GENETIC AND PHENOTYPIC IDENTIFICATION OF *MERCENARIA MERCENARIA*, *MERCENARIA CAMPECHIENSIS* AND THEIR HYBRIDS

By

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To my family

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The hard clam *Mercenaria mercenaria* supports a $65 million-dollar aquaculture industry in the U.S. Sustainability of this industry depends on the development of genetically improved broodstock for high quality seed production, and accurate identification of species and varieties is needed to produce genetically stable families or lines. However, another southern hard clam species *Mercenaria campechiensis* is naturally distributed in the U.S. southeast coast and sympatric with the aquaculture species. Furthermore, these two species have no reproductive isolation, and their hybrids are fertile. The goal of this study was to develop an accurate, fast, and reliable genotypic tool to recognize *M. mercenaria* and *M. campechiensis* and their hybrids by associating with the phenotypic scoring. Phenotypic scoring revealed that clams from Maine were 100% *M. mercenaria*, while samples from Bradenton Beach, Florida were 100% *M. campechiensis*. Samples from all the other locations showed mixed phenotypic characteristics. PCR length polymorphism of 16S rRNA, 18S rRNA, COI, ITS1 and ITS2 genes were not sensitive to identify the two species. RFLP-PCR analysis was proved to be an effective method to identify two species and their hybrids. The accurate and efficient way to identify clam species and hybrids developed in this study

can overcome the confusions arising in clam aquaculture, assist the clams breeding program and improve the conservation implication of these species, especially *M. campechiensis* which are now difficult to find.

CHAPTER 1
INTRODUCTION

General Information

Classification

The northern hard clam *Mercenaria mercenaria* and southern hard clam *M. campechiensis* belong to the phylum of Mollusca (Schumacher, 1817) and the family Veneridae as classified by Keen (1969). The classification is in a controversy in terms of its generic and subfamily placement (Harte, 2001). Even though they are considered as two distinct species today, they were recently considered as one by Fischer-Piette and Vukadinovic (1977). Currently, the widely-accepted classification is as follows (Harte, 2001):

Kingdom	Animalia
Phylum	Mollusca
Class	Bivalvia (Bonnai 1681)
Subclass	Heterodonta (Neumayr 1883)
Order	Veneroida (Adams & Adams 1857)
Super Family	Veneroidea (Rafinesque 1815)
Family	Veneroidae (Rafinesque 1815)
Subfamily	Chioninae (Frizzell 1936)
Genus	*Mercenaria* (Schumacher 1817)
Species	*Mercenaria mercenaria* (Linnaeus 1758)
	Mercenaria campechiensis (Gmelin 1791)

Distribution

The early fossil records of *M. mercenaria* during the Upper Miocene reveal their distribution from Massachusetts to Florida (Dall 1902, Palmer 1927). The recent distribution is from the Bay of Chaleur, Gulf of St. Lawrence, and Sable Island south to the Florida Keys (Harte 2001). *M. mercenaria* has been introduced to other areas for aquaculture in the United States, including Humboldt Bay, California (Murphy 1985), Washington State (Hanna 1966), and Puerto Rico (Juste & Cortes 1990), and to other countries, including England and France during the mid to late nineteenth century and early twentieth century (Heppell 1961) and China in 2000s (Hadley & Coen 2005). In addition, this species has been recorded in Dutch waters (Kaas 1937) and from Belgium (Tebble 1966). For *M. campechiensis*, its distribution extends from the Chesapeake Bay to Florida, Texas, and the Yucatán Peninsula to Cuba (Andrews 1974). Some attempts to introduce this clam species to other areas failed to maintain self-sustaining population. These two hard clam species do not occupy reproductive isolation. Therefore, they are readily hybridized in certain areas where they co-distributed (Dillon & Manzi 1992, Florida Fish and Wildlife Conservation Commission 2015).

Reproduction mode and life cycle

Adults of *Mercenaria* clams are dioecious (Eversole 2001). However, in early stages, *M. mercenaria*, *M. campechiensis,* and their hybrids exhibit consecutive hermaphroditism (Loosanoff 1936, Loosanoff 1937, Dalton & Menzel 1983). Sex determination of adult *Mercenaria* through shell morphology or tissue weight (Belding 1931) is impossible. The reliable method to detect sexes is microscopic observation of the released gametes after spawning. At younger ages, males exceed females in

number (Loosanoff 1937), and thus the sex ratios of some young populations are skewed. Sexual maturity of clams is based on size, age, food, and environments. Usually, *M. mercenaria* reaches sexual maturity at 3 years old in northern states (Stanley & DaWitt 1983) and 1 year in southern states (Kraeuter & Castagna 2001).

Adult clams release gametes through excurrent siphon. After releasing, gametes fertilize in external waters, and cell division occurs. Depending on water temperature, embryos can develop to free swimming trochophore larvae (with cilia) and then D-stage larvae (with shells) within 18-48 hr, and start feeding. After about two weeks (depending on water temperature) of free swimming stage, larvae grow and go through a metamorphosis process, and turned into juveniles (spat) which start to settle on the bottom for burrowing life style.

Hard clam farming process

Clam aquaculture industry in the U.S. is completely reliant on commercial hatchery seed production, and includes the following major stages:

Hatchery seed production

Hatchery seed production is a process conducted in a completely controlled condition with broodstock selection and maturation, spawning, gamete fertilization, larval culture, post-set culture, algal culture, and spat harvest (about 1 mm). Spawning of clams are usually induced by thermal treatment (T = 28-30 °C) in individual containers or mass spawning in a common container. Fertilized eggs are then hatched in filtered sea water at a density of 50-100 eggs/mL till the veliger stage is observed which is typically 24 hours (Hadley & Whetstone 2007). Generally, veliger larval stage can last 7 to 21 days to reach metamorphosis depending on the food quality and quantity and the culture temperature. Veliger larvae kept in static water, and daily water

change and feeding are recommended. Larvae reaching metamorphosis (called pediveliger) are indicated by the emergence of the foot, disappearing of the velum, and appearing of the gills and siphon; then the pediveliger larvae turn into juveniles (the stage called setting) (Hadley & Whetstone 2007). Post-set seed are susceptible to unstable environments, and usually kept in downwellers in hatcheries for another 20-30 more days until reaching 1 mm to outdoor nursery system (http://shellfish.ifas.ufl.edu/industry/).

Nursery

Nursery stage is the intermediate stage preparing small clam seed for growout phase. The systems for nursery are typically land based up-wellers or raceways to allow the spat reaching 5-6 mm shell length which is the minimum desired seed size for grow-out. This stage usually takes about 6-12 weeks depending on the water temperature and food availability.

Grow-out

In Florida, leasing of estuarine or coastal submerged lands for grow-out and monitoring of coastal waters for shellfish harvest is carried out by Division of Aquaculture, the Florida Department of Agriculture and Consumer Services. Soft bags made of a polyester mesh material are commonly used for clam grow-out. The bags are stacked together by using PVC pipes to position on bottom. Galvanized wire and plastic netting are used over the bags to ensure the protection from predators. The bag culture method usually a two-step process. First 16 ft^2 small mesh (3-4 mm) bags are used for nursing about 10,000 - 15,000 seeds of 5-6 mm in shell length. After reach a size of 12-15 mm (usually after 3 to 6 months), seeds are shifted to mesh bags of 9 to 12 mm in size with the stocking density of 800 to 1,400 per bag.

Harvest

After reaching the desired marketing size (it usually takes 12-18 months in Florida), clams are harvested. After cleaning, grouping, counting, categorizing by size, packing, and labeling, live clams are transported to the market nationwide.

Mercenaria mercenaria, M. campechiensis and Their Hybrids

Hard clams *M. mercenaria* is one of the commercially important aquaculture species in the U.S. east coast and Florida west coast (Harte 2001), accounting for a $65 million-dollar (sales value) industry (Yang et al. 2016). To sustain this large aquaculture industry, development of genetically improved broodstock through breeding programs (such as selective breeding, hybridization, polyploidy) is needed to identify superior varieties for high quality seed production. To initiate a breeding program for any species, accurate identification of broodstock species and varieties is the first step to produce genetically stable families or lines. This is specifically true for the hard clams because there are two species co-occurred in the U.S. southeast coast: the northern hard clam *M. mercenaria* and the southern hard clam *M. campechiensis*.

Naturally *M. mercenaria* is distributed along the Canada and U.S. east coast from the Gulf of St. Lawrence to the Florida Keys (Harte 2001), and *M. campechiensis* is distributed from the Chesapeake Bay to Florida (east and west coast), Texas, and the Yucatán Peninsula to Cuba (Andrews 1994). Between these two hard clam species, hybridization exists in the natural co-occurring areas (Dillon & Manzi 1992, Coen et.al 2008), and their hybrid offspring can survive to adult stage and produce gametes (Eversole, 1987). Both species and their hybrids have been reported in South Carolina (Dillon 1992, Dillon & Manzi 1992), and hybrids were reported in majority of the clams collected from the Indian River Lagoon, Florida (Dillon & Manzi 1992).

Hybridization itself is an important evolutionary process which plays a major role in genetic variation within species and often leads to complex interaction between natural selection and gene flow (Harrison 1983, Barton & Hewitt 1985). In general, hybrids of *M. mercenaria* and *M. campechiensis* display a wide variety of morphological variations including a mixture of sculptures, interior color, and thickness of the valve (Harte 2001).

Besides natural hybridization, artificial hybridization between these two hard clam species has been conducted in early 60-70's with parents from different environments, and offspring have been produced and deployed in different locations for growth comparison (North Carolina, Maine) (Loosanoff 1954, Chestnut et al. 1956, Haven & Andrews 1956, Menzel 1962, Menzel & Menzel 1965, Menzel 1977). The F1 and F2 hybrids were also successfully backcrossed (Loosanoff, 1954, Menzel 1977). During 2008-2009, reciprocal single-parent hybridization of these two species was conducted by using farmed individuals of *M. mercenaria* and wild catch individuals of *M. campechiensis* (http://shellfish.ifas.ufl.edu/projects/genetic-stock-improvement-of-hard-clams/hybridization/). Although hybrid offspring were observed to have improved performance compared to the offspring *M. mercenaria*, parental clams (at least one) in two of the crosses were later detected as hybrids by allozyme marker analysis. This called attention to the necessity for clam species and hybrid identification.

For the aquaculture populations in the U.S., it is believed to be *M. mercenaria*. However, with almost thirty years of intensive farming, especially in Florida west coast, it is possible that the farming populations have mixed with the local *M. campechiensis* due to their co-occurrence in Florida west coast and other southern states. Therefore, identification of these two species and their hybrids is in critical need for any breeding

activities. In addition, recognition of *M. campechiensis* is necessary for conservation actions of this once-thriving, but now difficult-to-find species in Florida, especially the Florida west coast.

Phenotypic Criteria for Species Identification of Hard Clams

Morphologically, *M. mercenaria* and *M. campechiensis* have similar typical venerate characteristics including three cardinal teeth in each valve, a pallial sinus, a lunule (the heart shaped feature of the shell (Figure 1-1; Figure 1-2) and escutcheon, and concentric sculpture (Harte 2001). There are prominent concentric rings over two equal size, elliptical, and thick valves (Eversole 1987).

The shells themselves hold adductor muscle pairs (Figure 1-2A) on each side of the shell which are used to open the shells as they relaxed and a prosogyrous umbo is located at hinge area to join the valves. Slightly arch shaped cardinal teeth (Figure 1-2A) are located on wide hinge plate are solid and dorsally attached to the umbone. While the dorsal connection is maintained by dark brown external ligament (Figure 1-2B) locate below the anteriorly-inclined umbo, the heart-shaped lunule (Figure 1-2B) is located at the opposite side (Hadley & Coen 2005). The pallial line (Figure 1-2A) which is located on the interior of each valve and anterior and posterior adductor muscle scars where the adductor muscles attach, are prominent in hard clams. The pallial sinus is a triangular shaped protrusion (Figure 1-2A) from the pallial line, and the apex of the sinus is leaning to the lower half of the anterior adductor muscle scar.

Generally, the criteria to distinguish *M. mercenaria* and *M. campechiensis* (Figure 1-3) are the following four phenotypic observations (Abbott 1974): 1) Lunule shape. In *M. mercenaria* the width of lunule is at least about its height, but for *M. campechiensis* the width is much narrower than its height; 2) Color of the nacre on inside of the shells.

Generally, pure white nacre of *M. campechiensis* is distinct from purple nacre of *M. mercenaria;* 3) Concentric ridges. Thickness of the concentric ridges located on outside of the shell surface of *M. mercenaria* tend to be thinner than that of *M. campechiensis.* In addition, due to erosion *M. mercenaria* has smooth patches on shell and *M. campechiensis* has incomplete erosion (Dillon & Manzi, 1989), and 4) Anterior side of the concentric ridges. Sharper and more pronounced anterior concentric ridges of *M. campechiensis* is prominent over the less pronounced anterior ridges of *M. mercenaria.*

Although these phenotypic criteria have been established and used for hard clam species identification, the high similarities often make the species identification super difficult, especially on the adult stage when their phenotypic characteristics were worn out due to their burrow-in-mud life style (Dillon & Manzi, 1989, Andrews1974, Abbott & Morris 2001). Both hard clam species are usually buried in mud/sand flats in a depth up to 10 m, and adult shells turn into dirt gray in color because of the siltation (Stewart 1996). In addition, hybrid individuals in some geographic locations, where *M. mercenaria* and *M. campechiensis* co-exist, show intermediate characteristics, and thus are even more difficult to identify with phenotypic observation only. The mixture of characteristics has led to a suggestion that the two taxa may be only subspecies or forms (Abbott 1974). Therefore, only phenotypic characters are not sufficient to identify these two clam species and their hybrids.

In addition to morphology observation, protein polymorphism (isozymes) has been developed in these two species for genetic structure analysis (Dillon 1985). Although seven enzyme loci have been identified with high polymorphism, they were

only used for determination of the allele frequencies in *Mercenaria spp*, but not for species identification (Pesch 1974).

Restriction Fragment Length Polymorphism (RFLP)

RFLP is a genetic marker developed in 1974 by using restriction enzymes to digest DNA and generate DNA fragment polymorphism in size and number among individuals, populations, and species (Grodzicker et al. 1974). Traditionally, RFLP analysis is time consuming and labor intensive, and requires Southern blot (Southern 1976) to separate fragments where digested genomic DNA run on an agarose gel and then use specific probes to detect hybridization after transfer to a membrane (Liu & Cordes 2004). Nowadays, the complicated southern blotting process has been replaced with polymerase chain reaction (PCR), and thus RFLP analysis is becoming more convenient with. To date, RFLP-PCR technique has been applied on species and hybrid identification in several shellfish species, such as razor clams for analysis of the 5S rDNA to identify *Ensis silique, Solen marginatus, E. arcuatus, E. macha*, *E. directus,* and their hybrids (Tajes & Meändez 2007), scallops for analysis of ITS region to recognize species (Pinon et al. 2002), and mussels for species discovery (Santaclara et al. 2006).

