

Fall 2004

Population Genetics Analysis of the Grass Shrimp Palaemonetes Pugio Using Single Strand Conformation Polymorphism

Melody A. Flowers

Follow this and additional works at: <https://digitalcommons.georgiasouthern.edu/etd>



Part of the [Life Sciences Commons](#)

Recommended Citation

Flowers, Melody A., "Population Genetics Analysis of the Grass Shrimp *Palaemonetes Pugio* Using Single Strand Conformation Polymorphism" (2004). *Electronic Theses and Dissertations*. 738.

<https://digitalcommons.georgiasouthern.edu/etd/738>

This thesis (open access) is brought to you for free and open access by the Jack N. Averitt College of Graduate Studies at Georgia Southern Commons. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Georgia Southern Commons. For more information, please contact digitalcommons@georgiasouthern.edu.

POPULATION GENETICS ANALYSIS OF THE GRASS SHRIMP *PALAEMONETES*
PUGIO USING SINGLE STRAND CONFORMATION POLYMORPHISM

by

MELODY A. FLOWERS

(Under the Direction of Oscar J. Pung and Quentin Fang)

ABSTRACT

Population genetics studies reveal significant information concerning an organism that lead to a better understanding of the microevolutionary forces acting upon the organism. Little is known about the genetic structure of grass shrimp, *Palaemonetes pugio*, populations or the effects the parasite *Microphallus turgidus* has on it. The objective of this study was to determine the amount of genetic diversity of the shrimp populations based on locality and parasite load. In order to examine the genetics of *P. pugio*, shrimp DNA was extracted and scanned using single strand conformation polymorphism. Results revealed *P. pugio* populations are highly conserved among localities. This suggests a significant amount of gene flow is taking place. Results also indicate there is no correlation between the most common host haplotype and parasite density, as shown in previous studies on host-parasite interactions. Further studies in this field will provide clearer answers in understanding the genetics of *P. pugio*.

INDEX WORDS: *Palaemonetes pugio*, *Microphallus turgidus*, Host-parasite interaction, Single-strand conformation polymorphism, Gene flow

POPULATION GENETICS ANALYSIS OF THE GRASS SHRIMP *PALAEMONETES*
PUGIO USING SINGLE STRAND CONFORMATION POLYMORPHISM

by

MELODY A. FLOWERS

B.S., Lee University, 1999

M.S., Georgia Southern University, 2004

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial
Fulfillment of the Requirements for the Degree

MASTERS OF SCIENCE

STATESBORO, GEORGIA

2004

© 2004

Melody A. Flowers

All Rights Reserved

POPULATION GENETICS ANALYSIS OF THE GRASS SHRIMP *PALAEMONETES*
PUGIO USING SINGLE STRAND CONFORMATION POLYMORPHISM

by

MELODY A. FLOWERS

Major Professors: Oscar J. Pung
Quentin Fang

Committee: Lance Durden

Electronic Version Approved:
December 2004

ACKNOWLEDGMENTS

This research was funded by stipends awarded to me by the College of Science and Technology Academic Excellence Committee of Georgia Southern University and to O. Pung from the Faculty Research Committee of Georgia Southern University. I owe special thanks to Dr. Oscar Pung and Dr. Quentin Fang for never giving up on me and providing guidance while working on my research. I am very grateful to my third committee member, Dr. Lance Durden for his editorial assistance with this manuscript. Dr. Steve Vives and Dr. Danny Gleason were a big help with the shrimp collection. In addition, I want to thank Mr. Brad Grinstead, Ms. Alyssa Kunz and Mr. Michael Pung for their assistance in collecting shrimp. I also cannot forget Dr. Guang Xu for his expertise and much needed advice in lab. I could not have made it through with my sanity if it were not for my family and friends who have provided me with words of encouragement along the way. Thank you to all for seeing me through!

TABLE OF CONTENTS

	Page
TITLE PAGE.....	ii
COPYRIGHT.....	iii
APPROVAL PAGE.....	iv
ACKNOWLEDGMENTS.....	v
CHAPTER	
I. Introduction.....	1
II. Materials and Methods.....	10
III. Results.....	18
IV. Discussion.....	21
REFERENCES.....	26
APPENDIX.....	37

INTRODUCTION

Grass shrimp, *Palaemonetes* spp., are decapod crustaceans belonging to the family Palaemonidae that are found in abundance in estuaries and marshes along the North Atlantic and Gulf coasts of the U.S.A. (Williams, 1984). Two species, *Palaemonetes pugio* and *Palaemonetes vulgaris*, constitute an important part of the tidal marsh ecosystem in the southeast U.S.A. They consume detritus and transfer energy between multiple trophic levels (Welsh, 1975). These shrimp are also known to feed on microalgae (Morgan, 1980), meiofauna and small infaunal polychaetes and nematodes (Sikora, 1977; Bell and Coull, 1978; Chambers, 1981) and even on small invertebrates such as mysids (Morgan, 1980). While these shrimp feed on a number of organisms, they are also prey to a variety of fish species and other aquatic carnivores (Anderson, 1985).

Palaemonetes pugio and *P. vulgaris* are relatively small, transparent to yellowish brown crustaceans, usually not exceeding 50 mm in total length. Grass shrimp often reside around oyster reefs and fouling organisms on docks and pilings (Heard, 1982). *Palaemonetes pugio*, however, may prefer muddy substrates (Khan et al., 1995). Distributional patterns of the shrimp may depend on photoperiod and tidal cycles (Anderson, 1985). Studies on their movement reveal that *P. pugio* and *P. vulgaris* migrate seaward (downstream) with the current during ebb tides and migrate upstream into tidal creeks during incoming tides (Antheunisse et al., 1971; Shenker and Dean, 1979; Sikora, 1977; Kneib, 1987a, 1987b)

Grass shrimp distribution and abundance are also affected by dissolved oxygen levels. Under controlled laboratory conditions, *P. pugio* had a higher survival rate than *P. vulgaris* when oxygen concentrations were lower than 1 ppm (Pomeroy and Wiegert, 1981). In fact, studies show that *P. pugio* is an oxyconformer. As oxygen levels decrease, *P. pugio* decreases oxygen uptake as well (Welsh, 1975; Dillon, 1983).

Many toxicology studies have been conducted on grass shrimp. Eisler (1969) examined the effects of 12 insecticides on grass shrimp and concluded that the shrimp are more sensitive to organophosphorous insecticides than organochlorine insecticides. In a later study, Eisler (1971) revealed that heavy metals such as cadmium, mercury, and chromium are acutely toxic to grass shrimp. As a result, the shrimp are not recommended for use as heavy metal bioassay test organisms due to their resistance (Vernberg et al., 1977). There are several detrimental effects heavy metals have on the shrimp including loss of a predator response (Barthalmus, 1977), developmental abnormalities in larvae (Shealy and Sandifer, 1975), and reduced tolerance to changes in salinity (Middaugh and Floyd, 1978).

Factors affecting grass shrimp growth include water temperature (Anderson, 1985) and salinity (Alon and Stancyk, 1982; Wood, 1967). Grass shrimp are eurythermal. For example, *P. pugio* can survive in water temperatures ranging from 5 to 38°C (Wood, 1967; Christmas and Langley, 1973), but survival is optimal between 18 and 25°C (Wood, 1967). *Palaemonetes pugio* can survive in brackish water ranging in salinity from 0 to 55 ppt but are normally found in a smaller range of 2 to 36 ppt. *Palaemonetes vulgaris* is more tolerant of high salinity water, while *P. pugio* can tolerate low salinity water better. Studies indicate that *P. pugio* and *P. vulgaris* survive best,

mature earlier on, and pass through fewer larval stages at optimal salinity (Sandifer, 1973; Floyd, 1977).

A number of microorganisms are known to parasitize grass shrimp. These include microsporidia (Street and Sprague, 1974), a bopyrid isopod (Richardson, 1905), and microphallid trematodes (Heard, 1967; Stunkard, 1979). *Palaemonetes pugio* is of particular interest because it is the principal crustacean host for the digenetic trematode *Microphallus turgidus* in the southeastern U.S.A. (Heard, 1967; Heard and Overstreet, 1983; Pung et al., 2002). The range of *M. turgidus* extends from New Jersey to Louisiana (Heard, 1976) and it is the only trematode reported in grass shrimp in the southeastern United States (Heard, 1967; Bridgeman, 1969; Pung et al., 2002). Migratory birds are thought to be the primary definitive hosts of the parasite, although shrimp eating mammals such as raccoons and rats are also frequently infected (Heard and Overstreet, 1983). *Microphallus turgidus* itself is susceptible to infection by a haplosporidian (Sprague, 1970).

Studies along the Georgia coast show that prevalence, intensity, abundance, and density of the metacercariae of *M. turgidus* are higher in *P. pugio* than in *P. vulgaris*. The prevalence of *M. turgidus* was highest in *P. pugio* when the salinity exceeded 19 ppt and the parasite's intensity was greatest in *P. pugio* in the salinity range of 20-29 ppt. Results also showed a positive correlation between parasite density and the host's body weight, indicating that the increasing intensity of *M. turgidus* does not affect the survival of the grass shrimp (Pung et al., 2002).

Behavioral alterations of *P. pugio* due to infection of *M. turgidus* have also been documented. Kunz et al. (2003) investigated the effects of *M. turgidus* on the predation,

swimming endurance, and activities of *P. pugio*. The study revealed that predators, in this case mummichogs (*Fundulus heteroclitus*), would prey on highly infected shrimp (>30 parasites/shrimp) more so than on uninfected shrimp. It also showed that swimming endurance for infected shrimp was significantly lower than for uninfected shrimp. There was also a significant decrease in activity for uninfected shrimp when a predator was present, whereas when a predator was absent, the infected shrimp had a significantly lower level of activity. This study indicates that *M. turgidus* affects the behavior and activity of *P. pugio*.

Although the ecological importance of *P. pugio* is clear, there is nothing known about the population biology of this shrimp. Studies in the area of population genetics reveal information about the genetic structure of a population by examining the distribution of alleles within and among populations (Slatkin, 1985). The study of population genetics is important for several reasons. First, the examination of genetic variation provides insight into the microevolutionary forces acting upon that population. These forces include the movement of alleles between populations (gene flow), random changes in the allele frequency of a finite population (genetic drift), mutation and natural selection. While genetic diversity of local populations is manifested through mutation, genetic drift and local adaptation (Slatkin, 1987), migration (gene flow) maintains the genetic and phenotypic homogeneity of a species (Slatkin, 1985). Second, it reveals the potential for evolutionary change and adaptation. Finally, assessing the amount of genetic variation within a population of organisms could determine the manner in which new species arise (Russell, 1994). All of these reasons illustrate the value of studying population genetics.

In addition, there is little known concerning the interaction between *P. pugio* and *M. turgidus*. There is much that can be learned through the study of the genetics of host-parasite associations. It is a widely known fact that parasites influence the genetic composition of natural animal communities and therefore can affect the selection pressures acting on the parasites themselves (Webster et al., 2001). Host-parasite interactions generate and maintain genetic polymorphism (May, 1985), which introduces the possibility that if there is an increase in parasite load, there could likewise be an increase in the host's genetic diversity (Webster et al., 1998).

According to the Red Queen hypothesis, parasites are more likely to infect the common host genotype, which leads to selection for sexual reproduction and local adaptation by the parasite population (Lively and Dybdahl, 2000). Dybdahl and Lively (1998) conducted a series of studies on a similar system involving an undescribed *Microphallus* species and its freshwater snail host, *Potamopyrgus antipodarum*. Their results showed that the common clonal host genotypes were more susceptible to infection by the parasite than the rare genotypes.

Molecular Ecology

The study of population genetics often focuses on analyses of the mitochondrial genome since it is maternally inherited, lacks recombination, and evolves as much as three to five times faster than the nuclear genome. For a good population study, the gene that is examined must not have a mutation rate that is neither too high nor too conserved. The 16S rRNA gene meets these criteria since it is a part of the mitochondrial genome

and is conserved in some regions while also maintaining a significant amount of variation in other regions (Moritz et al., 1987).

Recent advances in molecular biology have greatly improved studies in the areas of population genetics, evolution, and ecology. The development of the polymerase chain reaction (PCR) in the 1980's provided scientists with an inexpensive way to obtain numerous copies of DNA in a matter of hours (Mullis, 1990). Because of the value of PCR, it has been frequently coupled with other molecular techniques and applied to many different areas of biology. Some of these techniques have been used to detect high-resolution mutations in genes. Single strand conformation polymorphism (SSCP) is a common example of these techniques.

Single strand conformation polymorphism (Orita et al., 1989) has been applied to many different fields including medical research (Cotton, 1997), conservation genetics (Girman, 1996), and ecology (Lessa and Applebaum, 1993). The underlying concept is that denatured DNA from different individuals migrates on a non-denaturing gel at different rates based on its conformation in its single-stranded state. The secondary and tertiary conformations that are formed depend on several factors: the length and sequence composition of the DNA strand as well as the location and number of regions of base pairing (Bøgh et al., 1999). PCR-SSCP is a sensitive means for detecting mutations and polymorphisms (Hayashi, 1992). This technique is simple, inexpensive, and requires only conventional molecular equipment to carry out the procedures. When first used in studies on *Escherichia coli* F1-ATPase (Kanazawa et al., 1986), the technique required radioactivity. In the following years, nonradioactive methods were developed using staining buffers such as silver staining (Hayashi, 1992) and ethidium bromide. Whether

radiolabeling primers or using silver staining or ethidium bromide, these methods are crucial for detecting the presence of deoxyribonucleic acid (DNA) bands and therefore, variation on a gel. Its accuracy is an asset as well. Nucleotide changes can be detected >80% of the time for gene fragments less than 400 bp in length (Hayashi, 1991). As the length of the gene increases, though, the sensitivity decreases.

