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Preliminary Characterization of Three Populations of the Freshwater Sponge, Clypeatula cooperensis, using RAPD-PCR

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Preliminary Characterization of Three Populations of the Freshwater Sponge, *Clypeatula cooperensis*, using RAPD-PCR.

Submitted in partial fulfillment of the Requirements for Graduation with Honors to the Department of Natural Sciences at Carroll College, Helena, Montana

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April 10, 2000
This thesis for honors recognition has been approved for the Department of Natural Sciences by:

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April 10, 2000
Acknowledgments

I have several people to thank who have been of tremendous help to me in the completion of this project. First, thanks to Linda Beischel and the Shodaire Genetics Laboratory for their help in determining the DNA concentration of my samples. Linda was also a great source of information throughout the laboratory phase of this project and a knowledgeable and willing reader for my final paper. Thanks also to Dr. Gerald Shields for agreeing to read my paper. His experience and knowledge of molecular genetics has been of immense help to me as well as a valuable addition to Carroll College. Thank you Dr. Grant Hokit for supervising the senior research class last fall. Your insight and motivation were both of great help to me and this project. To the "sponge team" (Joel Edminster and Mark Nance), thanks for your help in sample collection and for a very entertaining summer. Finally, thank you Dr. John Addis for your support, advice and dedication to this project. The daily assistance you gave me throughout the summer and the countless number of drafts you read this spring have been greatly appreciated. This project has been a wonderful learning opportunity.
Abstract

In order to relate the three known populations of the freshwater sponge, *Clypeatula cooperensis*, to one another, sponge DNA was subjected to RAPD-PCR. DNA was extracted using a Puregene kit from three specimens collected from each population. Following PCR optimization, DNA was amplified using random primers (OPA-10, 11, 12, 13, 18, and 20). Although reproducibility was a problem, preliminary results suggest that variability within populations is as high as variability among populations, raising the possibility that the three populations are genetically identical. This is an unexpected finding because one population is geographically isolated from the other two. If correct, one explanation is that the three populations have only recently become separated.
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Introduction

*Clupeatula cooperensis* is a newly discovered freshwater sponge species about which relatively little is known (Peterson and Addis, 2000). It is a nongemmulating sponge that overwinters in a regressed state and apparently reproduces only sexually. To date, the species has only been found in three western Montana lakes: Coopers, Salmon, and Blanchard Lakes. Blanchard and Salmon Lake are two miles apart, connected by the Clearwater River. Coopers Lake is an isolated lake, located approximately 20 miles to the east of the other two.

With such restricted distribution, questions about genetic structure of the populations arise. Generally, there is little knowledge of the genetic structure of sponge populations. Previous attempts to assess genetic variability in sponges have made use of histocompatibility bioassays in which failure of sponge pieces to fuse is taken as evidence of genetic difference (Markenich and Francis, 1991; Neigel and Avise, 1985). This technique has been applied to both freshwater and marine sponges and has led to the identification of several strains within species. One shortcoming of this approach is that fusion of pieces cannot be equated with genetic identity, since genetic variability outside the histocompatibility system goes undetected (Stoddart and Ayre, 1985).

Another means of evaluating genetic variability is provided by RAPD (Random Amplified Polymorphic DNA)-PCR, a technique that has been successful in determining the relatedness within species (for example, see Yu and Pauls, 1994; Leal et al., 1994). This technique has also been used in a previous study of freshwater sponges. In 1999, Redman attempted to characterize *C. cooperensis* and other freshwater sponge species using the
RAPD technique. In his study, he was able to distinguish *C. cooperensis* from *Ephydatia muelleri*, another freshwater sponge.

The objective of my research was to explore the differences, if any, among the three known populations of *C. cooperensis* using the RAPD-PCR technique. My working hypothesis was that sponges from Blanchard and Salmon Lakes would be more closely related to each other than to samples from the isolated Coopers Lake.
Literature Review

*Clypeatula cooperensis*

*Clypeatula cooperensis* differs from other species of freshwater sponges in western Montana in occupying a protected microhabitat on the undersides of logs and rocks and in not producing gemmules (Peterson and Addis, 2000). The three most common species of freshwater sponges in western Montana, *Ephydatia muelleri, Spongilla lacstris,* and *Eunapius fragilis,* grow preferentially on the sides and upper surfaces of their substrates and die back during the winter after producing gemmules (Barton and Addis, 1997). *C. cooperensis* survives the winter intact (Peterson and Addis, 2000).

