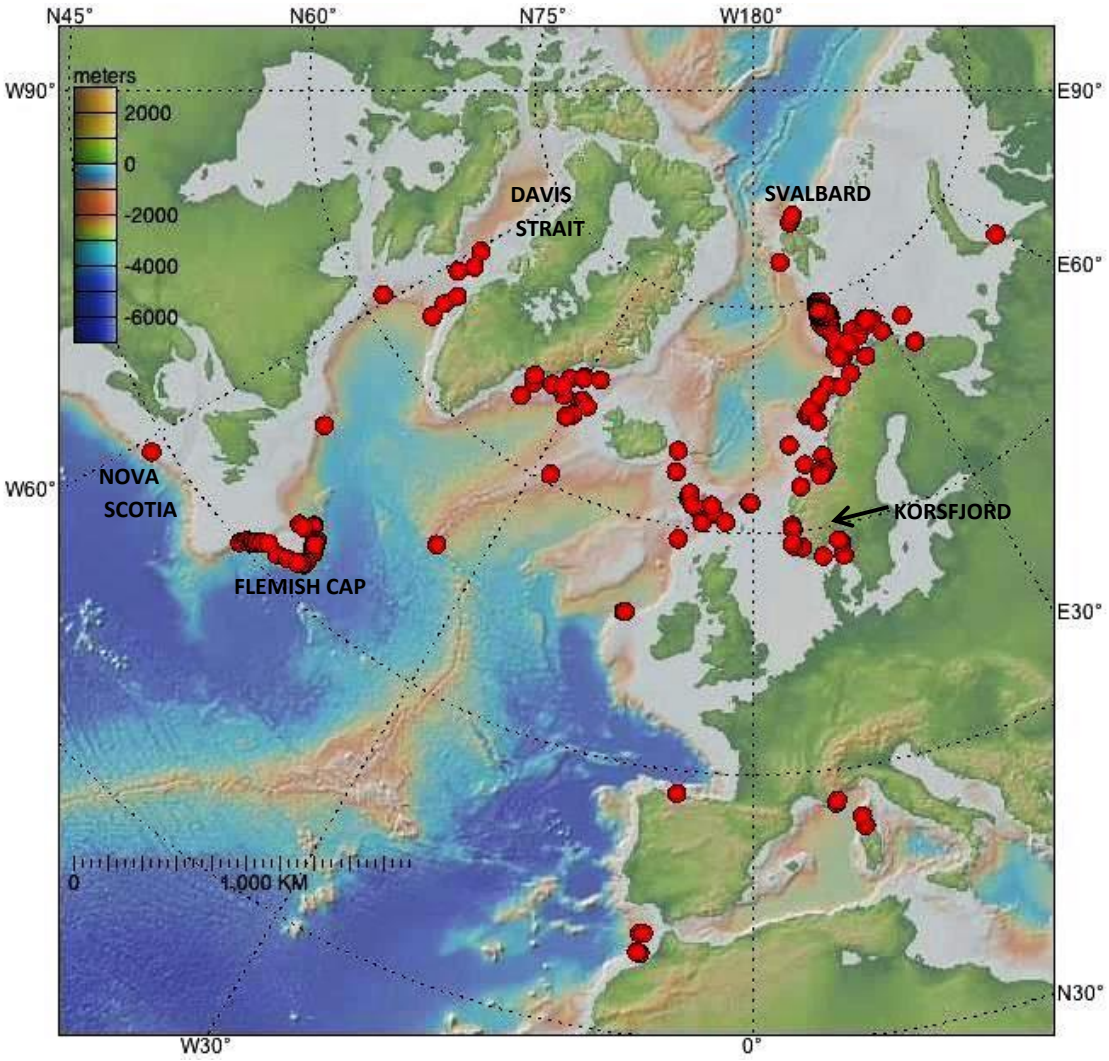


Figure 3. Distribution of *Geodia barretti* (modified from Cárdenas et al., 2013)

Genetic markers: Exon-Primed Intron-Crossing

Allozymes, nuclear gene sequences, mitochondrial DNA, and microsatellites are some examples of molecular markers that have been used for sponge population studies. Microsatellites have been the best option so far because they are highly polymorphic in the number of repeats and are co-dominant. Additionally, they have been extensively used for various genetic analyses. One drawback is that microsatellites require extensive development effort and they are species specific; they have only been developed for eight sponge species, not including *G. barretti* (Uriz & Turon, 2012). Furthermore, when looking at the evolutionary relationships between alleles, microsatellites are also at disadvantage for being susceptible to homoplasy. For example, if two microsatellite alleles are of identical

size, this can be wrongly attributed to common descent, leading to biased population genetic estimates (Anmarkrud et al., 2008).