The use of PCR-SSCP does have its disadvantages. For a high degree of accuracy, it can only be used to analyze nucleotide substitutions in gene fragments less than 500 bp in length. Because of its sensitivity, mutations may not be detected if conditions are not optimized (Hayashi, 1992). In some cases, there is even the possibility that PCR-SSCP cannot differentiate between different base substitutions at the same nucleotide position (Sheffield et al., 1993). It is also a time consuming process and not an appropriate technique to use for phylogenetic analysis at the family level or above.

Due to the sensitivity of PCR-SSCP, optimizing the conditions for the reagents and procedures is crucial. There are many components to consider such as the running buffer concentration (Girman, 1996) and the addition of neutral compounds. Glycerol is a commonly used neutral compound (Glavac and Dean, 1993) that is thought to help stabilize the DNA in its single-stranded conformation state (Hayashi and Yandell, 1993). The two most important components to consider, though, are the gel temperature and the concentration of polyacrylamide in the gel. The success of PCR-SSCP relies on the gel remaining at a constant, cold temperature (Girman, 1996). This is to ensure that the single-stranded DNA stays in its folded structure. Sensitivity is best when a high concentration of acrylamide (8-10%) is coupled with low crosslinking (1.3-2.6%)

(Glavac and Dean, 1993). Crosslinking is reduced with the addition of bisacrylamide and thereby improves band resolution (Girman, 1996).

Shrimp Population Genetics

A number of studies have been performed on the genetic structure of shrimp populations. Hurwood et al. (2003) conducted a study on the genetic variation of the freshwater shrimp *Paratya australiensis*, which included an analysis of mitochondrial DNA. The study involved a collection of these shrimp from headwater sites in two major river drainages in Australia. The results showed a significant amount of variation occurred between sub catchments within a single drainage system and the least amount between streams within sub catchments. This indicates that gene flow was constricted between sites yet a significant force between streams within sub catchments.

In another study involving the brine shrimp *Artemia sinica*, results revealed a moderate amount of genetic differentiation among nine populations examined throughout central and northeast China. In addition, a positive correlation existed between genetic distance and geographical distance. Interestingly, sites separated by as much as 100 kilometers had no genetic differentiation at all. Overall, the study showed a relatively low amount of differentiation, suggesting good dispersal capabilities of the resting stages (diapausing cysts) of the shrimp. This passive dispersal was most likely due to wind, waterfowl, or humans (Naihong et al., 2000).

Many population studies have also been conducted on commercial shrimp due to their economic importance. One such study involved the pink shrimp *Pandalus borealis* Krøyer found in many countries of the Pacific and Atlantic basins. Shrimp collected

from the Sea of Japan, Okhotsk Sea, and Bering Sea exhibited genetic homogenization within seas yet a sufficient amount of variation between basins (Kartavtsev et al., 1993). In another study assessing the genetic differentiation of two species of penaeid shrimp from the Gulf of California, de la Rosa-Vélez et al. (2000) revealed little variation in *Penaeus californiensis* among sites while *Penaeus stylirostris* showed a cline like pattern of genetic variability from north to south within the gulf. The study also characterized genetic similarities among and within species. While similarities among subpopulations existed in both species, subpopulations of *P. californiensis* were more genetically similar than the subpopulations of *P. stylirostris*.

Research Objectives

There is much to learn about the population genetics of *P. pugio* as well as the genes governing its interaction with *M. turgidus*. The significance of this information is clear. The goals of this project were to (1) develop a system that effectively examines the genetic structure of populations of *P. pugio* and (2) examine the population genetics of *P. pugio* based on two factors: locality and parasite (*M. turgidus*) prevalence and density. Although movement is somewhat limited within populations of *P. pugio*, there is an expectation that these populations will be genetically similar due to the drifting current over time. Therefore, there is also the prediction that genetic distance does not increase with geographical distance. There is also an expectation that there is a positive correlation between the density of *M. turgidus* and the most common haplotype of *P. pugio*, a hypothesis generated from the information given on the Dybdahl and Lively studies (Dybdahl and Lively, 1998; Lively and Dybdahl, 2000).

MATERIALS AND METHODS

Collection and Dissection

Grass shrimp, *Palaemonetes pugio* and *P. vulgaris*, were collected from several different localities along the coast of Georgia, Florida and South Carolina (Table 1, Figure 1). Dip nets were used to catch the shrimp in tidal rivers and creeks adjacent to marshes. The location, salinity in parts per thousand (ppt) and date of collection were recorded. The shrimp were transported to a laboratory in aerated water from the collection site. Shrimp were maintained in artificial sea water (salinity at 25 ppt) and fed once a day on a diet of tropical fish flake food. Shrimp were sorted by species and then separated from the shrimp infected with *Microphallus turgidus*. Infection prevalence was calculated by computing the percentage of shrimp infected. This was calculated by dividing the number of infected shrimp by the number of uninfected shrimp. Infection density was calculated by recording the number of *M. turgidus* found per centimeter of shrimp body length. The shrimp were kept alive for 3 wk to ensure complete development of the parasite. After 3 wk, infected shrimp were killed by freezing at -80°C and stored at that temperature until needed.

Prior to dissection, the shrimp were removed from the -80°C freezer and immediately stored on ice. In order to avoid DNA cross-contamination, each shrimp was placed in a separate petri dish containing 0.7% saline solution and dissected using clean instruments. Petri plates were discarded after each use. All dissecting instruments were initially sterilized by washing with soapy water, followed by soaking in bleach and then

autoclaving. This process was repeated after each set of shrimp was dissected. In between each shrimp dissection, the instruments were washed in distilled water followed by a wash in 95% ethanol followed by flame sterilization. A small amount of shrimp tissue was placed in sterile 1.5 ml microcentrifuge tubes containing 50 μ l of lysis buffer (4.5 M guanidine thiocyanate, 25 mM sodium citrate, and 0.5% n-lauroyl-sarcosine).

DNA Extraction

DNA was extracted using a modified version of the protocol described by Doyle and Doyle (1990). A clean pestil (sterilized in the same manner as the dissecting instruments), was used to grind the shrimp tissue in a microcentrifuge tube containing 50 μ l of lysis buffer. The pestil was then rinsed within the tube with 100 μ l of the buffer to ensure that all of the DNA was included after which 400 μ l of cetyltrimethylammoniumbromide buffer (CTAB) was added to the tube. The samples were then incubated in a 65°C water bath for 20 min, and vortexed every five minutes. After the samples were removed from the water bath, 500 μ l of chloroform: isoamyl alcohol (24:1) were added. The samples were then mixed and centrifuged for 5 min at 14,000 rpm. The supernatant fluid, which contained DNA, was then transferred to a second set of 1.5 ml microcentrifuge tubes to which 500 μ l of chloroform was added and centrifuged again for 5 min. Supernatant fluid was transferred to a third set of 1.5 ml microcentrifuge tubes. Next, 3M sodium acetate equivalent to one-tenth the volume of the supernatant fluid was added. Cold 100% isopropanol was then added to each tube. The amount added was equivalent to the sum of the volumes for the supernatant fluid and

the sodium acetate. To precipitate DNA, the samples were then placed at 4°C for at least 15 min.

Next, the samples were centrifuged for 10 min at 14,000 rpm. The supernatant was poured off and the pellet containing DNA was washed in 1 ml of cold 100% ethanol. The samples were centrifuged again for 4 min and dried in a DNA Speed Vac Drier (Savant Instruments, Inc., Farmingdale, New York) on medium power for 45 sec. Next, 30 µl of 10 mM Tris HCL (pH 8.0) was added to dissolve the pellet.

To verify the presence of DNA, the samples were loaded onto a 1.2% agarose gel that had been placed in a gel tray containing 1X TAE with 0.0005% ethidium bromide and electrophoresed at 70 V for one hour (8 µl of loading buffer to 3 µl of parasite DNA). Gels were viewed under ultraviolet (UV) light to confirm the presence of bands which indicated a successful DNA extraction. The gels were then photographed for record keeping. Following extractions, the DNA was stored at -20° C (short term use) or -80° C (long term use).

Primer Design

Primers were developed to amplify a portion of the 16S rDNA region of the mitochondrial genome. A total of 13 DNA sequences were downloaded from Genbank including those for *Macrobrachium atactum* (Accession #AF374468), *Palaemonetes atrinubes* (Accession #AF439520) (Murphy and Austin, 2003), *M. australiense* (Accession #AF374467), *M. australiense* (Accession #AF439521) (Murphy and Austin, 2003), *M. intermedium* (Accession #AF374466), *M. intermedium* (Accession #AF439515) (Murphy and Austin, 2003), *M. intermedium* (Accession #AF439516)

(Murphy and Austin, 2003), *M. nipponense* (Accession #AF373611) (Hwang et al., 2001), *M. rosenbergii* (Accession #AF439522) (Murphy and Austin, 2003), *P. australis* (Accession #AF439517) (Murphy and Austin, 2003), *Palaemon serenus* (Accession #AF374465), *Palaemon serenus* (Accession #AF439519) (Murphy and Austin, 2003), and *Paratya australiensis* (Accession #AF374469). The sequences were aligned using the Genetic Data Environment (GDE) software package (Smith et al., 1994). The primers were developed from the alignment.

The primers flanking the 16S rDNA region were as follows: (1) SHR 16S 80F, 5' CAT AGT MAR TAG TCT TTT AAT and (2) SHR 16S 463RC, 5' MTY TTA ATT CAA CAT CGA GGT. The primers amplified a 383 base pair portion of the 16S rRNA gene. To ensure the primers were not amplifying *M. turgidus*, they were tested on metacercarial cysts that did not include shrimp tissue.

Primers were constructed using the computer program Primer Analysis (Lynnon Corporation, 2001). This program selected primers based on optimal primer conditions including GC content, primer length, and annealing temperature. It also checked for the possibility of primer dimers and hairpin structures formed by the primers.

Polymerase Chain Reaction

PCR amplifications were performed using a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Watertown, Massachusetts) in 25 μ l volumes. To increase volume and confirm accurate amplification, duplicates were made of each sample. Therefore, there were 50 μ l total of each sample. The PCR reagents were mixed together in a 1.5 ml tube (for 4 samples of 25 μ l each: 66.5 μ l of dH₂O, 10.0 μ l of 10X Buffer, 2.5 mM MgCl₂,

200 μM of each dNTP, 0.2 μM of the forward primer, 0.2 μM of the reverse primer, and 2.5 U of Taq DNA polymerase) and 24.5 μl of the mix was pipetted into each of the 0.5 ml PCR tubes. Afterwards, 0.5 μl (approximately 0.1-0.5 μg) of DNA template was added to the tubes. The samples were then amplified under the following conditions: 94°C, 20 sec (initial denaturation), 49°C, 20 sec (annealing), and 72°C, 25 sec (extension). This was repeated for 3 cycles followed by 37 cycles of 94°C, 20 sec (denaturation), 54°C, 20 sec (annealing), and 72°C, 25 sec (extension). There was a final extension at 72°C for 10 min.

To avoid DNA contamination, all reagents were mixed together under a sterile hood and PCR supplies were regularly treated with UV light. The reaction mixture minus the DNA template served as a negative control for each set of reactions. To check for DNA amplification, the samples were loaded onto a 1.2% agarose gel and electrophoresed at 70V for one hour (5 μl of loading buffer to 5 μl of DNA template; 5 μl of loading buffer to 3 μl of 1 kb marker). Gels were viewed under UV light to confirm DNA amplification and photographed for record keeping. The remaining PCR product was stored at -20°C until further use.

Single Strand Conformation Polymorphism (SSCP)

The remaining PCR products underwent the process known as single strand conformation polymorphism (SSCP). To cast an SSCP gel, 14.0 ml. of acrylamide stock solution (38:1 acrylamide:bis-acrylamide) was mixed with 45.5 ml. of distilled water, 7.0 ml. of 5X TBE buffer, and 3.0 ml. of glycerol to make a 7.5% acrylamide solution. To polymerize the solution, 88.0 μl . of 25% of ammonium persulfate (APS) and 88.0 μl . of

TEMED was added. The solution was mixed well and poured between two sets of two glass plate sandwiches using a Bio-Rad Protein II xi cell (Bio-Rad, Hercules, CA). Gel polymerization was complete in 3-4 hours but was usually left overnight to ensure that the gel had solidified.

In the morning, samples were prepared by adding 4.0 μ l of SSCP loading buffer (98% formamide, 0.25% bromophenol blue) to 10 μ l of PCR product. The samples were then denatured at 98°C for 5 minutes, followed by snap cooling on ice for 30 minutes. The samples were loaded into wells in a vertical gel chamber and electrophoresed at 150 volts, 40 milliamps, 10 watts, and 15-16°C for 22-24 hours. Each gel was then stained with 0.0036% ethidium bromide for 30 minutes followed by destaining in water for 5-7 minutes. The gels were then transferred to a transilluminator and bands were viewed under UV light and photographed for record keeping.

Several steps were modified or added in order to obtain optimal results for band resolution. Initially, the primers were added in low concentrations to the samples as suggested by Almeida et al. (1998) and the PCR products were diluted 1:2 with distilled water, but later replaced by a dilution in 0.5X TBE buffer. Early tests revealed that this weakened the appearance of bands. Various temperatures were tested as well to determine optimization for keeping the DNA strands separated. The temperatures ranged from 4°C to 16°C, with the best results obtained at 15-16°C. The ethidium bromide concentration was increased as well in order to achieve better band resolution. Initially, the gels were stained in 0.0018% ethidium bromide, but this was later increased to 0.0036% with the addition of destaining in water afterwards.

