GENETIC INVESTIGATIONS OF GRASS SHRIMP (*PALAEMONETES SPP.*) POPULATIONS ALONG THE GULF OF MEXICO COAST

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Genetic Investigations of Grass Shrimp (*Palaemonetes spp.*) Populations Along the Gulf of Mexico Coast

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Salt marshes serve as essential habitat for many organisms. Grass shrimp of the genus *Palaemonetes* encompass approximately 25 to 70 percent of nekton within salt marsh edge habitat, serving as prey items for commercially and recreationally important fish species and playing an integral role in the breakdown of detrital materials. Morphological identification of species within the genus *Palaemonetes* is challenging; therefore, development of reliable genetic methods of species identification would be useful. Unambiguous identification of individual species is crucial in population studies to eliminate the inclusion of cryptic species. The objective of this study was to develop a reliable genetic assay for identification of *Palaemonetes spp*. High Resolution Melting Analysis (HRMA) of amplified products was used to genotype mtDNA haplotypes. Amplicons melted for the three species between 75°C - 77°C and each species displayed distinct melting profiles. *P. vulgaris* melted at the lowest temperature, followed by *P. intermedius*, with *P. pugio* melting at the highest temperature. The assay designed in this study will be used in future saltmarsh ecology studies involving *Palaemonetes spp.,* eliminating time-consuming and error-prone morphological identification. It will also reduce the potential

inclusion of conspecifics that would affect the measures of differentiation and variability in genetic population studies.

DEDICATION

I'd like to dedicate this work to my brother. He was the biggest supporter of all my career goals. He may not be here to read this and tell me what a nerd I am, but I know that he's watching over me every single day.

Phillip Charles Westmoreland

September 18, 1985 – December 10, 2015

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NOMENCLATURE

A	Adenine
BP	Base Pair
C	Cytosine
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
G	Guanine
G-C	Guanine-Cytosine
GOM	Gulf of Mexico
HRM	High Resolution Melting
HRMA	High Resolution Melting Analysis
MEFGEN	Molecular Ecology and Fisheries Genetics
mtDNA	Mitochondrial DNA
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SNPs	Single Nucleotide Polymorphisms
T	Thymine
TAMUG	Texas A&M University at Galveston

CHAPTER I

INTRODUCTION

Salt marshes serve as ecologically critical environments for many species of invertebrates, fish, and migratory birds (Rozas and Zimmerman 2000), and are deemed essential fish habitat due to their role in feeding and reproduction (Baker et al. 2013). Since more than half of the human population lives within 75 miles of the coast, and coastal areas are sinks for runoff pollutants from point and nonpoint sources, there is major concern for the health of coastal areas. Assessment and monitoring of the overall ecological condition of these coastal areas can be facilitated through studies of bioindicator species (Key et al. 2006).

Grass shrimp of the genus *Palaemonetes* are considered one of the most ecologically important species of the East and Gulf Coast estuaries in the United States, making them a favorable choice to use as a bioindicator (Anderson 1985). Nekton density within salt marshes is typically high to support a range of consumers (Rozas and Reed 1993), and members of the genus *Palaemonetes* encompass from 25 to 70 percent of the nekton found in marsh edge habitats (Rozas and Zimmerman 2000). The Gulf of Mexico marsh system is home to three species of *Palaemonetes*: *P. pugio*, *P. vulgaris*, and *P. intermedius* (Key et al. 2006). Productivity of *P. pugio* ranges from 9 to 16 grams dry weight/m^2/year, which is equivalent to productivity reported for the Mummichog (*Fundulus heteroclitus*), a killifish that is commonly employed as a bioindicator of saltmarsh health (Strange et al. 2002). With such a high rate of productivity, any significant changes in the size of *P. pugio* populations should impact the entire salt marsh ecosystem (Key et al. 2006, Strange et al. 2002). Further, because of their ability to uptake toxic materials NOAA also considers grass shrimp as an ecotoxicological indicator

species (Parry 2006). Suter and Rosen (1988) found that estuarine crustaceans are more sensitive to environmental toxins than estuarine fish species, such as *F. heteroclitus* and the sheepshead minnow (*Cyprinodon variegatus*).