As a codominant marker, RFLP can uncover both alleles of an individual and these markers make scoring easy due to the large size differences. However, the sequence data for PCR (or probes for Southern blot analysis) and relatively low level of polymorphism are downsides of RFLP marker, and sometimes create difficulties and time inefficient. Therefore, this low power in revealing genetic variation is a major reason for RFLP markers to have been replaced by recently developed high

polymorphism markers, such as microsatellite marker and single-nucleotide polymorphism marker (SNP) (Liu & Cordes 2004).

In this study, the genes commonly used for phylogenic analysis, including internal transcribed spacer (ITS), 18S ribosomal RNA,16S ribosomal RNA, and mitochondrial cytochrome C oxidase subunit I (COI), were used to develop genotypic analyses to identify the two hard clam species and their hybrids. ITSs are non-coding regions including ITS1 which is located between 18s and 5.8s rRNA genes, and ITS2 which is located between 5.8S and 28S rRNA genes. Divergence compared to their flanking regions and easy amplification make this gene a good candidate in taxonomy and phylogeny studies (Cheng et.al 2006). For mollusks, ITS sequences of Veneridae clams have been studied by Cheng et.al (2006) and ITSs have been used for oyster species identification (Wang & Guo 2008). The gene of 18S ribosomal RNA, a component of the small ribosomal subunit (40S), is composed of highly conserved flanking regions and repetitive arrangement in sequences (Field et al. 1988), and make it one of the most frequently used genes in phylogenetic studies (Meyer et al. 2010) and used for phylogeny analysis of mollusks including *M. mercenaria* (Winnepenninckx et al. 1994, Dreyer et al. 2003, Taylor et al. 2007, Espineira et al. 2009). The gene of 16S rRNA, a large ribosomal subunit, is a fast-evolving mitochondrial gene that has been used extensively and successfully in molluscan phylogenic and taxonomic analysis (Hoeh et al. 1997, Cooley and Ó Foighil 2000, Canapa et al. 2003). Mitochondrial gene COI is flanked by conserved regions providing base to design primers for PCR. This region is widely used as DNA barcoding (Hebert et al. 2003a) and encoded subunits for cytochrome transport chain. COI gene have been used for molluscan shellfish studies in

many species (Mikkelsen et al. 2007, Johnson et al. 2008, Chen et al. 2011, Zou et al. 2011, Layton et al. 2014).

The goal of this study was to develop an accurate, fast, and reliable genotyping tool to recognize *M. mercenaria* and *M. campechiensis* and their hybrids by associating with the phenotypic scoring. The supporting objectives were: 1) collection of clam samples known as *M. mercenaria*, *M. campechiensis* and unknown samples from different geographic locations; 2) phenotypic scoring of each clam based on the four described phenotypic characters; 3) identification of two hard clam species using PCR length polymorphism of ITS1, ITS2, mitochondrial 16S ribosomal RNA, 18S ribosomal RNA and COI genes; 4) analysis of restriction fragment length polymorphism of amplified DNA fragments of clams, and 5) establishment of an identification method for *M. mercenaria*, *M. campechiensis* and their hybrids using a combined approach of RFLP-PCR analysis and phenotypic scoring.

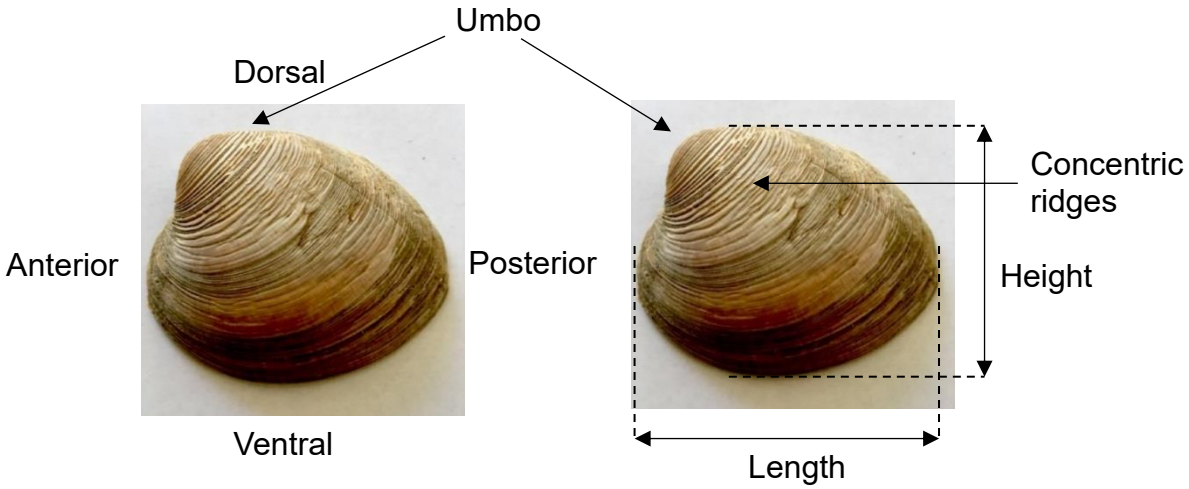


Figure 1-1. Lateral view of the shell valve of a Veneridae clam (*Mercenaria campechiensis*) (Photo courtesy of author)

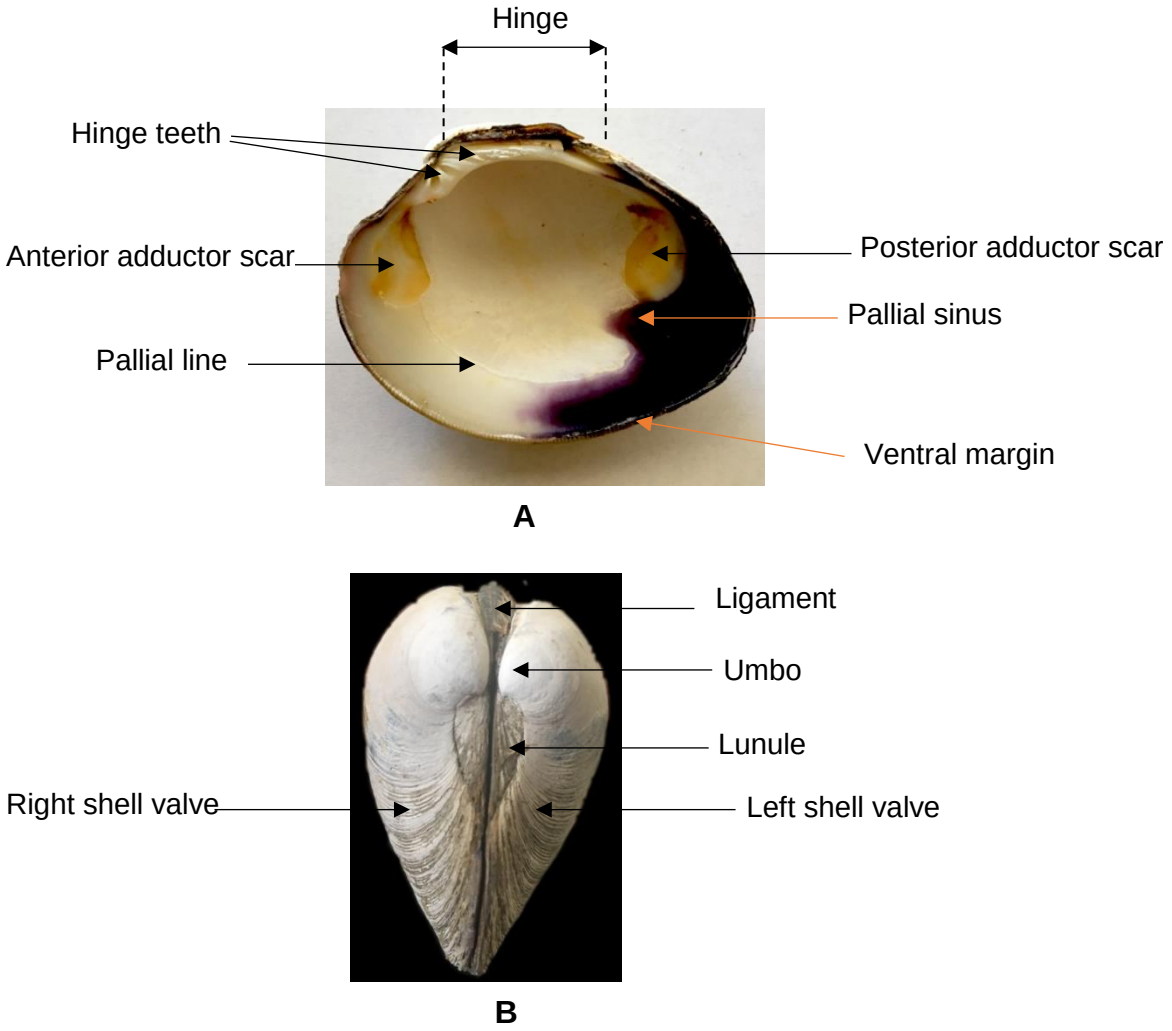


Figure 1-2. Features of the internal structure and the external dorsal view of the clam shell; A) Internal structure of the shell valve of a Veneridae clam (*Mercenaria mercenaria*); B) Dorsal view of a Veneridae clam (*Mercenaria campechiensis*) (Photo courtesy of author)

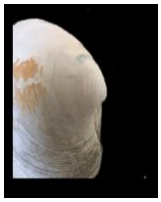
Character	*M. mercenaria*	*M. campechiensis*
Lunule	 Heart shape and width is ≥ its height	 Heart shape and width is < height
Color of the nacre	 White to cream in color and very often with deep purple staining	 Interior of the shell is entirely white in color
Concentric ridges	 Pronounced on the sides and very low to absent in the middle	 Equally pronounced over the entire shell
Anterior side of the concentric ridges	 Less sharp and less pronounced	 Sharper and more pronounced
Notata of the shell	 Present in some clams	 No notata in wild or aquacultured species

Figure1-3. Phenotypic differences in *M. mercenaria* and *M. campechiensis* (Photo courtesy of author)

CHAPTER 2
MATERIALS AND METHODS

Sampling Sites

Clam samples were collected from Harpswell, Maine, Westbath, Maine, Martha's Vineyard, Massachusetts, Savannah, Georgia, Cedar Key, Florida, Saint James city, Florida, and Bradenton beach, Florida. Among these samples, the clams from Maine were wild populations and known as *Mercenaria mercenaria*, and the clams from Bradenton beach, Florida were also from wild populations and known as *Mercenaria campechiensis*. The species of the clams from other locations were not sure due to their locations of distribution or the presences of clam farming near the collection sites (Table 2-1). The two samples from Saint James city, Florida were the third generation of a wild *M. campechiensis* brood (personal communication with the two clam farmers). Upon arriving to the laboratory (Gainesville, Florida), clams were temporarily kept in a recirculating system with bead filter and UV light until processing for phenotypic scoring and fixation of tissue sampling for DNA/RNA analysis.

Sample Processing

Clam individuals were processed with the following procedure: measurement of body weight and body sizes (length, height, and width) using a Vernier caliper (CEN-TECH digital caliper, Camarillo, California), photography of clam morphology (outside shell surface and the shape of lunule), dissection of tissue samples for fixation (see Tissue Sample Fixation section for details), measurement of shell weight, photography of the insides of shells (presence of purple color), and archive of the shells.

Tissue Sample Fixation

After opening the clams, gills and gonads (about 1 cm^3) were dissected from each individual and immediately submerged in RNAlater® stabilizing solution (Ambion, Thermo Fisher Scientific, Waltham, Massachusetts) in 1.5-ml micro-centrifuge tubes by following the manufacturer's instruction. These samples were stored at room temperature for 24 hours, and then moved in -20°C freezer for storage until use. Mantle, gill, adductor muscle, siphon, and foot (Figure 2-1) (about 1 cm^3) from each clam were sampled and fixed in 96% ethanol separately in 1.5-ml micro-centrifuge tubes for DNA extraction. For better fixation, the tissues were cut into small pieces, and ethanol was changed three times in two-day interval. In this study, adductor muscle fixed in ethanol was used for DNA extraction and genotypic analysis.

Phenotypic Identification

Based on the shell morphology described in several early references (Abbott 1974, Dillon & Manzi 1989), the following phenotypic scores were used in this study for identification of *M. mercenaria* and *M. campechiensis*:

1. Narrow or wider shape of the Lunule (Figure 2-2 A);
2. Presence or absence of purple color inside the shell (Figure 2-2 B);
3. Presence of pronounced anterior concentric ridges or not (Figure 2-2 C);
4. Presence of concentric ridges or smooth area on the shell (Figure 2-2 D).

DNA Extraction

DNA extraction was performed by using QIAGEN DNeasy® Blood & Tissue Kit (Venlo, Netherlands) following the manufacturer's instruction with minor modification, and by using Glass Fiber Plate DNA extraction protocol following the instruction in the reference (Ivanova et al. 2006). In plate extraction, 50 µl of Lysis Mix (5 ml of Vertebrate Lysis Buffer and 0.5 ml of Proteinase K) was added to each well of 96-well microplate

and 2-3 mm^3 of ethanol preserved tissue was added. Samples were incubated at 56°C for a minimum of 6 hours, preferably overnight. 100 µl Binding Mix was added to each sample and lysate (~150 µl) was transferred from microplate wells into the wells of the Glass Fiber plate (PALL) placed atop a deepwell block. Wash step #1 was carried out with 180 µl of Protein Wash Buffer and followed by Wash step #2 with 750 µl of Wash Buffer twice. After the Incubation at 56°C for 30-45 minutes, metal PALL collar was placed on a collection microplate and Glass Fiber plate was placed on top. DNA was eluted by adding 100 µL of Buffer AE to the center of the spin column membrane to keep DNA concentrated and was incubated for 5 min at room temperature (20-25°C) to remove excess ethanol. Prior to use buffer ATL and AE were warmed in the water bath to dissolve precipitations which is important for increasing the yield.

Choice of Genes and Identification of Primers for PCR Amplification

Based on the previously published literature, the following genes were chosen for analysis: 1) Internal Transcribed Spacer (ITS); 2) 18S ribosomal RNA gene; 3) 16S ribosomal RNA gene, and 4) Mitochondrial cytochrome C oxidase subunit I gene (COI). Primers for these candidate genes were selected per the published references (Table 2-2). All the primers were purchased from IDT (Integrated DNA Technologies, Coralville, Iowa).

