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Mitochondrial DNA Inheritance in Bivalves: A Comparative Study Involving the Unique System of Doubly Uniparental Inheritance

Sarah Heishman
Coastal Carolina University

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Abstract

A comparative study of Doubly Uniparental Inheritance (DUI) in the bivalve mollusks *Mytilus edulis* and *Geukensia demissa* has yielded unanticipated results. Around the world, it has been reported that members of the taxonomic family *Mytilidae* (along with the families of *Unionidae* and *Veneridae*) consistently exhibit DUI. However, the hard-to-place *Geukensia demissa*, which is a member of this family, has had varying reports of its DUI status. Most reports involving *G. demissa* vary with the location in which it is being studied, which prompts more questions than it answers. Due to many months of unsuccessful DNA purification attempts, sequencing and an actual determination and subsequent comparison of DUI within these two species failed to occur. What successfully occurred in this study was the inducement of reproduction in *Geukensia demissa* in tanks in a laboratory, and a successful purification of DNA from *G. demissa* followed by amplification of mtDNA using species-specific primers.

Introduction

Until recently, it has been believed that there is a strict maternal inheritance of mitochondrial DNA (mtDNA) in animals. The idea that only the female's mitochondrial lineage and genome is passed from parent to offspring is an unusually simple model that has had a huge impact on the fields of population genetics, evolutionary genetics, molecular ecology, and forensic science. Mitochondrial DNA is much smaller than the other DNA found in animals, called nuclear (or chromosomal) DNA. Chromosomal DNA is so named because it is

located on the chromosomes that are located in the nucleus. This chromosomal DNA makes up the vast majority of a cell's genome. Even genes that code for events that occur in the mitochondria (such as those for proteins that function in this organelle) are found in chromosomal nuclear DNA. The smaller size and simple inheritance combination present in mtDNA has made it the ideal candidate for modeling in population and evolutionary genetics, as well as molecular ecology (White *et al.*, 2008). This is why the scientific world was forced to pay attention when it was discovered that several species of bivalves, along with some other animals, do not follow this pattern of strict maternal inheritance.

Instead, these species of bivalves have been found to inherit mtDNA through a system known as doubly uniparental inheritance, or DUI. In DUI, unlike conventional mtDNA inheritance, two mitochondrial lineages (which include mitochondrial genomes) are transmitted to the organism from both its parents, not just the mother. Passamonti and Ghiselli describe this process in their 2009 paper by explaining that it is called DUI because two mitochondrial lineages (along with their mitochondrial genomes) are inherited. To avoid confusion, what is inherited from the mother is called the F or F-type (F standing for female), and what is inherited from the father is called the M or M-type (M standing for male). What is particularly interesting about DUI is that within it, the M and F sequences show up to 30% sequence divergence. An interesting point is that DUI is also a sex-determining mechanism (Passamonti and Ghiselli, 2009).

Burzyński *et al.* describe this process in greater detail in their 2003 paper. In this paper, DUI is once again described as a mechanism through which some

species of bivalves inherit a combination of M and F mtDNA. Because of this, these organisms are considered to be mosaics. Mosaics are organisms whose cells contain different genetic sequences, which is caused by a mutation during early development. If the fusion of two genetically distinct embryos at an early developmental stage were the cause, the individual would be considered a chimera. In the case of the aforementioned bivalves, they are mosaics because their mtDNA is a combination of their mother's and father's mtDNA, and the offspring express characteristics of both parents' mtDNA. However, these bivalves are a unique type of mosaic. They are unique because both male and female offspring have the non-coding region from the male mitochondrial genome. Another unique characteristic of these bivalves is that both sexes are transmitted through sperm, like the M genome. This occurs through a process known as female masculinization. Female masculinization is a type of role reversal in which the F genome invades the M transmission route and replaces the previous M genome with its own (F) information. There are two main consequences of this role reversal. The first consequence is that the M to F sequence divergence is set to zero for the following generation (since no M sequence is being input). The other consequence, which is more of a long-term effect, is that males are usually heteroplasmic (meaning they have normal and mutant mtDNA molecules within a single cell) for both genomes and females are homoplasmic (meaning they have only one type of mtDNA molecule in a cell) for the F genome (Burzyński *et al.*, 2003). The other way that DUI can occur is

through general recombination during development (as typically occurs in mosaics).