Grass shrimp are also an integral part of the salt marsh food chain. *Palaemonetes spp*. serve as an important food source for fish such as spotted sea trout (*Cynoscion nebulosus)*, sand sea trout (*C. arenarius)*, sheepshead (*Archosargus probatocephalus)*, black drum (*Pogonias crom*is), and southern flounder (*Paralichthys lethostigma)*, which are highly sought sport fishes (Overstreet and Heard 1982). Because grass shrimp are detritivores, their feeding activity serves to accelerate the breakdown of organic matter, and as prey items, they serve to transfer energy from producer and decomposer levels to higher trophic levels (Welsh 1975; Anderson 1985). This combination of traits ensures that data on *Palaemonetes* populations is valuable for assessments of the condition of salt marshes and for informing ecological approaches to salt marsh management (Parry 2006).

Visual identification of species within the genus *Palaemonetes* is challenging, since it's based on the character state of extremely small morphological features on the rostrum and dactylus that must be observed under a dissecting microscope (**Fig. 1a-b**) (Anderson 1985).

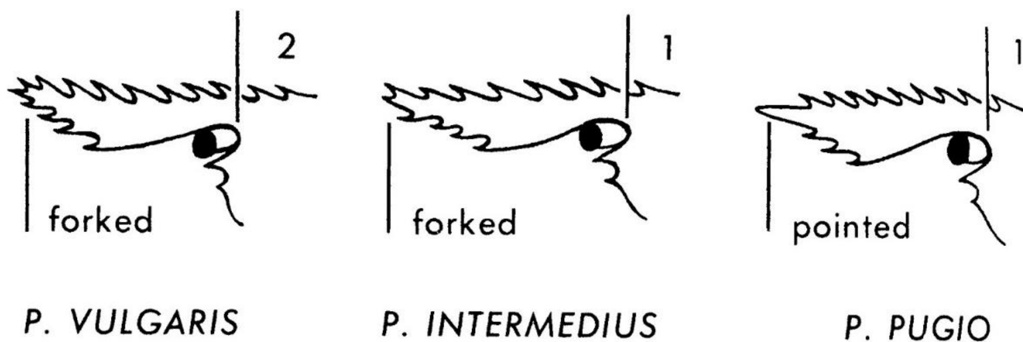

Figure 1a: Diagram showing rostrum characteristics of *Palaemonetes spp*. (Anderson 1985)

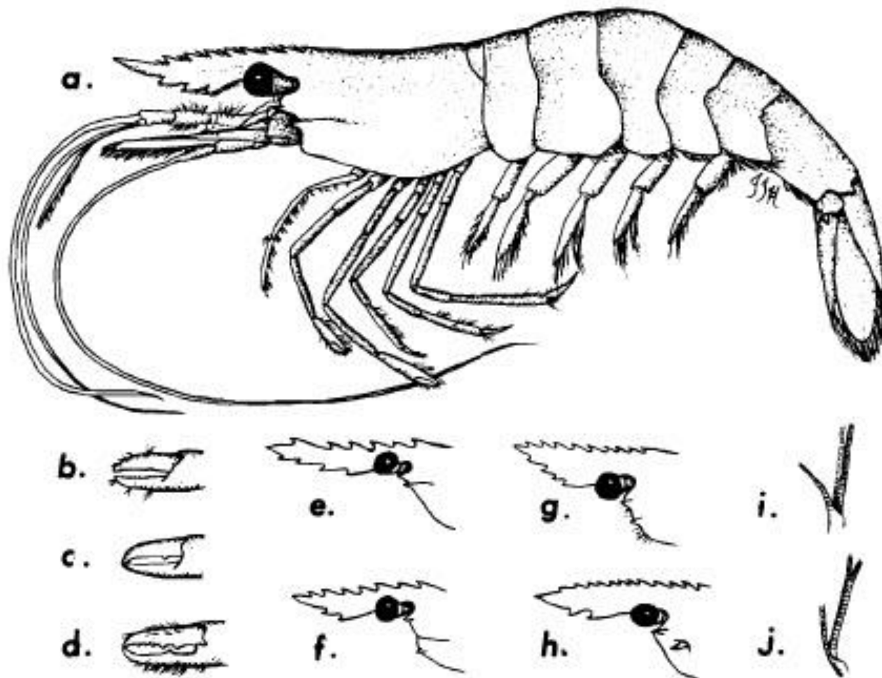

Figure 1b: General morphology of *Palaemonetes spp.* Note dactylus shapes b) *P. pugio* c) *P. intermedius* d) *P. vulgaris* (Anderson 1985).