Amplification of Genes by PCR (Polymerase Chain Reaction)

PCRs were set up in a 25-µL volume reaction composed of 1.5 µL genomic DNA (20 ng/µL), 5.0 µL of 5 × buffer, 2.5 µL $MgCl_2$ (25 mmol/L), 0.5µL dNTP (10 mmol/L each), 2.5 µL each primer (10 µmol/L), 0.25 µL Promega™ GoTaq™ Flexi DNA Polymerase (5 U/µL), and ddH_2O to 25µL. Amplifications were conducted in a Mastercycler Pro S thermal cycler (Eppendorf, Hamburg, Germany). The reaction cycle

was set as follows: initial denaturation at 95 ℃ for 2 min, followed by 35 cycles of denaturing for 30s at 95℃, annealing for 30s at specific temperature to each primer, extension for 20s or 30s at 72℃, and a final extension for 5 min at 72℃.

Gel Electrophoresis

To obtain enough resolution for PCR product separation, the following factors for gel electrophoresis were tested: concentration of agarose (1, 1.5, and 2.5%), voltage (90, 100, and 110V), and time duration (1, 2, and 3 hr). Sodium Boric Acid (SBA) was used instead of Tris acetic acid disodium EDTA (TAE) and Tris boric acid disodium EDTA (TBE) (Brody and Kern, 2004). Also, Low EEO agarose and submerged gel electrophoresis were tested. Primers were screened basing on the amplification of PCR products on the gel. All PCR amplified products of the selected genes were finally analyzed by 1% w/v agarose gel electrophoresis.

DNA Sequencing

For sequencing, PCR products were firstly cleaned with ExoSAP-IT™ (ThermoFisher Scientific, Waltham, Massachusetts) to remove any unconsumed dNTPs and primers remaining in the PCR product mixture. The total volume of reaction mixture was 7 µL with 5 µL of PCR product and 2 µL of ExoSAP. Reaction was performed in Eppendorf Mastercycler Pro S thermo cycler with the program set at 37 °C for 15 min followed by 80 °C for 15 min. Cleaned PCR products were sequenced by using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Bio System, Foster City, California). Cocktail reaction mixture was prepared depending on the number of samples and per sample volume used as following: Reaction mix 1.5 µL, 5× sequencing buffer 1.00 µL, Primer 0.16µL, ddH_2O 6.34µL. Cycler sequencing program was performed in the following settings: at 96° C for 1 min, at 96° C for 30 seconds, at 52° C

for 15 seconds, at 60° C for 4 min. Products were cleaned with Sephadex and sequenced using Applied Biosystems 3130xl Genetic Analyzer sequencer. Ten clams from each species were sequenced for ITS1, ITS2 and 16S rRNA genes. Sequences were analyzed by Geneious software (http://www.geneious.com/).

PCR-RFLP Analysis

To create species-specific PCR products, restriction enzymes were used to digest the genome DNA. Possible restriction enzymes were identified by aligning the DNA sequences for each locus (ITS1, ITS2 and 16S) using the software VIRS (visual tool for identifying restriction sites in multiple DNA sequences). All the enzymes were purchased from NEB (New England Biolabs, Ipswich, Maine).

The PCR amplified products of targeted genes were digested with restricted enzymes identified by software VIRS (Table 2-3). The digestion reaction was conducted in 50 µL of total volume containing 3 µL of diluted PCR product (PCR product: water = 1:1), 2 µL of enzyme, and 5 µL of the recommended buffer for each enzyme and 40 µL of ddH_2O in a Thermocycler with programs per the protocol for each enzyme . The digestion products were loaded onto a 1% agarose gel, stained with ethidium bromide and visualized with an ultraviolet light transilluminator (ENDURO™ GDS Gel Documentation System, Thomas Scientific Swedesboro, New Jersey)

Table 2-1. The number and geographic locations of the hard clams (Veneridae) collected for phenotypic score and genotypic analysis for species and hybrid identification.

Species	Number	Population/Origin	Wild/culture	GPS location
Mercenaria mercenaria	10	Martha's Vineyard, Massachusetts	Wild	41°22'03.1"N 70°39'11.8"W
	26		Farmed	
	50	Savannah, Georgia	Wild	31°59'45.8"N 81°00'56.9"W
	28	Harpswell, Maine	Wild	43°47'26.1"N 69°57'33.7"W
	41	Westbath, Maine	Wild	43°52'32.7"N 69°51'20.2"W
	26	Saint James, Florida	Farmed	26°29'55.8"N 82°03'52.2"W
	30	Saint James, Florida	Farmed	26°29'58.4"N 82°03'52.4"W
	4	Cedar Key, Florida	Farmed	29°08'59.3"N 83°01'50.4"W
Mercenaria campechiensis	41	Bradenton beach, Florida	Wild	27°27'55.9"N 82°41'46.1"W

Table 2-2. Candidate genes, related primers, and the annealing temperatures used for PCR amplification for the northern hard clam *Mercenaria mercenaria* and southern hard clam *Mercenaria campechiensis*

Gene	Primer Name	Sequences (5' to 3') (Forward/reverse)	*Temp (^{0}C)	Reference
ITS	ITS1-F	GGTGAACCTGCGGATGGA	55	Cheng et.al
	ITS1-R	GCTGGCTGCGCTCTTCAT	55	(2006)
	ITS2-F	ATGAAGAGCGCAGCCAGC	58	
	ITS2-R	GGCTCTTCCCGCTTCACTC	58	
18S	18S-F	CTGGTTGATYCTGCCAGT	52	Winnepenninck
	18-R	CYGCAGGTTCACCTACRG	52	x et.al (1998)
16S	16Sar	CGCCTGTTTATCAAAAACAT	48	Palumbi (1996)
	16Sbr	CCGGTCTGAACTCAGATCACGT	48	
	16SL3-Ven	GCAAYGAGAGTTGTRCTAAGGTAGC	58-61	Kappner &
	16SH1-Ven	ATAATCCAACATCGAGGTCGCAAA	58-61	Bieler (2006)
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	48	Folmer et.al
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	48	(1994)
	mtCOIbF*	TTTTCTATTTGGGCAGGTCT	50	Baker et.al
	mtCOIbR*	CCTAACCCTACAGGATCAAAA	50	(2008)
	COIF-ALT	ACAAATCAYAARGAYATYGG	47	Mikkelsen et al.
	COIR-ALT	TTCAGGRTGNCCRAARAAYCA	47	(2006)
	COIMIDF	ATRMTNGGNGGDTTYGGNAAYTG	55	Mikkelsen et al.
	COIMIDR	GGRTANABDGTYCANCCNGTNCC	55	(2006)
	LCO1490-Ven	ATTATTCAGAACCAATCATAAAGATATTGG	50-55	Kappner &
	HCOI-900Ven	TGTAGGAATAGCAATAATAAAAGTTAC	50-55	Bieler (2006)

*Temp.=Annealing temperature for each primer in centigrade
**These labels were used for the primers only in current study

Table 2-3. Restriction enzymes used in the study for RFLP-PCR analysis and Possibility of using those Restriction enzymes in species identification

Species for Gene Sequences	Locus	Cutting sites based on sequence data	Enzyme	Fragments created		Genotypic analyses for species identification
				Mm	Mc	
M. mercenaria	ITS1	1	BpmI	2	3	Yes
		2	BsoBI	3	3	No
		2	BstAPI	2	2	No
	16S	2	RsaI	1	1	Yes
M. campechiensis	ITS1	1	AccI	3	3	No
		2	BmpI	2	3	Yes
	ITS2	1	StyI	1	2	Yes

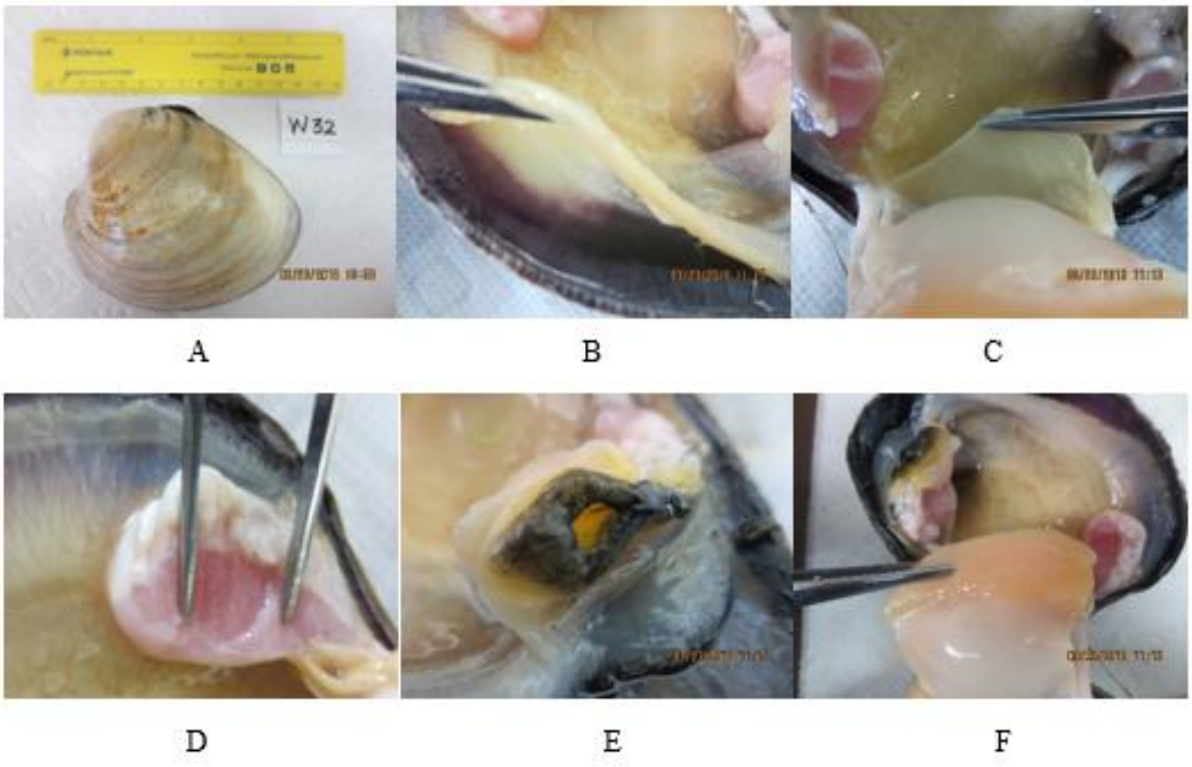


Figure 2-1. The sampling and tissue fixation process from hard clams for DNA analysis. A) Photography of the outside shells. B) Mantle. C) Gills. D) Adductor muscle. E) Siphon. F) Foot (Photo courtesy of author)

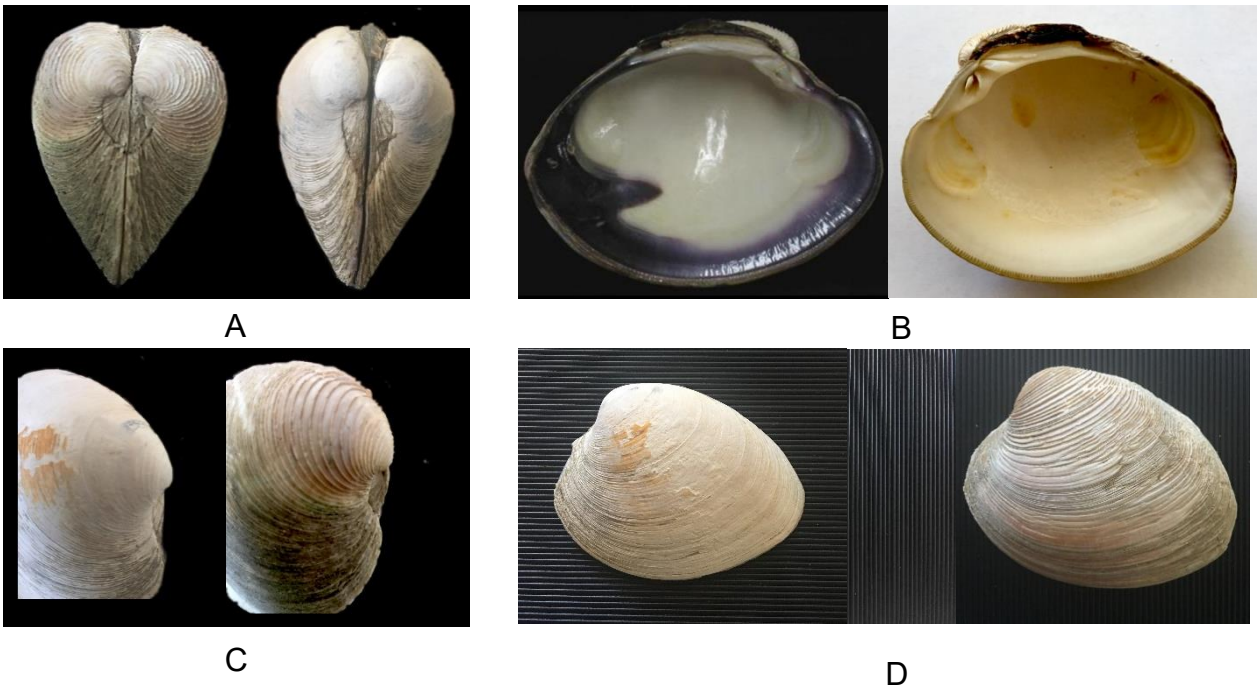


Figure 2-2. Phenotypic identification of the northern hard clam *M. mercenaria* (left column) and the southern hard clam *M. campechiensis* (right column). A) Wide and the narrow shaped lunules. B) Presence and absence of the purple color inside the shell. C) Presence and absence of pronounced anterior concentric ridges. D) Presence of the smooth shell surface and the concentric ridges (Photo courtesy of author)

CHAPTER 3
RESULTS

Phenotypic Scoring

Based on the phenotypic criteria (Figure 1-1), clam samples from Harpswell, Maine and West Bath, Maine were phenotypically identified as 100% *M. mercenaria*, while samples from Bradenton beach, Florida were phenotypically scored as 100% *M. campechiensis*. Samples from all the other locations showed mixed phenotypic characteristics of both species (Table 3-1).

PCR Amplification of Selected Gene Regions

Amplifications of 16S rRNA, 18S rRNA, COI, ITS1 and ITS2 gene regions from genome DNA were successful in both species (Figure 3-1). However, no length polymorphism was observed for tested genes and all gene regions were appeared to be the same size for both species. Each gene region showed different lengths: 16S rRNA - 500bp, 18S rRNA - 1500bp, COI - 900bp, ITS1 - 800bp, and ITS2 - 550bp (approximately).

