

Characterization and Identification of Long Island  
Sound Harbor Seals by Microsatellite  
Amplification

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# **characterization and identification of long island sound harbor seals by microsatellite amplification**

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# **PURPOSE**

- To evaluate the feasibility of DNA extraction and isolation from fecal matter.
- To evaluate the genetic variation of microsatellites of the DNA from Harbor Seals (*Phoca vitulina concolor*) from different locations within Long Island Sound

# **abstract**

Seal fecal samples were opportunistically obtained from Long Island Sound and desiccated. The DNA of these samples was then isolated using a Qiagen DNA stool kit. The DNA samples were quantified by spectrophotometry. The average amount of DNA in each was assessed in  $\mu\text{g}$ . Samples from Norwalk contained an averaged  $13.9\mu\text{g}$ , where as the samples from great gull island averaged \_\_\_\_  $\mu\text{g}$ .

The DNA samples then underwent amplification for microsatellites using Polymerase Chain Reaction. This served to amplify the specific microsatellites of the seal DNA. The samples were then electrophoresed so that the length of their microsatellites could be contrasted. Once viewed under ultraviolet light, the microsatellites indicated limited distinguishable diversity among the different samples.

# introduction

## Harbor Seals (*Phoca Vitulina concolor*)

Harbor Seals belong to the Sub Order Pinnipedia, which includes sea lions, fur seals, walruses and seals. 33 species of organisms exist within this sub order and are thought to be evolved from a bear-like ancestor that moved into the water at some point around 23 million years ago. Two endangered species of Pinnipedia exist in the United States today: Stellar Sea Lions and Hawaiian Monk Seals. Pinnipedia are often distinguished by their head, which is dog-like in appearance, and the W shape formed by their nostrils.

The Harbor Seal (*Phoca vitulina concolor*) is the most common type of seal found in Long Island Sound. Previously hunted to near extinction, the Harbor Seals are now protected by the Marine Mammal Protection Act of 1972, which restricts hunting of the seals to Alaskan natives, and the Harbor Seal population has made a significant rebound. The seals migrate from Maine in the winter to reach a warmer climate and then return in the summer. Their coats vary from light gray or tan to brown and red, with black or light spots. The coat often looks dark when wet, but can be very light when dry (<http://www.mmsc.org/info/seal-harbor.html>). The males are about 6 feet long and can weigh up to 250 pounds, which is consistently larger than the females who only reach about 5 feet in length. Unlike many seal species, the harbor seal pup has a coat that closely resembles the adult coat. Some have a longer, softer white or gray coat (lanugo) when born, but they shed that coat within about 10 days. These pups are born from late April to mid-June and weigh about 21 pounds with a length of about 2.5 feet (The Marine Mammal Center, 2002). Wild harbor seals consume about 6 to 8 percent of their body weight in food per

day, depending on the nutritional value of the food being eaten. Their diet varies greatly depending on location, and includes a wide variety of fish, cephalopods, and crustaceans. Their average life span is around 25 years, although some have lived for over 30 years. However, males tend to live shorter lives, probably due to the added physical stress of fighting during breeding season. Harbor Seals are often known to “haul out”, or drag themselves onto rocks where they lie and bathe in the sun. Reefs, sand and gravel beaches, sand and mud bars, and glacial and sea ice are commonly used for hauling sites (Kinkhart, 2002). Although they are fairly clumsy on land, hauling out provides the seals with an opportunity to congregate, give birth, care for their pups and rest. The seals are usually seen hauled out in groups, possibly to reduce the amount of time each individual must spend scanning for threats.

## Microsatellites

Microsatellites are short sequences of mono, di, tri, or tetra nucleotide repeats of variable length distributed widely throughout the genome. Using PCR primers to the unique sequences upstream and downstream of a microsatellite their location and polymorphism can be determined. (CancerWEB project, 2003). These short strings of repeated nucleotide combinations are commonly used as a genetic marker when comparing the primary and secondary structures of DNA. Widely dispersed among eukaryotic genomes, microsatellites appear in their primary structures as repeated nucleotide segments called “DNA repeats” like so; CTGCTGCTGCTG, in which this microsatellite would be referred to as (CTG)<sup>4</sup>.