Gel Scoring

The single stranded DNA bands from the polyacrylamide gels were scored using a transparent ruler. Bands having an identical banding pattern were assigned a letter ranging from A to F. Specifically, haplotypes were classified according to band location and number per individual. For accuracy, a sample of known haplotype from a previously run gel was added to the group to use as a standard to compare with the other samples.

Statistical Analysis

The sites were divided into regions based on location and proximity to one another. The relationship between prevalence/density and haplotype frequency was determined using one-way ANOVA in the JMP program (version 3.1 1995, SAS Institute Inc., Cary, NC). A p-value less than 0.05 indicated a significant correlation between parasite prevalence or intensity and host haplotype frequency. The haplotypes that appeared less than 5 times in all populations were grouped into a rare haplotype category. The JMP program was also used for the G-test to determine the haplotype frequency among sites and among regions. A p-value less than 0.05 indicated the haplotype frequency significantly differed among sites and/or regions.

The haplotype relative frequency and diversity index (Nei, 1987) was calculated using Arlequin version 2.0 (Schneider et al., 2000), a software program that analyzes population genetics data. Sites were grouped according to region for analysis. The Arlequin program was also used to analyze shared haplotypes within and between populations, an analysis of molecular variance (AMOVA) and population comparisons. The AMOVA analysis (Weir and Cockerham, 1984; Excoffier et al., 1992; Weir, 1996)

revealed information such as Wright's F-statistics (F_{ST}) and variance components. Significance for the F-statistics and variance components were tested using a permutational analysis described by Excoffier et al. (1992). The number of permutations was set at 1023. The population comparisons included a study of pairwise differences and a distance matrix.

Isolation by distance was analyzed by testing the correlation between pairwise F_{ST} values and geographical distance. Geographical distance was determined using Geographic Distance (<http://www.indo.com/distance>). Significance of the correlation was determined using a Mantel test (Mantel, 1967; Smouse et al., 1986). Arlequin was also used for the Mantel test. The number of permutations was again set at 1023. A neighbor-joining tree was also constructed from the genetic distance using the Molecular Evolutionary Genetics Analysis (MEGA) (Kumar et al., 1993).

RESULTS

A total of 301 *P. pugio* shrimp were analyzed from the 10 collection sites. High molecular weight bands indicated DNA was successfully extracted from the shrimp (Fig. 2). Primers developed for the 16S rRNA gene amplified a 383 base pair region within the mitochondrial genome (Fig. 3). SSCP bands were successfully displayed on a 7.5% polyacrylamide gel (Fig. 4) and results revealed six haplotypes (A to F) in all.

16S rDNA Haplotype Frequencies

Haplotype A was the dominant haplotype, appearing in 81% of the populations while haplotype B was the second most frequent, present in 13% of the populations. Haplotypes C through F were rare haplotypes distributed among the sites. The haplotype frequencies ranged from 0.000 to 1.000 (Table II). Haplotype diversities varied at sites, ranging from 0.00 at Moon River, GA and Lazaretto Creek, GA to 0.54 at St. Mary's River, GA (Table III). The G-test revealed that haplotype frequency distributions differed significantly both among sites (G-value = 165.0; P-value <0.01) as well as among regions (G-value = 124.3; P-value <0.01). However when charting the haplotype frequencies against the river systems, there does not appear to be a significant difference among the sites (Fig. 5). Further tests will indicate if this really is the case.

Genetic Structure of *P. pugio* Populations

When the sites were grouped by region in the AMOVA analysis, results revealed that most genetic variation occurred within populations at a single site with a rate of 66.56% and a F_{ST} value of 0.33, indicating a significant amount of variation. The least amount of variation appeared among sites with a rate of 10.52% (Table IV; $F_{CT} = 0.11$). The F-statistic values were significant for all sources of variation with the exception of among sites (Table IV; $p = 0.11 \pm 0.01$; $F_{CT} = 0.11$). The pairwise comparisons between F_{ST} values and geographic distance revealed a positive correlation at all sites at the 16S rRNA locus (Table V; Fig. 6; $p < 0.05$; $r^2 = 0.1945$; $y = 0.01 + 0.001$).

The topology of the neighbor-joining tree revealed that the populations were closely related to one another (Fig. 7). The tree was rooted at the Stono River, SC location since it was the northernmost point of collection. The tree showed that the closest neighbors, or operational taxonomic units, were Lazaretto Creek, GA and Moon River, GA with no genetic distance between the two. There was little difference between the sites at Stono River, SC, Combahee River, SC, Lazaretto Creek, GA, and Moon River, GA with branch lengths ranging from 0.00 to 0.02. There was only a difference of 0.04 between Stono River, SC and Ogeechee River, GA. The remaining sites had a larger distance between them, with the greatest genetic distance being between the Altamaha River, GA and St. Mary's River, GA. The branch length connecting these river systems was measured to be 0.37. Even though Sister's Creek, FL was the southernmost point of collection, the tree does not indicate that it was the farthest removed genetically. In fact, there was little difference between Sister's Creek and Little Satilla River, GA. The branch length connecting these two river systems was only 0.05.

Prevalence/Density of *M. turgidus* and *P. pugio*'s Haplotype Frequency

Analysis of the relationship between the mean density of *M. turgidus* at the sites and *P. pugio*'s haplotype frequency revealed that there was a significant correlation between the parasite's density and haplotype F of the host (Fig. 8; $p = 0.05$; $r^2 = 0.39$; $y = 16.6 - 2.3$ abundance). Results indicated that as the density of *M. turgidus* increased, there was a significant decrease in the appearance of haplotype F. However, a correlation between parasite density and any other haplotype frequency was not observed. Results also reveal no link between parasite prevalence and host haplotype frequency.

DISCUSSION

Genetic Structure Based On Locality

Haplotype A was clearly the most commonly shared haplotype and was the dominant haplotype at all of the sites except one, which indicates good dispersal capabilities of *P. pugio*. This is not surprising given that there are no apparent barriers hindering the dispersal ability of *P. pugio*. It has been noted that marine organisms are more structured genetically (genetically similar) than previously thought due to their high dispersal potential and apparent lack of barriers to dispersal in the ocean (Knowlton 1993; Avise 1994; Palumbi 1997; Benzie 1999a; Briggs 1999).

This also suggests a sufficient amount of gene flow has taken place. According to Hurwood et al. (2003), the strong presence of haplotype sharing is a direct result of contemporary gene flow. To further support this, both Lazaretto Creek, GA and Moon River, GA were fixed for haplotype A. However, there is a possibility that the current population structure results from historical gene flow due to retention of ancestral genes. This may be the case in isolated populations where gene flow was once occurring and genetic drift has not allowed for fixed differences among these populations. A minimum spanning cladogram, which displays the relationship between mtDNA haplotypes, may better determine what type of gene flow is taking place.

Slatkin (1987), though, mentions an important point when estimating levels of gene flow. Besides taking into consideration the dispersal rates, successful breeding remains a factor as well, and this may be difficult to assess. There are two distinct

methods for determining levels of gene flow among populations. The one employed in this study is referred to as the indirect method, which examines allele frequencies or DNA sequence differences to determine gene flow. Slatkin recommends using this method jointly with the direct method, which uses estimates of dispersal distances and breeding success of the dispersers to imply the amount of gene flow taking place. This is essential since each method yields different information.

Another indication that gene flow impacts the genetic structure of these populations is the significant amount of genetic variation within geographic localities. The genetic variation within the populations appears to be more highly structured than the other groups. AMOVA analysis shows a significant amount of genetic differentiation within the populations with an F- statistic value of 0.33. In addition, the overall haplotype diversity is low. The F- statistic among the populations is lower (0.11) and the p-value indicates there are no significant differences among these populations. This suggests that through migration, new alleles have been introduced to the gene pool.

It is interesting to note, though, that analyses for the pairwise F_{ST} matrix and isolation by distance indicate there is a significant correlation between geographic distance and genetic distance. This would suggest that the populations are differentiated and gene flow does not play a significant role. Further tests should be performed in order to better determine if this really is the case.

Habitat size may also play a significant role in determining the population's genetic structure. The sites sampled are not small, isolated areas but wide and open and therefore increase the potential for a large number of individuals to inhabit the area. This might decrease the chances of other random processes taking place that promote genetic

diversity. Among these are genetic drift, which would result in genetic differentiation over time in a small population, and founder events, where a small group of genetically similar individuals migrate to inhabit a previously unoccupied location.

Perhaps the most interesting result is that the southernmost locality, Sister's Creek, is not the farthest removed genetically. When examining the neighbor-joining tree, St. Mary's River, GA appears as the farthest removed. The branch length separating St. Mary's from its closest neighbor, the Altamaha River, GA, is 0.37. Geographically, there is no difference that should distinguish St. Mary's from the other sites sampled. Grobler and Mokgalong (2002) suggest several reasons why this may have occurred. This may have been a peculiarity that happened among this particular sampling site as opposed to a general characteristic that is representative of this species. Unequal distribution of haplotype frequencies across the distribution area may produce misleading results if there is a single sampling at this location. In order to better examine the population structure, there should be further analysis of the spatial as well as temporal scales of the populations. Grobler and Mokgalong (2002) point out that a single sampling in time may very likely lead to erroneous results. This may have been further complicated by the low number sampled at this site ($n = 26$).

Genetic Structure Based On Parasite Prevalence and Density

The study revealed a significant relationship between the density of *M. turgidus* and a specific *P. pugio* haplotype. However, there was not a significant correlation between *M. turgidus* and the most common *P. pugio* haplotype. On the contrary, the only significant relationship seen was between *M. turgidus* and one of the rare haplotypes. As

the density of *M. turgidus* increased, the frequency of one of the rare host haplotypes decreased. In addition, there was no connection between the prevalence of *M. turgidus* and any *P. pugio* haplotype. This seems to contradict the Red Queen hypothesis. This may not necessarily be the case, though.

Another possibility is that this rare haplotype may have been the common haplotype at one point. As previously stated, parasites are under strong selection to infect the most common local host haplotype. In their constantly evolving relationship, the parasite drives down the common host genotype/haplotype while a rare host genotype/haplotype emerges to escape infection. This may be what is being seen here. There is the possibility that the rare haplotype here may have been the common host haplotype at one time and selection has decreased the frequency of it (Lively, 2001; Lively and Dybdahl, 2000). This conclusion could be better assessed in a time series study.

Future Studies

Developing a system to examine the genetics of *P. pugio* creates many new opportunities for more in depth analyses of the host and its interaction with *M. turgidus*. First, in order to gain a better understanding of the genetics of *P. pugio* and the driving forces shaping the populations' genetic structure, it is essential to expand this study on a spatial and temporal scale. A time related study will provide a clear concept of the genetic structure of these populations. Secondly, better information can be obtained from additional loci and an increase in sample size. Thirdly, a co-evolution study in this host-parasite system would yield answers to whether or not this system follows the Red Queen

hypothesis. Finally, there should be markers selected to examine the nuclear genome. A study by Castella et al. (2000) showed that female philopatry (breeds close to or in the natal area) in *Myotis* bats resulted in high population structure when examined using mtDNA. However, the results were the opposite when nuclear markers were used due to widespread dispersal by males. In order to better assess the population genetics of *P. pugio* and its interaction with *M. turgidus*, these factors must be taken into account.

WORKS CITED

- Almeida, T.A., V.M. Cabrera, and J.G. Miranda. 1998. Improved detection and characterization of mutations by primer addition in nonradioisotopic SSCP and direct PCR sequencing. *BioTechniques* **24**: 220-221.
- Alon, N.C. and S.E. Stancyk. 1982. Variation in life-history patterns of the grass shrimp *Palaemonetes pugio* in two South Carolina estuarine systems. *Marine Biology* **68**: 265-276.
- Anderson, G. 1985. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Gulf of Mexico) – grass shrimp. United States Fish and Wildlife Services Biology Report 82(11.35) U.S. Army Corps of Engineers, EL-82-4. 19pp.
- Antheunisse, L.J., J.J. Lammens and N.P. Van den Hoven. 1971. Diurnal activities and tidal migration of the brackish water prawn *Palaemonetes varians* (Leach) (Decapoda, Caridea). *Crustaceana* **21**: 203-217.
- Avise, J.C. 1994. Molecular markers, natural history, and evolution. Chapman and Hall, New York.
- Barthalmus, G.T. 1977. Behavioral effects of mercury on grass shrimp. *Marine Pollution Bulletin* **8**: 87-90.
- Bell, S.S. and B.C. Coull. 1978. Field evidence that shrimp predation regulates meiofauna. *Oecologia* **35**: 141-148.
- Benzie, J.A.H. 1999a. Genetic structure of coral reef organisms-ghosts of dispersal past. *American Zoologist* **39**: 131-145.