Optimization of Gel Resolution with Running Time and Agarose Concentration

Among the running time tested, 40 min – 1 hr showed better separation. Although longer electrophoretic time tested could increase the separation of DNA fragments, electrophoresis above 2 hr caused DNA fragments to migrate off the gel and resulted high heat. Voltages tested (90V, 100V, and 110V) did not cause significant difference in resolution. However, DNA fragments traveled faster through the gel at 110V without gel melting and DNA band smearing or distortion. DNA fragments in the gels prepared with low electroendosmosis (EEO) agarose were smeared compared to the regular agarose (Figure 3-2 C). Among the agarose concentrations tested (1%,

1.5%, and 2.5%), 1% provided the better separation of DNA fragments. Electrophoresis with buffer sodium boric acid (SBA) produced smear ladder (Figure 3-2 A), and failed to contribute for a better resolution to separate PCR products (Figure 3-2 B).

RFLP Analysis

Sequence data from each gene for both species were listed in Appendix. Based on these sequence data, the candidate restriction enzymes were selected using VIRS based on the cutting sites for each species. Digestion profiles of these restriction enzymes created different DNA fragments for each locus (Table 2-3).

For ITS1, restriction enzyme digestion profile of BpmI generated two restriction fragments for *M. mercenaria* and three restriction fragments of *M. campechiensis,* allowing the distinguish of the two clam species (Figure 3-3A). Restriction profiles generated by BsoBI, BstAPI, and AccI did not generate restriction fragments that can be used to identify two clam species (Figure 3-3).

For 16S rRNA locus, restriction enzyme RsaI generated two different size fragments in two clam species (Figure 3-3C) allowing species identification.

For ITS2, restriction enzyme StyI digestion generated two fragments for *M. campechiensis*, a fragment for *M. mercenaria* and three fragments for the hybrids (Figure 3-3H). In Figure3-3H, *M. campechiensis* (cB18) and *M. mercenaria* (M5) were the controls and unknown sample (G18) was identified as a hybrid which clearly indicated with 3 bands. Thus, StyI for ITS2 locus proved to be the suitable enzyme from the RFLP analysis as it allowed to identify two species and their hybrids.

Association of Phenotypic Scoring and RFLP Analysis

Samples from Harpswell (Maine), West Bath (Maine) and Cedar Key (Florida) were identified as 100 percent *M. mercenaria* by both phenotypic scoring and RFLP

analysis. Sample from Bradenton Beach, Florida were identified as 100 percent *M. campechiensis* by both methods. All the other samples identified through phenotypic scoring generated different results from the RFLP analysis. When using phenotypic scoring, clams with intermediate characters were identified as hybrids. However, most of them were identified as one or the other species through RFLP analysis (Table 3-2). Based on these association analyses, phenotype presence or absence of concentric rings was proved to be more reliable for species identification than others.

Table 3-1. Phenotypic scoring of hard clams collected from different geographical locations based on the morphology criteria (Figure 1-1)

Location	Number	Condition	Phenotypic character				Species identified		
			Narrow lunule	Purple color inside	Concentric ridges on shell	Sharp anterior ridges	*Mm*	*Mc*	*Unknown*
Harpswell, Maine	28 Wild	Yes	-	28	-	-			
		No	28	-	28	28	28	-	-
		INTMD[1]	-	-	-	-			
West Bath, Maine	41 Wild	Yes	-	41	-	-			
		No	41	-	41	41	41	-	-
		INTMD	-	-	-	-			
Martha's Vineyard, Massachusetts	10 Wild	Yes	2	5	-	-			
		No	8	2	10	10	7	-	3
		INTMD	-	3	-	-			
	26 Farmed	Yes	7	1	-	2			
		No	19	8	33	34	4	-	22
		INTMD	-	17	3	-			
Savannah, Georgia	50 Wild	Yes	9	10	-	2			
		No	41	22	23	24	4	-	22
		INTMD	-	18	2	-			
Cedar Key, Florida	4 Farmed	Yes	-	4	-	-			
		No	4	-	4	4	4	-	-
		INTMD	-	-	-	-			
Bradenton Beach, Florida	41 Wild	Yes	41	-	41	41			
		No	-	41	-	-	-	41	-
		INTMD	-	-	-	-			
St. James City, Florida	30 Farmed	Yes	28	2	30	30			
		No	2	15	-	-	14	-	16
		INTMD	-	13	-	-			
	26 Farmed	Yes	22	1	26	26			
		No	4	14	-	-	12	-	14
		INTMD	-	11	-	-			

*INTMD: Intermediate.

** *Mm*: *Mercenaria mercenaria*

***Mc*: *Mercenaria campechiensis*

Table 3-2. Phenotypic scoring and genotypic analysis results of the study (Mm=*M. mercenaria*, Mc=*M. campechiensis*)

Sample	Measurements			Phenotypic scoring (%)				Genetic/RFLP analysis (%)			
	Length	Height	Width	Sample size	Mm	Mc	Hybrids	Sample size	Mm	Mc	Hybrids
Harpswell, Maine	89.6±7.2	75.8±6.1	47.4±3.9	28	100	-	-	10	100	-	-
West Bath, Maine	89.5±5.8	76.2±4.8	46.3±4.0	41	100	-	-	10	100	-	-
Martha's Vineyard, Massachusetts	49.5±7.1	42.1±6.4	26.8±3.6	10*	70	-	30	4	100	-	-
	30.8±5.7	26.5±5.0	16.6±3.3	26	78	-	22	2	100	-	-
Savannah, Georgia	48.6±0.3	42.6±8.9	27.2±5.9	50	44	-	56	11	91	-	9
Cedar Key, Florida	36.1±6.1	29.8±4.7	17.4±3.7	4	100	-	-	4	100	-	-
Bradenton beach, Florida	69.6±11.7	65.1±10.9	43.4±7.1	41	-	100	-	20	-	100	-
Saint James, Florida	52.5±7.0	44.6±6.5	29.1±4.6	26	-	46	54	5	-	100	-
Saint James, Florida	58.0±10.8	50.9±8.9	33.6±6.5	30	-	47	53	3	-	100	-

*Individuals collected from wild population

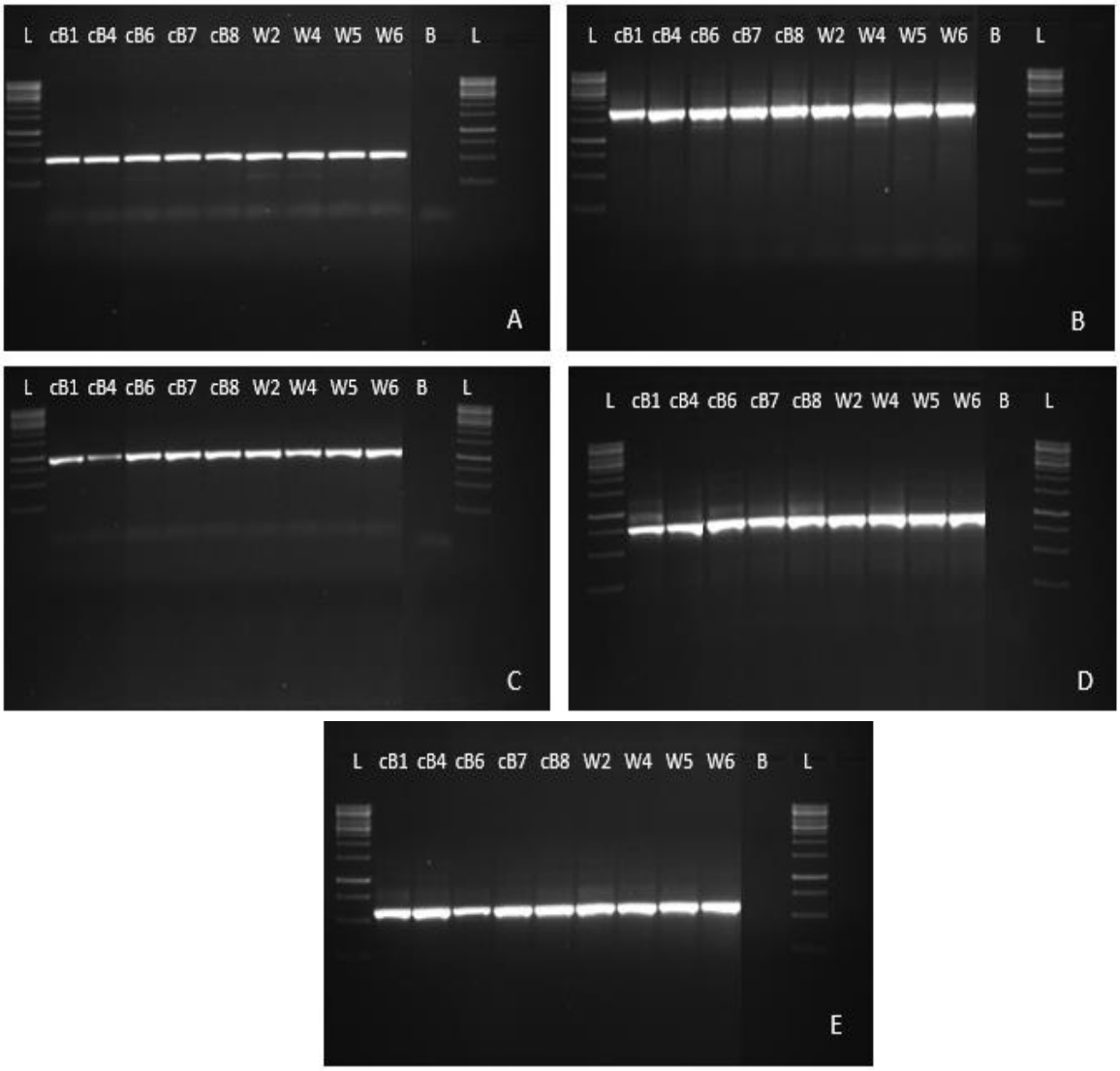


Figure 3-1. PCR amplification of loci from the northern hard clam *M. mercenaria* (W) and the southern hard clam *M. campechiensis* (cB). A)16S rRNA. B)18S rRNA. C)COI (cytochrome C oxidase subunit I). D)ITS1. E) ITS2.
Note: A ladder (L) of 1000bp and a blank lane (B) was run as controls

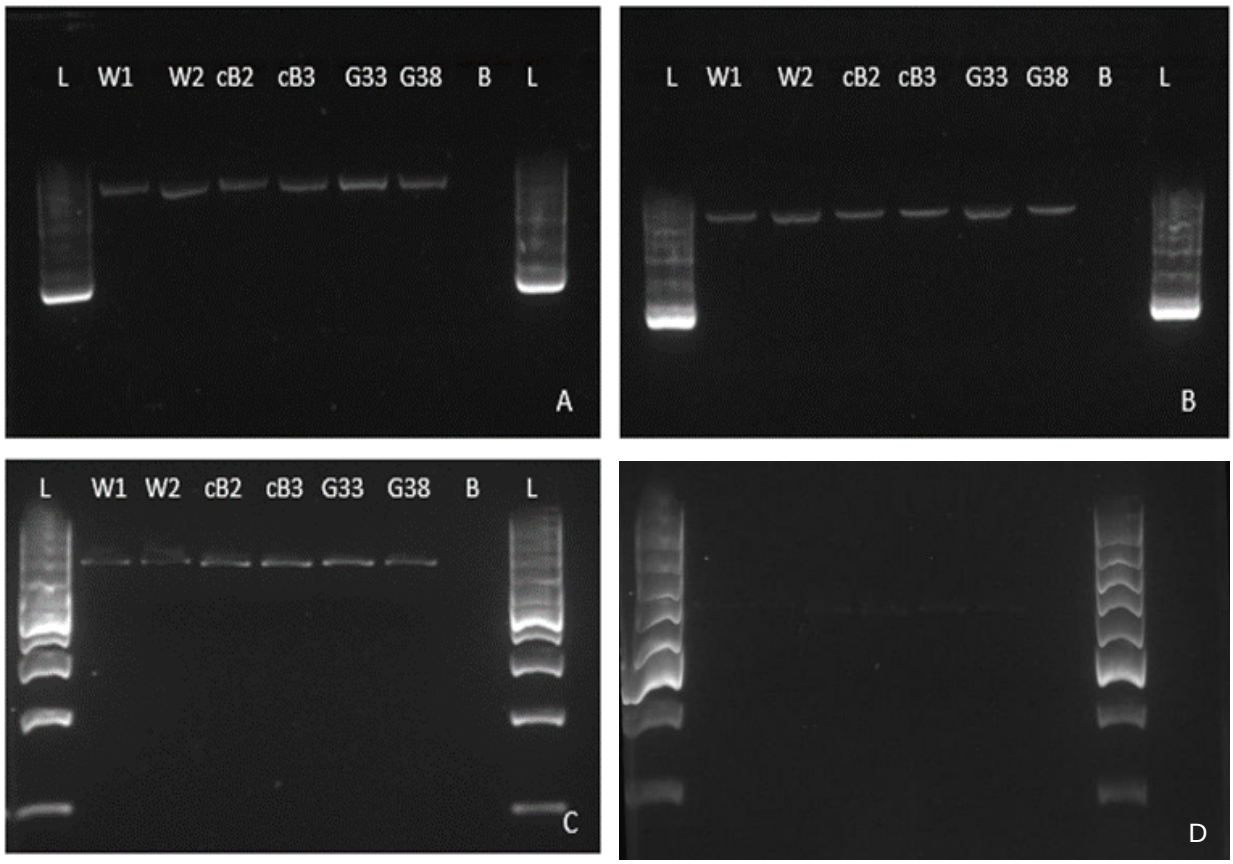


Figure 3-2. Factors tested for optimizing electrophoresis gel resolution for analysis of PCR amplification products. A) Smeared ladder when using Sodium Boric Acid as the buffer. B) Smeared ladder in Submerged TBE gel electrophoresis. C) Smeared ladder when using Low EEO agarose. D) Unclear DNA fragments in a gel run for 3 hours