Microsatellites are commonly referred to as “junk” DNA, in the sense that these repetitious DNA bands usually don't have any measurable effect on phenotype of an organism. In contrast, the importance of microsatellites is observed through an organism vs. organism

relationship. Repeated sequences can occur as a tandem array. Such sequences, called Variable Number Tandem Repeats (VNTRs), are unique to each person and are the basis for the precise DNA fingerprinting used in forensics (Frank, *Microsatellite instability*). The polymorphism of the microsatellites is used for comparison between organisms to view genetic variation.

Variation in microsatellite length suggests the variation in organism genetics.

To visibly read and evaluate DNA variation, a method called PCR (polymerase chain reaction) is used. PCR is a molecular technique that Two synthetic oligonucleotide primers, which are complementary to two regions of the target DNA to be amplified, are added to the target DNA, in the presence of excess deoxynucleotides and Taq polymerase, a heat stable DNA polymerase. In a series of temperature cycles, the target DNA is repeatedly denatured, annealed to the primers and a daughter strand extended from the primers. As the daughter strands themselves act as templates for subsequent cycles, DNA fragments matching both primers are amplified exponentially, rather than linearly (*Medical Oncology*, 2003). As PCR is taking place, the specific primers being amplify the specific microsatellites intended for isolating. As the DNA is denatured, the tertiary and quaternary structures of the DNA is broken down to its simplest form to the primary structure, and the primers then locate the DNA sequence, and amplify the sequences.

## Polymerase Chain Reaction

PCR or Polymerase Chain Reaction is a technique that allows scientists to single out and amplify specific sequences of DNA millions of times in just a few hours. It is a technique used widely in studies of biology, genetic engineering and disease, forensics, medicine, cloning, and molecular evolution. By using relatively small samples of DNA, replicates can be produced rapidly for many purposes. For example, in the case of forensics,

investigators can find a small drop of blood at the scene of the crime. Even though there is very little DNA in a dried drop of blood, by using PCR and DNA polymerase a small sample of DNA can be multiplied creating a large sample that may be easily analyzed. The tiny sample is placed in a test tube, and DNA polymerase along with the other components of PCR are added. The sample is then heated up momentarily, and the two strands of DNA separate. Then DNA polymerase builds a new double helix from each strand. These two copies are then heated, and duplicated, yielding four copies. After many times, a large quantity of identical DNA strands are produced. Our own DNA polymerases, and those from most organisms, would be destroyed by the heating step in this process because of the denature of proteins at high temperatures. But today, DNA polymerase from *Thermus aquaticus*, a bacterium that lives in hot springs, is used. The benefit of using the *Thermus aquaticus* DNA polymerase is that the enzyme is capable of remaining active after repeated heating during amplification.

Polymerase is an enzyme that catalyses the repair and formation of DNA and RNA. It catalyses the synthesis of the new DNA strand by utilizing the parental strand as a template. In addition to the replication of chromosomes, DNA polymerases are also needed in other processes like DNA repair, transcriptional and cell cycle control, and DNA recombination. Every time a cell divides, DNA polymerase duplicates all of its DNA, and the cell gives one copy to each daughter cell. In this way, genetic information is passed from generation to generation.

There are other ingredients in the process of PCR as well. Primers (also known as oligonucleotides) play a vital role in PCR. After the two strands of the template DNA are split apart, the copying of the target sequence cannot begin if there is just a single strand of DNA sitting there. There has to be a double-stranded section for the polymerase to start from. The primers attach to either end of the target sequence. Chemical or pH buffers are used in order to

make sure the solution is not too basic or acidic which could result in inaccurate copies of DNA. Magnesium Chloride is added to ensure that DNA polymerase functions properly. Two other enzymes, gyrase and hexicase are used as well. Gyrase helps break apart the DNA when heated and hexicase keeps the double helix open so it can be copied. PCR is executed in a machine called a thermocycler, which heats and cools samples of DNA on a block.