All the functions of DUI are interesting and important in and of themselves. Even so, they are becoming increasingly important as scientists continue to discover the roles that mitochondria play in animals. Passamonti and Ghiselli mention this in their 2009 paper as well, and some of the functions of mitochondria that they mention are signaling, fertilization, development, differentiation, ageing, apoptosis, and sex determination (including the origin of sex). As can easily be seen, these are all functions that are fundamental to life itself.

In order to continue studying these important roles of mtDNA and how it is inherited, it is important to know the species of animals in which DUI has consistently been identified. Breton *et al.* describe in their 2009 paper many of the bivalve species in which DUI has been discovered. These species include, but are not limited to *Mytilus edulis*, *Mytilus galloprovincialis*, *Mytilus trossus*, *Venerupis philippinarum*, *Lampsilus ornata*, *Hyriopsis cumingii*, *Cristaria plicata*, *Inversidens japanesis*, *Venustaconcha ellipsiformis*, *Quadrula quadrula*, *and Pyganodon grandis*. In general, these species are marine mussels, marine clams, and freshwater mussels from all over the world. They serve various ecological and economic functions that range from general consumption to freshwater pearl producers.

The species of concern for this study were *Mytilus edulis* (a marine mussel commonly known as the blue mussel) and *Geukensia demissa* (an estuarine mussel commonly known as the ribbed mussel).

Mytilus edulis is a prominent littoral mussel that is widely distributed, with a life cycle typical of intertidal marine invertebrates that have extended larval dispersal and sedentary adulthood (Koehn *et al.*, 1976). They have historically spanned from the Arctic to North Carolina, as was recorded in 1889. A study in the late 1950s found that *M. edulis* north of Cape Hatteras, NC survived year-round, while south of that location the majority of the population died by mid-summer. A following study published in 1960 determined that these die-offs south of Cape Hatteras were due to a vastly different temperature profile as compared to north of the cape. In 2005, mussels both north and south of Cape Hatteras experienced mass mortality events by early July. This same study concluded that at the time, the southern range of *M. edulis* had retreated to 350 meters north of Cape Hatteras. The study determined that the southern range of this species is limited by an intolerance to high summer temperatures, which result in adult mortality events. Specifically, consecutive aerial exposures of *M. edulis* to temperatures greater than or equal to 32°C will result in mussel death (Jones *et al.*, 2010).

Mussels are widespread on the coasts of many nations globally and are one of the most harvested and commercialized bivalve mollusks. Their affinity for mild climate zones in the northern and southern hemispheres expedite their cultivation (Rego *et al.*, 2002). Within this commercialized industry of shellfish

cultivation, the marine mussel *Mytilus edulis* dominates (Bendezu *et al.*, 2005). One reason for this is that mussels are harvested for their highly valued meat taste and texture; for *M. edulis* from Prince Edward Island, Canada, this is especially true.

Mytilus edulis belongs to the family Mytilidae. This family, along with Unionidae and Veneridae, is a family of mollusk bivalves that has consistently been shown to exhibit DUI all over the world (Breton *et al.*,2006, 2009; Cao *et al.*, 2004; Hoeh *et al.*, 1997; Passamonti, 2007; Soroka, 2008; Burzynski *et al.*, 2003). Mytilidae is the best-studied family for DUI. This is likely because *Mytilus* mussels serve as an excellent model for studying the evolutionary forces that are operating on the mitochondrial genome. They enable this because through them, different levels of divergence can be studied (Breton *et al.*, 2006).

Mytilus edulis served as the control for this study for the aforementioned reasons, and because it is the species from Breton *et al.*'s 2009 paper that is easiest to procure in South Carolina. Again, it is acting as the control because its DUI has already been established and documented.

Geukensia demissa is found from southeast Canada to Florida and the Gulf of Mexico. Within this range, it is predominantly found in estuarine salt marshes (Franz, 1996). Due to its ability to withstand salinities ranging from 4-42‰, this species is able to survive in a multitude of environmental conditions (Wilbur, 1987). Similarly, *G. demissa* is able to withstand greater temperatures than other mussels, including *M. edulis*. *Geukensia demissa* is able to endure

temperatures that are less than 45°C. However, days on which the maximum temperature is greater than or equal to 45°C, the risk of mortality greatly increases (Jost and Helmuth, 2007). The ability of *G. demissa* to survive such extreme fluctuations and ranges in salinity and temperature are what allow this species to survive in a wide variety of habitats. This can make it difficult to classify as a mussel. It is this reason that in some literature *Geukensia demissa* is considered to be a marine mussel (Theologidis *et al.*, 2008; Hoeh *et al.*,1997) and other times it is considered to be a freshwater mussel (Soroka, 2008). These variations in tolerance and habitat are also responsible for this species' ability to exhibit characteristics of both marine and freshwater mussels. The studies conducted by Theologidis *et al.* in 2008 and Hoeh *et al.* in 1997 also described *Geukensia demissa* as being a member of the Mytilidae family, which means that it follows many of the previously discussed attributes of this family.