- Benzie, J.A.H., S.E. Ballment, A.T. Forbes, N.T. Demetriades, K. Sugama, Haryanti, and S. Moria. 2002. Mitochondrial DNA variation in Indo-Pacific populations of the giant tiger prawn, *Penaeus monodon*. *Molecular Ecology* **11**: 2553-2569.
- Bøgh, H.O., X.Q. Zhu, B.Z. Qian and R.B. Gasser. 1999. Scanning for nucleotide variations in mitochondrial DNA fragments of *Schistosoma japonicum* by single-strand conformation polymorphism. *Parasitology* **118**: 73-82.
- Bridgman, J.F. 1969. Life cycles of *Carneophallus chanophallus* n. sp. and *C. basodactylophallus* n. sp. (Trematoda: Microphallidae). *Tulane Studies in Zoology and Botany* **15**: 81-105.
- Briggs, J.C. 1999. Coincident biogeographic patterns: Indo-West Pacific Ocean. *Evolution* **53**: 326-335.
- Brouwer, K.C., P. Ndhlovu, A. Munatsi and C.J. Shiff. 2001. Genetic diversity of a population of *Schistosoma haematobium* derived from schoolchildren in east central Zimbabwe. *Journal of Parasitology* **87**: 762-769.
- Castella, V., M. Ruedi, L. Excoffier, C. Ibanez, R. Arlettaz and J. Hausser. 2000. Is the Gibraltar strait a barrier to gene flow for the bat *Myotis myotis* (Chiroptera: Vespertilionidae)? *Molecular Ecology* **9**: 1761-1772.
- Chambers, R. 1981. Seasonal feeding and distribution of *Palaemonetes pugio* and *P. vulgaris* in Great Sippewissett salt marsh. *The Biological Bulletin (Woods Hole)* **161**: 324.
- Christmas, J.Y. and W. Langley. 1973. Estuarine invertebrates, Mississippi. Pp. 255-319 in J.Y. Christmas, ed. *Cooperative Gulf of Mexico estuarine inventory and study, Mississippi*. Gulf Coast Research Laboratory, Ocean Springs, Miss.

- Cotton, R.G.H. 1997. Mutation detection. Oxford University Press, Oxford.
- Curtis, J. and D.J. Minchella. 2000. Schistosome population genetic structure: when clumping worms is not just splitting hairs. *Parasitology Today* **16**: 68-71.
- De la Rosa-Vélez, J., R. Escobar-Fernández, F. Correa, M. Maqueda-Cornejo and J. de la Torre-Cueto. 2000. Genetic structure of two commercial penaeids (*Penaeus californiensis* and *P. stylirostris*) from the Gulf of California, as revealed by allozyme variation. *Fishery Bulletin* **98**: 674-683.
- Dillon, T.M. 1983. Oxygen consumption in the shrimp, *Palaemonetes pugio*, exposed to fluctuating temperatures and food contaminated with the diaromatic petroleum hydrocarbon, dimethylnaphthalene. *Estuarine Coastal Shelf Science* **16**: 403-413.
- Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15.
- Dybdahl, M.F. and C.M. Lively. 1998. Host-parasite coevolution: evidence for rare advantage and time-lagged selection in a natural population. *Evolution* **52**: 1057-1066.
- Eisler, R. 1969. Acute toxicities of insecticides to marine decapod crustaceans. *Crustaceana* **16**: 302-310.
- Eisler, R. 1971. Cadmium poisoning in *Fundulus heteroclitus* (Pisces: Cyprinodontidae) and other marine organisms. *Journal of Fishery Research Board Canada* **28**: 1228-1234.
- Excoffier, L., P. Smouse, and J. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **131**: 479-491.

Floyd, W.R. 1977. The effects of temperature and salinity on the larval development of the grass shrimp *Palaemonetes pugio* reared in the laboratory. Virginia Journal of Science **28**: 92.

Geographic Distance. Retrieved October 2003, from <http://www.indo.com/distance/>

Girman, D.J. 1996. The use of PCR-based single-stranded conformation polymorphism analysis (PCR-SSCP) in conservation genetics. Pp. 167-182 in T.B. Smith and R.K. Wayne, eds. Molecular genetic approaches in conservation. Oxford University Press, New York.

Glavac, D. and M. Dean. 1993. Optimization of the single-strand conformation polymorphism (SSCP) technique for detection of point mutations. Human Mutation **2**: 404-414.

Grobler J.P. and N.M. Mokgalong. 2002. Patterns of genetic heterogeneity in *Neutraclinostomum intermedialis* (Digenea: Clinostomatidae): geographical and temporal considerations. African Zoology **37**: 55-60.

Hayashi, K. 1991. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods **1**: 34-38.

Hayashi, K. 1992. PCR-SSCP: A method for detection of mutations. Genetic Analysis, Techniques and Applications **9**: 73-79.

Hayashi, K. and D.W. Yandell. 1993. How sensitive is PCR-SSCP? Human Mutation **2**: 338-346.

Heard, R. W. 1967. Some helminth parasites of the clapper rail, *Rallus longirostris* Boddaert, from the Atlantic and Gulf Coasts of the United States. M.S. Thesis, University of Georgia, Athens, 32 pp.

- Heard, R. W. 1976. Microphallid trematode metacercariae in fiddler crabs of the genus *Uca* Leach, 1814 from the northern Gulf of Mexico. Ph.D. Dissertation, University of Southern Mississippi, 179 pp.
- Heard, R.W. 1982. Guide to common tidal marsh invertebrates of the northeastern Gulf of Mexico. Mississippi-Alabama Sea Grant Consortium, MASGP-79-004. 82 pp.
- Heard, R. W. and R. M. Overstreet. 1983. Taxonomy and life histories of two North American species of "*Carneophallus*" (= *Microphallus*) (Digenea: Microphallidae). *Proceedings of the Helminthological Society of Washington* **50**: 170-174.
- Hurwood, D.A., J.M. Hughes, S.E. Bunn and C. Cleary. 2003. Population structure in the freshwater shrimp (*Paratya australiensis*) inferred from allozymes and mitochondrial DNA. *Heredity* **90**: 64-70.
- Hwang, U.W., C.J. Park, T.S. Yong and W. Kim. 2001. One-step PCR amplification of complete arthropod mitochondrial genomes. *Molecular Phylogenetics and Evolution* **19**: 345-352.
- Kanazawa, H., T. Noumi and M. Futai. 1986. Analysis of *Escherichia coli* mutants of the H(+)-transporting ATPase: determination of altered site of the structural genes. *Methods in Enzymology* **126**: 588-594.
- Kartavtsev, Y.P., K.A. Zgurovsky and Z.M. Fedina. 1993. Spatial structure of the pink shrimp *Pandalus borealis* Krøyer, 1838 from the far-eastern seas as proved by methods of population genetics and morphometrics. *Journal of Shellfish Research* **12**: 81-87.
- Khan, R. N., Knowlton, R. E., Merchant, H. C. 1995. Effects of homogeneous and heterogeneous substrates on the distribution of *Palaemonetes*. *The Journal of the Elisha Mitchell Scientific Society* **2**: 83-95.

- Kneib, R. T. 1987a. Predation risk and use of intertidal habitats by young fishes and shrimp. *Ecology* **68**: 379-386.
- Kneib, R. T. 1987b. Seasonal abundance, distribution and growth of postlarval and juvenile grass shrimp (*Palaemonetes pugio*) in a Georgia, U.S.A., salt marsh. *Marine Biology* **96**: 215-223.
- Knowlton, N. 1993. Sibling species in the sea. *Annual Review of Ecology and Systematics* **24**: 189-216.
- Kumar, S., K. Tamura, and M. Nei. 1993. MEGA: Molecular Evolutionary Genetics Analysis, version 1.0. The Pennsylvania State University, University Park, PA.
- Kunz, A.K. and O.J. Pung 2004. Effects of *Microphallus turgidus* (Trematoda: Microphallidae) on the predation, behavior, and swimming stamina of the grass shrimp *Palaemonetes pugio*. *Journal of Parasitology* **90**: 441-445.
- Lessa, E.P. and G. Applebaum. 1993. Screening techniques for detecting allelic variation in DNA sequences. *Molecular Ecology* **2**: 119-129.
- Lively, C.M. 2001. Trematode infection and the distribution and dynamics of parthenogenetic snail populations. *Parasitology* **123**: S19-S26.
- Lively, C.M. and M.F. Dybdahl. 2000. Parasite adaptation to locally common host genotypes. *Nature* **405**: 679-681.
- Lydeard, C., M. Mulvey, J.M. Aho, and P.K. Kennedy. 1989. Genetic variability among natural populations of the liver fluke, *Fascioloides magna*, in the white-tailed deer, *Odocoileus virginianus*. *Canadian Journal of Zoology* **67**: 2021-2025.
- Lynnon Corporation. Retrieved October 2003, from <http://www.lynnon.com/>

- Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**: 209-220.
- May, R.M. 1985. Host-parasite associations: their population biology and population genetics. In *Ecology and Genetics of Host-parasite Interactions*, eds Rollinson, D. & Anderson, R.M. pp. 243-262. London: Academic Press.
- Middaugh, D.P. and G. Floyd. 1978. Effect of prehatch and posthatch exposure to cadmium on salinity tolerance of larval grass shrimp, *Palaemonetes pugio*. *Estuaries* **1**: 123-125.
- Morgan, M.D., 1980. Grazing and predation of the grass shrimp *Palaemonetes pugio*. *Limnology and Oceanography* **25**: 896-902.
- Moritz, C., T.E. Dowling and W.M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics* **18**: 269-292.
- Mullis, K.B. 1990. The unusual origin of the polymerase chain reaction. *Scientific American* **262**(4): 56-65.
- Murphy, N.P. and C.M. Austin. 2003. Molecular taxonomy and phylogenetics of some species of Australian palaemonid shrimps. *Journal of Crustacean Biology* **23**: 169-177.
- Nadler, S.A. 1995. Microevolution and the genetic structure of parasite populations. *Journal of Parasitology* **81**: 395-403.
- Naihong, X., E. Audenaert, J. Vanoverbeke, L. Brendonck, P. Sorgeloos and L. de Meester. 2000. Low among-population genetic differentiation in Chinese bisexual *Artemia* populations. *Heredity* **84**: 238-243.

- Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.
- Olson, P.D., T.H. Cribb, V.V. Tkach, R.A. Bray and DTJ Littlewood. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology* **33**: 733-755.
- Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-stranded conformation polymorphisms. *Proceedings of the National Academy of Science USA* **86**: 2766-2770.
- Palumbi, S.R. 1997. Molecular biogeography of the Pacific. *Coral Reefs* **16**: S47-S52.
- Pomeroy, L.R. and R.G. Wiegert. 1981. *The ecology of a salt marsh*. Springer-Verlag, New York.
- Pung, O.J., R.N. Khan, S.P. Vives, and C.B. Walker 2002. Prevalence, geographic distribution, and fitness effects of *Microphallus turgidus* (Trematoda: Microphallidae) in grass shrimp (*Palaemonetes* spp.) from coastal Georgia. *Journal of Parasitology* **88**: 89-92.
- Rannala, B.H. 1991. Evidence for host allozymes present on electrophoretic gels of trematode parasites (Digenea: Plagiorchiiformes). *Journal of Parasitology* **77**: 805-808.
- Richardson, H. 1905. *A monograph on the isopods of North America*. U.S. National Museum Bulletin **54**: 1-717.
- Russell, P.J. 1994. *Fundamentals of Genetics*. HarperCollins College Publishers, New York.
- SAS Institute Inc. 1995. *JMP Statistics and Graphics Guide, Version 3.1* SAS Institute Inc., Cary, North Carolina.

- Sandifer, P.A. 1973. Effects of temperature and salinity on larval development of grass shrimp *Palaemonetes vulgaris* (Decapoda, Caridea). U.S. National Marine Fisheries Service Fisheries Bulletin 71: 115-123.
- Schneider, S., D. Roessli, and L. Excoffier. 2000. Arlequin: A software for population genetics data analysis. Ver 2.000. Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva.
- Schulenburg, J.H. and J.W. Wagele. 1998. Molecular characterization of digenetic trematodes associated with *Cyathura carinata* (Crustacea: Isopoda) with a note on the utility of 18S ribosomal DNA for phylogenetic analysis in the Digenea (Platyhelminthes: Trematoda). International Journal for Parasitology **28**: 1425-1428.
- Shealy, M.H. and P.A. Sandifer. 1975. Effects of mercury on survival and development of larval grass shrimp *Palaemonetes vulgaris*. Marine Biology 33: 7-16.
- Sheffield, V.C., J.S. Beck, A.E. Kwitek, D.W. Sandstrom, and E.M. Stone. 1993. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. Genomics **16**: 325-332.
- Shenker, J.M. and J.M. Dean. 1979. The utilization of an intertidal salt marsh creek by larval and juvenile fishes: abundance, diversity and temporal variation. Estuaries **2**: 154-163.
- Sikora, W.B. 1977. The ecology of *Palaemonetes pugio* in a southeastern salt marsh ecosystem, with particular emphasis on production and trophic relationships. Ph.D. Dissertation. University of South Carolina, Columbia, 122 pp.

- Sire, C., J. Langed, V. Barral and A. Théron. 2001. Parasite (*Schistosoma mansoni*) and host (*Biomphalaria glabrata*) genetic diversity: population structure in a fragmented landscape. *Parasitology* **122**: 545-554.
- Slatkin, M. 1985. Rare alleles as indicators of gene flow. *Evolution* **39**: 53-65.
- Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. *Science* **236**: 787-792.
- Smith, S.W., R. Overbeek, C.R. Woese, W. Gilbert and P.M. Gillevet. 1994. The genetic data environment: an expandable GUI for multiple sequence analysis. *Computer Applications in Bioscience* **10**: 671-675.
- Smouse, P.E., J. C. Long and R. R. Sokal. 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic Zoology* **35**: 627-632.
- Sprague, V. 1970. Some protozoan parasites and hyperparasites in marine decapod crustacea. pp. 416-430. In S.F. Snieszko (ed.): *A Symposium on Diseases of Fishes and Shell Fishes*. American Fisheries Society Special Publication 5, Washington, D.C.
- Street, D. A., and V. Sprague. 1974. A new species of *Pleistophora* (Microsporidia: Pleistophoridae) parasitic in the shrimp *Palaemonetes pugio*. *Journal of Invertebrate Pathology* **23**: 153-156.
- Stunkard, H. W. 1979. The morphology, life-history, and taxonomic relations of *Odhneria odhneri* Travassos, 1921 (Digenea: Microphallidae). *Biological Bulletin* **156**: 234-245.