Population genetics studies require unambiguous species identification to avoid introducing potential biases in allele frequencies. Similarly, the accidental inclusion of misidentified species within a species-specific study could hinder managerial controls and conservation efforts (Bickford et al. 2006). Population studies of members of the genus *Palaemonetes* have provided valuable ecological information about salt marsh habitats, due to their abundance, high productivity, their role as a potential bioindicator, and their contribution to nutrient cycling. However, members of this genus are difficult to identify based upon morphological traits, and the full potential of their value in ecological and ecotoxicological studies can only be realized if reliable methods for species-level identification is obtained. Genetic identification facilitates the identification of cryptic or hard-to-distinguish species, and

new technologies, such as high resolution melting analysis (HRMA), can be used as a fast, inexpensive, high throughput alternative for species identification. HRMA detects single nucleotide polymorphisms (SNPs) and small deletions in a fragment of amplified DNA by using a comparison of fluorescence of a saturating dye as a function of temperature (**Fig. 2;** Smith et al. 2009).

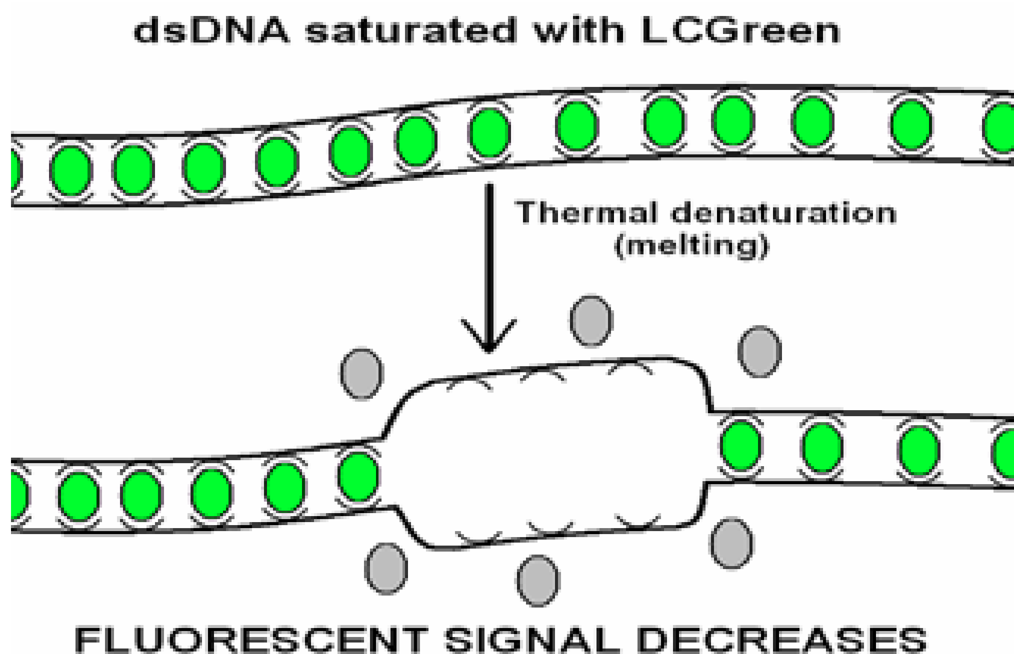

Figure 2: Illustration of the HRMA process

Alleles differing by one single nucleotide substitution produce unique melting curves, which can be compared with reference samples (Graham et al. 2005). The use of a saturating DNA dye also enhances the detection of heteroduplexes, which are formed when the complementary strands of the alternative alleles in a heterozygous individual hybridize producing a distinct melting curve shape that is easily distinguishable from homoduplex strands (Palais et al. 2005). This facilitates the scoring of heterozygous individuals. The process of HRMA takes approximately 15-20 minutes from PCR to scoring. Since the entire process is

carried in single closed tube assay it eliminates the potential for cross-contamination and the need for multiple steps using different platforms to score alleles (Smith et al. 2009). HRMA has been successfully used to identify marine species such as Istiophorid billfishes, tunas, and Penaeid shrimp (Alvarado-Bremer 2010; Smith et al. 2009), as well as arthropods and spiders (Winder et al. 2011). Although the method is reliable and inexpensive, wide use of this technology among wild populations is still not as popular as expected despite the importance of analyzing SNPs within this field (Smith et al. 2009).