One variability between *G. demissa* and other members of the Mytilidae family (which includes the *Mytilus* subspecies {spp.}) is that the literature varies in its description of DUI within this species. Some studies have reported discovering DUI in *G. demissa*, while others deny its presence in this species. Most studies either describe *G. demissa* in strictly ecological terms rather than molecular ones. A large portion of papers that do associate molecular tests with *G. demissa* still relate those findings to ecology. One such study to take the latter approach is Díaz-Ferguson *et al.*'s 2010 study that compared the genetic profile of *G. demissa* and five other salt marsh community members across four distinct geographical regions.

The combination of *Geukensia demissa*'s ready availability in South Carolina fringe salt marshes, the small amount of strictly molecular studies conducted on this species, and the lack of certainty about its method of mitochondrial DNA inheritance in South Carolina populations specifically are what made it an ideal experimental species for this study.

Along with these reasons, both species used in this study were selected because of their ecological and/or economic importance. This study seeks to discover if the mtDNA in these species is inherited strictly maternally or if it follows the process of DUI.

Methods

Obtaining samples

When possible, the bivalves were collected from the field at a time when they were particularly reproductively active. This was dependent on the individual species and their locations, however both species of mussels do exhibit seasonal patterns of reproduction (Franz, 1996). A study of *Mytilus edulis* in Chester Basin, Nova Scotia, Canada collected their mature specimen over the summer (Breton *et al.*, 2006). This was relevant to when *M. edulis* specimen for this study were collected because Nova Scotia and Prince Edward Island (where this study's *M. edulis* were from) are neighboring Canadian islands. Since the *M. edulis* cannot be collected locally (due to the heat limitations that were previously

described), they were purchased from Philips' Seafood Market in Myrtle Beach and used to validate the methods.

Due to *Geukensia demissa*'s larger southern range (compared to *M. edulis*), it was collected locally at Vereen Park in Little River, SC (33.88485°N, 78.5972°W). Specifically, the *G. demissa* were collected at the low marsh borderline within the fringing salt marsh at this location. The location within the salt marsh (high marsh versus low marsh) is important to note, because mussels in these locations have very different reproductive and maturation attributes. One study that examined these differences was D.R. Franz's 1996 study in Jamaica Bay, New York. Franz determined that low marsh populations of *G. demissa* will become mature after two full years of growing, and at smaller shell lengths than high marsh mussels. He also determined that high marsh mussels will become mature up to a full year after low marsh mussels in the same area (only 15 meters horizontal difference). This reaffirms a common finding in scientific literature that there is a difference between the two study species; *Geukensia demissa* maturation (and subsequent reproduction) is determined by shell length, while *Mytilus edulis* maturation and reproduction are determined by growth rate (decreased growth rate in *M. edulis* results in reproduction in many populations of this species).

The length at which *G. demissa* become mature is location-driven. For example, a population of *G. demissa* in North Carolina became mature at a shell length of 20mm (Franz, 1996), while a researcher who frequently conducts studies approximately two hours south of this study's sampling location at Baruch

Marine Lab (in Georgetown, South Carolina) has found that *G. demissa* specimens at this location typically reach maturity at approximately 70mm. This sizing is consistent with other researcher's classification of *G. demissa* at this location; Jost and Helmuth classified *G. demissa* into size classes at this locale for their 2007 study. This size is in the middle of their "medium" size class.

Taking all of this information into consideration, only mussels greater than or equal to 74mm were used for this study; however, some mussels closer to the bottom of Jost and Helmuth's "medium" size group (6-8cm) were also collected. These smaller mussels would be used after all of the larger mussels had been analyzed, if necessary.

Gender determination and obtaining DNA

Once the specimens had been collected, their gametes (eggs from the females and sperm from the males) were then extracted via a thorough flushing of the mantle tissue with sterile seawater. Specimens were sexed via microscopic examination of mantle-extracted material. Samples were kept separate as soon as their sex was determined.