Generally, the availability of nuclear markers that are polymorphic enough and suitable to use across different phyla is nowadays limited, especially for non-model organisms. **Exon-Primed Intron-Crossing (EPIC)** markers could be a possible solution to this problem. These markers amplify introns, which are non-coding, highly variable, genomic regions. By using potentially universal primer pairs on highly conserved exon sequences, introns can be found and amplified for a wide phylogenetic range, making it possible to carry out population genetic and phylogeographic studies on species from which no genomic data is available (Chenuil et al., 2010).

In a **polymerase chain reaction (PCR)** survey of 50 introns in different phyla, including Porifera, 13 loci appeared likely to contain an intron (Gérard et al., 2013). Of those 13 loci, 10 were considered to be promising, which means they offered remarkably good amplification results for all individuals of the species. The sponge species that was tested in the survey was *Aplysina cavernicola,* which belongs to the same class as *G. barretti,* but not to the same order.

Aims of the project

This project tries to answer the following questions:

- Can we identify readily amplified EPIC markers in *Geodia barretti*?
- If so, are the EPIC markers variable within *Geodia barretti*?
- If the EPIC markers show variability, can they tell something about the genetic connectivity of the *Geodia barretti* specimens?

Materials and Methods

Finding introns

Sample dataset

Table 1. List of sponge DNA extractions and the localities they originate from.

Extraction #	Locality
5	Svalbard
9	Svalbard
17	Svalbard
18	Svalbard
19	Svalbard
23	Newfoundland
32	Korsfjord
60	Nova Scotia
91	Mediterranean Sea
96	Flemish Cap
97	Flemish Cap
102	Davis Strait
117	Flemish Cap
118	Flemish Cap
122	Flemish Cap
132	Flemish Cap
138	Flemish Cap
143	Curacao
150	Korsfjord
192	Korsfjord
205	Korsfjord

The DNA extractions of 21 sponge specimens (Table 1) were already available at the Evolutionary Biology Centre of Uppsala University. Extractions were made within the past two years; see appendix for more details.

List of primers

Ten universal primers were chosen from a previous study, which were successfully amplified on a sponge (*Aplysina cavernicola)* (Gérard et al., 2013). For the first part of the project, the goal was to see which introns could be successfully amplified in *G. barretti* using these ten universal primers. The same PCR program (Gérard et al., 2013) and PCR machine were used for the first round of testing.

Table 2. List of universal primer pairs used and their corresponding introns.

Primer name	Primer Sequence (5'→3')	Tm (°C)	GC-Content (%)	Intron
i1-F	GGCCTGTCCATGACAGAYTGGCAYYT	67.2	55.8	1b
i1-R2	CTATGTCTATACTCCATCGTCCAGATRAACTTGAA	66.5	38.6	
i5-F	ACACTGCCACCCGAGTACCCNATGAARCC	70.9	58.6	5b
i5-R2	AGAGACAGATTTTAGTTCCCACTTCAAACCTTC	65.8	39.4	
i8-F	CACCACTGGTCATATGGCAYGGNATG	68.0	53.6	8a
i8-R	TGATGGAGCCCATGCTTAATGGRTTRCARCA	67.5	46.8	
i12-F	GGACGACAAGAGTCTGAGGGTNTGGGARGT	70.9	56.7	12a
i12-R	GCATGCTGGGGTCTGCAATGTAYTTRAARTC	67.5	46.8	
i15-F	ACACCCTTGACGAGGGGCTGATHGARTTYGG	71.3	55.9	15a
i15-R	ACAGCTTGGCCAGAGTCTTCCACATNGCYTC	70.8	54.8	
i21-F	AACCGGTATACAATCCTTGTGGCAARTAYATGGT	67.1	41.2	21b
i21-R	CCCGGGAAATCATAGCCTCCCATGACYTTCATRTA	70.6	48.6	
i34-F	ATGTATGAACAGTTCCAGAATATCATGAARATGGG	65.4	35.7	34a
i34-R	TTCTTCATCCTTCATACTGTCCATRATNGTCAT	64.5	36.4	
i46-F	GAGGTGGATAAATACAGGGTGGARACNTGYTG	68.8	48.4	46a
i46-R	TCTCATGCTGCCCACGTAGGGARTARTARTTCT	68.9	47	
i50-F	GATGGAATCCATGTCTTGGTCAAYATGAAYGG	66.9	43.8	50a
i50-R	GTGACCGAGTCGGTGATCAGGTARTCCATRAA	69.5	50	
i56-F	CATCATCTTGGTCAGAATTTCTCCAARATGTTYGA	66.0	37.1	56a
i56-R	AACTCCCTTGAGTTCCCAATGRTTRAAYTTCCA	66.4	40.9	