- Vernberg, W.B., P.J. DeCoursey, M. Kelly and D.M. Johns. 1977. Effects of sublethal concentrations of cadmium on adult *Palaemonetes pugio* under static and flow-through conditions. *Bulletin of Environmental Contamination and Toxicology* 17: 16-24.
- Webster, J.P. and M.E.J. Woolhouse. 1998. Selection and strain specificity of compatibility between snail intermediate hosts and their parasitic schistosomes. *Evolution* 52: 1627-1634.
- Webster, J.P., C.M. Davies and J.I. Hoffman. 2001. Population genetics of the schistosome intermediate host *Biomphalaria pfeifferi* in the Zimbabwean highveld: implications for co-evolutionary theory. *Annals of Tropical Medicine and Parasitology* 95: 203-214.
- Weir, B. 1996. Intraspecific differentiation. In Hillis, D.M., C. Moritz, B.K. Mable, eds. *Molecular Systematics*, 2nd ed. Sinauer Assoc. Inc, Sunderland, MA.
- Weir, B.S. and C.C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358-1370.
- Welsh, B. L. 1975. The role of grass shrimp, *Palaemonetes pugio*, in a tidal marsh ecosystem. *Ecology* 56: 513-530.
- Williams, A.B. 1984. Shrimps, lobsters, and crabs of the Atlantic coast of the Eastern U.S., Maine to Florida. Smithsonian Institution Press, Washington, D.C. 550 pp.
- Wood, C.E. 1967. Physioecology of the grass shrimp *Palaemonetes pugio*, in the Galveston Bay estuarine system. *Contributions in Marine Science University of Texas* 12: 54-79.

APPENDIX

Population Genetics of *Microphallus turgidus*

Numerous genetic studies have been conducted on parasite populations. Several of these studies have focused on digenetic trematodes (Brouwer et al., 2001; Grobler and Mokgalong, 2002; Rannala, 1991; Schulenburg and Wagele, 1998; Lydeard et al., 1989). The study of parasite populations is important for a number of reasons. First, analyzing the genetic variation of parasite populations contributes to a better understanding of the epidemiology of disease transmission (Sire et al., 2001). Second, examining the genetic diversity at each stage of the parasite's life cycle is crucial in accounting for the origin of diversity and how that diversity is maintained in a population (Curtis and Minchella, 2000). Finally, an analysis of the microevolution of a parasite population yields information about population parameters such as genetic drift, gene flow, natural selection and other features that influence genetic evolution (Nadler, 1995).

Like its second intermediate host, *Palaemonetes pugio*, nothing is known about the population genetics of *Microphallus turgidus*. Therefore, the focus of this research was twofold: **1)** develop a system to examine the genetic structure of populations of *M. turgidus* and **2)** determine the extent of genetic variability among populations from South Carolina, Georgia, and Florida, within populations from a single locality, and within a single *P. pugio* shrimp. Information provided in this study will open the doors for further genetic studies with the parasite as well as its interaction with *P. pugio*.

The shrimp collection, dissection, and DNA extraction is documented in the materials and methods section of this work. In order to allow for the complete development of the parasite, the shrimp were kept alive for 3 wk. This was to ensure that

only a single parasite was extracted at a time from the shrimp. Due to the small size of the parasite, there were many occasions where no bands appeared on the gel from the DNA extraction process. It is most likely that when bands did appear, they represented shrimp DNA rather than parasite DNA.

In order to run the polymerase chain reaction (PCR), several genes were initially analyzed for the study. The first gene considered was the 28S rRNA gene. Primers designed for this gene were based on microphallid data from Olson et al. (2003). Preliminary data revealed promising results and therefore two molecular techniques were utilized to further assess the genetic variation of populations of *M. turgidus*.

One such technique was restriction fragment length polymorphism (RFLP). The *Taq* I enzyme was tested but revealed no variation. While RFLP analysis was being conducted, randomly amplified polymorphic DNA (RAPD) markers were also being studied. Several markers were tested at once including A12, G11, Z08, and A02. The G11 primer produced the most DNA bands, indicative of a strong primer. However, since RAPD primers “scan” an entire genome for sequences and DNA was extracted from the host as well as the parasite, there was no way to distinguish between the two in terms of DNA bands on the gel. Therefore, RAPD primers could not be used for this study. Before further tests could be performed, DNA from individual parasites was purified and sent to the University of Maryland Biotechnology Institute for sequencing. Upon receiving the sequences, it was determined that not enough variation existed between the individual parasites and therefore the 28S rRNA gene was too conserved to use for this study (Appendix 1).

To find a more suitable gene to use for analysis, attention focused on selecting a gene from the mitochondrial genome. The mitochondrial genome has proven to be effective for population studies due to its high evolving rate, maternal inheritance, and lack of recombination. Primers were developed simultaneously for two genes: the cytochrome *c* oxidase subunit I (COI) gene and the 16S rRNA gene. The primers designed for the COI gene proved to be ineffective. Results showed the primers were amplifying a 300 base pair fragment, whereas they should have been amplifying a 500 base pair fragment. Therefore, further studies centered on the 16S rRNA gene. While PCR reactions were being run, DNA from individual parasites was purified and sequenced. Results revealed that the primers designed for this gene were in fact amplifying the host, *P. pugio*, and not the parasite.

Another set of primers was then designed for the COI gene of the mitochondrial genome. Primers for this gene were based on aligned sequences from Genbank for the trematodes *Schistosoma mansoni* (Accession # AF216698), *Schistosoma mekongi* (Accession # AF217449), *Schistosoma japonicum* (Accession # AF215860), *Paragonimus westermani* (Accession # AF219379), and *Fasciola hepatica* (Accession # AF216697). Several combinations of primers were tested, with one set of primers (MPCOI 310 F/ 1324 Rc) showing strong DNA amplification.

PCR amplifications were performed using a PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Watertown, Massachusetts) in 25 μ l volumes. The PCR reagents were mixed together in a 1.5 ml tube (for 4 samples of 25 μ l each: 66.5 μ l of dH₂O, 10.0 μ l of 10X Buffer, 2.5 mM MgCl₂, 200 μ M of each dNTP, 0.2 μ M of the forward primer, 0.2 μ M of the reverse primer, and 2.5 U of Taq DNA polymerase) and pipetting 24.5 μ l of

the mix into each of the 0.5 ml PCR tubes. Afterwards, 0.5 μ l (approximately 0.1-0.5 μ g) of DNA template was added to the tubes. PCR conditions were as follows: 4 cycles of 94°C-20 seconds, 49°C-20 seconds, and 72°C-25 seconds; 37 cycles of 94°C-20 seconds, 54°C-20 seconds, and 72°C-25 seconds; this was followed by a final extension at 72°C-10 minutes. Uninfected shrimp served as a negative control for the PCR reactions.

The results show stronger bands for the parasite DNA, indicating that the primers are amplifying the parasite and not the host (Appendix 2). To confirm this, DNA from individual *M. turgidus* would need to be purified and sequenced. Results would reveal whether or not the primers are truly amplifying *M. turgidus*. If in fact it is the parasite's DNA being amplified, this would enable further studies to be conducted on the genetics of *M. turgidus* as well as its interaction with *P. pugio*. At this point, however, the focus of the study had already shifted from the genetics of *M. turgidus* to the genetics of *P. pugio* so further work on the population genetics of *M. turgidus* was not continued.

Table I. Prevalence and density of *Microphallus turgidus* metacercarial cysts in *Palaemonetes pugio* collected in South Carolina, Georgia, and Florida for use in this study. Parasite prevalence and density are based on examination of at least 65 shrimp from each locality.

Region	Collection	Collection Date	Salinity (ppt)	Parasite density*	Parasite prevalence†
1	Stono River, SC	July 30, 2002	30	7.0	>90
	Combahee River, SC	July 30, 2002	20	7.0	>90
2	Lazaretto Creek, GA	October 20, 2001	26	1.0	54
	Moon River, GA	January 25, 2002	28	8.0	95
3	Ogeechee River, GA	August 23, 2001	20	8.0	92
	Broro River, GA	January 17, 2002	33	4.9	80
4	Altamaha River, GA	October 2, 2001	2.0	2.7	77
	Little Satilla River, GA	June 6, 2002	23	7.4	100
5	St. Mary's River, GA	June 6, 2002	11	1.3	89
	Sister's Creek, FL	June 6, 2002	32	0.2	21

* Number of metacercarial cysts/cm host body length

† Percent shrimp infected

Table II. Haplotype frequencies of 16S rDNA in *Palaemonetes pugio* populations.

Region	Site	n	Haplotype					
			A	B	C	D	E	F
1	Stono River	35	0.94	----	----	0.03	0.03	----
	Combahee River	26	0.96	----	----	----	----	0.04
2	Lazaretto Creek	31	1.00	----	----	----	----	----
	Moon River	30	1.00	----	----	----	----	----
3	Ogeechee River	31	0.90	0.07	----	----	----	----
	Broro River	32	0.84	0.13	0.03	----	----	----
4	Altamaha River	32	0.66	0.34	----	----	----	----
	Little Satilla River	28	0.82	0.18	----	----	----	----
5	St. Mary's River	26	0.08	0.62	----	----	----	0.31
	Sister's Creek	30	0.77	----	----	----	----	0.23

Table III. Haplotype diversity indices for 16S rDNA among *Palaemonetes pugio* populations.

Region	Site	Haplotype diversity
1	Stono River, SC	0.1126 +/- 0.0718
	Combahee River, SC	0.0769 +/- 0.0697
2	Lazaretto Creek, GA	0.0000 +/- 0.0000
	Moon River, GA	0.0000 +/- 0.0000
3	Ogeechee River, GA	0.1849 +/- 0.0905
	Broro River, GA	0.2802 +/- 0.0954
4	Altamaha River, GA	0.4657 +/- 0.0563
	Little Satilla River, GA	0.3042 +/- 0.0943
5	St. Mary's River, GA	0.5415 +/- 0.0750
	Sister's Creek, FL	0.3701 +/- 0.0841

Table IV. AMOVA analysis of the genetic structure of *Palaemonetes pugio* populations by geographic groups.

Region	Source of variation	Variance component	% Total variation	F-statistics	P-value
1 vs.2 vs. 3 vs.4 vs. 5	Among groups	0.01818	10.52	0.10525 F _{CT}	0.11046 +/- 0.01028
	Among populations within groups	0.03959	22.92	0.25614 F _{SC}	0.000 +/- 0.000
	Within populations	0.11497	66.56	0.33443 F _{ST}	0.000 +/- 0.000

Table V. F_{ST} -values for *Palaemonetes pugio* populations and geographic distance between sites. F_{ST} -values are represented by the lower diagonal matrix; the geographic distance (km.) is represented by the upper diagonal matrix. SR = Stono River, SC; CR = Combahee River, SC; LC = Lazaretto Creek, GA; MR = Moon River, GA; OR = Ogeechee River, GA; BR = Broro River, GA; AR = Altamaha River, GA; LSR = Little Satilla River, GA; SMR = St. Mary's River, GA; SC = Sister's Creek, FL. *** indicates $p < 0.05$.

Collection Site	SR	CR	LC	MR	OR	BR	AR	LSR	SMR	SC
SR	----	56	93	130	140	173	210	233	298	289
CR	-0.02	----	71	87	96	132	159	193	254	256
LC	0.01	0.01	----	50	61	86	135	142	209	196
MR	0.01	0.01	0.00	----	12	45	86	105	168	170
OR	0.00	0.00	0.04	0.04	----	37	74	97	159	164
BR	0.04	0.05	0.10	0.10	-0.01	----	59	61	125	127
AR	0.25***	0.25***	0.32***	0.32***	0.15***	0.08	----	70	103	141
LSR	0.08***	0.09	0.16***	0.16***	0.01	-0.03	0.04	----	68	73
SMR	0.67***	0.66***	0.73***	0.72***	0.60***	0.53***	0.32***	0.49***	----	76
SC	0.14***	0.11	0.21***	0.21***	0.10***	0.08***	0.16***	0.09***	0.48***	----

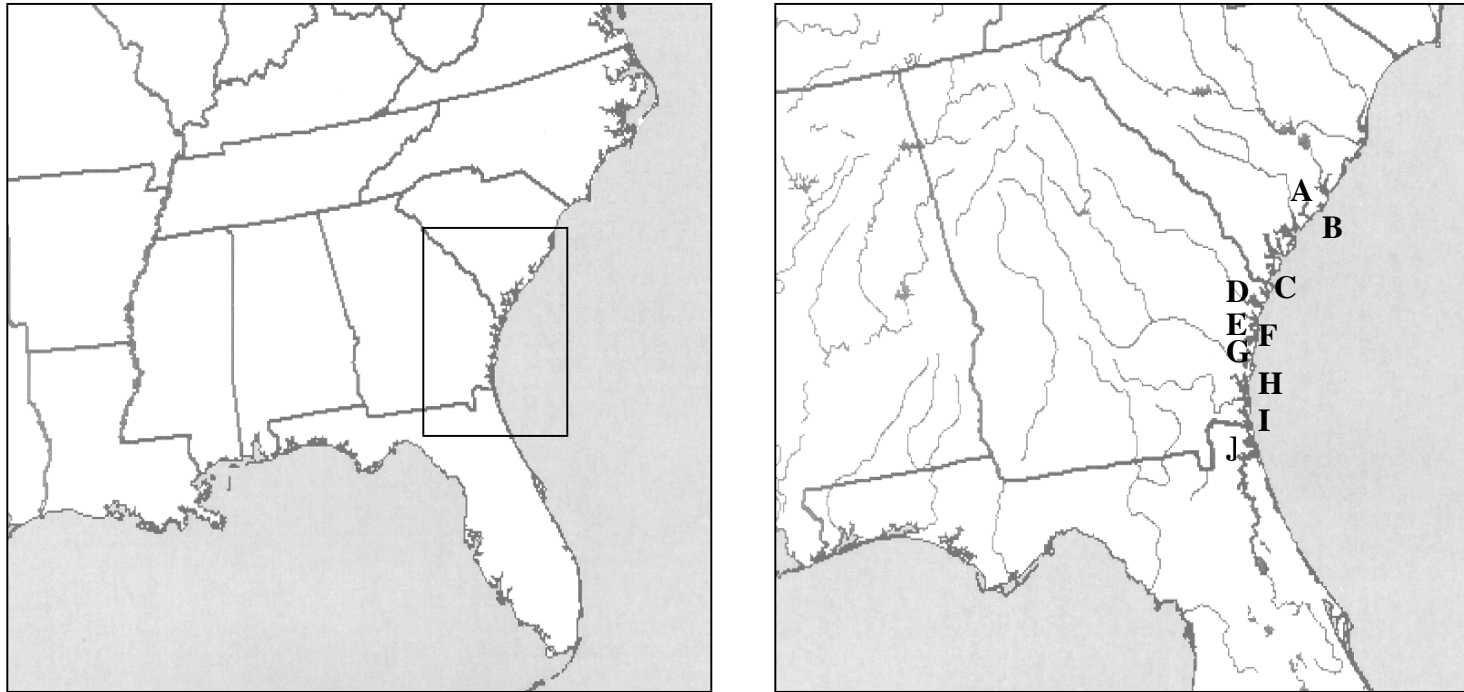


Figure 1. Map of *Palaemonetes pugio* collection sites. A) Stono River, SC; B) Combahee River, SC; C) Moon River, GA; D) Lazaretto Creek, GA; E) Ogeechee River, GA; F) Broro River, GA; G) Altamaha River, GA; H) Little Satilla River, GA; I) St. Mary's River, GA; J) Sister's Creek, FL.