The methods of sequence analysis and gamete extraction that were used in this study were based on those used by Burzyński *et al.* in their 2003 study of *Mytilus trossulus* (another member of the Mytilidae family).

An F genome was to be obtained from mtDNA in the eggs and a recombinant genome will be obtained from the sperm. A pure male sample was also to be obtained. Burzyński *et al.* confronted this problem in their 2003 paper by obtaining an M genome from Swansea Bay in South Wales (this pure sequence was from an *M. edulis* individual). After the gametes had been removed from their parent using sterile seawater, this mixture (of seawater and gametes) was pelleted by centrifugation at 4°C and 500rpm (revolutions per minute) for five minutes.

STE-100 buffer (0.1M NaCl, 1M EDTA, 0.05M Tris-HCl, pH 8.0) was used to resuspend the gametes; a wash step was included first, then the STE-100 buffer was used to resuspend the gametes a second time. They were then lysed with 0.3% SDS and 300 µg/mL Proteinase K at 56°C overnight (for 15.5 hours). Total DNA were obtained via phenol/chloroform extraction followed by ethanol precipitation. A TE buffer (1mM EDTA, 0.01M Tris-HCl, pH 8.0) at 10 µg/mL was used to suspend purified DNA.

Testing DNA Extraction

After the purification was complete, DNA extraction was tested by running the products, buffer, BSA, EcoRI, and water (combined in the appropriate amounts) on an agarose gel via electrophoresis after the mixture had been allowed to incubate at 37°C for one hour, then 70°C for ten minutes, and finally 4°C overnight.

EcoRI was used because it is a restriction enzyme that cuts DNA's sugar phosphate backbone at known sequences that are specific to this enzyme. It is important that the sequences (and their lengths) are known so that it can be determined if the desired target has been amplified. The sequence lengths are determined after amplification via agarose gel electrophoresis. This technique separates fragments and produces bands based on the fragments' size and charge. Since DNA is negative, it will run from the negative charge toward the positive charge on the gel. The sizes are separated by the gel because smaller fragments are better able to maneuver, and thus run farther on the gel than larger, less maneuverable fragments do. If the DNA were not digested by a restriction enzyme such as EcoRI, it would be too large to run at all, and would remain entirely in the sample well of the agarose gel.

If the extraction did not provide strong bands on the agarose gel, DNA was quantified using spectrophotometry at wavelengths of 260 and 280nm. The ratio of absorbance of the sample at 260nm to absorbance at 280nm was used to determine the sample's purity.

Agarose Gel Electrophoresis

A ladder was mixed using 4μL ladder DNA and 1μL SYBR Green Stain 1. 9μL PCR product DNA, 2μL of 6x Agarose gel loading dye, and 1μL SYBR Green Stain 1 were added to samples. All samples were vortexed for 2 seconds in order to thoroughly mix. Then, samples were centrifuged at max speed for approximately 15 seconds. Each sample was then loaded into its assigned well

in the agarose gel in its entirety. Once all samples had been loaded, an approximately 90 Volt electrical charge was run through the gel for approximately one hour (or until dye bands were at least ¾ of the way down the gel). Pictures of the gel were taken and analyzed to determine DNA extraction success.

Amplification of mtDNA

The forward primer (CBM) was AGAACGGCGTGAGCTAGTTC (16S rRNA gene, nucleotide positions 3313 to 3332), the reverse primer (CBM2) sequence was ACCTTCACCAGGCGTTTAAG (cytochrome b gene, nucleotide positions 4833 to 4814). This target sequence is common to both the M and F genome. Therefore, it will be amplified in both male and female specimens. *M. edulis*-specific primers M1 (AAACCCTTCGTCCACAAGG) and M2 (AGCCTTTTTGTCATCATTCTGT) were used to check for the presence of M genome characteristics. These techniques are the same as those used by Burzyński *et al.* in 2003. Oligonucleotides were manufactured by Sigma-Aldrich Corporation in The Woodlands, Texas.

PCR was conducted in 50µL total reactions, containing 2x PCR Master Mix, 2µL each of appropriate primers (one upstream primer and its complimentary downstream primer), 5µL diluted (1/10) DNA, and 16µL endonuclease-free water.