PCR Program

Based on the PCR program from Gérard et al. (2013) study, different temperatures and number of cycles were tested to see how the amplification results varied. For each sample, the PCR reaction contained 22 µl of distilled water, 1 µl of each primer (primers had been previously diluted to 10 µM) and one bead of PuReTaq™ Ready-To-Go™ PCR (GE Healthcare). The PCR program was a touch-down program, which means the annealing temperature was gradually lowered. The program was: 2'94°C; 14 x [1'94°C, 1'58°C to 45°C (- 1°C/cycle), 1'73°C], 25 x [40"94°C, 40"58°C, 1'72°C], 3'73°C; ∞ 6°C.

Electrophoresis

PCR products were checked with electrophoresis on 1.5% agarose gel along with a DNA size marker (GeneRuler™ DNA ladder mix). For each sample 3 µl of PCR product were used. The gel was run at 140 V for 30 minutes.

If the amplification products contained an intron, the bands in the agarose gel were expected to be generally larger than 300 base pairs (Chenuil et al., 2010).

Initially, all ten primers were tested on the same four extractions (9, 192, 205 and 32), plus a negative control. DNA extractions 192 and 205 were diluted 10 times due their high initial concentration. The primers and extractions that showed clear results were noted, and primers that clearly failed to amplify anything were discarded at this point. If a band was in the right position but appeared to be a little faint and/or multiple bands were present, re-amplification was done by directly dipping the tip of the pipette into the gel to take PCR product, and running it through the PCR program again, with 29 cycles instead of 39.

Purification and sequencing

The PCR products that appeared to contain an intron were selected and purified to get rid of primers, dNTPs, enzymes and short-failed PCR products. Purification was done by taking 10 µl of PCR product plus 3 µl of illustra™ ExoStar™ (GE Healthcare), which was diluted 8 times. The samples were centrifuged and run through a PCR program (see appendix).

Once the purification step was done, the samples were sent to Macrogen for DNA Sanger sequencing.

Software used

The sequences were processed and assembled using Staden Package (www.staden.sourceforge.net/), and then aligned with AliView version 1.01 (courtesy of A. Larsson, unpublished open source alignment program). The primers sites were trimmed from the sequences, which were then realigned.

Designing and testing new primers

Based on 3 introns that were successfully sequenced, new primers (Table 3) were designed with the hope that they would work on additional sponge samples. The primers (one forward and two reverse) were designed using AliView and ordered from Eurofins Genomics. Several primers were suggested by the program, but to ensure that the new primers would work well, preference was given to those primers which had higher GC-contents, longer sequences (at least 20 base pairs long) and higher melting temperatures (Tm)- or at least a Tm closer to the Tm of the 56a intron primers, around 66.0°C.

The new primers were first diluted to 10 µM. They were then tested on DNA extractions 192, 205, 32, 17, 19, 143 and 102, with changes in temperature and number of cycles in the PCR program, and in all possible combinations. Primer i56-F was included in these combinations to have more than one forward primer.

Electrophoresis was performed in the same way as previously described.