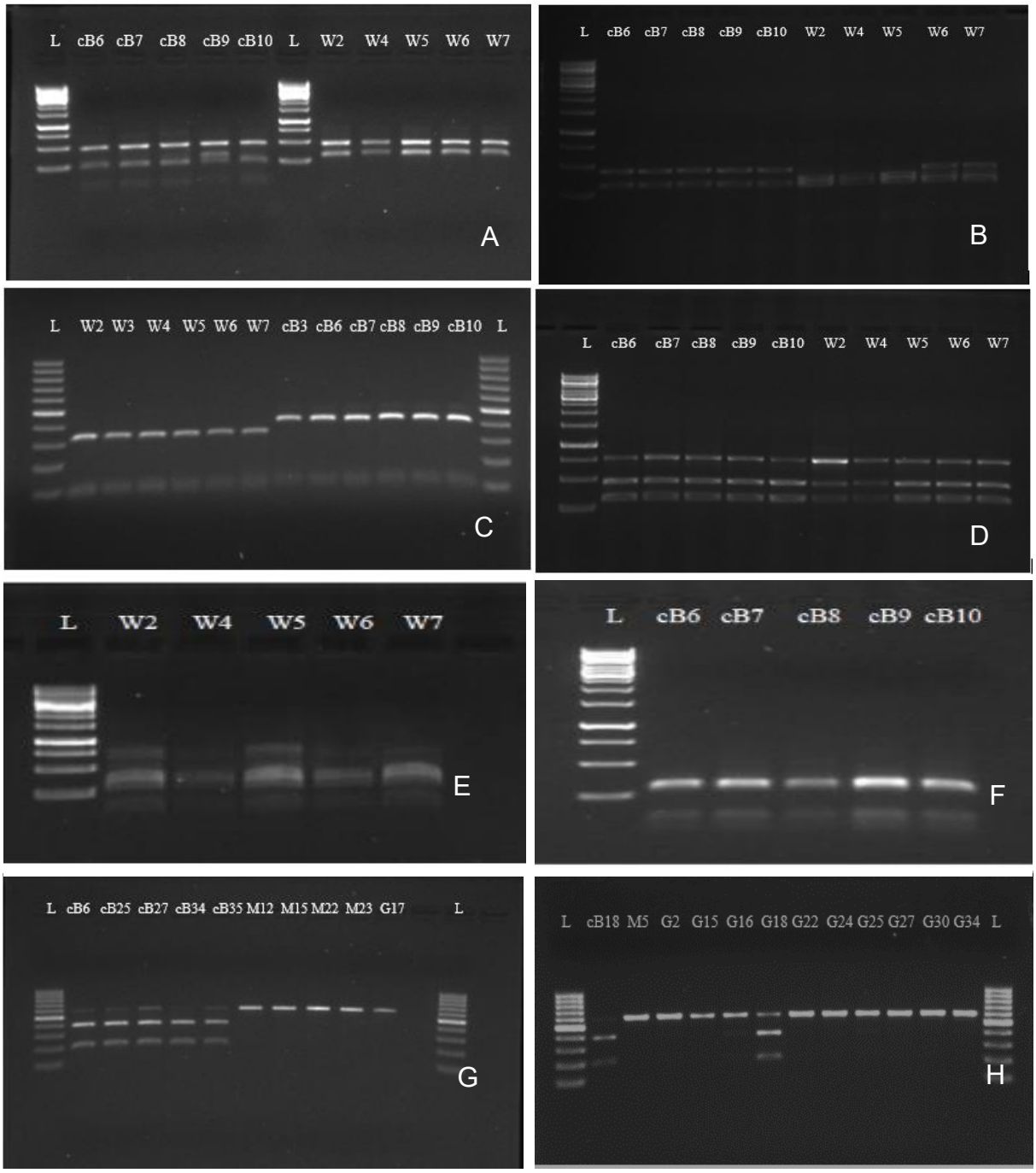


Figure 3-3. Restriction enzyme digestion profiles for the known hard clam species (A) BpmI; B) BstAPI; C) RsaI; D)AccI; E & F) BsoBI; G) StyI, and unknown clam samples (H) StyI
(cB=*M. campechiensis*; W & M= *M. mercenaria* G=Unknown)

CHAPTER 4
DISCUSSION

The Significance of Broodstock Management for Aquaculture Industry

Hard clams, including both *M. mercenaria* and *M. campechiensis,* are important fishery and aquaculture species in the U.S. Generally, the farming species is considered to be *M. mercenaria* in the U.S. including the southeast states where *M. campechiensis* are naturally distributed. Although *M. campechiensis* is not an aquaculture species, historically it has been abundant with a fishery landings about 5 million pounds in 1980's along the Florida east and west coast before the initiation of clam farming industry (https://publictemp.myfwc.com/FWRI/PFDM/). However, it is now becoming difficult to find wild populations of *M. campechiensis* along the Florida coast. With the increase of clam farming, it is highly possible that hybridization may occur between these two hard clam species in areas where they co-occurred. Confusion may happen due to wrong identification of species in certain situations especially in breeding programs. Therefore, precise detection of species and their hybrids is important for the comprehensive understanding of biology, conservation implications, and commercial aspects of the aquaculture industry.

Hybrids of closely related species share higher percentage of parental morphology showing intermediate characteristics of two parental species; thus, some hybrids may erroneously be used as broodstock for seed production. However, post-F1 hybrids could decrease the viability of offspring owing to excessive mortality rates triggering low production and in turn financial losses. Additionally, identification of pure and hybrid lineages is fundamental in developing policies for the conservation and management of native species (Allendorf et al. 2001). The encounter of hybrids with

native populations are problematic to forecast (Toledo-Filho et al. 1998), and pure species face an additional, high impact risk due to the genetic contamination of wild populations with high incidences of hybrids (Melo et al. 2009).

Currently in Florida, pure *M. campechiensis* is difficult to find which directly impacts conservation efforts and for the conservation it is important to identify pure species. Releasing of interspecific hybrids produced in aquaculture ventures could cause serious impacts on wild populations. Fertile hybrids of closely related species have a possibility of genetic introgression which may be a reason for the extinction of pure species from natural populations. And, at juvenile stage hybrids and pure species are often morphologically undifferentiated in breeding facilities, and can be easily blended species in natural environments.

Thus, close monitoring and accurate identification methods should be used to guarantee the reliability of pure-species in aquaculture breeding programs. In this study, one method for accurate and fast identification of two hard clam species and their hybrids was developed by using RFLP-PCR analysis and associated with phenotypic scoring.

Phenotypic Scoring of *M. mercenaria* and *M. campechiensis* Populations

According to the phenotypic scoring, majority of wild clam from northern states were identified as *M. mercenaria* while wild clams from Florida west coast were identified as *M. campechiensis*. Also, there were many clams with intermediate or mixing characteristics. Burrow-in-mud life style of these two clam species especially at adult stage can wipe out the established morphological identification characteristics (Dillon & Manzi 1989, Andrews 1974, Abbott & Morris 2001) or, in geographic locations where *M. mercenaria* and *M. campechiensis* co-exist, hybrid individuals could show

intermediate characteristics. In fact, the mixture of characteristics has led to suggestion that the two taxa may be only subspecies or forms (Abbott 1974). Therefore, only morphology and phenotypic observations are not sufficient to identify two species and their hybrids.

PCR Length Polymorphism

PCR length polymorphism of 16S rRNA, 18S rRNA, COI, ITS1 and ITS2 gene regions has been used in early studies as a reliable and easy method to identify species, such as ITSs for 12 common species of Ostreidae (Wang & Guo 2008), and 5S rDNA to distinguish *Solen marginatus* from four other razor clam species (Tajes & Meändez 2007). However, in this study, no length polymorphism was observed for tested genes, and all gene regions were appeared to be the same size for both species. Thus, increase of electrophoresis resolution was conducted by testing agarose concentration, type of agarose (low EEO agarose), voltage, running time, and buffer system and submerged gel electrophoresis based on previous studies (Brody & Kern, 2004). Although these strategies did not create expected gel resolution to distinguish the PCR length polymorphism of genes selected, optimum conditions for gel electrophoresis were established for the following electrophoresis analysis, which was 1% agarose gels in TBE buffer with 40-1hour running time and 110V.

Due to the small base pair differences of the gene tested between the two clam species, PCR length polymorphism was not able to distinguish species through regular agarose gel electrophoresis, and thus RFLP-PCR analysis was performed for the selected loci (ITS1, ITS2 and 16S rRNA).

RFLP Analysis

RFLP analysis is an approach by using restriction enzymes to digest DNA and create DNA fragments with varying sizes and number among individuals, populations, and species, and is the first generation of genetic marker (Grodzicker et al. 1974). For hybrid identification, RFLP is a prevalent marker especially after southern blotting is replaced by polymerase chain reaction (PCR) (Liu & Cordes 2004). For shellfish, PCR-RFLP analysis of 5S rDNA gene has been used on razor clams for identification of species *Ensis silique, Solen marginatus, E. arcuatus, E. macha, E. directus,* and their hybrids (Tajes & Meändez, 2007). And, the same technique was also used for recognition of four scallop species through analysis of ITS region (Pinon et al. 2002), and for discovering mussel species (Santaclara et al. 2006).

In current study, restriction enzymes, BpmI for ITS1, RsaI for 16S rRNA, and StyI for ITS2 could identify two species by generating different size or different number of fragments for each species. And, StyI for the locus ITS2 proved to be the best choice as it allowed to identify two species (producing a fragment for *M. mercenaria* and two for *M. campechiensis*) and their hybrids (producing 3 fragments).

Other Genetic Markers for Detecting Closely Related Species and Their Hybrids

Hybrids play an important role in altering the genetic structure of native habitats of parental species (Bartley et al. 2001), but accurate identification of hybrids is usually difficult, particularly for hybrids beyond the F1 generation (Sanz et al. 2009, Hashimoto et al. 2012), who may be affected by environmental factors such as temperature, salinity, dissolved oxygen (Lindsey 1988), and need more sensitive methods for species identification. Besides RFLP marker, other genetic markers could also serve as tools for species identification, such as random amplified polymorphic DNA (RAPD) for oyster

species identification (Klinbunga et al. 2000), amplified fragment length polymorphism (AFLP) for mollusk product identification (Maldini et al. 2006).

In addition, two highly polymorphism markers, microsatellites and single nucleotide polymorphism (SNP), were developed and have replaced the first generation of markers for species identification and other applications. Microsatellite markers are characterized with high abundance, high polymorphism, easy access, and capable of differentiating homozygous and heterozygous individuals (Miah et al. 2013), and have been developed for the hard clam (Wang et al. 2010) and have been used for larval identification of the European oyster *Ostrea edulis* (Morgan & Rogers 2001) and Zhikong scallop, *Chlamys farreri* (Zhan 2009). SNP is based on single nucleotide polymorphism, and thus they are sensitive and well suited for high-throughput large-scale genotyping analysis. The application of SNP marker will not only for species identification, but many other aspects; Haplotype mapping, Linkage Disequilibrium studies, disease diagnosis (Vignal et al. 2002).

Conclusion

Identification of two hard clam species and their F1 hybrids based on phenotypic scoring is useful but not completely reliable. PCR length polymorphism of 16S rRNA, 18S rRNA, COI, ITS1 and ITS2 genes were not sensitive enough to identify the two closely related hard clam species due to the low base pair size differences. RFLP-PCR analysis was proved to be an effective method to identify two species and their hybrids. Restriction enzymes, BpmI for ITS1, RsaI for 16S could identify two species, and StyI for nuclear gene region ITS2 can identify two species and their hybrids. The accurate, efficient, and fast way to identify clam species and hybrids can overcome the confusions arising in clam aquaculture, assist the clams breeding program (such as establishment

of pure lines for hybridization and genetic analysis), and improve the conservation implication of these species, especially *M. campechiensis* which are now difficult to find. Though there are recent advanced markers, such as SNP and microsatellites, RFLP markers are still useful in species and hybrid identification because of the low cost, easy to perform in simple laboratories and low skill requirements.

APPENDIX A
DNeasy® BLOOD & TISSUE KIT- QUICK START PROTOCOL

The DNeasy Blood & Tissue Kit (cat. 69504 and 69506) can be stored at room temperature (15–25°C) for up to 1 year.

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Re-dissolve any precipitates in Buffer AL and Buffer ATL (use a water bath)
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Equilibrate frozen tissue or cell pellets to room temperature.
- Preheat an incubator to 56°C.

Protocol

1. Tissue: Cut tissue (≤25 mg tissue/2 cubic mm) into small pieces, and place in a 1.5 ml microcentrifuge tube.
   - Add 180 µl Buffer ATL.
   - Add 20 µl proteinase K,
   - mix by vortexing
   - incubate at 56°C until completely lysed (overnight digestion)
   - Vortex occasionally during incubation.

** Warm buffer AE prior to use – to increase the yield

2. Briefly centrifuge the digested product to bring down the liquid from the lid
3. Add 200 µl Buffer AL. Mix thoroughly by vortexing
4. Briefly centrifuge and add 200 µl ethanol (96–100%). Mix thoroughly by vortexing (Should see the precipitation)
5. Pipet the mixture into a DNeasy Mini spin column placed in a 2-mL collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube (mixture refers to both the precipitation and the liquid)
6. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.
7. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube (repeat the step 7).
8. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
9. Elute the DNA by adding 200 µl (or 100 µl which can keep DNA concentrated) Buffer AE to the center of the spin column membrane. Incubate for 5 min at room temperature (15–25°C) (keep the lid close). Centrifuge for 1 min at ≥6000 x g.
10. Optional: Repeat step 8 for increased DNA yield.

APPENDIX B
GLASS FIBER PLATE DNA EXTRACTION PROTOCOL

DAY 1

1.Clean/sterilize the following:
-extraction bench (bleach, water, ethanol)
-extraction equipment (plates, scissors, scalpels, forceps, etc)
-reservoir and graduated cylinder (wash with Eliminase and RINSE WELL)

2.For 1 plate, mix 5 ml of Vertebrate Lysis Buffer (VLB - located in refrigerator door, heat using either hot plate (level 2 with stir) or incubator (56 C) until crystals are dissolved) and 0.5 ml of Proteinase K (20 mg/ml – located in extraction room freezer) in a sterile reservoir. Add 50 µl of Lysis Mix to each well of 96-well microplate ("chimney-well" plates from USA Scientific). Cover plate with cap strips (USA Scientific).

3.Add a small amount of tissue (2-3 mm3 of ethanol preserved tissue) to each well of 96-well plate. Flame all instruments between samples.

4.Briefly centrifuge plate to ensure that all samples are in the Lysis Mix. Incubate at 56°C for a minimum of 6 hours, preferably overnight. If tissue is particularly dense, agitation (vortexing entire plate followed by brief spin on centrifuge) aids in thorough digestion of tissue.

~~~~~~
Before starting Day 2, ensure you have the following quantities of chemicals available prior to starting otherwise you won't be able to finish (double volumes if extraction 2 plates):

-9.6 mL Binding Mix (BM)
-17.28 mL Protein Wash Buffer (PWB)
-72 mL Wash Buffer (WB)
-4.8 mL sterile H2O

DAY 2

1.Clean/sterilize the following:
-extraction bench (bleach, water, ethanol)
-reservoirs (one for each solution), graduated cylinders, deepwell block (wash with Eliminase and RINSE WELL)

2.Centrifuge sample plate at 1500 g for 15 sec to remove condensate from cap strips.

3.Add 100 µl Binding Mix (BM - located in refrigerator door) to each sample using multichannel pipette. Cover plate with cap strips. Shake vigorously for 10-15 seconds and centrifuge at 1000 g for 20 seconds to remove any sample from cap strips.
~~~~~~

4.Remove cap strips and use a multichannel pipette to transfer lysate (~150 µl) from wells of microplate into the wells of the Glass Fiber plate (PALL) placed atop a deepwell block. Seal the plate with self-adhering cover.