Thirty-three cycles were repeated, which included a denaturation of 94°C (for 3 minutes for the first cycle and 1 minute for the following cycles), annealing at 54°C (for 30 seconds), and extension at 72°C (for 1 minute, 30 seconds for the

first 32 cycles and 5 minutes for the last cycle). After the thirty-three cycles ran, the PCR machine will keep products at 4°C until turned off. This program was named “SNHE” and was stored in the PCR machine. PCR products will be tested using agarose gel electrophoresis.

A PCR program named “SNH2” that was identical to “SNHE” except for a 55°C annealing temperature was created to be used with species-determining primers. These primers were taken from “PCR Technique for the Identification of Mussel Species” (Rego *et al.*, 2002). The upstream primer called 580-1F (GAGCTGAGCGAGGAGA) and the downstream primer called 580-1R (ACCAGACTGCAACCTGA) were used in combination with their resultant fragment size to molecularly determine the identity of the species being tested.

Gradient PCR

A gradient PCR was conducted after unsuccessful PCR amplification in order to determine the specific annealing temperatures that would best amplify the target sequence using the CBM pair of primers. This was performed using 20μL total samples, consisting of 10μL 2x Master Mix, 1μL each of upstream and corresponding downstream primers, 2μL diluted (same as before) DNA, and 6μL endonuclease-free water. Gradient PCR was performed for thirty-three cycles, with a 94°C denaturation (for 3 minutes the first cycle and 1 minute for the subsequent cycles), annealing at 50°C to 60°C for 30 seconds (the temperature increased by {3/2}°C in each row from Row 1 to Row 8, over this temperature

range), and extension at 72°C for 1 minute, 30 seconds from cycles 1-32, and a final extension at this temperature for 5 minutes. Finally, products were kept at 4°C for 8 minutes. These gradient PCR products were then examined via agarose gel electrophoresis.

Chelex Purification

Chelex purification was used as another attempt at purifying *M. edulis* DNA. If successful, it would be a quick and easy purification technique that would be very affordable. This purification technique shares the first couple steps of flushing mantle tissue with approximately 10mL of sterile seawater, and then centrifuging at 500xgravity. However, this technique centrifuged at that speed for ten minutes instead of five. The supernatant was then decanted, and 500µL of 10% Chelex was added. This mixture was then heated for ten minutes at 95ºC. After its heat bath, the mixture was centrifuged at 14,000rpm for one minute, and 200µL of its supernatant was removed. This supernatant contained genomic DNA, and it was added to the PCR reaction.

Geukensia demissa Tank Set Up

Geukensia demissa specimens were not collected until early February. Since this is well outside of the species' peak reproductive season, it was necessary to keep them in tanks and try to trick them into believing that it was their peak reproductive time (which is early August). In order to accomplish this,

temperature and hours of light were slowly increased to reach those levels seen in South Carolina in August. Besides these two August-like conditions, a high availability of food was also necessary to be established. The high food availability was necessary because a high energy availability, coupled with a low stress level, is essential for reproduction to occur in *G. demissa*.

In order to establish this, ten gallon tanks were filled approximately half full with local estuarine water that had been collected with carboys. Water quality measurements (including temperature, salinity, and pH) were taken both before and after water was placed in the tank. Tank set up included a submerged thermometer, tank heater, and 1-2 air stones (depending on amount of water and biomass in tank). All measurements were recorded. After ideal temperature of 32°C was established, tank was kept at this temperature to expedite *G. demissa* gamete maturation. Salinity was kept in 15-39‰ range so animals would not undergo haline-induce stress. Specimens were fed Kent Marine PhytoPlex food (which consisted of aquacultured phytoplankton in solution) as needed. Feeding typically occurred once to twice a week. Above the tank set-up there was a Grow Green light that was programmed to increasing intervals of light to dark (to help simulate high summer conditions). **Figure 1** gives a visual as to how the *Geukensia demissa* maturation tank was set up.

Figure 1—*Geukensia demissa* tank set up. This figure shows how the tank, Grow Green light, food, water quality testing equipment, and microscope were set up in the lab space.

Checking for Maturity of Geukensia demissa gametes

Specimens were selected to be examined to determine if they were reproductively mature. The conditions under which the specimens had been living were observed and recorded. From the literature, it appeared that these conditions included large size (would have been classified in “large” size group by Jost and Helmuth in 2007) and close proximity to tank heater (which would mean that the specimen had experienced high, summer-like temperatures for the longest period of time). These mussels underwent the same gamete extraction

techniques as previously described. Color, thickness, and overall appearance of mantle tissue was noted. Flushed materials from the mantle were examined microscopically for the presence of mature gametes, which were expected to look similar to those of *M. edulis*. Once a true mature female was discovered, its conditions were observed and used as a measure of comparison for subsequent identifications. The most immediately obvious of these is that mature *G. demissa* oocytes are fluorescent yellow.

