Minimal nucleotide sequence divergence detected in the sea urchin *Strongylocentrotus droebachiensis* by direct sequencing of enzymatically amplified DNA

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ABSTRACT

The assumptions that have been made concerning mitochondrial DNA (mtDNA) variability have been based on studies on *Drosophila* and vertebrates. Recent studies on marine species show marked differences in comparison to terrestrial populations. Terrestrial populations tend to show considerable amounts of mtDNA variability accompanied by distinct population structure. My study is concerned with the variation present in the mitochondrial genome of *Strongylocentrotus droebachiensis* populations. This sea urchin has a distribution throughout the Arctic Ocean and in both the North Pacific and the North Atlantic Oceans. Based on the previous assumptions about mtDNA variation, we should expect large degrees of sequence difference due to the mere magnitude of geographic distances involved.

Using the polymerase chain reaction we have been able to determine the sequence of the ATPase 6 gene in the mitochondrial genome of six *S. droebachiensis* sea urchins from the North Atlantic. These sequences were compared both among themselves and to a *S. droebachiensis* urchin from Puget Sound, Washington. Of the approximately 1500 base pairs that were sequenced, only one base substitution was found. This indicates dramatic differences between the amounts of variation present in the sea urchin genome versus the greater diversity found in genomes previously studied.

INTRODUCTION

Mitochondrial DNA (mtDNA) has gained widespread use as a powerful tool for the evolutionary studies of animals (reviews in Avise *et al.*, 1986, Moritz *et al.*, 1986, Wilson *et al.*, 1985). MtDNA is a relatively small, circular molecule that is easily isolated and present in the cell at very high copy numbers. This type of DNA evolves quickly in comparison to nuclear DNA (Brown *et al.*, 1979) and therefore is useful for studying the relationships between closely related species (Wilson *et al.*, 1985). Its maternal mode of inheritance (Hutchinson *et al.*, 1974) also makes it useful for building geneological trees and for tracing migrations (Wilson *et al.*, 1985).

Much of the information to date concerning DNA variability has come from studies on *Drosophila* and vertebrates. These mitochondrial genomes are well characterized in comparison to what is known about genetic change in other taxa. Britten (1986) reported that the rates of change for single copy nuclear DNA differed across phylogenetic groups. He noted that primates and several bird lineages accumulated changes in their nucleotide sequence more slowly than rodents, sea urchins and *Drosophila*. Due to these rate differences, further investigation into species with markedly different habitats and life histories is necessary.

The life histories of marine invertebrates differ in many ways from terrestrial animals. Typical patterns of genetic variation therefore, may not be valid across taxa. In contrast to predominantly internal fertilization, characteristic of terrestrial vertebrates, sea urchins release eggs and sperm into the water column where external fertilization occurs (Schechter, 1959). As larvae, they are dispersed at the mercy of oceanic currents and may spend up to three months in the plankton, possibly settling thousands of kilometers from

their parents (Scheltema, 1983). As a result, sea urchin populations have extremely large ranges.

The temperate sea urchin *Strongylocentrotus droebachiensis* is the focus of this project. The genus *Strongylocentrotus* has been extensively studied and is well characterized in terms of its morphology, ecology (Mortensen, 1948, Jensen, 1974), development (Strathmann, 1987), and molecular biology (Jacobs *et al.*, 1988). The complete sequence of the mitochondrial genome for *S. purpuratus* has already been established (Jacobs *et al.*, 1988).

The distribution of *S. droebachiensis* is circumpolar, occurring throughout the arctic seas even on the northern coast of Greenland. In the Atlantic, it ranges south to northern Scotland and in North America, as far south as Chesapeake Bay. It is common in the Pacific region throughout the Bering Sea and as far south as Vancouver, British Columbia and Puget Sound, Washington (Jensen, 1974, Mortensen, 1943).

It is thought that *S. droebachiensis* originated in the north Pacific region and spread to the Atlantic via an easterly route (Mortensen, 1943). This is based on the fact that all eight members of the *Strongylocentrotus* genus are present in the Pacific, while *S. droebachiensis* and *S. pallidus* are the only two Atlantic species (Jensen, 1974).