Without gemmules for dispersal (see below), the question of how the three known populations of *C. cooperensis* are related to one another arose. A priori, it would be expected that the Salmon and Blanchard Lake populations would be closely related to one another since the lakes are connected by the Clearwater River. However, since Coopers Lake does not lie on the Clearwater and is situated approximately 20 miles to the east of the other lakes, it would be expected that this more isolated population would be different from the other two lakes.

*Sponge Dispersal*

Gemmules seem to be the most likely mechanism of dispersal of freshwater sponges among habitats because of their ability to withstand harsh
environments (Frost, 1991). They are created from a dedifferentiation process in which a resistant coat is formed around a group of cells (Frost, 1991).

In C. cooperensis, as a part of reproduction, larvae are produced and released into the open water, thus becoming a possible (although more fragile) means of dispersal. Frost (1991) believes that dispersal by larvae could only be within a short range and out-of-water dispersal would be almost impossible.

**RAPD-PCR**

Unlike traditional PCR that requires primers with specific target sequences, RAPD-PCR uses non-specific, random primers. RAPD primers are ten base sequences randomly generated so they require no previous knowledge of target DNA sequence, thus avoiding the time consuming and costly process of finding specific template sequences and the corresponding primers (Williams et al., 1993). There are numerous other benefits to this technique: it can be used on a broad range of species (Williams et al., 1993), the randomness of the primers leads to amplified sections on virtually every chromosome (Palumbi, 1996), and it has a relatively low cost (Hadrys et al., 1992).

The RAPD technique has been used in many areas of genetic study including genetic mapping and population genetics (Williams et al., 1993). RAPD fingerprinting can be used determine taxonomic relationships, observe gene flow, study kinship relationships, produce specific probes, and can even be used in mixed genome samples (Hadrys et al., 1992). RAPD-PCR is very useful in determining the relatedness of closely related individuals (Williams, 1993). Yu
and Pauls (1994) used RAPD-PCR to determine relatedness among populations of tetraploid alfalfa, thus breeders could prevent inbreeding by maximizing genetic distances between breeding pairs. Leal et al. (1994) used RAPD-PCR to distinguish between strains of the fungus, *Metarhizium anisopliae*. They were able to separate all but two of the isolates collected from several sources within four different countries. The potential ability of RAPD-PCR to distinguish between populations will be exploited in the analysis of the freshwater sponge samples of *Clypeatula cooperensis*. 
Materials and Methods

Three samples of freshwater sponge, *Clypeatula cooperensis*, were collected from each of 3 lakes: Coopers Lake (47°05'N/112°55'N), Salmon Lake (47°06'N/113°24'N), and Blanchard Lake (47°01'N/113°23'N). After collection, the samples were wrapped in aluminum foil, frozen on dry ice, and then stored at -70 °C.

**DNA isolation**

DNA from the frozen samples was isolated using a Puregene DNA isolation kit (Gentra). The small, frozen sponge samples were weighed and divided so that each piece weighed approximately 0.1 g. The sponge was immediately placed in a 1.5 mL sterile microfuge tube containing 600 μL of Cell Lysis solution. The sample was homogenized thoroughly with a pestle. Proteinase K solution, 3 μL, were added to the lysate, the tube was inverted 25 times to mix, and incubated at 55 °C for 3 hours. Tubes were inverted periodically during the incubation. After incubation, 3 μL of RNase A solution were added to the sample. The sample was mixed by inverting 25 times and then incubated at 37 °C for 60 min. The sample was cooled to room temperature, and 200 μL Protein Precipitation solution were added to the cell lysate. The sample was vortexed at high speed for 20 s and incubated on ice for 15 min. The sample was then centrifuged at 13,000 Xg for 3 min in order to form a large protein pellet. The supernatant containing the DNA was pipetted into a
sterile tube containing 600 μL of 100% isopropanol, and then inverted gently 50 times. The DNA was pelleted by centrifuging at 13,000 Xg for 1 min. The supernatant was pipetted off, leaving behind a small, white pellet. The tube was drained briefly by inverting it over clean absorbent paper. The pellet was washed by adding 600 μL of 70% ethanol and inverting several times. Again the sample was centrifuged at 13,000 Xg for 1 min. The supernatant was pipetted off and the pellet dried over clean absorbent paper for 15 min. The pellet was resuspended in 20 μL of double distilled water.