The objective of this study is to develop a reliable genetic assay for identification of the three common species within the genus *Palaemonetes.*

CHAPTER II

METHODS

2.1 Collection of reference samples and morphological identification

Grass shrimp specimens were collected using a dip net along marsh edge from locations along the Texas, Louisiana, and Mississippi coastline (**Fig. 3**). Samples were preserved in 70% ethanol. Morphological identification to species level was conducted on well-preserved, intact specimens using a dichotomous key for *Palaemonetes spp.* (Wood 1974, Anderson 1985).

Figure 3: Map of sample sites along the Northern Gulf of Mexico

The wide range of sample collection will be useful to determine the extent by which variation within-species could affect the reliability of the assay.

2.2 DNA extraction

DNA extractions followed the protocol outlined in Alvarado Bremer et al. (2010). Briefly, a 10-50mg piece of tissue was dissected from each organism and digested with proteinase-K, and DNA was precipitated with 70% ethanol. DNA pellets were then eluted in 100µL of sterile, double distilled water (ddH_2O). Extraction quality was assessed via gel electrophoresis in 1% Tris-acetate (TA) agarose gels, pre-stained with ethidium bromide (EtBr) and visualized in a UV trans-illuminator.

2.3 HRMA assay design

Twenty pre-identified individuals each from *P. pugio, P. intermiedius,* and *P. vulgaris* were selected for sequencing. A 300 bp fragment of the mitochondrial DNA (mtDNA) 16S RNA gene was targeted using primers F-16sRNA-PP (5' <u>**TCG**</u> CCT GTT TA**T** CAA AAA CAT 3') and R-16sRNA-PP (5' AGA TAG AAA CC**C** A**A**C <u>**C**</u>TG G 3'), which are versions of the crayfish universal primers 1471 and 1472 (Crandall and Fitzpatrick 1996) that have been modified to match *P. pugio* (i.e., altered nucleotides in bold, inserted nucleotides underlined).

PCR reactions were carried out in 12.5 µL volumes containing 1X EconoTaq Plus Green Master Mix (Lucigen), 0.1 µM of each primer, and 10 ng of DNA template. Thermocycling was performed in an Eppendorf Mastercycler (Eppendorf) with an initial denaturing step at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 sec, ending with a final extension step at 72°C for 3 minutes.

Amplicons were visualized in 2% TA agarose gels pre-stained with EtBr. Sequencing of the 16S RNA fragment was carried out in both directions, with reaction setups and thermocycling profiles as described in Cruscanti et al., (2015). Multiple sequence alignments

were carried out in Geneious Pro v.9.1.8 (Biomatters Ltd., Aukland, NZ). Fixed differences between species were identified to design short amplicon (SA) HRMAs (Smith et al. 2012).

2.4 Evaluation of HRMA primers

Samples that included the three species of grass shrimp were identified based upon morphological characters, and subsamples of randomly chosen individuals for each species, consisting of *P. pugio* (n=20), *P. intermedius* (n=20) and *P. vulgaris* (n=15) were selected at random to test the HRMA assay. Negative controls were included in all reactions. PCR reactions were carried out in 10 µL volumes in capillary tubes containing 1X EconoTaq Master Mix (Lucigen), 0.1µM of each primer in the pair, 0.5 µL of LCGreen, and approximately 10ng DNA template. A drop (~20 µL) of mineral oil was added to each reaction to prevent evaporative losses and ensure melting profile uniformity.

Thermocycling was performed in a RapidCycler II (Idaho Technology) with an initial denaturing step of 95°C for 5 minutes, followed by 55 cycles of denaturation 94°C for 0 seconds, annealing at 48°C for 0 seconds, and extension at 72°C for 20 seconds (temperature ramp = 2.0°C/sec), and a final denaturing step at 94°C, and cooling step at 40°C (temperature ramp = 9.9°C/sec) was performed. Melting analyses were conducted in an HR-1 High Resolution Melter (Idaho Technology). The melting protocol was set to measure fluorescence from 65°C to 85°C, with a temperature ramp of 0.20°C per second, and a cooling temperature between samples of 60°C. The resulting curves were analyzed in the HR1 Instrument Control software (Idaho Technology).