There are several steps that occur in the process of PCR. First off, the tube with all the ingredients is heated to a very high temperature (94-96°C) in the thermocycler in order to break apart or denature the DNA. Next, the temperature is lowered so that the primers can attach or anneal themselves to the single strands of the DNA that have been separated. Lastly, the tube is heated to a temperature somewhere between the first two, so that the copying of the DNA can begin. This process continues approximately 30-40 times.

# materials and procedure

## Sample collection:

Scat (fecal samples) were opportunistically collected from harbor seal haul out sites during low tide. When the tide comes in, it washes samples off the rocks, so samples can be discretely collected without fear of cross contamination. Samples were stored in zip-lock bags until preparation. Small samples (~8 cm<sup>3</sup>) were placed in glass jars with excess silica gel desiccant. The desiccant was blue in color and if it turned red, indicating saturation with water, more was added until the blue color remained.

Eight samples were collected from Norwalk and seven samples were collected from Great Gull Island.

## Isolation of Harbor Seal DNA:

A Qiagen DNA stool kit was used to isolate the DNA. 200 mg of seal scat was massed and placed in a 15 ml sterile culture tube. 1.6 ml of Buffer ASL was added to each tube and each tube was vortexed for 1 minute followed by a 1 minute centrifugation. 1.4 ml of the supernatant was put into a 1.5 ml tube and 1 InhibitEX tablet was added. Samples were vortexed for 1 minute, incubated at room temperature for 1 minute, and centrifuged for 3 minutes. The supernatant was transferred to another 1.5 ml tube and re-centrifuged for 3 minutes to remove any remaining solid material. The supernatant (600 µl) sample was transferred to another 1.5 ml

centrifuge tube and 0.25  $\mu$ l of Proteinase K + 600  $\mu$ l of Buffer AL was added to the sample. The sample was vortexed for 15 seconds, incubated for 10 minutes in a 70°C water bath and vortexed again for 15 seconds. 600  $\mu$ l of ethanol and 600  $\mu$ l lysate was added to a spin column and centrifuged for 1 minute. Filtered waste was discarded and this process was repeated until all of the supernatant was used. 500  $\mu$ l of Buffer AW was added to the column and centrifuged for 1 minute. 500  $\mu$ l of Buffer AW2 was added to the column and centrifuged for 3 minutes. The waste was discarded and 200  $\mu$ l of Buffer AE was added, incubated for 1 minute at room temperature and centrifuged for 1 minute to collect the DNA sample.

## Spectrophotometry:

Spectrophotometer was first calibrated using standard techniques. 100 $\mu$ l of DNA of each of 8 scat samples was added to a 1ml quartz cuvette along with 700 $\mu$ l of distilled H<sub>2</sub>O. The cuvettes were then inserted into the spectrophotometer and the absorbance was recorded at 260.00nm. Every absorbance unit indicated 50 $\mu$ g of DNA in the sample. Therefore, the weight of the DNA in each sample was calculated and recorded.

## Amplification of Microsatellites:

Two sets of primers were purchased for 2 microsatellite genes.

Pvc19 +      GGGTGAACAGGATTTATCC  
             -      GTGCTAGATAACAATCCTAC

Pvc 29 +      GGTTAATTGTGTTGTTTACATCT  
             -      AACCAGAAGAATAGAATTAGCAT

For each PCR sample, 0.25  $\mu$ l of Taq polymerase, 5  $\mu$ l of 10x buffer solution, 4  $\mu$ l dNTP bases, 5  $\mu$ l seal DNA, 5  $\mu$ l + primer, 5  $\mu$ l – primer, and 26  $\mu$ l of distilled H<sub>2</sub>O for a total of 50  $\mu$ l was added to a 200  $\mu$ l thin-wall microcentrifuge tube. Each seal DNA sample was used twice: once with the Pvc 19 primers and once with the Pvc 29 primers. Samples were loaded into a PerkinElmer GeneAmp PCR System 2400 thermal cycler. The machine was set to denature the DNA for 5 minutes at 94oC, then cycled 32 times under the following conditions: denaturation 94oC for 30 seconds, annealing 45oC for 30 seconds, extension 72oC for 30 seconds. A final extension for 7 minutes was done at 72oC. The cycler then stored samples at 4oC until they were retrieved for electrophoresis.