P. pugio DNA bands

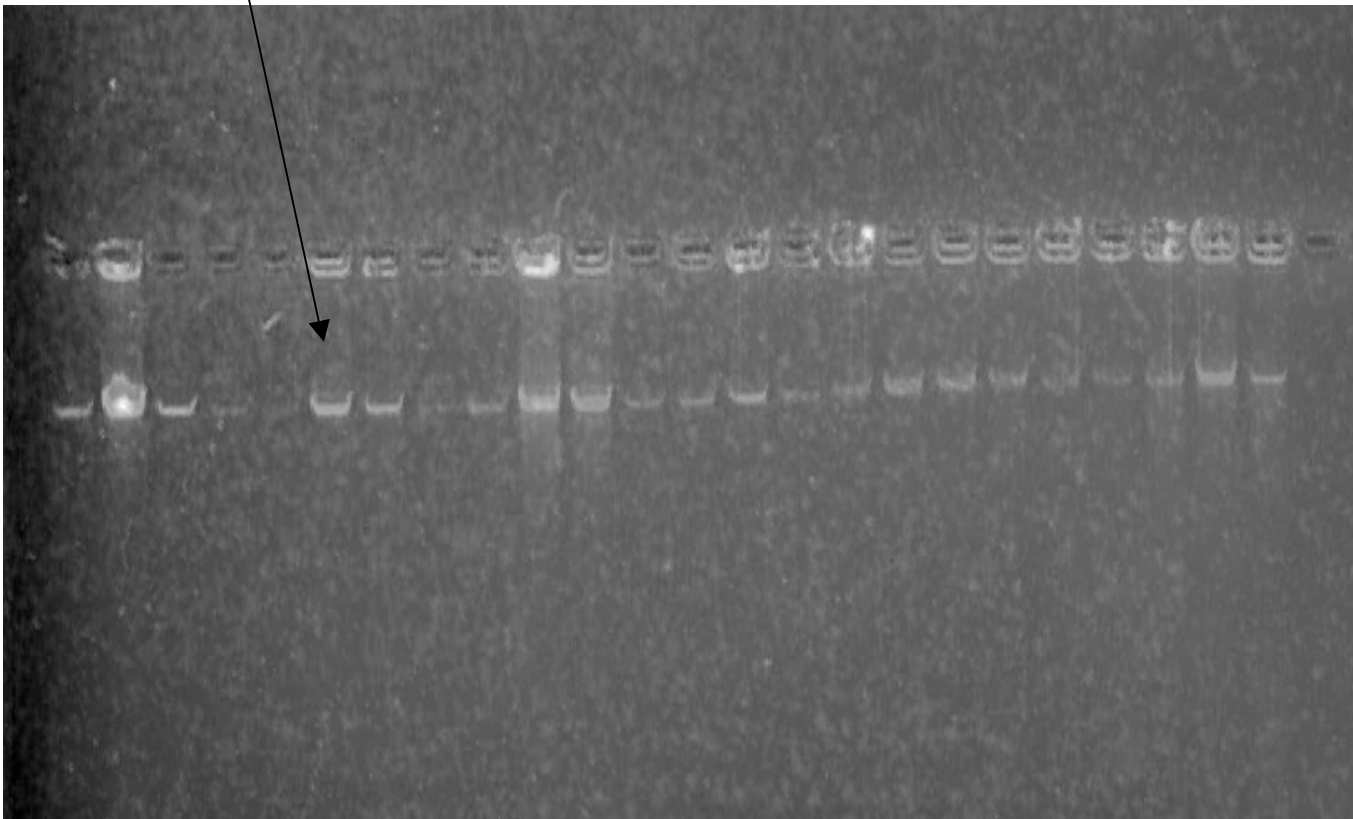


Figure 2. CTAB extraction of *Palaemonetes pugio* DNA on a 1.2% agarose gel.

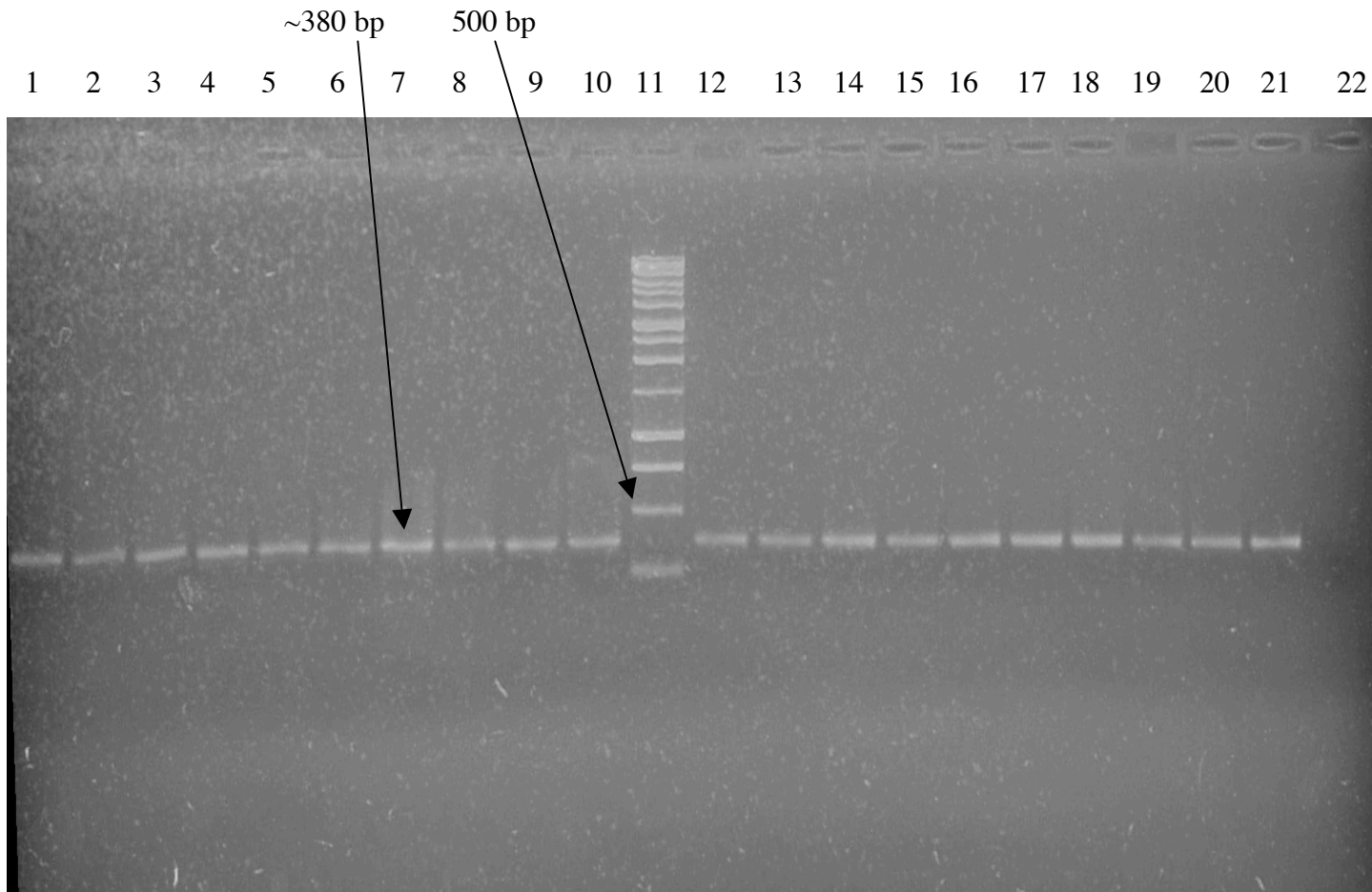


Figure 3. PCR gel revealing amplification of a partial segment of *Palaemonetes pugio* 16S rRNA gene within the mitochondrial genome. Lane 11 represents the 1 kb molecular marker; lane 22 represents the negative control.

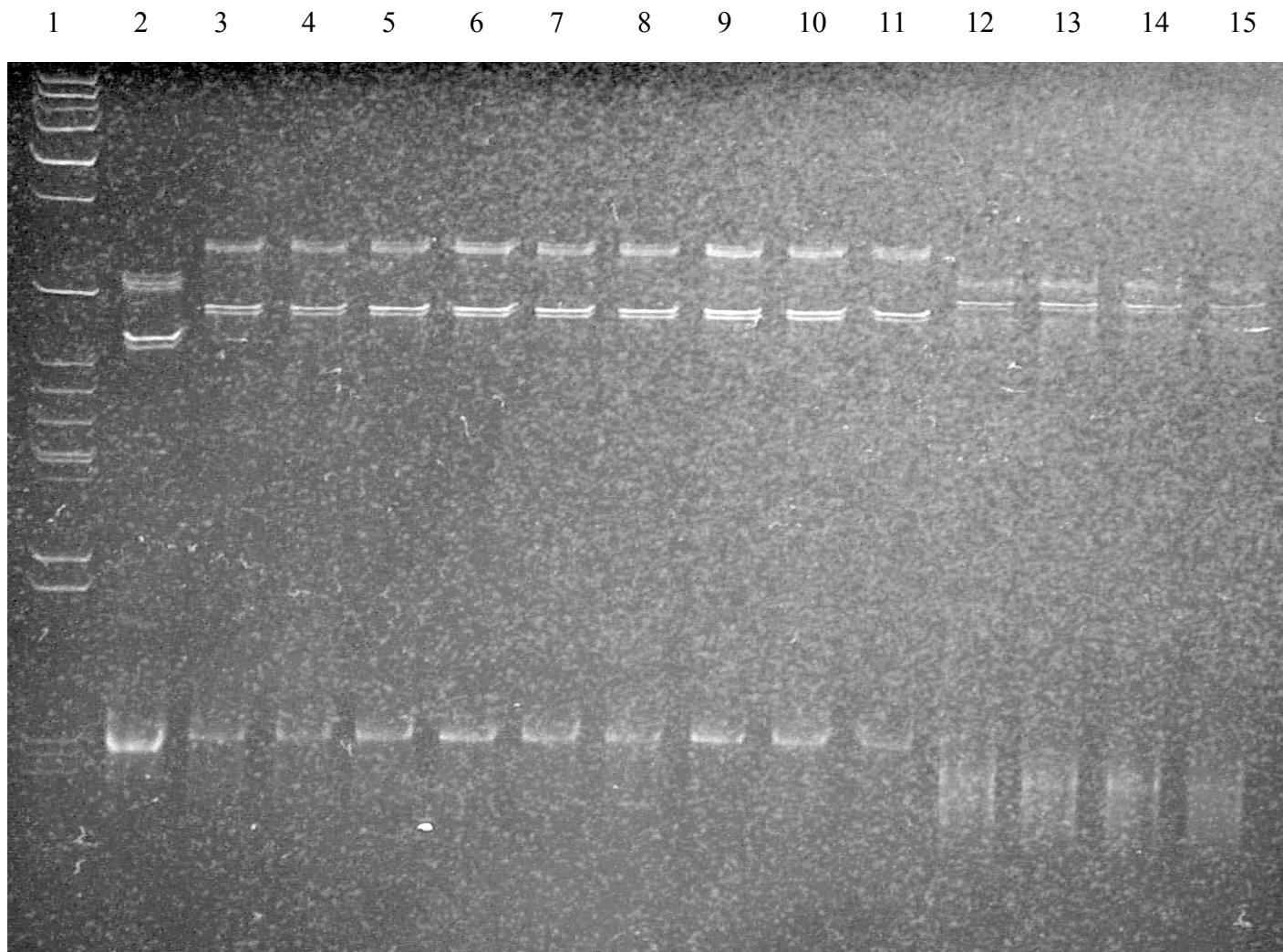


Figure 4. Single strand conformation polymorphism profile displayed on a 7.5% polyacrylamide gel. Lane 1 represents the 1 kb molecular marker.