At this point the samples were found to be turbid, suggesting there was protein contamination. Puregene's procedure for removing protein contaminants in the rehydrated DNA sample was then followed. Five parts Cell Lysis Solution were added for every 1 part water used to rehydrate DNA, then the sample was mixed. Proteinase K Solution was added to a final concentration of 100 μg/mL, and then incubated at 55 °C for 1 h. Two parts protein precipitation solution were added, and the sample was vortexed at high speed for 20 s. The sample was centrifuged at 13,000 Xg for 3 min. Supernatant was pipetted into a clean tube containing 6 parts of 100% isopropanol and mixed by inverting 50 times. DNA was pelleted by centrifuging at 13,000 Xg for 1 min. The supernatant was pipetted off and the pellet was dried briefly and then centrifuged at 13,000 Xg for 1 min. The pellet was dried for 15 min and then resuspended in 50 μL of water. The concentration of DNA was determined at the genetics laboratory at Shodair Children’s Hospital, Helena, MT using fluorescent dye (Hoechst 33258) and Hoefer fluorometer TKO100 according to the directions of the manufacture.
RAPD PCR

The RAPD method used was adapted from the procedures of Williams et al. (1990) and Nadeau et al. (1992). Six RAPD primers from Operon Technologies were used: OPA-10, OPA-11, OPA-12, OPA-13, OPA-18, and OPA-20. These were randomly generated decamers (Table 1). Several PCR conditions were varied in order to optimize the PCR products obtained. The various concentrations and amounts included: dNTP concentration, 100 µM and 200 µM; primer concentration, 0.1 µM, 0.2 µM, 0.4 µM and 1.0 µM; Taq polymerase amount, 0.5 unit and 0.75 unit; and template amount, 0.1 ng, 1 ng, 10 ng, 25 ng, and 50 ng. The best results obtained were with the PCR conditions as follows: 1x PCR Buffer (Perkin-Elmer), 2 mM MgCl₂, 100 µM dNTP, 0.5 units AmpliTaq DNA polymerase (Perkin-Elmer), 1 µM primer, 2.5 µL Rediload (Research Genetics), and 1 ng template. Each reaction was run in a total volume of 25 µL. A cocktail of primer, buffer, MgCl₂, dNTP, Rediload, and water was first prepared and distributed to the PCR tubes. The tubes were then placed over ice, and the separate templates were added. Taq polymerase was added last. The samples were run in a thermocycler (Perkins-Elmer) for 45 cycles. The sample was initially held for 5 min at 94 °C, then 1 min at 94 °C, 1 min at 35 °C, 2 min at 72 °C for 45 cycles, and then 7 min at 72 °C. The amplified products were then kept at 4 °C.
Electrophoresis

The PCR products were separated in a 1.8% agarose gel (wide range/standard 3:1 agarose, Sigma). This amount was chosen after testing concentrations of 1.0%, 1.6%, 1.8%, 2.0%, 2.4% and 2.5% agarose. The gel was placed in an electrophoresis chamber with TAE (Tris/acetate/EDTA) buffer. After separation, the gels were stained for 1 h in ethidium bromide. The gels were then viewed and photographed under UV light.
Results

Six RAPD primers were assessed (Table 1). From these only OPA-11 failed to give at least one PCR product. The products ranged in size from 1000 to just below 200 bp (Fig. 1, Table 2). OPA-20 yielded one product, which because of a problem with the initial ladder as well as the inability to obtain products in subsequent runs, could not be sized. Products obtained from primers OPA - 10, 12, and 18 showed some variation among lake populations as well as among the individual samples collected from each lake. One common product of 610 bp was found using OPA-10. An additional 400 bp product was in two of the three samples from Salmon Lake. OPA-12 showed one product at 530 bp in every sample except for one collected from Salmon Lake, no products were obtained from this sample using OPA-12. OPA-13 showed no variation within or among the populations. The same three products were found in all samples taken from the three lakes. Finally, OPA-18 resulted in the most variation. Products of 575 bp and 450 bp appeared in every sample. One sample from each of the three lakes had products of 355 bp and 250 bp, while Salmon and Blanchard Lakes had one sample with a product of 195 bp.