The purpose of this project is to determine the amount of intraspecific variation present in the mitochondrial genome of *S. droebachiensis*. Individuals collected from St. John's, Newfoundland will be compared to those found in Puget Sound, Washington. These localities are approximately 15,000 kilometers apart via the shortest water route through the Bering Sea and the Arctic Ocean (Palumbi and Wilson, 1989). The molecular region of interest is the ATPase 6 gene, a protein coding region of the genome. The ATPase 6 gene has a slightly higher average percentage (37.8%) of amino acid sequence

similarity among Xenopus, human, mouse and cow than the ATPase 8 gene (25.5%), but less homology (75.0%) than the cytochrome oxidase I gene (Jacobs *et al.*, 1988). The ATPase 8 and cytochrome oxidase I genes represent, respectively, the highest and lowest amounts of mean sequence similarity present in the protein coding regions of the sea urchin mitochondrial genome (Jacobs *et al.*, 1988). From this cross-taxa comparison, we can infer that the ATPase 6 gene is a highly variable region of the genome. I will be determining the DNA sequence changes and the resultant effects that this might have on the amino acid code. Based on the assumptions made about mtDNA evolution through previous studies, we would expect a high degree of variability due to the mere geographic distances and the region of the genome involved.

MATERIALS AND METHODS

POLYMERASE CHAIN REACTION

A new method called the Polymerase Chain Reaction (PCR) allows for rapid sequence determination directly from genomic DNA (Erlich *et al.*, 1988, Scharf and Erlich, 1988), eliminating many time consuming steps. PCR is an enzymatic in vitro procedure which allows for isolation and amplification of a specific segment of DNA, circumventing tedious manipulations of bacteria and phage. PCR involves two oligonucleotide primers that flank the region to be amplified and define the length of the product. Repeated cycles of denaturation, annealing of primers and extension by a DNA polymerase result in an exponential accumulation of specific target sequence (Erlich *et al.*, 1988). Isolation of a thermostable DNA Polymerase from the bacterium *Thermus aquaticus* has greatly enhanced the method. This polymerase retains its activity after heat denaturation of the template DNA and also has a higher temperature

optimum which significantly increases the specificity of the reaction (Saiki *et al.*, 1988).

A typical PCR cycle involves incubations at three different temperatures corresponding to the three steps in the amplification cycle. In the first step, the DNA strands are separated by heat denaturation at 94°C. The temperature is then lowered to about 50°C where two oligonucleotide primers, present in equal concentration, anneal to their complementary sequence on the template DNA. The primers orient themselves in the 5' to 3' direction as this corresponds to the direction that synthesis will take place in the next step. The annealing temperature can be varied anywhere from 40 to 60°C. If you are working with template that is not completely complementary to the primer, lowering the annealing temperature and thus lowering the specificity will increase chances of a good yield. Primer extension at 72°C is the third step of the cycle. This is the temperature at which the *Thermus aquaticus* polymerase has its maximum activity. The time spent at this temperature is determined by the length of the gene that is being amplified, usually one minute for every one thousand base pairs is allotted. After extension, the temperature is again raised to 92°C where the strands separate and begin the cycle over. The PCR cycle is usually repeated 50 times. With each cycle exponentially increasing the amount of DNA, large quantities of target sequence become available in an extremely short amount of time.

Besides being useful for amplifying large quantities of double stranded DNA, PCR can also be employed to produce the single stranded DNA needed for sequencing (Gyllensten and Erlich, 1988). By using an asymmetric ratio of the primers, usually 1:100, double stranded DNA is generated in the earlier cycles until the limiting primer is completely consumed. From that point on, single stranded DNA is produced as only one primer is present.