CHAPTER III

RESULTS

A segment 104 bp long contained within a 300 bp fragment of 16sRNA that was sequenced, revealing the presence of fixed differences between the three target species (**Fig. 4**). Some of these mutations included A/G transition at nucleotide position 24, but also included two transversions; one C/A/T substitution at nucleotide position 38, and a G/C at position 54, which were likely to result in observable changes in melting temperatures among species (**Fig. 4**). The flanking regions for these polymorphisms contained sequence that was conserved between all three species, making it appropriate for primer development. The resultant primers are as follows: Pala16s_HRMA1_F (5’ GGC TGG AAT GAA CGG CTG 3’), Pala16s_HRMA1_R (5’ GGG TCT TAT CGT CCC TTT AAA 3’).

Figure 4: Aligned consensus sequences for *P. intermedius, P. pugio,* and *P. vulgaris,* showing the 104bp fragment of 16sRNA used for development of HRM assay.

Amplicons melted for the three species between 75°C - 77°C and each species displayed melting distinct melting profiles. *P. vulgaris* melted at the lowest temperature, followed by *P. intermedius*, with *P. pugio* melting at the highest temperature. Since the differences in melting temperatures were relatively small (~0.5°C between each species), a difference curve using the intermediate species, *P. intermedius,* as a baseline provided the best method to distinguish the three species (**Fig. 5**).