Subsequently, the primer pair combination that seemed to be the most effective (i56-F and i56-R-Gb2) was applied to the whole dataset of DNA extractions. Specimen samples number 60, 91, 96, 97, 102, 117, 122 and 205 were re-amplified to ensure high quality sequencing results, whereas samples 5, 9, 18, 32, 150 and 192 were sent for gel extraction to Macrogen. All the samples were sequenced at Macrogen.

The resulting sequences were processed and assembled with Staden Package. The primer sites were trimmed from the sequences, which were realigned with AliView.

Table 3. List of new primers designed for higher quality amplification results. F: forward primer, R: reverse primer, and Gb: *Geodia barretti.*

Primer name	Primer Sequence (5'→3')	Tm (°C)	GC-Content (%)
i56-F-Gb	CATTGTCTTTGAGCACCCAGA	57.9	47.6
i56-R-Gb1	TTCCCAATGGTTGAATTTCCAGCC	61.0	45.8
i56-R-Gb2	TTGAATTTCCAGCCGGGAGAG	59.8	52.4

Analysis of the data

To finalize the methods, the sequences of 15 sponge samples were compared, and we ran a phylogenetic analysis to visualize the genetic connections among them.

Two different software were used: first PAUP* (Phylogenetic Analysis Using Parsimony) and then FigTree v.1.4.0.

PAUP* was used for creating a pairwise distance matrix and a phylogenetic tree based on maximum parsimony.

The first step for making the tree was making a stepwise addition heuristic search on PAUP with 10 replicates, which produced 14 equal trees. From the 14 trees, one strict consensus tree was made. Bootstrapping was then done for 10,000 times to check how much support there was for each grouping in the tree.

Results

Evaluation of the universal primers

Table 4. below gives an overview of how effective the primers were in the first round of PCRs. The results for each intron in each extraction were classified as promising (P), intron (I), and amplification (A), which is also how Chenuil et al. (2010) reported their results. Promising means the amplification was long enough to contain an intron and there was one clear band. Intron refers to amplicons that were the right size but faint or with multiple bands, and amplification means that the fragments amplified were too short to contain an intron (Chenuil et al., 2010). The touch-down PCR program described by Gérard et al. (2013) consistently showed the best amplification results compared to any other variations of that same program.

The design of the new primers was based on intron 56a, which appeared to be the most readily amplified for a greater number of DNA extractions. From the second round of PCRs with the new primers, primer pair i56-F with i56-R-Gb2 turned out to be the most effective and was therefore used on all the DNA extractions for the final PCR.

Table 4. A summary of the quality of the PCR amplifications for different introns and DNA extractions. A= amplification, I= intron, P= promising.

		Intron									
Extraction #		1b	5b	8a	12a	15a	21b	34a	46a	50a	56a
	9		I	A	A	A	A	P	A	A	P
	17		I			A	A	I		A	P
	19		I			A	A	I		A	P
	32		I	A	A	I	A	A	A	A	P
	96					A		A		A	
	97		A				A				
	102					A		A		A	I
	117					A				A	
	122					A		A		A	A
	143		A			I	A	A		A	I
	150	A	A				A				
	192	I	I	A	A	I	A	A	A	A	P
	205	I	I	A	A	A	A	A	A	A	A

Pairwise distances between the sponges

Figure 4 shows a matrix with pairwise distance comparisons between the final 15 sequences of the sponges. The values that are given are the number of character differences (nucleotide substitutions) between each two sequences. For example, there are three character differences between sequences 192 from Korsfjord and 102 from Davis Strait.

Extraction		192	122	60	96	102	91	32	19	18	5	9	150	205	17	97
192	Korsfjord															
122	Flemish Cap	1														
60	Nova Scotia	0	2													
96	Flemish Cap	0	2	0												
102	Davis Strait	3	7	4	4											
91	Mediterranean S.	2	5	3	3	4										
32	Korsfjord	0	4	2	2	5	3									
19	Svalbard	9	17	11	10	16	16	12								
18	Svalbard	9	17	11	10	16	16	12	0							
5	Svalbard	9	17	11	10	16	16	12	0	0						
9	Svalbard	10	17	12	11	16	16	12	11	11	11					
150	Korsfjord	9	16	11	10	15	15	11	12	12	12	3				
205	Korsfjord	9	16	11	10	15	15	11	12	12	12	3	0			
17	Svalbard	10	17	12	11	16	16	12	11	11	11	0	3	3		
97	Flemish Cap	22	26	23	22	27	27	23	24	24	24	20	19	19	20	

Figure 4. Pairwise distance matrix of the *Geodia barretti* specimens compared in this study. The number of character differences between the sequences of the specimens is given.