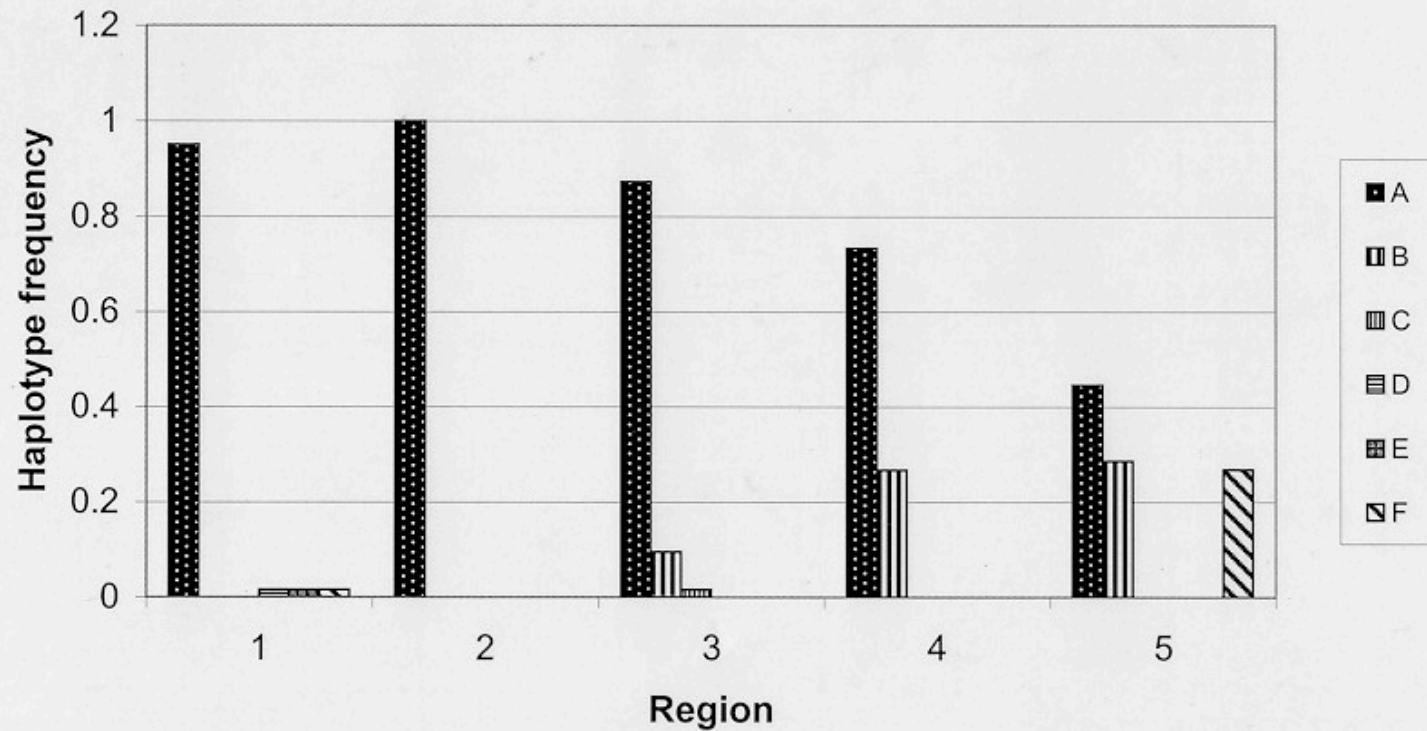


Figure 5. Haplotype frequencies for *Palaemonetes pugio* appearing in South Carolina, Georgia, and Florida. Region 1 = Stono River, SC; Combahee River, SC; 2 = Lazaretto Creek, GA; Moon River, GA; 3 = Ogeechee River, GA; Broro River, GA; 4 = Altamaha River, GA; Little Satilla River, GA; 5 = St. Mary's River, GA; Sister's Creek, FL.

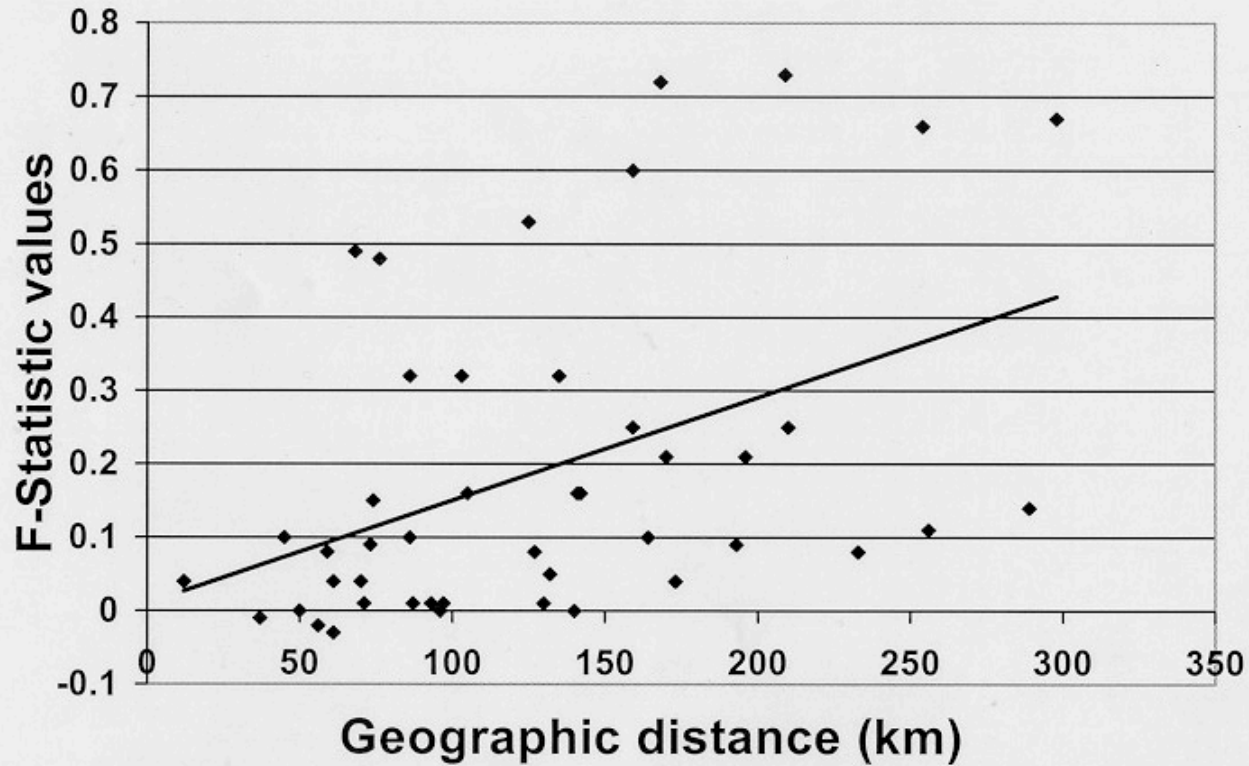


Figure 6. The relationship between *Palaemonetes pugio*'s F-statistic values and the geographic distance (km) of the collection localities; $p < 0.05$; $r^2 = 0.1945$; $y = 0.010 + 0.001x$.

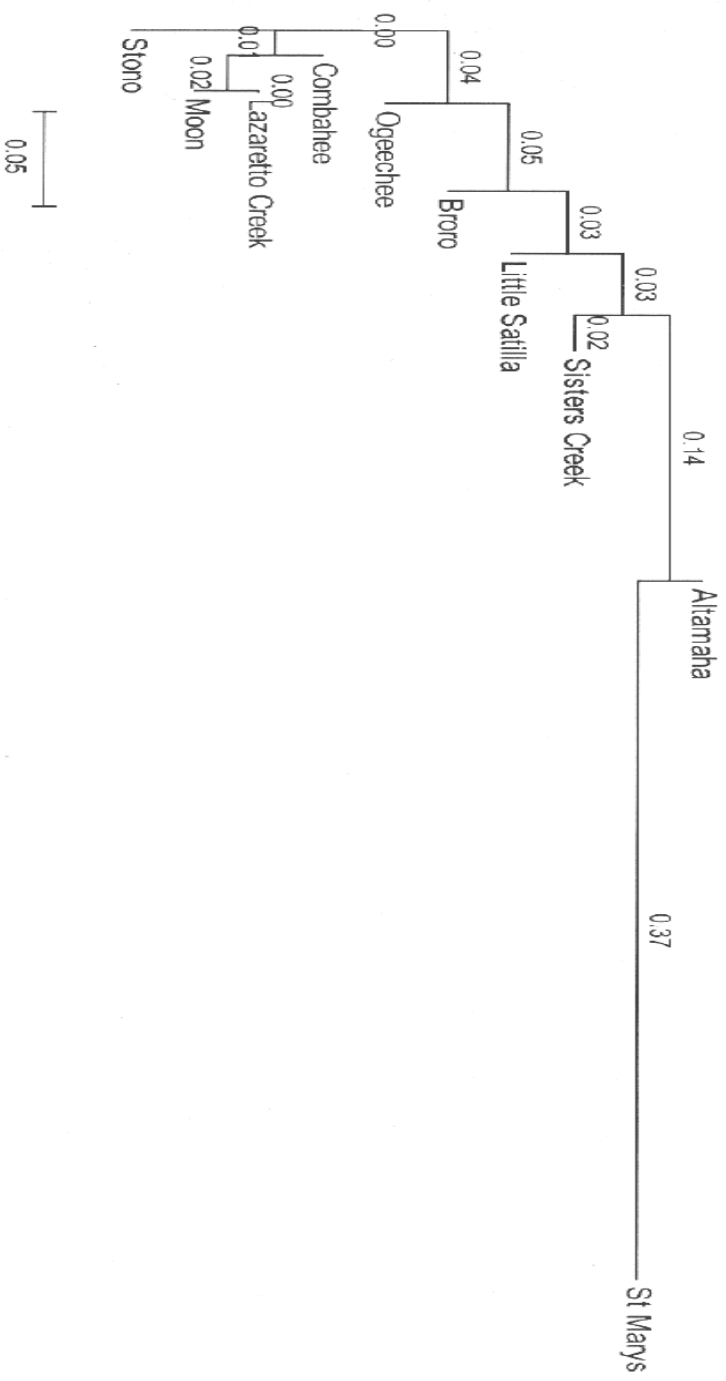


Figure 7. Neighbor-joining tree for 16S rDNA haplotypes of *Palaemonetes pugio* populations.