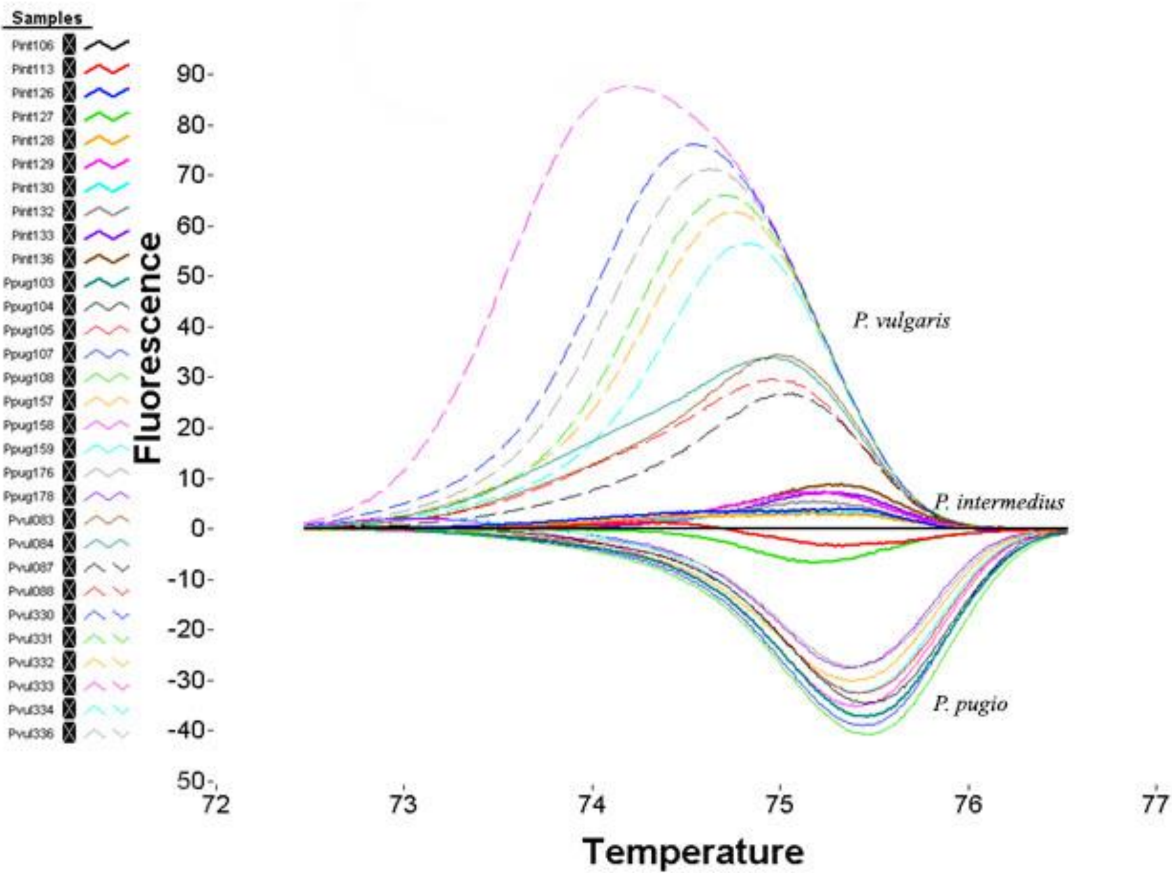

Figure 5: Difference plot of melting profile of a fragment ≈100 of the 16s mtDNA gene for representative samples of 10 individuals each for *P. intermedius* (Pint), *P. pugio* (Ppug), and *P. vulgaris* (Pvul).

Of the 20 specimens of *P. intermedius* tested, all successfully amplified and produced distinct curves (**Table 1**). Conversely, of the 20 specimens of *P. pugio* tested, 18 successfully amplified and produced distinct curves. Amplification success with , *P. vulgaris* was lower, with only 11 successful amplifications out of 15 specimens tested. However, despite of these amplification failures, the melting profiles (confirmed by sequencing) revealed instances of incorrect identifications based on morphology. Two specimens identified as *P. pugio* and four specimens identified as *P. intermedius* using morphological characters, were genetically identified as *P. vulgaris*. Note that the figure is only representative of a subsample (n=30, 10 of each species) due to software constraints (**Table 1; Fig. 6**)

Table 1: Success of samples tested using the SA-HRMA assay and percent error of morphological identification errors

	Number Tested	***Successful Amplifications***	***Morphological Mis-Identifications***	***% morphological identification error***
Palaemonetes intermedius	20	20	2	10
Palaemonetes pugio	20	18	4	22
Palaemonetes vulgaris	15	11	0	0