Phylogenetic tree

To summarize the 14 equally parsimonious trees obtained from the stepwise addition heuristic search, one strict consensus tree was made (Fig. 5).

The values on the branches of the consensus tree come from the bootstrap analysis, and they indicate how well-supported the groupings are. The highest value is 100, which means that the grouping is highly supported by the data we have. A value below 80 or even 90 suggests rather low support for a grouping. Notice that this tree is unrooted.

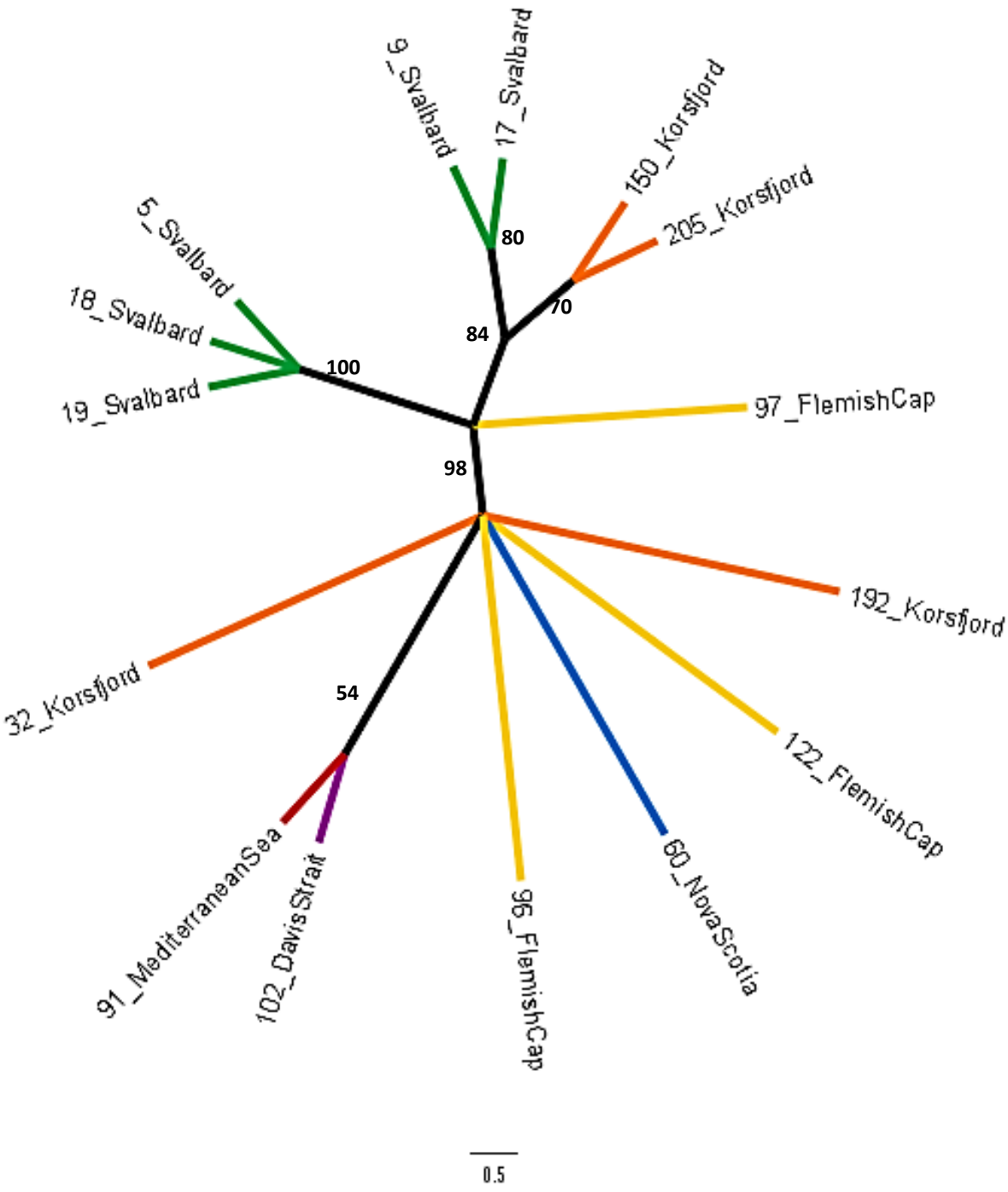


Figure 5.Strict consensus tree based on the 14 most parsimonious trees of *G. barretti* specimens. Bootstrap values are given on the branches of the tree.

Discussion

PCR troubleshooting

Overall, intron 56a was the most successful marker for a greater number of DNA extractions. In other words, only one out of the ten primer pairs worked remarkably well. In some cases it might have not been the primers that failed, but rather the quality of the DNA extracted, since not all sponge specimens were fixed the same way (see appendix for information about the fixations). Some of the extractions were very concentrated and needed to be diluted. A few extractions such as number 17 from Svalbard and 192 from Korsfjord were amplified quite well for different introns. Nonetheless, one good primer pair (i56- F and i56-R) was enough to obtain sequences that demonstrated variability between the samples, and by designing new specific primers, the resulting amplicons had a higher quality.

One interesting observation was the appearance of mixed double peaks for a few of the samples (e.g. in 192) in the chromatograms, which is probably an indication of heterozygosity; this means that more than one allele was present. To be able to see all the variants of an intron, cloning could be used to separate them. Furthermore, the discovery of heterozygosity in a population gives clues as to how structured that population is, so this could be a potential future study.

Pairwise distance matrix and phylogenetic trees

The pairwise distance matrix (Fig. 4) is one way of presenting the variation found among the sponge samples, and if it is compared to the phylogenetic tree, one can see that there is some concordance between them. Generally, the pairs of sponges with less character differences in the matrix are shown more closely related in the tree. However, the matrix and the tree were not made with the same methods, so they are not necessarily correlated; i.e. the tree is not based on the matrix.

All the trees that were produced are unrooted, which means they present information about the phylogenetic relationships but not about the order of descent (Hall, 2001). Even though this makes the tree less informative, it is more appropriate in this case because we don't