SANGER SEQUENCING

The DNA generated by PCR can be sequenced using the primer limiting in the amplification process (Wrishchnik *et al.*, 1987). The Sanger method of DNA sequencing involves the synthesis of a complementary strand of DNA which is terminated at various stages of extension. This results in a random population of DNA strands. The DNA is again heat denatured in order to insure that the DNA is single stranded. Primers are then allowed to anneal to the template by cooling to ambient temperature. The DNA is radiolabeled to facilitate visualization on an autoradiogram by incorporation of 35-SdATP into the synthesized strand. The extension reaction is terminated by its addition to four different reaction mixes, each which contain dideoxy nucleotides corresponding to each base - guanine, adenine, thymine and cytosine. These nucleotide analogs are readily incorporated into the synthesis reaction however, they lack the 3' hydroxyl group necessary for continued extension. These strands of varying length are then separated on a 8% polyacrylamide, 8M Urea gel. The result is a ladder from which the DNA sequence can be easily read.

S. droebachiensis was collected from both Puget Sound, Washington and St. John's, Newfoundland in 1988. Mitochondrial DNA was isolated by lysing the cell in a 1% SDS, 1M CsCl solution and purified by CsCl density ultracentrifugation as described by Palumbi and Wilson (1989).

This purified mtDNA was then amplified using the Polymerase Chain Reaction (Figure 1). Primers to amplify the ATPase 6 region were chosen from the published sequence of *S. purpuratus* (Jacobs *et al.*, 1988). Two different methods were used to obtain single stranded DNA. Amplifications were done both directly from the purified mitochondrial DNA, using an asymmetric ratio of

primers and from the double strand amplifications, by the addition of only one primer. This single stranded DNA was used as a template for sequencing.

RESULTS

MtDNA sequences of the ATPase 6 region from six Newfoundland *S. droebachiensis* (see Figure 2) were compared to each other, to a sequence obtained by Bailey Kessing (unpubl. data) for an individual from Puget Sound, Washington and to the *S. purpuratus* sequence published by Jacobs *et al.* (1988). Approximately 1500 base pairs have been sequenced from these six individuals. Of this entire data set, only one substitution at position 8831 in individual NFL34 was found. In comparing the Atlantic *S. droebachiensis* to the Pacific sequence, there was variation present again only at site 8831. The Pacific sequence at this position contained the same nucleotide as the NFL34 sequence. Comparisons between *S. droebachiensis* and *S. purpuratus* revealed 21 total substitutions. Of these 21, four of which resulted in a change in the amino acid code.

DISCUSSION

Given the wide geographical separation, the moderate variability of the ATPase 6 gene and the rapid mutation rate for mtDNA in general, we would expect a much greater amount of intraspecific diversity. This finding is consistent with the low intraspecific change observed by Palumbi and Wilson (1989). Using restriction fragment length polymorphism analysis, they reported a value of 0.1% intraspecific sequence divergence for *S. droebachiensis.* Although these results are both consistently low, restriction analysis is incapable of determining changes in the amino acid sequence and the changes

that occur at each codon position. However in our case, no differences were found in the amino acid code between Pacific and Atlantic *S. droebachiensis*. Both AGA and AGG, the only codon found altered, degenerately code for serine.

Based on results from their restriction fragment analysis, Palumbi and Wilson (1989) were able to assign six genotypes for *S. droebachiensis* (see Table 1). The Newfoundland urchins carried either genotype *a* or *f*. Genotype *a* was characterized as being the dominant Puget Sound genotype. Genotypes *a* and *f* differ only by a single HPA II restriction site. This difference was characterized as a restriction site gain for the Atlantic type *S. droebachiensis,* as it was found among urchins in Maine as well (Palumbi and Wilson, 1989).

It is surprising that the sequence data did not separate out with the restriction analysis. Restriction fragment analysis is capable of distinguishing sequences which differ by as little as 0.5% (Wilson *et al.*, 1985). Position 8831, however does not fall at a recognizable restriction site. Therefore, NFL34 still has restriction genotype *f*. Individual NFL27, which has the predominant Washington restriction genotype *a*, has sequence genotype 2. The HPA II restriction site must lie outside the area that has been sequenced. Future projects may focus in areas of the genome which do contain HPA II restriction sites in order to better assess the parallels between restriction analysis and sequence data in regard to *S. droebachiensis*.