5.Weigh Glass Fiber plate and deepwell block and create equal balance. Centrifuge at 2250 g for 6 minutes to bind DNA to the Glass Fiber membrane. Turn the incubator on and set to 56°C.

6.Wash step #1. Add 180 µl of Protein Wash Buffer (PWB - located in refrigerator door) to each well of the Glass Fiber plate. Seal with cover and centrifuge at 2250 g for 3 minutes.

7.Wash step #2. Add 750 µl of Wash Buffer (WB - located in extraction room freezer) to each well of Glass Fiber plate. Seal with cover and centrifuge at 2250 g for 6 minutes. Remove seal and let the plate sit for 30 seconds (this releases the vacuum in center columns). Seal with new cover, re-weigh balance, and centrifuge again at 2250 g for 6 minutes.

8.Remove self-adhering cover. Place Glass Fiber plate on top of a tip box. Incubate at 56°C for 30-45 minutes to evaporate residual ethanol. Place sterile H_2O in incubator at the same time.

9.Place a metal PALL collar on a collection microplate (Eppendorf twin tec plate) and place Glass Fiber plate on top (make sure plates are properly aligned). Place assembled plate on a 96-well rack to prevent cracking of the collection plate (tape plates and rack together). Dispense 30-60 µl of pre-warmed, sterile H_20 (depends on target concentration) directly onto the membrane in each well of the Glass Fiber plate and incubate at room temperature for 1-5 minutes. Seal Plate.

10.Create balance plate (same assembly as above) and centrifuge at 2250 g for 6 minutes to collect the DNA eluate. Ensure that all wells of the collection plate are equal height. Remove the Glass Fiber plate and discard (DO NOT DISCARD METAL PALL COLLAR!!!).

11.Cover DNA plate with cap strips (Fisher only! Do not use USA Scientific cap strips as they will shrink and pop off once frozen!!!). Nanodrop plate to determine DNA concentrations (e.g., fin clips averaged 350 ng/µl when using 30 µl sterile H20).

Square-blocks can be re-used.

APPENDIX C
GEL ELECTROPHORESIS PROTOCOL

1. Prepare the gel box for pouring – make sure no place to leave the gel, place the comb with desired number of wells
2. Measure the amount of Agarose for the gel
    - For big gel 1% - 2 grams of agarose + 200 mL TBE (Tris Borate EDTA)
    - For small gel 1% -1 gram of Agarose+ 100 mL TBE
3. Put the measured Agarose to the volumetric flask and add appropriate amount of TBE (to the eye level)
4. Give it a mix by hand and put inside the microwave- Turn the light off and check for the bubble forming, take the flask out, mix and see through the light and continue until no solid particles
5. Take out from the microwave and keep the flask at an angle, away from the body and add the stirrer
6. Add the desired amount of EtBr (Ethidium Bromide) – 2.5 µL to the big gel and 1.5 µL to the small gel
7. Place the flask on the mixer and set the mixer for very low speed
8. Take out the flask and check it on the hand, if can bear (should not be too cool), pour the gel slowly to the box
9. Keep the gel set aside for thickening – check occasionally,
    - By blowing- no movement
    - By light- wave forming
    - By milky color appearance

    } Gel is ready to use

10. Remove the comb slowly and place the gel inside the gel box
11. Mix the product and dye on a parafilm (typically 3µL dye and 5µL product) and load the mixed product to each well
12. Load the first well with the ladder (depend on the type of the sample ladder can be different, use 1Kb ladder for the current experiment)
13. Check the amount of TBE inside the gel box and Close the lid.
14. Connect the electric cables of the gel box to the power pack (+ to + and – to -) and set the Voltage and desired time to run the gel and press on

After running the gel

1. Place the gel on stage and hit acquire button and then illuminate
2. Take the picture and save
3. Should take the picture as soon as possible

APPENDIX D
POLYMERASE CHAIN REACTION (PCR PROTOCOL)

1.Check the profile of PCR machine prior to start adding chemicals and set up the machine profile according the protocol.

Name: Clam 16s		Primer					
1/5/2017							
sample nos:	SEE BACK						
					No. of rxns:	7	
Reagent	Stock []		Rxn []		Single Vol.		
Water					10.25	78.93	Water
Buffer	5	X	1	X	5	38.50	Buffer
MgCl	25	mM	2.5	mM	2.5	19.25	MgCl
dNTP's	10	mM	0.2	mM	0.5	3.85	dNTP's
primer-F	10	uM	1	uM	2.5	19.25	primer-F
primer-R	10	uM	1	uM	2.5	19.25	primer-R
Taq (Flexi Go Taq)	5	U/ul	1.25	U	0.25	1.93	Taq
DNA					1.5		
	Cocktail Volume:	23.5		Total Vol	25	180.95	ul
95C for 2 mins							
95C for 30s							
see back for 30s	x35						
72C for 20s							
72C for 5 mins							
4C hold							

Start adding chemicals

2.Use the water bath to thaw $MgCl_2$, dNTP, buffer and primers

3.After thawing keep primers and the reagents cold on a cold box

4.Take the tube stripes and keep on the cold box

5.Make the cocktail mix

-Add chemicals to a 1.5mL (according to the volume of cocktail) centrifuge tube in an expensive order.

- 1.Water
- 2. Buffer
- 3. dNTP

} Before adding mix all these reagents by vortexing

- 4.Primers – should not vortex, instead hand mix

6.Set everything prepared before taking Taq polymerase out from the freezer

- Taq is thick and should pause while pipetting to give enough time to draw the correct amount

- add to the cocktail and give it a pipette mix allowing Taq to mix well (bottom to top mixing)

7.Centrifuge and quick vortex mixing of cocktail

8.Add allocated amount of cocktail mix (above example 23.5 µL) to each tube

9.Add the amount of DNA to each tube (above 1.5 µL) and use cover stripe to cover the tubes.

10.Centrifuge prepared samples

11.Place in the thermo cycler and run the profile already set up

APPENDIX E
DNA QUANTIFICATION

Quantification by NanoDrop® ND-1000 Spectrophotometer

The NanoDrop is used to quantify genetic material. Although there are many different methods to quantifying DNA, the NanoDrop is a **spectrophotometer** that calculates absorbance of a sample across different wavelengths.

Protocol

1. Assemble cafeteria tray
    - Kim Wipes
    - 2µL pipette
    - pipette tips
    - gloves
    - USB drive
    - water and buffer tubes
    - samples

2. Open the NanoDrop software, "ND1000" on the desktop
    - Choose "Nucleic Acids"
3. Initialize the instrument
    - ensure upper and lower pedestal surfaces are clean by wiping with Kim Wipe
    - Place 2µL of NanoPure water on the lower pedestal
    - Lower the sampling arm and press OK
    - when it's done, wipe upper and lower pedestals with Kim Wipe
4. Calibrate the instrument
    - Place 2 µL of elution buffer on the pedestal
    - For Clark's lab, elution buffer is 10mM Tris-Cl, pH 8.4
    - Click "Blank"
    - when it's done, wipe upper and lower pedestals with Kim Wipe
5. Measure sample
    - Place 2 µL of sample on the pedestal
    - Enter sample ID
    - Click "Measure"
    - wipe upper and lower pedestals with Kim Wipe after each sample
    - Re-calibrate the instrument each 10 samples, or so, by going back to step 6 and clicking "Re-Blank" instead of "Blank"
6. Save data
    - Click "Show Report"
    - Click "Reports", "Save Report"
    - Click "Export Report Table Only"
7. Re-initialize the instrument each 30-50 samples, by going back to step 3
    - remember to save your data before your re-initialize
8. Clean up thoroughly! Other people use this instrument!
9. don't forget to enter data into spreadsheet

Purity Assessment

- ratio of sample absorbance at 230, 260 and 280nm is used to assess sample purity. The ratios and information relevant to our work with DNA is given below:

260/280 Ratio

- used to assess purity of DNA and RNA
- pure DNA: ~1.8
- <1.8 → residual reagent form extraction, low nucleic acid concentration (<10 ng/µL)
- >1.8 → not an issue!

260/230 Ratio

- used as a secondary measure of nucleic acid purity
- pure nucleic acid: 2.0-2.2
- <2.0 → residual guanidine
- >2.2 → blanked on dirty pedestal, inappropriate blank (should be same pH and ionic strength)

Plate Reading Protocol by Epoch Microplate Spectrometer

1. Turn on computer and BioTek Epoch machine (it doesn't matter which order).
2. Open Gen5 software from computer
3. Select "Read Now"
4. Select Take3 application "Nucleic Acid Quantification"
5. Select the following options
    - Take3 Plate: Take3 (20161)
    - Well Type: Microspots
    - Sample Type: dsDNA
6. Leave the Wavelengths box unchecked, unless you want more information
7. Select "Blanks" from the drop-down menu on Well-type
8. Select the microspots you will use for blanks by clicking on the circles
9. If you are blanking all sixteen microspots proceed to Read
10. Select "Samples" from the drop-down menu on well-type
11. Select the microspots you will use for samples
12. Wipe Take3 plate (microspots and glass) with a damp kim wipe to clean it
13. The plate is held shut by a magnet, so hold plate down with finger while gently lowering lid closed.
14. Place 2 µl of TE or water (depend on the solution used for DNA elution) on the Take3 plate for an initial blanking (The spot will hold from 2 to 10 µl)
15. Gently close lid
16. Select "Read", machine will make a little sound, door will open and window will tell you to put the plate in the tray
17. Place the Take3 plate in tray and select "OK"
18. Tray will close, machine will take the readings-microspots should be in green color (if red should continue blanking)

19. After reading, the program will show you the control CV's and will state whether the CV's are in an acceptable range or not. The CV measures the "Coefficient of variance" which basically tells you whether your multiple blanks are all giving similar readings. Generally, you want to have a CV below 10% or > 0.10. The program refuses to give you any values until you click "accept" for the CV values. If the program finds bad CV values, it won't let you continue
20. Add samples on the plate after cleaning with the silica free wipe. Continue to "read", the program will open excel and show you the readings for your samples. You can save or print this file, or just write down the numbers. If you have more samples, return to the Gen5 program and read the next plate.
21. The program will continue to add excel worksheets to the spreadsheet file until you click End of Batch.
22. When you click End of Batch the program will create a summary spreadsheet on the excel spreadsheet.
23. Save the excel spreadsheet or print it.

APPENDIX F
PROTOCOL FOR ENZYME DIGESTION

1. Sterilize the work bench with bleach, water and ethanol respectively
2. Check the thermo cycler and set the protocol per the enzyme
3. Take out the enzyme and the related buffer from the freezer and place on the ice block
4. Take the water out and place on the ice block
5. Place tube strip on a cold box
6. Add water and buffer to each tube per the given protocol (water should be added to make the total volume 50µL) and mix gently, then centrifuge

Restriction Enzyme	10 units is sufficient, generally 1µl is used
DNA	1 µg
10X NE Buffer	5 µl (1X)
Total Reaction Volume	50 µl
Incubation Time	1 hour*
Incubation Temperature	Enzyme dependent

* Incubation time and the temperature is Enzyme dependent

7. Add 3µL of DNA (depend on the enzyme can be different) to each tube and then add the amount of restriction enzyme
8. Cover the tubes and mix gently and centrifuge
9. Place the tubes in thermocycler and incubate by running the related profile
10. After incubation, can run on a gel to identify the reaction

APPENDIX G
PROTOCOL FOR SAMPLING AND FIXATION FOR DNA EXTRACTION

Reagents/Materials

- 96% ethanol (over 70% is fine)
- RNA later
- Bleach
- Deionized H_2O
- Vernier caliper
- Dissecting instrument
- Lab camera
- Beakers
- Balance
- Sampling trays
- Bunsen Flame
- Nasco Whirl-Pak™ Easy-To-Close Bags
- Paper bags

Color codes used to label samples

M	Mantle
D	Adductor muscle
S	Siphon
G	Gill
F	Foot

Procedure

1. Clean the bench top with bleach, water and ethanol
2. Clean the dissecting instrument with ethanol and flame
3. Take the length, height and width of the selected clam using a Vernier caliper in millimeters(mm)
4. Take the total body weight of the clam in grams(g) using a balance
5. Take a photograph for phenotypic identification (keep a scale and label) (umbo should be oriented left and top)
6. Shuck the clam and placed on a tray for sampling
7. Prepare 1.5 mL micro centrifuge tubes on a rack and fill with RNA later solution
8. Take small pieces of gill and gonads for RNA level analysis and fix in RNA later
9. Prepare 1.5 micro centrifuge tubes fill with 96%ethanol and use 2B pencil for labelling the tubes(color coded labels can be used on the top of the tube)
10. Take samples from mantle, gill, foot, adductor muscle and siphon of each clam and fix in 96% ethanol
11. Cut the tissues in to small pieces for better fixation
12. Remove the muscles from the shell , take muscle weight and keep muscles separately refrigerated in Nasco Whirl-Pak™ Easy-To-Close Bags
13. Place clam shells separately in labeled paper bags
14. Place samples in freezer boxes and keep inside the freezer for future analysis
15. Change the ethanol few times in two day interval for better fixation

APPENDIX H
SEQUENCING PROTOCOL

Clean the PCR Product with Exosap

1. Spin the PCR product down
2. Keep Exosap on an ice block
3. Add 5µL of PCR products to the tube strip
4. Add 2µL of Exosap to each tube
5. Mix and insert into cycler program already set
    - 37^0C for 15 min
    - 80^0C for 15 min

Big Dye Reaction-V3.1cycler Sequencing Kit (Applied Bio System)

1. Set up sequencing reaction plate with sample IDs
2. Pull Sephadax out to warm up to room temperature
3. Calculate the cocktail for the number of reactions (including 5% error)

Reagent	Adding order	Per 1 sample/µL	Per all samples (7 samples)	5% error	Total
Reaction mix	4	1.50	1.5×7=10.5	1.05	11.025
5× sequencing buffer	3	1.00	1.0×7=7.0		7.35
Primer	2	0.16	0.16×7=1.12		1.176
DdH_2O	1	6.34	6.34×7=44.38		46.599
		9.00			

- Add the inexpensive reagent first, this is for 1 primer of 1 locus, not a mix of primers
- Sequencing buffer located in fridge, mix thoroughly use mixer(vortex), hand mix by inverting, centrifuge

4. Add 9µL of the product to each tube
5. Cap the plate and centrifuge (label)
6. Run cycler sequencing program in thermos cycler