have enough information to make assumptions about the direction of evolution of the populations.

Regarding the methods for generating the tree, Maximum Parsimony (MP) was chosen over Maximum Likelihood (ML). MP produces trees with the minimum number of steps to explain the data from an alignment. Taxa that share a common characteristic will be assumed to have inherited that characteristic from a common ancestor. This leads to homoplasies, which can be regarded as extra steps that are needed to explain the data. By using parsimony, these steps are not seen in the tree, which is a disadvantage. On the other hand a ML method can be used, which is more time-consuming, but produces trees that maximize the probability of observing the data, and are usually more accurate because ML requires an evolutionary model to be specified. The likelihood of the resulting tree (usually one tree is produced) is also known, which is an advantage (Hall, 2001). Since the amount of data we had for this project was relatively small, and the goal was rather to show variation among sponge specimens from different locations, a MP method was favored.

From the consensus tree (Fig.5), one of the first things that can be observed is that some of the specimens which come from the same locality are grouped together, but some are not. Specimen 97 from the Flemish Cap is separated from specimens 122 and 96, which are also from the Flemish Cap. When we looked for further information about these Flemish Cap sponges, it was noted that specimen 97 is of a different *coxI* haplotype (a common genetic marker in animals) than the rest of the specimens (Cárdenas et al., 2013). It is interesting that the EPIC marker used in this project seems to support this difference.

Specimen 192 from Korsfjord appears to be quite distant from rest of the Korsfjord specimens, which was surprising at first, until looking back at the chromatograms and the alignment of the sequences, and making two observations. The first one is that the sequence for this specimen was shorter and contained more gaps compared to the other sequences. It is a possibility, that because of the poor quality of the sequence, the sponge has been inaccurately placed as an unresolved branch in the tree. The second observation was the presence of double peaks in the chromatogram of this sequence, which were very mixed. As it has been previously discussed, this could be a sign of heterozygosity, and maybe the

sequence we obtained was from a different allele than the other Korsfjord sequences. This is another potential reason why it appears disconnected in the tree.

