ASSESSMENT OF MULTIPLE PATERNITY FOR THE QUEEN CONCH, STROMBUS GIGAS

by

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A Thesis Submitted to the Faculty of

The Wilkes Honors College

in Partial Fulfillment of the Requirements for the Degree of

Bachelor of Arts in Liberal Arts and Sciences

with a Concentration in Marine Biology

Wilkes Honors College of

Florida Atlantic University

Jupiter, Florida

May 2008

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This thesis was prepared under the direction of the candidate's co-thesis advisors, Dr. Jon Moore and Dr. Donna Devlin, and has been approved by the members of his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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Acknowledgements

First and foremost, I would like to thank Dr. Donna Devlin for the guidance and knowledge she provided in completion of this project. As well, I thank Ellen Grefsrud, Dr. Edward Proffitt, and Amber Shawl for their assistance in the field and in the lab, and for their comments and suggestions towards writing the final report. I also thank Tiffany Gaston, Dr. Jon Moore, and Dr. Jim Wetterer for their review of my thesis. I would like to thank the Harbor Branch Oceanographic Institute for funding this research and the Florida Fish and Wildlife Service for providing access to the wild animals used in the study.

I would like to thank the faculty and staff from Wilkes Honor's College and Harbor Branch Oceanographic Institute for preparing me for this endeavor. In particular, I would like to thank Dr. Jon Moore for his guidance and instruction during my four years as an undergraduate student, and Dr. Edward Proffitt and Dr. Donna Devlin for introducing me to the world of professional science and allowing me to be a part of so many research projects.

ABSTRACT

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Title:	Assessment of multiple paternity for the queen conch, *Strombus gigas*
Institution:	Wilkes Honors College of Florida Atlantic University
Thesis Advisors:	Dr. Jon A. Moore and Dr. Donna Devlin
Degree:	Bachelor of Arts in Liberal Arts and Sciences
Concentration:	Marine Biology
Year:	2008

The commercially important queen conch, *Strombus gigas*, has been observed copulating with multiple partners and laying multiple egg masses during a reproductive season (Randall, 1964). While multiple paternity has been confirmed using microsatellite based genetic analysis for a variety of other gastropods, this technique has not been employed for *S. gigas.* Determining whether or not this species is capable of multiple paternity is important to understanding and maintaining genetic diversity of natural and captive populations. While an assessment of multiple paternity is the ultimate goal of this study, for my thesis, I have completed preliminary work which includes perfecting methods of tissue collection, DNA extraction, and DNA amplification with six non-labeled polymorphic microsatellite molecular markers, using cultured *Strombus gigas* animals. In addition, I collected tissue and extracted DNA from three wild *S. gigas* adult females and their egg masses from Pelican Shoal in the Florida Keys.

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Introduction

The queen conch *Strombus gigas* Linnaeus (1758) is a marine gastropod distributed in nearshore waters in Bermuda, the Bahamas, southern Florida, the southern Gulf of Mexico, the Caribbean and Brazil (Warmke and Abbott, 1961). Due to its decorative shell, palatable meat, and accessibility, the species has served as a symbol of cultural heritage, an essential source of protein, and a major economic resource for the people of more than 20 countries (Doran, 1958; Berg, 1976; Davis and Hesse, 1983; Thiele, 2001).

During the twentieth century, increased demand and technological advancements augmented the exploitation of *S. gigas* throughout the species' distribution (Randall, 1964). During the 1980's and 1990's population assessments in Belize, Turks and Caicos, Cuba, and the Florida Keys revealed that the stocks had been greatly depleted due to overexploitation (Berg, 1987; Hunt, 1987; Ferrer and Alcolado, 1994). Population decline in *S. gigas* affected the local fishing industries; fisheries in Jamaica (Aiken, et al., 2006) and Mexico (Patiño-Suárez et al., 2004) experienced closures. Within the United States, the Florida Keys commercial fisheries closed in 1975 and recreational take was prohibited in 1986. In 1992, *S. gigas* was added to Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

For species with small populations, such as *Strombus gigas*, it is important to understand existing levels of genetic diversity, since maintaining genetic diversity is essential to ensuring species survival (Crozier, 1992). Understanding the genetic flow from parents to offspring is integral in determining the genetic diversity of a

population, as it has consequences on effective population size (Sugg and Chesser, 1994). Field observations indicate that female *S. gigas* mate with multiple partners (Randall, 1964), yet whether multiple males are responsible for fertilizing the eggs in a single brood is unknown.