96°C	1 min
96°C	30 seconds
52°C	15 seconds
60°C	4 min
4°C	Hold

Clean the Cycler Sequencing Product with Sephadex

1. Prepare Sephadex
2. Snip off the end of 1mL pipette tip
3. Add 450 mL Sephadex solution to each well in sephadex plate
4. Spin down at 2500rpm for 3minutes
5. Dump water (Sephadex forms plugs in plate)
6. Add 20µL H_2O to 5µL sequencing reaction product
7. Pipette 25µL combined water and product on Sephadex column
8. Spin down at 2500rpm for 3 minutes (final volume should be appx. 20µL)
9. Place into ABI sequencer

ABI Sequencer

1. Prepare datasheet using template
2. Enter sample data in to ABI plate manager

APPENDIX I
SEQUENCING DATA FOR THE STUDY

Table I-1 DNA sequences of the hard clams used in the study

Locus	Species	Sequence
16S	*M. mercenaria*	GCTCGCCTGTTTTTACAAAAACATGGCTCTTTGGTGTTAGAAAT AAAGAGTCGGACCTGCCCGGTGAATGWAGTAAACGGTTGCAA CGAGAGTTGTACGAAGGTAGCGTGATAAGTTGTCTTTTGATTGG AGAATGGAATGAAGGGTTTGACGTAGAATTTCTTTTTTTGTAGA AGATACTAAAATTTCCTTCTAAGTGAAAAGGCTTAGGTTTTTRAA AAAGACGAGAAGACCCTGTCGAACTTGATTAGGAATTACTGATA ATAGGTGTTTAAAAGTTTTATTGGGGCAATATTAACTTAAAAAAC AGTTAATTTATTCATAGATCCTTTTTGAAAGAAAGAGGAAAAAGT TACCGCAGGGATAACAGCGTTATTTCTTCTAAGAGATCTTATTG AGGAAGAAGTTTGCGACCTCGATGTTGGATTATAGAAACTTTAT GGTGCAGCAGCTATAATTGTGAGACTGTTCGTCTTTTAATTCTA TACGTGATCTGAGTTCAGACCGGAGGACCTTTGTGGG
	M. campechiensis	CTCGCCTGTTTTTACAAAAACATGGCTCTTTGGTGTTAGAAATA AAGAGTCGGACCTGCCCGGTGAATGTAATAAACGGTTGCAACG AGAGTTGTACGAAGGTAGCGTGATAAGTTGTCTTTTAATTGGAG AATGGAATGAAGGGTTTGACGTGAAATTTCTTTTTTTATAAAAGG TACTAAAGTTTCCTTCTAAGTGAAAAGGCTTAGGTTTTTGAAAAA GACGAGAAGACCCTGTCGAACTTGATTAAGAATTRCTGGTTATA GATATTTRAAAGTTTTATTGGGGCAATATTAACTTAAAAAACAGT TAATTTATTCATAGATCCTTTTTGAAAGAAAGAGGAAAAAGTTAC CGCAGGGATAACAGCGTTATTTCTTCTAAGAGATCTTATTGAGG AAGAAGTTTGCGACCTCGATGTTGGATTATAGAAACTTTATGGT GCAGCAGCTATAATTGTGAGACTGTTCGTCTTTTAATTCTATAC GTGATCTGAGTTCAGACCGGA
COI	*M. mercenaria*	AAGTTACCGTTCTAAAAATAAGCACGACTATCCACGTTCATTCC TACTGTAAATATATGATGTCCTCAAACAATAAAACCTAAAACACC AATAGACAAAACCGCATACACTATAGGRACCTTTCCAAACAACT CATACTTYCCTCTACCAACTTTTACCACATGAGAAATAATCCCAA AAGCCGGTAAAATTAAAATATAAACCTCCGGATGACCAAAGAAT CAAAACAAGTGGACAAAAAGAATAGGATCACCTAACCCTACAG GATCAAAAAAAGAAGTATTAAAATTCCGATCAGTTAAAAGTATAG TTAAAGCCCCAGCCAAAACAGGCATTGCTACAATAAGAAGGAA CCCGGTTACAGCTACACATCAGACAAACATTCTAGTACGCAGC AACACCATAACACCCGGACGCATCAAGAAACTAGTTCTAACGAA ATTAATTGACGCCAAAATAGAAGATGCACCACCCACATGAAGAG AAAAAATAACATAATCCATTGARCTACCAGAATGAGAAAGAGCC CTAGACAGCGGAGGATAAATAGTTCACCCTGTTCCAGCTCCCC CATCTACATAAGCAGAACCTAATAACAAAAGCATTGACACTGGT AACAACCAGAAACTCAGATTATTCATYCGAGGAAACGCCATATC AGGCATAGTTAATATTAAAGGAACCAACCAATTCCCAAAACCYC CAATCATTATTGGCATAACTAGAAAAAAAATCATTACTAAACCAT GTGCAGTAACAATTAAATTATACAACTGCCCATCATCCAACATC TTTCCAGGTATAGCCAGTTCTATACGAATAATAACACTAAAAGC AGTACCCATTAGACCYGCCCAAATAG

Table I-1. Continued.

Locus	Species	Sequence
COI	*M. campechiensis*	TTACCGTTCTAAAAATAAGCACGACTATCTACATTTATTCCTACT GTAAACATATGATGCCCTCAAACAATAAAACCTAAAACACCAAT AGATAAGACTGCRTACACYATAGGAACTTTTCCAAACAACTCAT ATTTTCCTCTACCRACTTTCACTACRTGAGAAATRATCCCAAAA GCCGGTAAAATCAAAATATAAACCTCTGGGTGACCAAAGAAYCA AAACAAATGAACAAAAAGAATAGGATCRCCTAGTCCCACAGGAT CAAAGAAAGAGGTATTAAAATTTCGATCTGTTAAAAGYATAGTC AAAGCTCCRGCTAAGACAGGTATTGCTACAATAAGAAGRAAYC CAGTCACYGCAAYACATCAAACAAACATCCTAGTACGYAGCAAT ACTATAACACCCGGACGCATTAAAAAGCTAGTCCTAACAAAATT AATTGAYGCTAAAATAGAAGACGCACCACCTACATGAAGAGAG AAAATAACATAATCCATTGAACTACCAGAATGGGAAAGAGCTCT AGATAACGGGGGATAAATAGTTCACCCTGTRCCAGCTCCCCCA TCTACATAAGCAGAACCTAGTAACAAAAGCATTGAYACTGGTAA CAATCAAAAACTTARRTTATTCATCCGAGGAAATGCCATATCAG GCATAGTTAACATCAAAGGAACCAACCARTTTCCAAAACCYCCA ATCATCATTGGTATAACTAGAAAAAAAATYATCACTAAACCATGA GCAGTAACAATTAAATTATACAACTGCCCATCATCTAATATCTTY CCAGGCATAGCCAATTCTATACGAATAATAACACTAAAAGCAGT ACCTATTAATCCCGCTCAAATAGAAAAAA
ITS1	*M .mercenaria*	TCTTCATCGACGCACGAGCCGAGTGATCCACCGCATAGAGTTG TCTCAGTTTTGAACAGGGATCGACCGCTCGCGCAACACGAGCG GAAGTCCTGCGCCCTGGCTCCCGAGAGTACGGATCCAGAGAC TTGCGTTCAAGTGTCGCCGGGCACCGAGATGCCCGTTGACGG TTGCACCACAGAGTGTGTGGTTCTGCTGTGAAAACAGAATTCC CCCTCCCAAAGGAGGGGAAAAACGGGGCGGATGTCCCGGCAA GACGGCGCCGAGGCCCAGCGTGCCAGACCGCAGTCGAGTAG WCCGCAGTCCCTGCCCATTTAGGCGGCACGAGGGCCCTTCTT CCGTCCCCCCTGGGGACGATCACTTTAAACCGGAATCGCCTRT CCCGTCGTCCGTCCCGCAAAGGGACCCCGGTCGGGTGGCTCC TGGAGGGAAACCGGTACCCCACTGCTCGGACTGTCTCGGGCT GGGAAGACCCGCGATCGCGCCGTGCGACCGCCGCCCGYGTG GGTCGTTGATGCCGCGGCCTGGGTCGGCCGGTCCCGTTTTGA TCAGGAACGGAACCGAAGCCTGGCGGCCGGGCGAGAGGCTG GGCATGGGCACTCTCTTGCGGGMACGTCCACGGCGTGAATCG CCCTTGGGACGCCCCGACTTTGTTTTTCTGTGCAATATATGCTA GTTTATAGACGCCCCCCGGCAAAGGTAGCAGTCTTGTTTCTGT AATGATCCATCC

Table I-1. Continued.

Locus	Species	Sequence
ITS1	*M. campechiensis*	TCTTCATCGACGCACGAGCCGAGTGATCCACCGCATAGAGTTG TCTCAGTTTTGAACAGGGATCGACCGCTCGCGCAACACGAGCG GAAGTCCTGCGCCCTGGCTCCCGAGAGTACGGATCCAGAGAC TTGCGTTCAAGTGTCGCCGGGCACCGAGATGCCCGTTGACGG TTGCACCACAGAGTGTGTGGTTCTGCTGTRAAACAGAATTCCC CCTCCTATAAAAGGAGGGGAAAAACGGGGCGGATGTCCCGGC AAGACGGCGCCGAGGCCCAGCGTGCCAGACCGCAGTCGAGTA GACCGCAGTCCCTGCCCATTTAGGCGGCACGAGGGCCCTTCTT CCGTCCCCCCTGGGGACGATCACTTTAAACCGGAATCGCCTGT CCCGTCGTCCGTCCCGCAAGGCCGTCGCCTCGGGGACCCCGG TCGGGTGGCTCCTGGAGGGAAACCGGTACCCCACTGCTCGGA CTGTCTCGGGCTGGGAAGACCCGCGATCGCGCMGTGCGACCG CCGCCCGTGTGGGTCGTTGATGCCGCGGCCTGGGTYGGCCGG TCCCGTCTTGATCAGGAACGGAACCGAAGCCTGGCGGCCGGG CGAGAGGCTGGGCATGGGCACTCTCTTGCGGGRACGTCCACG GCGTGAATCGCCCTTGGGACGCCCCGACTTTGTTTGCAATGTG CGTTTTTGCTAGTTTATAGACGNCCTCCAGCAAAGGTAGCAGTC TTGTTTCTGTAATGATCCATCC
	M. mercenaria	CAGCCAGCTGCGTGAATTAATGTGAATTGCAGGACACACTGAA CATCGACACCTTGAACGCACATTGCGGCTCTGGCTCACTGCCA GAGCCACGCCTGTCCGAGGGTCGGCGAACAAGTCATCGCCCG AGACCGATTCACTTCGGTCTGCYGGGCGCGTTGGCGAGTCGC GCGGGCACAGACCCGCCCGTCCGCCGTAGACTTCAGCCTCTC TCACGGCGGCCGAGCGAAGCGGCGCGGGGACAGGGCTCGAA CGGGCCTTCTGTCTGGCGCACGTCTGCGACGGAAACGAAGCG GACGACCTTCGCTGGAGTCACCRGCGTCCCCWCTGCGGGGAG GGAGAGCGCGACTACTSTGTCGCGGACCAGGGACAAWGCCAG CCGTCCAAAGGGGAGAGCTGGGCCGACCGGGGGAGACCCCG AGATCACGGCCCGKKCTTTTCCTCGCGGYAGAATGCTYGGCAG TCTCGCACATCCGACCTCGGATCAGACGGGGATACCCGCTGAA TTTAAGCATATCAGTAAGCGGAGGAAAAGAAACTAACCAGGATT
ITS2		CCCTCAGTAACGGCGAGTGAAGCGGG
	M. campechiensis	CAGCCAGCTGCGTGAATTAATGTGAATTGCAGGACACACTGAA CATCGACACCTTGAACGCACATTGCGGCTCTGGCTCACTGCCA GAGCCACGCCTGTCCGAGGGTCGGCGAACAAGTCATCGCCCG AGACCGATTCACTYCGGTCTGCTGGGCGCGTTGGCGAGTCGC GCGGGCAYAGACCCGCCCGTCCGCCGTAGACTTCAGCCTCTC TCACGGCGGCCGAGCGAAGCGGCGCGGGGACAGGGCTCGAA CGGGCCTTCTGTCTGGCGCACGTCTGCGACGGAAACGAAGCG GACGACCTTCGCTGGAGTCACCAGCGTCCCCTTGCGGGGAGG GAGAGCGCGACTCNGTGTCGCGGACCAGGGACAAAGCCAGCC GTCCAAGGGAGAGCTGGGCCGACCGGGGGAGACCCCGAGAT CACGGCCCGTGCTTTCTCGCGGYAGAAGCKTGGCWGTCTCGC ACATCCGACCTCGGATCAGACGGGGATACCCGCTGAATTTAAG CATATCAGTAAGCGGAGGAAAAGAAACTAACCAGGATTCCCTC AGTAACGGCGAGTGAAGCGGG

LIST OF REFERENCES

Abbott, R. T.1974. *American Seashells; The Marine Molluska of the Atlantic and Pacific Coasts of North America*. No. Ed 2. Van Nostrand Reinhold.pp.182

Abbott, R.T. & P.A. Morris. 2001. *A field guide to shells: Atlantic and Gulf Coasts and the West Indies*. Houghton Mifflin Harcourt.

Allendorf, F.W., R.F. Leary, P. Spruell & J.K. Wenburg.2001. The problems with hybrids: setting conservation guidelines. *Trends in Ecology and Evolution* 16 (11): 613–622.

Andrews, J. 1971. *Sea shells of the Texas coast*. University of Texas Press. p.285.

Bartley, D.M., K. Rana & A.J. Immink.2001. The use of interspecific hybrids in aquaculture and fisheries. *Reviews in Fish Biology and Fisheries* 10:325–337.

Barton, N.H. & M. H. Godfrey.1985. Analysis of hybrid zones. *Annual review of Ecology and Systematics* 16.1: 113-148.

Belding, D.L. 1931. *The scallop fishery of Massachusetts: including an account of the natural history of the common scallop* (No. 3). Commonwealth of Massachusetts, Department of Conservation, Division of Fisheries and Game, Marine Fisheries Section.

Brody, J. R. & S. E. Kern.2004. Sodium boric acid: a Tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36(2):214-217.

Canapa, A., S. Schiaparelli, I. Marota, & M. Barucca.2003. Molecular data from the 16S rRNA gene for the phylogeny of Veneridae (Mollusca: Bivalvia). *Marine Biology* 142(6):1125-1130.

Chen, J., Q. Li, L. Kong & X. Zheng.2011. Molecular phylogeny of venus clams (Mollusca, Bivalvia, Veneridae) with emphasis on the systematic position of taxa along the coast of mainland China. *Zoologica Scripta* 40(3): 260-271.