Using both the restriction data from Palumbi and Wilson (1989) and the sequence data from this study, we can calculate an estimate of the probability that a nucleotide position may differ between identical sequences. The sea urchin mitochondrial genome is 15650 base pairs long (Jacobs *et al.*, 1988). Adding the number of sites screened in the restriction fragment analysis to the number of bases sequenced, gives us a total of 674 base pairs or approximately 4.3% of the genome that has been accounted for. There were no

cleavage sites in the region that was sequenced. The probability, at a 95% confidence level, that the sequences will be the same across the rest of the genome is $\pm$ 0.000076. Using a divergence estimate of 1-2% per million years, the estimated rate of nucleotide substitution for most mitochondrial DNAs (Wilson *et al.*, 1985), the calculated maximum time since divergence from identical genotypes yields a time frame of between 3,750 and 7,500 years.

This exceeding low value, on an evolutionary time scale, may indicate two things. Firstly, that there has been and possibly still exists considerable gene flow between the Pacific and Atlantic *S. droebachiensis* populations. High degrees of sequence homology over such vast distances, are an indication that population structure in the marine environment is very different from that in the terrestrial realm. Usually, within terrestrial species, there exists a high level of polymorphism, with marked geographic differentiation (Avise *et al.*, 1986, Moritz *et al.*, 1986, Vawter and Brown, 1986).

Secondly, sea urchin mtDNA may merely evolve at slower rates than in other species studied so far. Perhaps the sea urchin mitochondrial genome is under greater constraints than the genomes previously studied. High conservation in this particular molecule may be due to functional constraints that are variable across taxonomic groups. It has also been recognized that stabilizing selection acts to prevent change at certain functional nucleotide or amino acid sequences. Substitutions are also much rarer at enzyme active sites than elsewhere in the genome (Goodman, 1978).

Sea urchin mtDNA diversity is strikingly low. This observation may be explained by frequent dispersal by females or a recent bottleneck in the population (Moritz *et al.*, 1987). Sea urchins are prone to both of these situations. There is documented evidence of widespread population crashes of sea urchins (North and Pearse, 1970, Lessios *et al.*, 1985) as sea urchin

populations are known to be unstable. *S. droebachiensis* populations in Nova Scotia, Canada underwent a series of mass mortalities in the years 1980-1982 (Miller and Colodey, 1983, Scheibling and Stephenson, 1984). Mortality in some areas was as high as 70% (Scheibling and Stephenson, 1984). In sea urchins also, fecundity is enormous. A female may release three to six million eggs at a time into the water column (Strathmann, 1987).

In general reference to sea urchin interspecific diversity, comparisons between *S. purpuratus* and *S. droebachiensis* yielded 21 total substitutions, four of which resulted in amino acid substitutions (about 18%). *S. purpuratus* and *S. droebachiensis* differ in their mtDNA sequences by about 6.1% (Vawter and Brown, 1986). A comparison between human, chimp and gorilla sequences, which differ by approximately the same amount, show that almost 30% of the substitutions that occur result in amino acid changes (Brown *et al.*, 1982). The sea urchin situation is more similar to the *Drosophila* case where, for the ATPase 6 gene 13% of the substitutions are replacement changes (Brown, 1985).

Intraspecific diversities for other marine animals with planktonic dispersal modes are found to be low as well. *S. franciscanus* (Vawter and Brown, 1986), tropical sea urchins (Palumbi and Metz, unpubl. data), and several marine fish (Avise *et al.*, 1986), all have low diversity in comparison to terrestrial populations. Although this study was effective in revealing an almost total lack of differentiation between Atlantic and Pacific populations, more studies concerning mtDNA diversity in other high dispersal marine species are required in order to discern the extent of the interaction between life histories and population biology.

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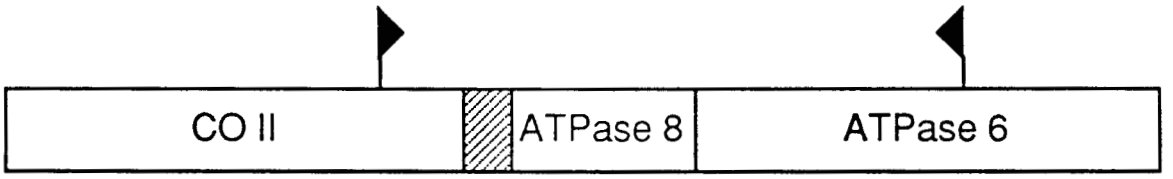

Figure 1. Region of the sea urchin mitochondrial genome amplified by the polymerase chain reaction. Flags indicate the primer orientation and direction of synthesis. The shaded area is the Lysine transfer RNA coding region.