The increasing availability of molecular genetic techniques has made investigating genetic relationships, such as parentage, feasible. Microsatellite molecular markers have been used to examine paternity for many polyandrous gastropod species (Patterson et al., 2001; Mäkinen et al., 2007; Walker et al., 2007), with multiple paternity being observed in many cases. For example, Patterson et al.(2001) used microsatellite based analysis to show that as many as five sires contributed to a given brood from the land snail *Littorina obtusata*.

This study is an integral step in a longer term project to use eight recently developed polymorphic microsatellite molecular markers for *Strombus gigas* (Zamora-Bustillos et al. 2007) to determine if multiple paternal partners contribute to single egg masses of the species. For my thesis, I used cultured juveniles to perfect methods of tissue collection, DNA extraction, and amplification of DNA with primers for all eight of the *S. gigas* microsatellite markers. During the summer of 2007, with the help of colleagues, I also collected three wild maternal adults and a portion of the eggs from their egg masses from Pelican Shoal in the Florida Keys. I then successfully extracted high quality DNA from these wild caught specimens; this DNA will be used in combination with the optimized microsatellite markers in the ongoing study to determine if the progeny of the wild maternal adults show evidence of multiple paternity.

Materials and Methods

Wild *Strombus gigas* populations are protected in Florida, so we could not sacrifice wild conchs for tissue samples. Therefore we first tested different methods of tissue collection and genomic DNA extraction and amplification using cultured juvenile *S. gigas* obtained from Oceans, Reefs and Aquariums in Fort Pierce, Fl. We then used the most successful methods for wild *S. gigas* from the Florida Keys.

Tissue Collection

Laboratory

We removed foot (~.20 g) and eye-stalk (~1.0 g) tissue from cultured *S. gigas* individuals. For additional analysis, we removed foot (~.20 g) and siphon (~1.0 g) tissue from the cultured *Strombus alatus.* The *Strombus alatus* siphon tissue was collected from a sacrificed animal. Because live conchs can fully retract into their shell, soft tissue removal is difficult. Therefore to collect tissue, we attempted to relax live conchs by submerging them in magnesium chloride solution (30 g/L) for 30-min (Acosta-Salmón and Davis 2007). Once a conch was relaxed, one researcher forcibly pulled the conch's operculum out of the shell until its foot tissue was exposed, at which point a second researcher used a scalpel to quickly excise a small piece of tissue. Pulling the conch from their shells remained difficult, as they were not fully relaxed. We put each tissue sample in a 1.5 mL microcentrifuge tube and placed them on dry ice until transfer to a -80°C freezer. We monitored recovery of the conchs, and found that within a week's time, the conchs that had foot tissue removed were moving around their tank, sifting through the sand using their siphon. However, given the same amount of recovery time, the conch that had one of its eyes removed, though

alive, remained tucked inside its shell. We determined removal of foot tissue to be the preferred method, since conchs experiencing removal of this tissue type appeared to recover more quickly.

Figure 1.—Map indicating site of field collection, Pelican Shoal.

Field

On 29 June 2007, we located a large group of adult female *S. gigas* on the seafloor (~5 m) at Pelican Shoal (24.500N, 81.629W) in the lower Florida Keys (Figure 1). We collected three females in the process of laying eggs, and brought them and their egg masses aboard our boat. We attempted to relax the adults as described above, but after 30-min, the conchs continued to resist our attempts to pull them from their shells; this was likely due to the larger size of the wild adults in comparison to the cultured juveniles. We increased exposure time to ~2 hrs and added more magnesium chloride (unknown final concentration), but were only able to collect foot tissue from two conchs and a small piece of operculum from a third. We