Reproducibility was a problem. Several attempts were made to repeat the PCR series using OPA-13 and OPA-18. No products were found in any sample using OPA-13. Five of nine samples gave identical products using OPA-18, but no products were obtained from the other four samples. For these five samples the same products shown in the first trial appeared again.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>GC Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA-10</td>
<td>GTGATCGCAG</td>
<td>60</td>
</tr>
<tr>
<td>OPA-11</td>
<td>CAATCGCCGT</td>
<td>60</td>
</tr>
<tr>
<td>OPA-12</td>
<td>TCGGCATAG</td>
<td>60</td>
</tr>
<tr>
<td>OPA-13</td>
<td>CAGCACCCAC</td>
<td>70</td>
</tr>
<tr>
<td>OPA-18</td>
<td>AGGTGACCCTG</td>
<td>60</td>
</tr>
<tr>
<td>OPA-20</td>
<td>GTTGCATCC</td>
<td>60</td>
</tr>
</tbody>
</table>
Figure 1. *Sponge DNA amplified by primer OPA-18*. Replication was performed in presence of 1 μM primer, 1 ng template, and 100 μM dNTP. Ladder (Research Genetics Gelmarker) for sizing the base pairs, was 20% ladder, 10% 10X loading buffer. Open arrows indicate common bands at 450 and 575 bp. Additional bands are at 355, 250, 195 bp on a 1.8% agarose gel in a TAE electrophoresis buffer.
<table>
<thead>
<tr>
<th>Band Size&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Coopers</th>
<th>Salmon</th>
<th>Blanchard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>610</td>
<td>2/2</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>400</td>
<td>0/2</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Primer 12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>530</td>
<td>3/3</td>
<td>2/2</td>
<td>3/3</td>
</tr>
<tr>
<td>Primer 13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>650</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>325</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Primer 18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>575</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>450</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>355</td>
<td>1/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>250</td>
<td>1/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>195</td>
<td>0/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>

<sup>a</sup> base pairs
<sup>b</sup> fraction of sample showing amplified product
Discussion

PCR products obtained for all samples from the three populations showed similar banding patterns. The variability was as great within each population as among the different populations, raising the possibility that the three populations are genetically identical. Yet, some genetic difference between the populations would be expected. First, Coopers Lake is not connected to the other two lakes by a common waterway. Thus gene flow would be very difficult. Secondly, all material flowing between Blanchard and Salmon Lakes flows in one direction, so material could be carried downstream from Salmon to Blanchard Lake but would not be able to move in the opposite direction. It is possible that the limited variability seen here occurs because the populations have only recently separated from each other or some other main colony. If they are recently separated one of the three known populations could be the original colony, but it is also possible that all three originated from another still undiscovered source.

Although, over 40 lakes in western Montana have been surveyed for freshwater sponges (Addis, personal communication), and *C. cooperensis* has only been found in the three lakes included in this study. For this reason the existence of an undiscovered source population which could have been a founding colony seems unlikely.

It must be emphasized that these findings are preliminary results. In this experiment reproducibility was a problem because many trials resulted in no
products. After several attempts only one primer (OPA-18) gave results twice, although the same products were obtained both times.

In a previous study of freshwater sponges of western Montana, Redman (1999) used the RAPD-PCR procedure. He also obtained results using primer OPA-13. Although he obtained three bands with this primer, they were not the same size as the bands I found. He had three consistent products of 450, 800, and 1100 base pairs, while mine, although seeming to correspond to this pattern, were smaller at 325, 650, 1000 base pairs. The PCR conditions for his experiment as compared to mine were different, and variation in conditions may lead to variable results. Thus, the different sizes could be attributed to this fact.

Concentration of the PCR reagents seemed to be most critical in obtaining appropriate results. The concentration of the dNTP and the template were especially important. My best results occurred when the dNTP concentration was at 100 µM, simply doubling this to a second commonly used concentration of 200 µM resulted in no banding. A test gel of the isolated DNA suggest it was intact DNA and of high molecular weight. Palumbi (1996) suggested that with high molecular weight template DNA, the concentration of DNA should be decreased. I decreased the amount of template from 25 ng suggested by Williams (1993) or 10 ng suggested by Redman (1999) to 1 ng of template. I achieved much better results using this method.

As stated before these are preliminary results so additional research is needed. Further tests are needed to confirm that the populations are indeed indistinguishable. These tests could include simply using many more RAPD
primers, or if more genomic information became available for *C. cooperensis*, a site-specific primer could lead to more specific results. The small sample size of this study is a concern. There are not an abundant number of sponges in any of the three lakes. For a preliminary study only a few sponges were sampled, so as to not adversely affect the populations. In an in-depth study, sampling a larger number of individuals would be a necessity.

I have proposed a few explanations for the source of the population variability. Each of these relies on some sort of distribution. Thus, possible explanations as to how three apparently indistinguishable populations appeared in three separate lakes should be explored. As stated before, *Clypeatula cooperensis* does not produce gemmules so dispersal of the sponges into other lakes via a third party (e.g., humans or birds) would be difficult. Dispersal of the larvae is possible but long-range dispersal of any kind is unlikely. Although it is not known at this time how the separate populations were established, further research will most likely find that the populations can all be traced back to an original colony. The new colonies might not have been established long enough to show any genetic variability. This study could serve as a baseline for research of these sponges in the future. It may be possible to use these populations to observe how populations begin to diversify from each other, and thus of importance to population ecology.
Literature Cited