Purification of Geukensia demissa DNA

QIAGEN DNeasy Blood and Tissue Kit (catalogue number 69504) was used to purify DNA from mature specimens. The procedure for "Purification of Total DNA from Animal Blood or Cells; Spin Column protocol" in the accompanying handbook was followed. Within this protocol, the optional step to obtain maximum DNA yield was followed.

Results and Discussion

Although the methods of Burzyński *et al.* in their 2003 paper seemed to be very straightforward and capable with the materials available at Coastal Carolina University, using these methods proved to be ineffective. They were ineffective because although purification of DNA was successful, it proved impossible to obtain the desired target sequence after PCR amplification of the purified DNA. This failed PCR amplification implied either the presence of a PCR-inhibiting molecule in the preparation, or the primers were unable to anneal to a

complimentary sequence. If the latter explanation were true, it would mean that the specimen from which the DNA had been purified was not the species for which the primer was designed (*M. edulis*). The repeated failure to obtain an amplified DNA sequence, especially after the purified DNA had been stored for several weeks, resulted in the numerous subsequent tests and purification methods used in this study.

For example, the second set of primers (580-1F and 580-1R) were procured in order to determine if the specimens that had been obtained from the seafood market were actually *M. edulis*. The sequences came from Rego *et al.*'s 2002 study, and the idea of using molecular means as a way to identify *M. edulis* was also supported by Bendezu *et al.*'s 2005 study on *Mytilus* spp. in Irish waters. Specifically, the 2002 study conducted by Rego *et al.* showed that *M. edulis*, whether from Prince Edward Island (Canada) or Ría de Arousa (Spain), will exhibit either a 400-450bp band or a 1300bp band. If the specimen exhibited a 555bp band, it would be *Mytilus galloprovincialis*. This knowledge can then be used to identify the species.

Due to the amount of time spent trying to obtain a pure *M. edulis* DNA sample with valid methods, the prime reproductive season of *Geukensia demissa* (from May to October) passed by without obtaining any specimen. In the interest of pushing forward with the project and hopefully obtaining some usable data, the decision was made to push onto *Geukensia demissa* and collect samples from the field, even though it was early February.

In order to obtain usable, mature gametes from the February *G. demissa*, additional literature research on *G. demissa* growth, reproduction (natural and induced), and mortality was conducted. Although no studies that induced *G. demissa* reproduction were found, there was a plethora of information on them, since they are such a widespread and ecologically important species.

One very helpful study in understanding *G. demissa* growth and reproduction was David Franz's 1996 study on the size and age at first reproduction of *G. demissa* in a New York salt marsh. This study reported the notable differences in these areas between low marsh mussels and high marsh mussels. Since the *G. demissa* collected in this study were on the borderline low marsh (meaning that they were obtained from the boundary between the low marsh and the beginning of the high marsh), Franz's study provides valuable insight on attributes of each population that this study's *G. demissa* specimens possess. Franz's study also helped to establish ways to expedite *G. demissa* maturation because he observed that the low marsh mussels' accelerated growth rate and maturation is due to their increased amount of time to filter food out of the water column (since high tide would reach and last longer for these mussels as compared to the high marsh mussels) and the increased amount of food still in the water when it reaches the low versus high marsh mussels. The difference in food availability was also observed by Hillard and Walters in 2009.

Jost and Helmuth, in their 2007 study on how temperature affected mortality in *G. demissa*, made some observations on food availability as well. These and other observations in Jost and Helmuth's study were particularly

helpful for this study. They related food availability and temperature regulation not only to placement in the marsh (high, low, middle), but also to body placement/orientation and presence (or lack) of vegetation. Size of organisms as compared to their experience of heat was also observed. This too was very helpful in researching *G. demissa* maturation in the lab, because they found that two organisms that are undergoing the same exact conditions and are identical in every regard except for size will experience very different body temperatures. Another very helpful piece of information from this study was the observation that a submerged mussel's body temperature will be very nearly identical to that of the water surrounding it. This was extremely helpful because the *G. demissa* kept in tanks in this study were submerged at all times except when their tanks were being cleaned. Removing the specimens until their tank water returned to as near the temperature that the old water had been was very useful in preventing gains made prior to tank cleaning from being lost.