Figure 2. Mitochondrial DNA sequences of the ATPase 6 gene for six individual *S. droebachiensis* from Newfoundland (NFL). The reference sequence is a *S. droebacheinsis* from the Friday Harbor Laboratories, Puget Sound, WA (FHL). Numbering is done according to the sequence for *S. purpuratus* (Jacobs *et al.*, 1988). Sequence homology is indicated by a dot (·).

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8721                TC GTA TTT ATC TAC CCC GTA AAA TGA   FHL236
                                       ... ... ... ... ...   NFL22
     ... ... ... ... ... ... ... ... ... ... ... ... ...   NFL26
     ... ... ... ... ... ... ... ... ... ... ... ... ...   NFL32
     ... ... ... ... ..G ... ..T ... ... ... ... ..T       PURP

8757 GCC CCA TCT CGA TTC CAA TCT GTT TGG CTT GGT TTT   FHL236
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL22
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL26
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL32
                     ... ... ... ... ... ... ... ...   NFL40
     ... ... ... ... ... ... ... A.. ... ... ... ...   PURP

8793 CGA GAA AAC ATC TTA GAG ATG ATC TTC CAG AAA ACC   FHL236
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL22
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL26
                                                 ...   NFL27
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL32
               . ... ... ... ... ... ... ... ... ...   NFL34
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL40
     ..G AG. ... ... ... ..A ... ..T ... ... ... ...   PURP

8829 AGA CCT AAA ACT GCC CCT TGA GCA GGC TTG ATA GCA   FHL236
     ..G ... ... ... ... ... ... ... ... ... ... ...   NFL22
     ..G ... ... ... ... ... ... ... ... ... ... ...   NFL26
     ..G ... ... ... ... ... ... ... ... ... ... ...   NFL27
     ..G ... ... ... ... ... ... ... ... ... ... ...   NFL32
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL34
     ..G ... ... ... ... ... ... ... ... ... ... ...   NFL40
     ... ... ... ... ... ... ..G ... ... ..A ... ...   PURP
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8865 AGA GTC TTT GTC CTC ATT TTA TCT GTT AAC GTT CTA   FHL236
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL22
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL26
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL27
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL32
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL34
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL40
     G.. ... ... ... ..T ... ... ... ... ... ..C T.G    PURP

8901 GGC CTC TTT CCC TAT GCT TTT ACG GCC ACA AGA CAC   FHL236
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL22
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL26
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL27
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL32
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL34
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL40
     ... ..T ... ... ... ... ... ... ... ... ... A..    PURP

8937 ATA TCC TTG ACC TAC AGA TTG GGT TTC CCT CTA TGA   FHL236
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL22
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL26
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL27
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL32
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL34
     ... ... ... ... ... ... ... ... ... ... ... ...   NFL40
     ... ... ... ... ... ... C.A ... ... ... ..T ...    PURP

8973 ATG GCA GTA AAA ATT CTA GGT TTC TAC C             FHL236
     ... ... ... ... ... ... ...                       NFL22
     ... ... ... ... ... ... ... ... .                 NFL26
     ... ... ... ... ... ... ...                       NFL32
     ... ... ... ... ... ... ... ... .                 NFL34
     ... ... ... ... ... ... ... ... ... .             NFL40
     ... ... ... ... ... ... ... ... ... .              PURP
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Table 1. Restriction genotypes assigned by Palumbi and Wilson (1989) and corresponding sequence genotypes for the ATPase 6 gene.

INDIVIDUAL	RESTRICTION GENOTYPE	SEQUENCE GENOTYPE
FHL236	a	1
NFL22	f	2
NFL26	f	2
NFL27	a	2
NFL32	f	2
NFL34	f	1
NFL40	not avaliable	2