Cheng, H., D. Xia, T. Wu, X. Meng, H. Ji & Z. Dong.2006. Study on sequences of ribosomal DNA internal transcribed spacers of clams belonging to the Veneridae family (Mollusca: Bivalvia). *Acta Genetica Sinica* 33(8): 702-710.

Chestnut, A. F., W. E Fahy & H. J. Porter.1956. Growth of young *Venus mercenaria*, *Venus campechiensis* and their hybrids. Proe. natn. Shellfish. Ass. 47:50-56

Cooley, L.R. & D.Ó. Foighil. 2000. Phylogenetic analysis of the Sphaeriidae (Mollusca: Bivalvia) based on partial mitochondrial 16S rDNA gene sequences. *Invertebrate Biology* 119(3):299-308.

Dall, W.H. 1902. *Synopsis of the family Veneridae and of the North American Recent species*. US Government Printing Office. 26: 335-412.

Dalton, R. & W. Menzel.1983. Seasonal gonadal development of young laboratory: spawned southern (*Mercenaria campechiensis*) and northern (*Mercenaria mercenaria*) quahogs and their reciprocal hybrids in northwest Florida. *Journal of shellfish research* 3: 11-17.

Dillon, R. T.1985.Correspondence between the buffer systems suitable for electrophoretic resolution of bivalve and gastropod isozymes. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* 82(4):643-645.

Dillon, R. T.1992. Minimal hybridization between populations of the hard clams, *Mercenaria mercenaria* and *Mercenaria campechiensis*, co-occurring in South Carolina. *Bulletin of Marine Science* 50(3):411-416.

Dillon Jr., R. & J. Manzi. 1989.Genetics and shell morphology in a hybrid zone between the hard clams *Mercenaria mercenaria* and *M. campechiensis*. *Marine Biology* 100.2: 217-222.

Dillon Jr., R. & J. Manzi.1992. Population Genetics of the Hard Clam, *Mercenaria mercenaria*, at the Northern Limit of Its Range. *Can. J. Fish. Aquat. Sci*. 49(12): 2574-2578.

Dreyer, H., G. Steiner & E.M. Harper.2003. Molecular phylogeny of Anomalodesmata (Mollusca: Bivalvia) inferred from 18S rRNA sequences. *Zoological Journal of the Linnean Society* 139(2): 229-246.

Espineira, M., N. González-Lavín, J. M. Vieites & F. J. Santaclara.2009. Development of a method for the genetic identification of commercial bivalve species based on mitochondrial 18S rRNA sequences. *Journal of Agricultural and Food Chemistry* 57(2): 495-502.

Eversole, A.G.1987.*Species Profiles: Life Histories and Environmental Requirements of Coastal Fishes and Invertebrates (South Atlantic) Hard Clam*. CLEMSON UNIV SC.

Eversole, A.G.2001. Reproduction in *Mercenaria mercenaria*.*Developments in Aquaculture and Fisheries Science* 31: 221-260.

Fernández-Tajes, J. & J. Méndez.2007. Identification of the razor clam species *Ensis arcuatus, E. siliqua, E. directus, E. macha*, and *Solen marginatus* using PCR-RFLP analysis of the 5S rDNA region. *Journal of agricultural and food chemistry* 55(18):7278-7282.

Field, K. G., G. J. Olsen, D. J Lane, S. J. Giovannoni, M. T. Ghiselin, E. C. Raff, & R. A. Raff.1988. Molecular phylogeny of the animal kingdom. *Science* 239(4841): 748-753.

Florida Fish and Wildlife Conservation Commission. Commercial Fisheries Landings Summaries. Accessed February 2017.Available at: https://publictemp.myfwc.com/FWRI/PFDM/

Hadley, N. & L. Coen.2005. Hard clams. *South Carolina State Documents Depository*.

Hadley, N. H. & J. M. Whetstone.2007. Hard clam hatchery and nursery production. *Southern Regional Aquaculture Center, Publication 4301*.

Hanna, G. D.1966. Introduced mollusks of western North America. - *Occ. Pap. Calif. Acad. Sci.* 48:t-108

Harrison, R.G.1983. Barriers to gene exchange between closely related cricket species. I. Laboratory hybridization studies. *Evolution* 37.2: 245-251.

Harte, M. E. 2001. Systematics and taxonomy. *Developments in Aquaculture and Fisheries Science* 31: 3-51.

Hashimoto, D.T., J.A. Senhorini, F. Foresti & F. Porto-Foresti. 2012. Interspecific fish hybrids in Brazil: management of genetic resources for sustainable use. *Reviews in Aquaculture* 4(2): pp.108-118.

Haven, D. & J. D. Andrews.1956. Survival and growth of *Venus mercenaria*, *Venus campechiensis* and their hybrids in suspended trays on natural bottoms. *Proceeding of the National Shellfisheries Association* 47:43-49

Hebert, P. D., A. Cywinska & S. L. Ball.2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B: Biological Sciences 270(1512): 313-321.

Heppell, D.1961. The naturalization in Europe of the quahog, *Mercenaria mercenaria* (L.). *J. Conch*. *25*: 21-34.

Hoeh, W. R., D. T. Stewart, C. Saavedra, B.W. Sutherland & E. Zouros.1997. Phylogenetic evidence for role-reversals of gender-associated mitochondrial DNA in *Mytilus* (Bivalvia: Mytilidae). *Molecular Biology and Evolution* 14(9): 959-967.

Ivanova, N. V., J. R. Dewaard & P. D. Hebert.2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular ecology notes* 6(4): 998-1002.

Johnson, S. B., A. Warén & R. C. Vrijenhoek.2008. DNA barcoding of Lepetodrilus limpets reveals cryptic species. *Journal of Shellfish Research* 27(1): 43-51.

Juste, V. & R. Cortes.1990. Distribution and biological aspects of the hard clam *Mercenaria mercenaria* (Linnaeus), *M. Mercenaria* notata (Say), and *M. campechiensis* (Gmelin) in Puerto Rico. *Caribb. J. Sci.* 26: 136-140.

Kaas, P. 1937.*Venus mercenaria* L., een nieuwe mollusk voor de Nederlandshe Fauna. *Basteria* 2 (4):58-60

Klinbunga, S., P. Ampayup, A. Tassanakajon, P. Jarayabhand & W. Yoosukh.2000. Development of species-specific markers of the tropical oyster (*Crassostrea belcheri*) in Thailand. *Marine biotechnology* 2(5): pp.476-484.

Kraeuter, J.N. & M. Castagna. 2001. *Biology of the hard clam* (Vol. 31). The 1st Edition. Elsevier Science B.V., Amsterdam, the Netherlands. Pp 772.

Layton, K. K., A. L. Martel & P. D. Hebert.2014. Patterns of DNA barcode variation in Canadian marine molluscs. *PLoS One* 9(4): p.e95003.

Lindsey, C.C. 1988. 3 Factors Controlling Meristic Variation. *Fish physiology* 11: pp.197-274.

Liu, Z. J. & J. F. Cordes.2004. DNA marker technologies and their applications in aquaculture genetics. *Aquaculture* 238(1):1-37.

Loosanoff, V. L. 1936. Temperature and hibernation of hard shell clam (*Venus mercenaria*). *Fisheries Service Bull*. No. 252: p.4.

Loosanoff, V. L.1937. Seasonal gonadal changes of adult clams, *Venus mercenaria* (L.). *The Biological Bulletin 72*(3): 406-416.

Loosanoff, V.L.1954.New advances in the study of bivalve larvae. *American Scientist* 42.4: 607-624.

Lopez-Pinon, M. J., A. Insua & J. Mendez.2002. Identification of four scallop species using PCP and restriction analysis of the ribosomal DNA internal transcribed spacer region. *Marine Biotechnology* 4(5): 495-502.

Maldini, M., F.N. Marzano, G.G. Fortes, R. Papa & G. Gandolfi, 2006. Fish and seafood traceability based on AFLP markers: Elaboration of a species database. *Aquaculture* 261(2): 487-494.

Melo, M.C., C. Salazar, C.D. Jiggins & M. Linares.2009. Assortative mating preferences among hybrids offers a route to hybrid speciation. *Evolution* 63(6): 1660-1665.

Menzel, R. W.1962. Seasonal growth of the northern and southern quahogs *Mercenaria mercenaria* and *M. campechiensis* and their hybrids in Florida. Proc. natn. Shellfish Ass. 52:37-46

Menzel, R. W. 1977. Selection and hybridization in quahog clams. Proc. Wld Maricult Soc. 8:507-521

Menzel, R. W. & M.Y. Menzel.1965. Chromosomes of two species of quahogs and their hybrids. Biol. Bull. mar. biol. Lab., Woods Hole 129:181-188

Meyer, A., C. Todt, N.T. Mikkelsen & B. Lieb.2010. Fast evolving 18S rRNA sequences from *Solenogastres* (Mollusca) resist standard PCR amplification and give new insights into mollusk substitution rate heterogeneity. *BMC Evolutionary Biology* 10(1):70.

Miah, G., M. Y. Rafii, M. R.Ismail, A. B. Puteh, H. A. Rahim, K. N. Islam, & M. A. Latif.2013. A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance. *International journal of molecular sciences* 14(11): 22499-22528.

Mikkelsen, N. T., C. Schander, & E. Willassen.2007. Local scale DNA barcoding of bivalves (Mollusca): a case study. *Zoologica Scripta* 36(5) :455-463.

Morgan, T. & A. Rogers.2001. Specificity and sensitivity of microsatellite markers for the identification of larvae. *Marine Biology* 139(5): 967-973.

Murphy, R. C.1985. Factors affecting the distribution of the introduced bivalve, *Mercenaria mercenaria*, in a California lagoon—the importance of bioturbation. *Journal of Marine Research 43*(3): 673-692.

Palmer, K. V. W. 1927. The Veneridae of Eastern America, Cenozoic and Recent. *Palaeontographica Americana* 1(5): 209-522

Pesch, G.1974. Protein Polymorphisms in the Hard Clams *Mercenaria mercenaria* and *Mercenaria campechiensis*. *Biological Bulletin* 146(3): 393.

Santaclara, F. J., M. Espiñeira, A. G. Cabado, A. Aldasoro, N. Gonzalez-Lavín & J. M. Vieites.2006. Development of a method for the genetic identification of mussel species belonging to *Mytilus, Perna, Aulacomya*, and other genera. *Journal of Agricultural and Food Chemistry* 54(22):8461-8470.

Sanz, N., R.M. Araguas, R. Fernández, M. Vera, & J.L. García-Marín. 2009. Efficiency of markers and methods for detecting hybrids and introgression in stocked populations. *Conservation Genetics* 10(1): 225-236.

Stanley, J.G. & R. DeWitt.1983. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (North Atlantic)—hard clam. *U.S. Fish and Wildlife Service, Biol. Rep*. 82: 19 pp.

Stewart, V. Sea-Stats No. 7 – Clams. Accessed November 2015.Available at: http://www.epa.gov/gumpo/seast07.html.

Taylor, J. D., S. T. Williams, E. A. Glover & P. Dyal.2007. A molecular phylogeny of heterodont bivalves (Mollusca: Bivalvia: Heterodonta): new analyses of 18S and 28S rRNA genes. *Zoologica Scripta* 36(6): 587-606.

Tebble, N.1966. British Bivalve Seashells: A Handbook for Identification. The British Museum (Natural History), London, Publication, Number 647: pp 212.

Toledo–Filho SA, L.F Almeida–Toledo, F. Foresti, D. Calcagnotto, S.B.A Fonteles–Santos & G. Bernardino.1998. Cadernos de Ictiogenética 4: *Programas Genéticos de Seleção, Hibridação e Endocruzamento Aplicados à Piscicultura*. Universidade de São Paulo, São Paulo, 56 pp.

UF IFAS University of Florida. Online resource guide for Florida Shellfish Aquaculture. Accessed March 2017. Available at: http://shellfish.ifas.ufl.edu/industry/

UF IFAS University of Florida. Online resource guide for Florida Shellfish Aquaculture. Genetic Stock Improvement of Hard Clams Projects.Accessed March 2017. Available at: http://shellfish.ifas.ufl.edu/projects/genetic-stock-improvement-of-hard-clams/hybridization/

Vignal, A., D. Milan, M. SanCristobal & A. Eggen.2002. A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution* 34(3): 275-306.

Wang, Y. & X. Guo.2008. ITS Length Polymorphism in Oysters and its Use in Species Identification. *Journal of Shellfish Research* 27(3): pp.489-493.

Wang, Y., A. Wang, X. Guo. 2010. Development and characterization of polymorphic microsatellite markers for the northern quahog *Mercenaria mercenaria* (Linnaeus, 1758). *Journal of Shellfish Research*, 29(1):77-88.

Winnepenninckx, B., T. R. D. W. Backeljau, & R. De Wachter.1994. Extraction of high molecular weight DNA from molluscs. *Trends in Genetics* 9:407.

Yang, H., L.N. Sturmer & S.Baker.2016. Molluscan Shellfish Aquaculture and Production. http://edis.ifas.ufl.edu/fa191

Zhan, A., J. Hu, X. Hu, Z. Zhou, M. Hui, S. Wang & Z. Bao.2009. Fine-scale population genetic structure of Zhikong scallop (*Chlamys farreri*): do local marine currents drive geographical differentiation? *Marine Biotechnology* 11(2): 223-235.

Zou, S., Q. Li, & L. Kong.2011. Additional gene data and increased sampling give new insights into the phylogenetic relationships of Neogastropoda, within the caenogastropod phylogenetic framework. *Molecular Phylogenetics and Evolution* 61(2):425-435.

BIOGRAPHICAL SKETCH

Erangi Heenkenda was born and grew up in Sri Lanka. She obtained her bachelor's degree in fisheries and aquatic sciences with a first-class honors from University of Ruhuna, Sri Lanka and she received the Vice Chancellor's gold medal for the best student at the convocation. In 2015, she could secured 1 out of 3 Fulbright Scholarship Awards for Masters for the country and started her master's study in fisheries and aquatic sciences at University of Florida (UF). Since then, she has been working on projects related to the clam and oyster genetics, cryopreservation, aquaculture and restoration.

Besides her academic achievements, as a Fulbright Scholarship student she represented her country as an ambassador. She was volunteering at Fishing for Success program and Family Fishing day program at UF Fisheries. And, she volunteered for Florida 4-H program, activities by Florida Youth Institution, oyster restoration program at the Chesapeake Bay, Cedar key Sea Food Festival and several other activities. She was also selected as an advisor for new Fulbright scholars. Erangi received Scarborough-Maud Fraser Award at the International Center of UF for her academic excellence and dedication to community activities.

She received her Master of Science majoring fisheries and aquatic sciences from the University of Florida in the summer of 2017. Her future ambition is to serve the entire world with her fullest effort by cooperating research knowledge and willingness to engage in community service.