This study found that there were the fewest deaths and appeared to be the most mussel "contentment" (majority of mussels were open and filtering, water was clear, food consumption was slightly increased, etc.) when the water temperature was kept at 32°C. As per Neufeld and Wright's 1996 on salinity change and cell volume, along with Wilbur's thesis research in 1987, tanks were kept within the 4-42‰ range. Most commonly, the salinity would be near 15‰ when the water was changed, and it often got up to 37‰ before it got changed at the end of the week.

Yet another helpful piece of information from Jost and Helmuth's 2007 study was that one of *G. demissa*'s important ecological roles is alleviating nutrient deficiencies via deposition of nitrogenous wastes on the sediment surface, in the form of their pseudo-fecal casts. More extensive observations on the relationship between nitrogen loads and *G. demissa* were made by Chintala *et al.* in 2006. They observed that the increased nitrogen levels caused by anthropogenic activity result in increased food sources for *G. demissa*. Both phytoplankton growth and abundance and particulate organic material (POM) abundance are increased along with increased nitrogen; phytoplankton and POM are food sources of *G. demissa*. This increased growth of food sources and in turn *Geukensia demissa* result in increased reproduction. Thus, a suggestion for future research would be to include consideration of nitrogen content of food for mussels when trying to accelerate their maturation and reproduction in the lab.

Another consideration to be given to mussel food in the lab in future studies is the exact species of phytoplankton that are in it. Ren *et al.* considered this in their study of assimilation efficiency of nine different phytoplankton species in the mussel *Perna canaliculus* in 2006. In accordance with the results of their study, a diet that consists of dinoflagellates as compared to flagellates or diatoms will result in significantly higher assimilation (84.5% as compared to 77.9% or 61.7%, respectively). Such specific considerations were not given to the food selected in this study; however, it would be a great improvement to future studies to make such a consideration.

Another great addition to future studies that was not available during this study is a description of the various stages of oogenesis and spermatogenesis in mussels. This process is similar amongst mussel species. Yurchenko and Vaschenko described these processes in their 2010 paper on *Modiolus kurilensis* (a marine mussel). Ç ek and Şereflişan describe these processes in *Unio terminalis delicates* (a freshwater mussel). Having descriptions and pictures of what these immature stages, of which there are five in each and which each have their own names, look like available in the lab when determining if the gametes are mature enough to obtain pure DNA from them would be extremely helpful. This would be helpful not only so that immature gametes could be identified, but also so that the scientist would have an idea as to how long it would be until the gametes were mature.

Even though this study was unable to determine if DUI is the mechanism of mitochondrial inheritance in *G. demissa*, it was able to finally obtain a usable, long-lasting DNA purification from *G. demissa* using the QIAGEN DNeasy Blood and Tissue Kit's procedure for "Purification of Total DNA from Animal Blood or Cells; Spin Column protocol". This was a major breakthrough in the project, but it occurred near the end of the available research period. **Figure 2** shows the successfully purified and amplified DNA. As is shown in the figure, in Lanes 2 and 3, the chromosomal DNA was successfully amplified via PCR (using "SNH2") and species-specific primers 580-1F and 580-1R. As can clearly be seen, the DNA under these conditions was successfully amplified. Figure 2 also clearly shows that in Lanes 4 and 5DNA sequences common to M & F genomes

of *M. edulis* were not amplified. These had undergone all of the same conditions as that in Lanes 2 and 3, except that the primers used were CBM and CBM2; these primers were specifically designed to amplify *M. edulis* mtDNA, not *G. demissa* mtDNA. These results for Lanes 4 and 5 were anticipated, because even though both of the test species were members of the family *Mytilidae*, they are not any more closely related, and although they cohabitate in the Northeast United States of America, *G. demissa* has a much larger overall range and has the ability to inhabit many more habitats than *M. edulis* does.