also collected foot mucus samples from two of the females by scraping a pipette tip along the exposed foot. Armbruster et al. (2005) found foot mucus to be a non-destructive source of DNA from the land snail *Arianta arbustorum*. We placed the foot tissue and mucus samples into 95% ethanol or on dry ice until transferring to a -80°C freezer. We used the two different methods, so that when later extracting from the tissue, we could determine which method was best at preserving the quality of the DNA. We collected a small section of egg mass from the three females, and placed each into a separate bag containing seawater. Post-collection, the adults and their remaining eggs were immediately returned to the water. Upon transfer to HBOI, we froze seven eggs from each egg mass section for later DNA extraction (a technique that had not been attempted with *S. gigas*). My collaborators cultured the remaining eggs at the ORA aquaculture facility at HBOI, keeping the eggs of different maternal families separated. Unfortunately, once the eggs had progressed to the larval stage, there was very high mortality (unknown cause). We were able to collect and freeze eighteen of the remaining larvae, however the larvae from the three maternal families were accidently mixed during collection; therefore their usefulness for this project may be compromised.

DNA Extraction

We first tested different methods of DNA extraction with the cultured conch samples. We used three DNA extraction kits: Qiagen® DNeasy Plant Mini Kit (QPM), Qiagen® DNeasy Blood and Tissue Kit (QBT), and the Cartagen® Genomic DNA Extraction Kit for Plants (CGP). We chose plant extraction kits since conchs often have secondary chemicals that interfere with extraction (Morales, 2004). The

QBT kit was utilized based on recommendations from Qiagen®. Since we had more foot tissue samples than other tissue types, we used foot tissue to compare DNA yields of the three kits. We used a Nanodrop® ND-1000 UV-Vis Spectrophotometer to measure the concentrations of DNA extracted from foot tissue using the three different kits.

For the Florida Keys samples, we only used the QBT and QPM kits for extraction, since these kits were more successful than the CGP when extracting from cultured conch tissue. We extracted DNA from the foot tissue samples using both kits while the operculum was extracted from using only the QBT kit. All of the eggs from the three wild maternal adults were extracted using the QPM kit, since Morales (2004) had success extracting from *S. gigas* eggs using extraction kits designed for plants and we did not have cultured eggs available to test different kits. We used a Nanodrop® ND-1000 UV-Vis Spectrophotometer to measure DNA concentration .

In total I collected and tested DNA extraction techniques for nine different types of *Strombus spp* tissue: cultured *S. gigas* foot and eye-stalk, wild *S. gig*as foot, mucus, operculum, eggs and larvae, and cultured *S. alatus* foot and siphon

Optimizing Microsatellite Primers

In fall 2007, we optimized the eight polymorphic microsatellite molecular markers specific to *Strombus gigas*, previously established by Zamora-Bustillos et al. (2007). Due to our limited number of samples collected from the Florida Keys, we used cultured conch DNA (from foot tissue only) to optimize the protocol of Zamora-Bustillos et al. (2007) using unlabeled microsatellite primers. The PCR amplification was programmed for 5 min at 95 °C for initial denaturation, followed by 30 cycles

involving a denaturation step at 95 °C for 30 s, 30 s at annealing temperature, and extension at 72 °C for 30 s. The final extension cycle was for 5 min at 72 °C. We used electrophoresis to examine the products of microsatellite PCR. LE Agarose gels were run at 60 volts for ~1.5 hrs. We captured the gel under ultraviolet light using the Analyst® Investigator/Plus System (Fotodyne). By observing the banding on the gel, we were able to visualize how well the microsatellites were annealing to the cultured conch DNA. Because amplification of the DNA at the annealing temperatures (Table 2) recommended by Zamora-Bustillos et al. (2007) was not always successful, we ran PCR for each microsatellite at six different annealing temperatures (50.0°C, 55.0°C, 60.0°C, 60.6°C, 65.2°C, and 67.6°C). We ran the gradient using a temperature gradient thermocycler at the United States Department of Agriculture Lab in Fort Pierce, Fl. The products of the PCR were separated using electrophoresis so that we could visually determine the optimal annealing temperatures for each microsatellite.