## Gel Electrophoresis

A 2% w/v agarose gel in 1xTBE (Tris Borate EDTA) Buffer was prepared and 6.5  $\mu$ l of PCR sample was combined with 3.5  $\mu$ l of loading buffer (bromophenol blue, xylene cyanol FF).

Samples were electrophoresed at 100 v for 1 hour. Gels were stained with Ethidium Bromide and viewed under UV transillumination.

## Resolving of DNA bands

Successful PCR products were electrophoresed on a 4.5% Agarose MS (micro screening) gel which provided a more accurate display of microsatellite length. The Agarose MS gel allows the samples to move slower and shows a  $\frac{3}{4}$  band size increase over the 2% agarose gel.

# CONCLUSION

In conclusion, despite difficulty the seal DNA from each of the samples was isolated and purified until pure DNA was successfully extracted. Feces are complex assemblages of biomolecules and contain substances known to interfere with DNA analysis (Coltman, YEAR). The isolated DNA from the fecal matter demonstrated that although scat sampling may not be the most efficient direct form of DNA extraction (in comparison to that of skin or blood samples), it is possible and yields efficient DNA extraction. As these samples were taken opportunistically without bothering the seals, no harm was done.

Spectrophotometer results indicated that samples from Long Island Sound contained an average of 13.9  $\mu\text{g}$ , whereas samples from Great Gull Island averaged \*\*\*\*\* DNA respectively.

Furthermore, PCR techniques used insured the successful amplification of the seal DNA microsatellites and not the DNA of any other organisms that might have been present in the fecal material. The specific primers used, (*Pvc 19/29*) complemented the DNA of harbor seals, and could only amplify that of specific seal microsatellites. This amplification successfully eliminated the need for analysis of any access material and insured that only the seal DNA was used. To test the results of the samples after PCR, electrophoreses went underway to compare DNA length of the seal microsatellites. The migration patterns of the PCR samples through the Agarose gel served to verify this. After electrophoresis, the *Pvc 19* primer displayed high levels of heterozygosity.

The linear proximity of the samples within the Agarose gel after the electrophoreses, showed that microsatellite length did not vastly vary amongst the different seals DNA. After

electrophoreses, the bands migrated in no distinct pattern and resulted in random distribution. The data advocates that due to the random gel alignments, along with the low genetic variation of microsatellites, the harbor seals appear to express similar genetic characteristics but do not show patternistic signs of direct biological relations. The data also conveys the hypothesis that harbor seals in general show low signs of variation amongst their populations in a given area. Although seal samples were taken amongst different areas in Long Island Sound, this suggests that samples taken within a small geographical radius equates to the idea that seals in similar areas share similar genetic traits and have possible family ties.

The data may have resulted in this way due to the habitual cyclic natural patterns of harbor seals. Harbor seals live in close groups and migrate north to Maine in the summer, and return to Long Island Sound in the winter. This continuous cycle has been done for generations, suggesting common ancestry. This kinship would suggest little genetic diversity among the seals themselves, which was displayed through the similarity in microsatellite length and is exactly what the electrophoresis results suggested.

Certain samples did not appear on the gel at all. This can be attributed to several factors. Human error during the PCR process could easily have caused temperature fluctuation, which would prevent the primers from annealing correctly. Also, limited size of the fecal samples themselves may have contributed to a lack of sufficient DNA.

All in all, a great experience was accompanied by great results. While breaking new ground in the procedures of amplification and purification of DNA, this experiment has shown a new humane way to study and analyze seals. Through studying fecal material, just enough DNA can be isolated to learn a treasure trove of information about seals all without the slightest harm done to them.

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