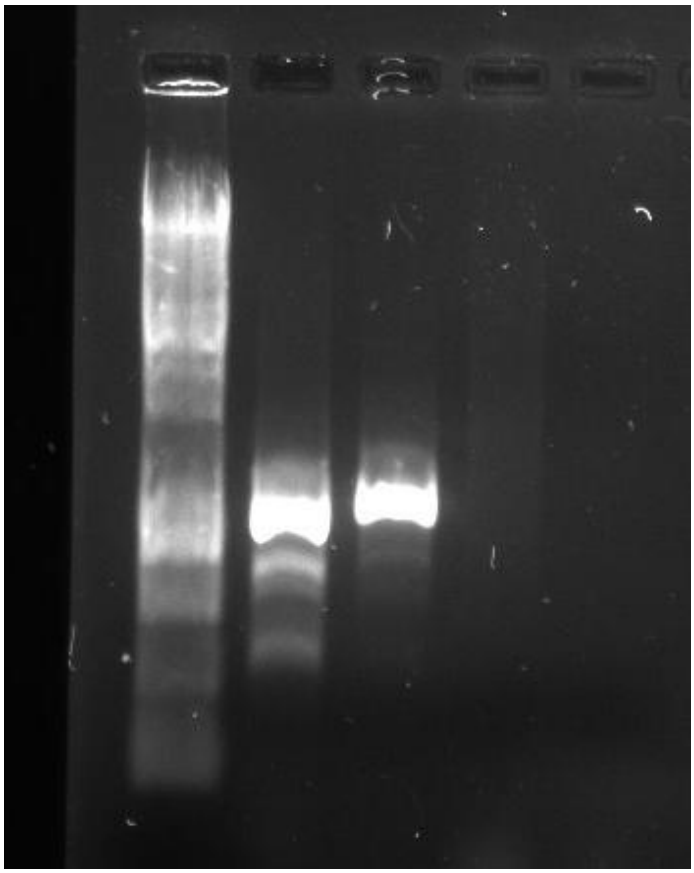

Figure 2—DNA purification after using the QIAGEN DNeasy Blood and Tissue Kit. This figure shows the results of purification using the aforementioned kit, followed by PCR amplification using the "SNH2" cycle. Lane 1 contained the ladder, Lanes 2 and 3 contained mtDNA amplified using the species-specific 580 pair of primers (from Rego *et al.*, 2002), and Lanes 4 and 5 contained the mtDNA that failed to be amplified after PCR amplification with the CBM pair of primers (from Burzyński *et al.*, 2003).

An excellent future study would be to examine the two species and several other local species of bivalves found in the fringing salt marshes of South Carolina to determine if they exhibit DUI, using this means of purification. This would be particularly useful because of the common observation of differences within the same species depending on geographic location. Díaz-Ferguson *et*

al.'s 2010 study examined this to a more specific degree, amongst six North American Atlantic salt marsh community members. They broke the Atlantic coast up into four distinct regions based on their specific attributes; these regions were the Upper Virginian, Lower Virginian, Georgia-South Carolina, and Florida. When compared, it was found that *Geukensia demissa* exhibited significant differentiation between the northernmost populations and all the other regions. Díaz-Ferguson *et al.* also determined that latitudinal shifts in ecological interactions are environmentally rather than heritably adapted. Their study also brought up the point that 42 North American species showed a significant nucleotide diversity increase from north to south. All of these facts, in addition to the variable and, in some cases, absent information on DUI in many species of bivalves lead to the strong potential for future research.

Conclusions

This study has uncovered the potential for future studies in the mitochondrial DNA inheritance in bivalves. Particular bivalves of interest would be those that have large geographic ranges, ecological importance, economical importance, and/or varying reports of inheritance from around the world. Due to the high polypeptide content of bivalves (as a side effect of their high mucus content), traditional DNA purification methods are not very effective. However, this study discovered that the QIAGEN DNeasy procedure for "Purification of Total DNA from Animal Blood or Cells; Spin Column protocol" works extremely well at purifying long-lasting DNA from bivalves. Another great success of this

project was the discovery that it is possible to induce reproductive maturation of *G. demissa* (and likely other *Mytilus* spp.) in tanks in the laboratory under the correct conditions. For *G. demissa*, these conditions were tank temperature of 38ºC, salinity between 14‰ and 38‰, and 11.5 hour “day” (Grow Green light on) coupled with a 12.5 hour “night” (Grow Green light off) light cycle. It is possible that a further increase day to night ratio would expedite reproductive maturation even more than in this study.

Although attempts at amplifying *M. edulis* mtDNA target sequences were unsuccessful, *G. demissa* amplification with CBM primers (CBM and CBM2) was successful. This latter amplification was only possible due to the combination of the successful tank reproductive maturation and QIAGEN DNeasy Blood and Tissue Kit purification coupled with the best PCR program found in the study (program “SNH2”)

This knowledge, coupled with the sizable knowledge base of related information, lays the foundation for future studies.

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