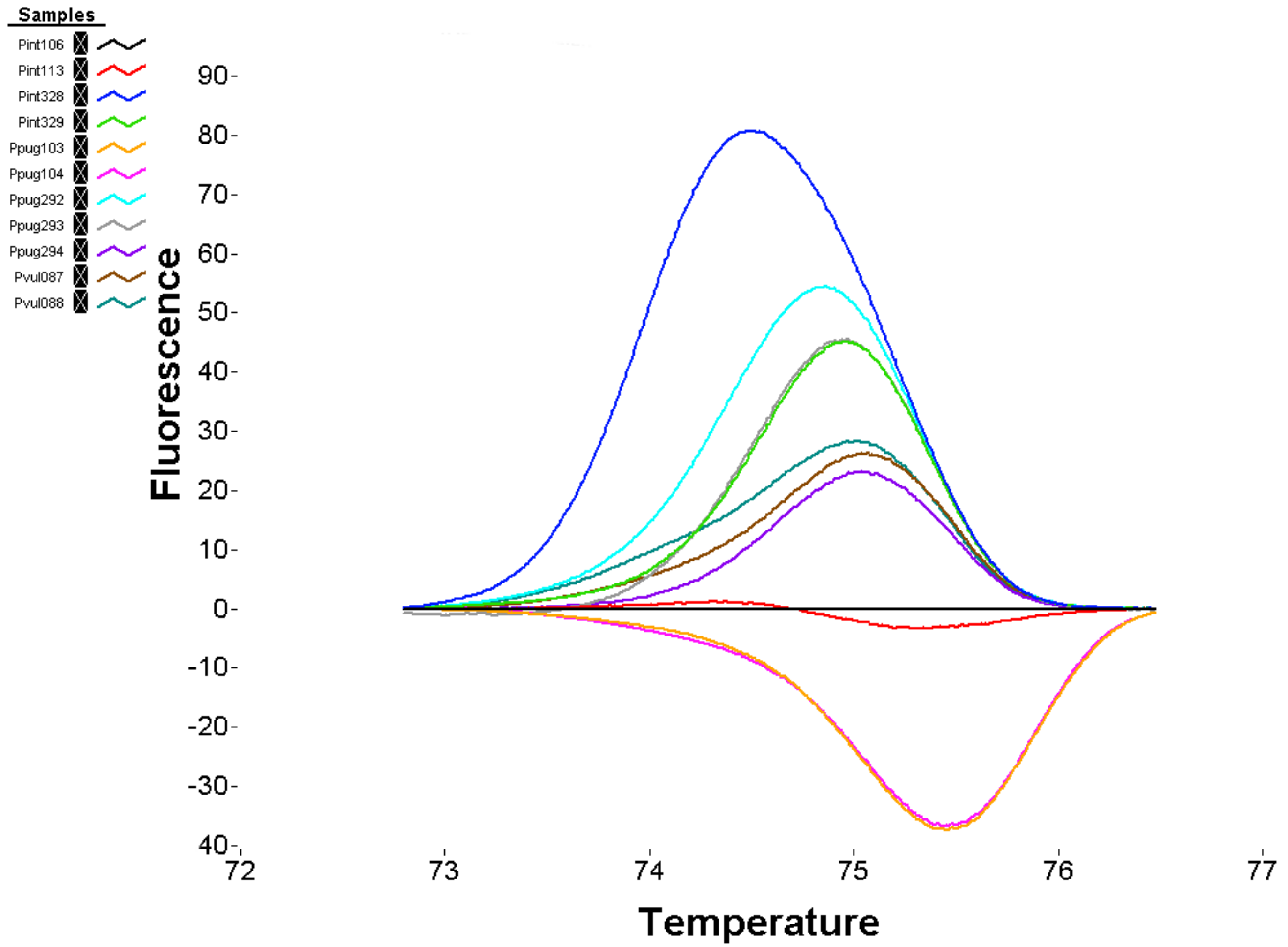

Figure 6: Melting curves of five mis-identified *Palaemonetes* specimens (Pint328, Pint329, Ppug292, Ppug293, Ppug294), plotted against positive controls for two of each species (Pint106, Pint113, Ppug103, Ppug104, Pvul087, Pvul088).

CHAPTER IV

CONCLUSION

The HRM assay designed to identify three species of grass shrimp belonging to the genus *Palaemonetes* was successful. Each of the three species tested produced diagnostic melting curve shapes that also differed in melting temperatures. Calculated differences in melting temperature between the three species matched expectations based on the differences in the G-C content when sequences were aligned for primer design, with the sequences containing higher G-C melting at higher temperatures. The HRM assay also revealed that some specimens identified to species based on morphology had been misidentified. This assay represents an improvement over morphological identification methods.

Relying solely morphological identifications can have important implications towards genetic population studies where the differences in allele frequency among samples may be biased by the inclusion of specimens of another species among the samples compared. The inclusion of multiple species within a sample could also result in errors in the measures of genetic variation within populations. Accordingly, when cryptic species are present, it is important to unambiguously identify each specimen in each sample prior to conducting a genetic population study to prevent introducing biases in the measure of genetic differentiation, and in the estimates of effective population size and other parameters. While HRMA has been utilized in several laboratory settings (Reed et al. 2007, Wittwer et al. 2003) it has rarely been used as a genotyping tool for wild populations despite its effectiveness (Smith et al. 2009, Alvarado-Bremer et al. 2010, Meisterzheim et al. 2012).

HRMA represents a valuable alternative for species identification because it is accurate, time and cost effective, and minimizes the potential error of cross-contamination, making it ideal for study of model species in the laboratory and of wild populations. Research conducted on wild populations of other species (Smith et al. 2009, Alvarado-Bremer et al. 2010, Meisterzheim et al. 2012) have demonstrated that the utility of HRMA to identify species and to characterize variation among populations. Adoption of this technique in future genetic based ecology studies could prove highly beneficial to researchers.

The assay designed in this study will be used in future salt marsh ecology studies involving *Palaemonetes spp.,* which will eliminate the need for time-consuming and error-prone morphological identification. It will also reduce the potential inclusion of conspecifics which would affect the measures of differentiation and variability in population studies. This ease of genotyping by HRMA could also link species within the genus *Palaemonetes* to other ecology-based studies. The use of stable isotopes could elucidate potential feeding patterns of *Palaemonetes spp.* and determine habitat usage. With grass shrimp being one of the most important prey items of consumers within salt marsh ecosystems, it is important that these populations be properly assessed. Understanding the genetic makeup and distribution of these organisms will help researchers understand the ecology of commercially and recreationally important species found within salt marshes.

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