Results

DNA Extraction

DNA extraction from conch foot tissue was most successful using the QBT and QPM kits (Figure 2). Using the QBT and QPM kits, we were able to successfully extract DNA from all nine tissue types (Table 1). The QBT kit effectively extracted DNA from the cultured *S. gigas* eye stalk and foot tissue, wild *S. gigas* larvae and operculum, and from the cultured *S. alatus* siphon sample. The QPM kit successfully extracted DNA from cultured *S. gigas* eye stalk and foot tissue, wild *S. gigas* eggs, foot, larvae and mucus, and also from cultured *S. alatus* foot and siphon samples. The greatest DNA concentrations were yielded when extracting with the QBT kit from the

S. alatus siphon (152.6 ng/µL) and the cultured *S. gigas* eye (145.9 ng/µL). The higher concentrations of DNA using these tissue types may be due to their larger mass in comparison to the other samples; foot tissue samples were ~.20 g, but siphon and eye tissue were ~1.0 g.

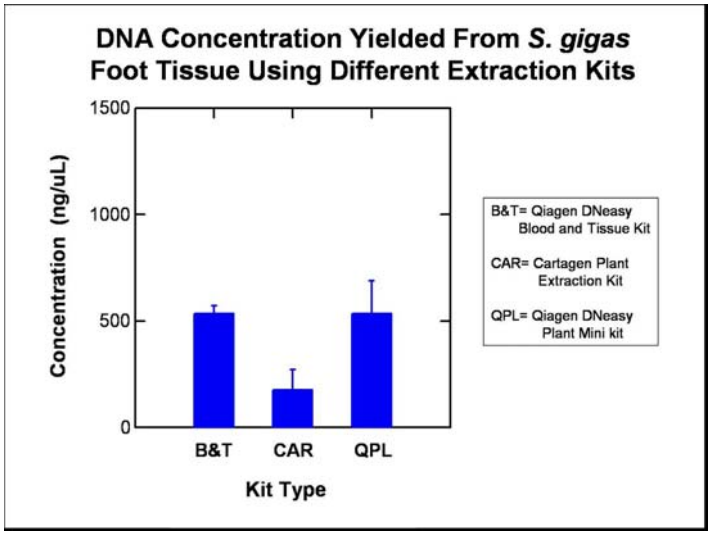

Figure 2. Comparison of resulting concentrations of *Strombus gigas* DNA among three different genomic DNA extraction kits.

Table 1

Tissue collected and DNA extracted

	Qiagen® DNeasy Plant Mini Kit		Qiagen® DNeasy Blood and Tissue Kit		Cartagen® Genomic DNA Extraction Kit for Plants	
	Samples	DNA Concentration	Samples	DNA Concentration	Samples	DNA Concentration
Cultured *S. gigas*						
Eye Stalk	1	18.5	1	145.9	0	0
Foot	2	28.6	4	56.3	4	17.65
Wild *S. gigas*						
Egg	20	9.4	0	0	0	0
Foot	2	105.6	3	49.9	0	0
Larvae	6	9.8	8	3.8	0	0
Operculum	0	0	1	4.6	0	0
Mucus	2	5.4	0	0	0	0
Cultured *S. alatus*						
Foot	1	54.7	0	0	0	0
Siphon	1	100.7	1	152.6	0	0

**DNA concentrations (ng/µL) are averages*

Strombus gigas foot tissue was the only type of tissue preserved using both the 95% ethanol and the -80°C freezer. The difference in DNA concentration yielded using the different forms of preservation was not statistically significant (one-way ANOVA, $P \geq 0.05$).

DNA Amplification

For microsatellites 1, 4, and 5, we observed optimal annealing temperatures that differed from those found by Zamora-Bustillos et al. (2007) (Table 2). For microsatellite 1, we found that a lower annealing temperature improved DNA amplification, but for microsatellites 4 and 5, the optimal annealing temperatures were higher than those of Zamora-Bustillos et al. (2007). For microsatellite 2, 6, and 7, we observed that optimal annealing temperatures matched those of Zamora-Bustillos et al. (2007), microsatellite 6 and 7 successfully amplified at other annealing temperatures as well (Table 2). We have yet to complete successful optimization of microsatellites 3 and 8.