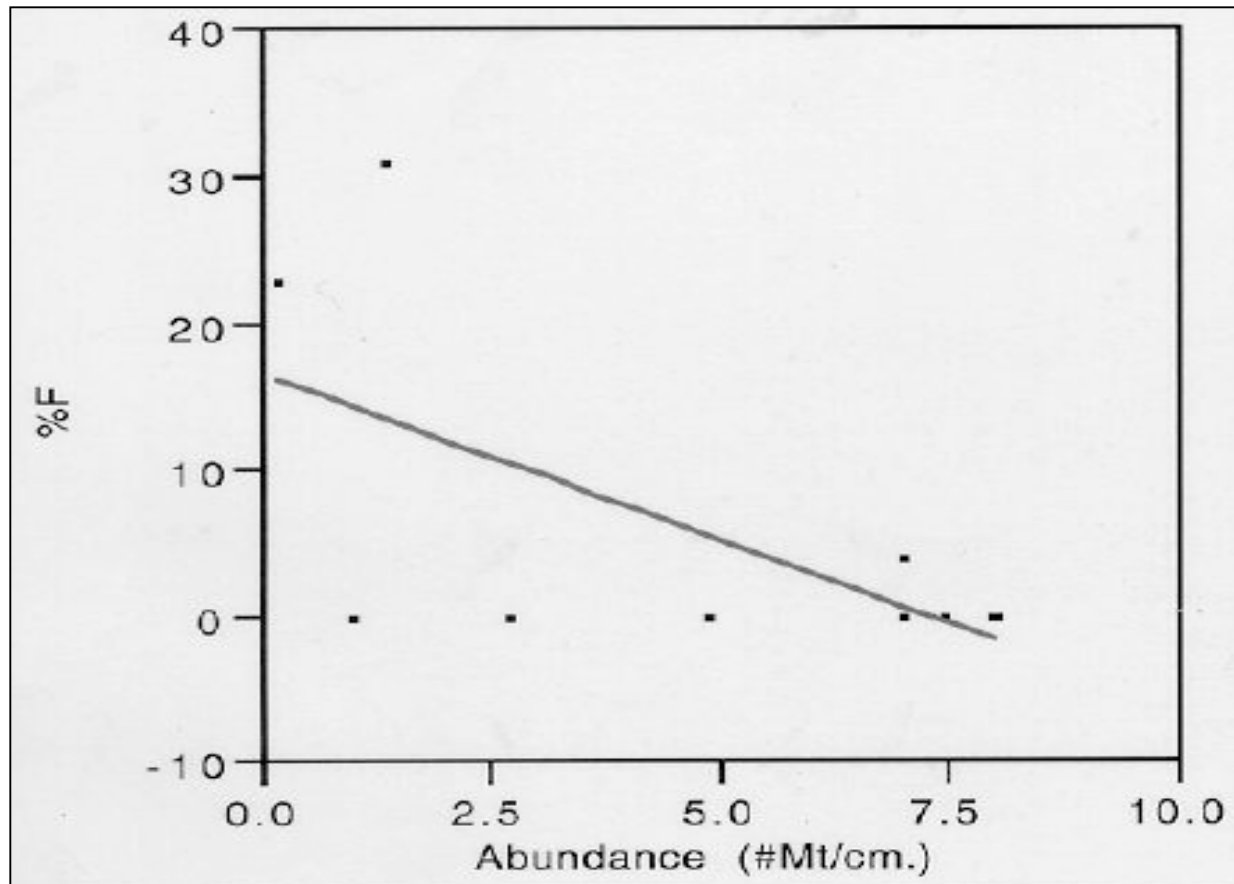


Figure 8. Density of *Microphallus turgidus* (#cysts/cm.) plotted against haplotype frequency F of *Palaemonetes pugio*; $P = 0.05$; $r^2 = 0.39$; $y = 16.6 - 2.3 \text{ abundance}$.


```

52_P0062-Mt      290 CTTTTTCAGAGTAGTCACCACGACCGGCATTGCTGTCTGGCTGGTGCGGTTAAACCGGTC 349
53_P0063-Mt      301 CTTTTTCAGAGTAGTCACCACGACCGGCATTGCTGTCTGGCTGGTGCGGTTAAACCGGTC 360
55_P0064-Mt      301 CTTTTTCAGAGTAGTCACCACGACCGGCATTGCTGTCTGGCTGGTGCGGTTAAACCGGTC 360
Clustal Consensus 285 ***** 344

50_P0061-Mt      351 TTGCATTTCCCTTGTGGTTTTGCTTGGTCGGGATGGCAGGTAGCTCGTTGGCTAGCTTGT 410
57_P0068-Mt      351 TTGCATTTCCCTTGTGGTTTTGCTTGGTCGGGATGGCAGGTAGCTCGTTGGCTAGCTTGT 410
52_P0062-Mt      350 TTGCATTTCCCTTGTGGTTTTGCTTGGTCGGGATGGCAGGTAGCTCGTTGGCTAGCTTGT 409
53_P0063-Mt      361 TTGCATTTCCCTTGTGGTTTTGCTTGGTCGGGATGGCAGGTAGCTCGTTGGCTAGCTTGT 420
55_P0064-Mt      361 TTGCATTTCCCTTGTGGTTTTGCTTGGTCGGGATGGCAGGTAGCTCGTTGGCTAGCTTGT 420
Clustal Consensus 345 ***** 404

50_P0061-Mt      411 GGTTTCGCTGCAGGCATCGGCTTTCGAGTGTAAATCAGCTTACCGTATCGGTTCTGTGCAGT 470
57_P0068-Mt      411 GGTTTCGCTGCAGGCATCGGCTTTCGAGTGTAAATCAGCTTACCGTATCGGTTCTGTGCAGT 470
52_P0062-Mt      410 GGTTTCGCTGCAGGCATCGGCTTTCGAGTGTAAATCAGCTTACCGTATCGGTTCTGTGCAGT 469
53_P0063-Mt      421 GGTTTCGCTGCAGGCATCGGCTTTCGAGTGTAAATCAGCTTACCGTATCGGTTCTGTGCAGT 480
55_P0064-Mt      421 GGTTTCGCTGCAGGCATCGGCTTTCGAGTGTAAATCAGCTTACCGTATCGGTTCTGTGCAGT 480
Clustal Consensus 405 ***** 464

50_P0061-Mt      471 GTGTCGGAGATGGCGGCTTGTGTGTGCGTGCGTGCCATGTTAGCCGACGTGTTTCGAGATC 530
57_P0068-Mt      471 GTGTCGGAGATGGCGGCTTGTGTGTGCGTGCGTGCCATGTTAGCCGACGTGTTTCGAGATC 530
52_P0062-Mt      470 GTGTCGGAGATGGCGGCTTGTGTGTGCGTGCGTGCCATGTTAGCCGACGTGTTTCGAGATC 529
53_P0063-Mt      481 GTGTCGGAGATGGCGGCTTGTGTGTGCGTGCGTGCCATGTTAGCCGACGTGTTTCGAGATC 540
55_P0064-Mt      481 GTGTCGGAGATGGCGGCTTGTGTGTGCGTGCGTGCCATGTTAGCCGACGTGTTTCGAGATC 540
Clustal Consensus 465 ***** 523

50_P0061-Mt      531 TGGTTGTTTTGTTGCCTGAATGCAGGCCTGATGATGACTCGGATGCGTTGTGTTGACGGT 590
57_P0068-Mt      531 TGGTTGTTTTGTTGCCTGAATGCAGGCCTGATGATGACTCGGATGCGTTGTGTTGACGGT 590
52_P0062-Mt      530 TGGTTGTTTTGTTGCCTGAATGCAGGCCTGATGATGACTCGGATGCGTTGTGTTGACGGT 589
53_P0063-Mt      541 TGGTTGTTTTGTTGCCTGAATGCAGGCCTGATGATGACTCGGATGCGTTGTGTTGACGGT 600
55_P0064-Mt      541 TGGTTGTTTTGTTGCCTGAATGCAGGCCTGATGATGACTCGGATGCGTTGTGTTGACGGT 600
Clustal Consensus 524 ***** 583

50_P0061-Mt      591 TGCGTGTGTGACACGTTATGAGCCAAAAGTCTGTGGTGTAGTGGTAGACTATCCACCTGA 650
57_P0068-Mt      591 TGCGTGTGTGACACGTTATGAGCCAAAAGTCTGTGGTGTAGTGGTAGACTATCCACCTGA 650
52_P0062-Mt      590 TGCGTGTGTGACACGTTATGAGCCAAAAGTCTGTGGTGTAGTGGTAGACTATCCACCTGA 649
53_P0063-Mt      601 TGCGTGTGTGACACGTTATGAGCCAAAAGTCTGTGGTGTAGTGGTAGACTATCCACCTGA 660

```

```

55_P0064-Mt      601 TCGTGTGTGACACGTTATGAGCCAAAAGTCTGTGGTGTAGTGGTAGACTATCCACCTGA 660
Clustal Consensus 584 ***** 643

50_P0061-Mt      651 CCCGTCTTGAAACACGGACCAAGGAGAGTAACATGTGCGCGAGTCATTGGGCGTTACGAA 710
57_P0068-Mt      651 CCCGTCTTGAAACACGGACCAAGGAGAGTAACATGTGCGCGAGTCATTGGGCGTTACGAA 710
52_P0062-Mt      650 CCCGTCTTGAAACACGGACCAAGGAGAGTAACATGTGCGCGAGTCATTGGGCGTTACGAA 709
53_P0063-Mt      661 CCCGTCTTGAAACACGGACCAAGGAGAGTAACATGTGCGCGAGTCATTGGGCGTTACGAA 720
55_P0064-Mt      661 CCCGTCTTGAAACACGGACCAAGGAGAGTAACATGTGCGCGAGTCATTGGGCGTTACGAA 720
Clustal Consensus 644 ***** 703

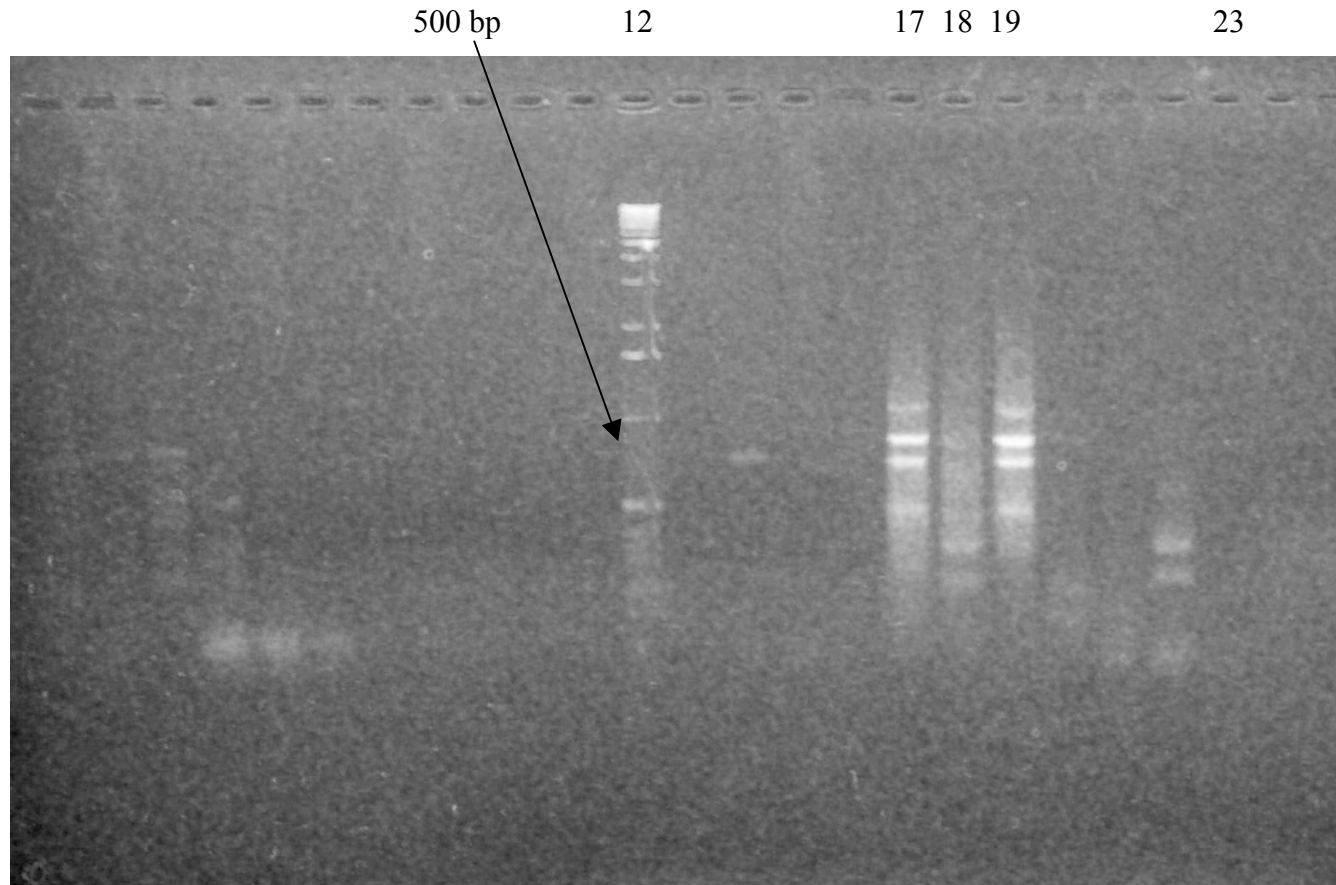
50_P0061-Mt      711 ACCCATAGGCGAAGTGAAAGTGAAGGTTTGGCTTGTCCAGACTGAGGTGAGATCCTGTCC 770
57_P0068-Mt      711 ACCCATAGGCGAAGTGAAAGTGAAGGTTTGGCTTGTCCAGACTGAGGTGAGATCCTGTCC 770
52_P0062-Mt      710 ACCCATAGGCGAAGTGAAAGTGAAGGTTTGGCTTGTCCAGACTGAGGTGAGATCCTGTCC 769
53_P0063-Mt      721 ACCCATAGGCGAAGTGAAAGTGAAGGTTTGGCTTGTCCAGACTGAGGTGAGATCCTGTCC 780
55_P0064-Mt      721 ACCCATAGGCGAAGTGAAAGTGAAGGTTTGGCTTGTCCAGACTGAGGTGAGATCCTGTCC 780
Clustal Consensus 704 ***** 763

50_P0061-Mt      771 TTTCTCATGCGCGGTACCACCAAGCTTCGAGCGGCAGGCGCATCACCGGCCCGTCCCATG 830
57_P0068-Mt      771 TTTCTCATGCGCGGTACCACCAAGCTTCGAGCGGCAGGCGCATCACCGGCCCGTCCCATG 830
52_P0062-Mt      770 TTTCTCATGCGCGGTACCACCAAGCTTCGAGCGGCAGGCGCATCACCGGCCCGTCCCATG 829
53_P0063-Mt      781 TTTCTCATGCGCGGTACCACCAAGCTTCGAGCGGCAGGCGCATCACCGGCCCGTCCCATG 840
55_P0064-Mt      781 TTTCTCATGCGCGGTACCACCAAGCTTCGAGCGGCAGGCGCATCACCGGCCCGTCCCATG 840
Clustal Consensus 764 ***** 823

50_P0061-Mt      831 ACAATTGTTTTCGATCATTACCCAGTCGGGGCGGAGCATGAGCGTACATGTTGAGACCCG 890
57_P0068-Mt      831 ACAATTGTTTTCGATCATTACCCAGTCGGGGCGGAGCATGAGCGTACATGTTGAGACCCG 890
52_P0062-Mt      830 ACAATTGTTTTCGATCATTACCCAGTCGGGGCGGAGCATGAGCGTACATGTTGAGACCCG 889
53_P0063-Mt      841 ACAATTGTTTTCGATCATTACCCAGTCGGGGCGGAGCATGAGCGTACATGTTGAGACCCG 900
55_P0064-Mt      841 ACAATTGTTTTCGATCATTACCCAGTCGGGGCGGAGCATGAGCGTACATGTTGAGACCCG 900
Clustal Consensus 824 ***** 883

50_P0061-Mt      891 AAAGATGGTGAACATATGCTTGTGC- 914
57_P0068-Mt      891 AAAGATGGTGAACATATGCTTGTGC- 914
52_P0062-Mt      890 AAAGATGGTGAACATATGCTTGTGCA 914
53_P0063-Mt      901 AAAGATGGTGAAC----- 914
55_P0064-Mt      901 AAAGATGGTGAAC----- 914
Clustal Consensus 884 ***** 897

```



Appendix 2. Various primer combinations tested for *Microphallus turgidus*. Lane 12 represents the 1 kb molecular marker; lanes 17 and 19 represent *M. turgidus* DNA; lane 18 represents uninfected *Palaemonetes pugio* DNA; lane 23 represents the negative control lane.