Another unexpected grouping is the one of specimens 102 and 91, from Davis Strait and the Mediterranean Sea, respectively. The bootstrap analysis does not support this grouping strongly (a value of 54 is considered to be rather low). Nevertheless, if they really are more closely related, a few guesses can be made to explain this. Even though the two localities are very far away from each other, we don't know how far the larvae of a sponge could travel in a strong deep-sea current. Little is known about sponge larvae, and based on what we do know, it seems unlikely that a larva could travel such a long distance because the planktonic stage is usually short (Brusca & Brusca., 1990). Another possibility is that the populations have been in contact by the dispersal of larvae via ballast water. More specimens need to be studied to verify this grouping.

When looking through the 14 trees of the heuristic search, the arrangement of the nodes and branches did not vary a lot; some of the groups were consistently formed the same way (all the Svalbard specimens, and specimens 150, 205, 117 and 97), which is what we see in the consensus tree. The lengths of the branches are an important aspect of the tree because they reflect the number of nucleotide substitutions in a sequence.

One thing to keep in mind is that the number of sample specimens from each locality was uneven, so to truly investigate the connections between the populations that these specimens represent, more samples need to be tested and sequenced.

Conclusion

EPIC markers proved to be useful in this preliminary study, as variability was found among the *G. barretti* specimens. The possibility of designing new primers based on the initial sequences obtained by using the universal primers is an effective way of increasing the PCR success rate with EPIC markers. For a more complete phylogeographic study, a larger number of specimens should be sampled and sequenced to get a more accurate idea of how the populations are connected to each other. The connectivity of the populations could give us some information about the dispersal capabilities of the larvae and how much they can travel under different environmental conditions, which could be an interesting investigation for the future.

Acknowledgements

I would like to thank Mikael Thollesson for giving me the opportunity to work on this project, Astrid, Allison & Sarina for giving me tips and tricks, and special thanks to Paco Cárdenas for guiding me throughout the 10 weeks of this project, passing on his sponge knowledge and letting me use some of his material.

References

Anmarkrud JA, Kleven O, Bachmann L, Lifjeld JT. 2008. Microsatellite evolution: Mutations, sequence variation, and homoplasy in the hypervariable avian microsatellite locus HrU10. *BMC Evolutionary Biology* 8: 138.

Brusca RC, Brusca GJ. 1990. *Invertebrates*. Sunderland, Mass: Sinauer Associates.

Cárdenas P, Rapp HT, Klitgaard AB, Best M, Thollesson M, Tendal OS. 2013. Taxonomy, biogeography and DNA barcodes of *Geodia* species (Porifera, Demospongiae, Tetractinellida) in the Atlantic boreo-arctic region: Revision of Atlantic boreo-arctic *Geodia*. *Zoological Journal of the Linnean Society*, 169: 251-311.

Chenuil A, Hoareau TB, Egea E, Penant G, Rocher C, Aurelle D, Mokhtar-Jamai K, Bishop JD, Boissin E, Diaz A, Krakau M, Luttikhuizen PC, Patti FP, Blavet N, Mousset S. 2010. An efficient method to find potentially universal population genetic markers, applied to metazoans. *BMC Evolutionary Biology* 10: 276.

Genta-Jouve G, Thomas OP. 2012. Sponge Chemical Diversity. *Advances in Marine Biology*. Elsevier, 183–230.

Gérard K, Guilloton E, Arnaud-Haond S, Aurelle D, Bastrop R, Chevaldonné P, Derycke S, Hanel R, Lapègue S, Lejeusne C, Mousset S, Ramšak A, Remerie T, Viard F, Féral JP, Chenuil A. 2013. PCR survey of 50 introns in animals: Cross-amplification of homologous EPIC loci in eight non-bilaterian, protostome and deuterostome phyla. *Marine Genomics* 12: 1–8.