Table 2

Optimal PCR annealing temperatures (°C) for microsatellites using cultured conch DNA

Microsatellite	Zamora-Bustillos et al. (2007)	FAU-HBOI Lab
1	63	55.0 or 60.6
2	60	60.6
3	62	undetermined
4	62	67.6
5	60	65.2 or 67.6
6	60	60.6 or 65.2
7	60	55.0 or 60.0
8	59	undetermined

In order to ensure that the amplification process would also work with DNA from the Florida Keys tissue samples, we ran a PCR reaction with a Florida Keys foot tissue sample at microsatellite 1 (Zamora-Bustillos et al., 2007). We used 60.6°C the optimal annealing temperature for cultured conch in our lab. Post-PCR, we ran an LE agarose gel, and were able to visually determine that the Florida Keys DNA sample amplified successfully.

Discussion:

We successfully extracted DNA using both of the Qiagen® extraction kits. Though Morales (2004) recommended the use of DNA extraction kits designed for plants, we had equal success when extracting with the Qiagen® DNeasy Plant Mini Kit and the Qiagen® DNeasy Blood and Tissue kit. Where as Morales (2004) designed a complicated DNA extraction technique for *S. gigas*, our success with these commercially packaged kits should make DNA extraction from the queen conch less intimidating to future researchers.

The extraction of DNA from *S. gigas* operculum and mucus are novel for the species. There are no existing records of using operculum as a source of DNA. Foot mucus was used to collect DNA from *Arianta arbustorum* in 2006. The concentration of DNA extracted from *A. arbustorum* foot mucus was similar to the concentration of DNA we were able to extract from *S. gigas* foot mucus in this study. Before *S. gigas* operculum or foot mucus is used as a source of DNA for other genetic studies, we must determine whether the DNA extracted from them can be amplified.

If DNA from queen conch operculum and/or foot mucus is successfully amplified, this may increase the number of future genetic studies involving wild

queen conch permitted by the state of Florida, as small collections from either source may pose less harm to a conch's health. Finding sources of DNA other that foot tissue is especially important for this species because it is evident from our study that the magnesium chloride solution (30 g/L) method used for relaxation of captivity reared *S. gigas* (Acosta-Salmón and Davis 2007), is not adequate for inducing relaxation of fully grown wild adults in the field. Another relaxation method, Nembutal (sodium pentobarbitone) at 22.5°C (Aquilina and Roberts 2000), works for *Haliotis iris* (abalone) and may also be worth exploring.

The Florida fighting conch, *Strombus alatus*, has received little attention from the scientific community. Natural populaltions of *S. alatus* in some regions of the Indian River Lagoon have declined recently (personal communication Edward Proffitt with Paul Mikkelson), making it important to gain an understanding of the genetic flow of their populations as well. We were able to successfully extract DNA from *S. alatus*, which had previously not been attempted. If the DNA extracted from *S. alatus* can be amplified using the same microsatellites designed for *S. gigas*, future genetic studies of the species will be possible. Even if the DNA we extracted from the *Strombus alatus* siphon amplifies successfully, this will not be the preferred method for tissue collection, as it requires sacrificing animals.

The collection and extraction of DNA from wild *Strombus gigas* samples will be used in the assessment of paternity to follow. We have extracted DNA from three mothers and seven progeny from each. The next step in the project will be to produce microsatellite fragments for each of the wild *S. gigas* specimens that I extracted DNA from. This will require separate PCRs for each specimen with primers pairs (one pair

fluorescently labeled) for each of the eight microsatellites. These PCRs will be run at the optimal annealing temperatures I identified in this study. Then the PCR products will be run on an ABI 310 automated DNA Sequencer to separate the microsatellite molecular markers. We will then analyze the data by comparing microsatellite markers from each maternal specimen and its progeny to determine if our samples indicate evidence of multiple paternity in *S. gigas*.

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