Goeji, J. M. 2009. Element cycling on tropical coral reefs: the cryptic carbon shunt revealed. Unpublished thesis, University of Groningen.

Hall BG. 2001. *Phylogenetic trees made easy: a how-to manual for molecular biologists*. Sunderland, Mass: Sinauer Associates.

Leys SP, Hill A. 2012. The Physiology and Molecular Biology of Sponge Tissues. *Advances in Marine Biology*. Elsevier, 1–56.

Radax R, Rattei T, Lanzen A, Bayer C, Rapp HT, Urich T, Schleper C. 2012. Metatranscriptomics of the marine sponge Geodia barretti: tackling phylogeny and function of its microbial community: Metatranscriptomics in *Geodia barretti*. *Environmental Microbiology* 14: 1308–1324.

Spetland, F., Rapp H.T., Hoffmann, F., Tendal, O. S. 2007. Sexual Reproduction of *Geodia barretti* Bowerbank, 1858 (Porifera, Astrophorida) in two Scandinavian fjords. In: Custódio MR, Lôbo-Hajdu G, Hajdu E, Muricy G (eds). Porifera research: biodiversity, innovation and sustainability. Série Livros 28. Museu Nacional, Rio de Janeiro.

Thacker RW, Freeman CJ. 2012. Sponge–Microbe Symbioses. *Advances in Marine Biology*. Elsevier, 57–111.

Todt C, Cárdenas P, Rapp HT. 2009. The chiton *Hanleya nagelfar* (Polyplacophora, Mollusca) and its association with sponges in the European Northern Atlantic. *Marine Biology Research* 5: 408–411.

Uriz MJ, Turon X. 2012. Sponge Ecology in the Molecular Era. *Advances in Marine Biology*. Elsevier, 345–410.

Van Soest, R.W.M; Boury-Esnault, N.; Hooper, J.N.A.; Rützler, K.; de Voogd, N.J.; Alvarez de Glasby, B.; Hajdu, E.; Pisera, A.B.; Manconi, R.; Schoenberg, C.; Janussen, D.; Tabachnick, K.R., Klautau, M.; Picton, B.; Kelly, M.; Vacelet, J.; Dohrmann, M.; Cristina Díaz, M.; Cárdenas, P. (2014) World Porifera database. Accessed at http://www.marinespecies.org/porifera on 2014-05-20

Wörheide G, Dohrmann M, Erpenbeck D, Larroux C, Maldonado M, Voigt O, Borchiellini C, Lavrov DV. 2012. Deep Phylogeny and Evolution of Sponges (Phylum Porifera). *Advances in Marine Biology*. Elsevier, 1–78.

Appendix

Table A1. Information on the DNA extractions of the specimens used in this project.

Geodia barretti specimens				
Extraction #	Fixation	Locality	1st Dilution of DNA (µl)	DNA concentration (ng/µl)
5	ethanol 96%	Svaldbard	100	32.5
9	ethanol 96%	Svaldbard	100	65.2
17	ethanol 96%	Svaldbard	100	105
18	ethanol 96%	Svaldbard	100	72
19	ethanol 96%	Svaldbard	100	115
23	ethanol 70%	Newfoundland	100	-
32	ethanol 70%	Korsfjord	100	57.4
60	ethanol 70%	Nova Scotia	100	-
91	ethanol 70%	Mediterranean Sea	100	12.9
96	ethanol 70%	Flemish Cap	100	2.5
97	ethanol 70%	Flemish Cap	100	20
102	ethanol 70%	Davis Strait	100	100
117	ethanol 70%	Flemish Cap	100	12.6
118	ethanol 70%	Flemish Cap	100	-
122	ethanol 70%	Flemish Cap	100	5.7
132	ethanol 70%	Flemish Cap	100	-
138	ethanol 70%	Flemish Cap	100	-
143	ethanol 70%	Curacao	100	160
150	RNA later	Korsfjord	100	394
192	RNA later	Korsfjord	100	244
205	RNA later	Korsfjord	100	360

Table A2. ExoStar™ PCR program used for the purification of PCR products.

Purification PCR Program		
Steps	Temperature (°C)	Time (min)
1 Cycle		
1	37	15
2	80	15
∞ 8°C		