CRYPTIC SPECIES ANALYSIS OF *AUSTROBILHARZIA VARIGLANDIS* AND *MESOSTEPHANUS APPENDICULATUS* IN THE SALT MARSH GASTROPOD, *CERITHIDEA PLICULOSA*, IN GALVESTON BAY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Cryptic Species Analysis of *Austrobilharzia variglandis* and *Mesostephanus appendiculatus* in the Salt Marsh Gastropod, *Cerithidea pliculosa*, in Galveston Bay. (May 2015)

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Parasites, in particular trematodes (Platyhelminthes: Digenea), play major roles in the population dynamics and community structure of invertebrates on soft-sediment mudflats (Leung et al. 2009). *Austrobilharzia variglandis* and *Mesostephanus appendiculatus* are two species of trematodes that are known to infect the plicate horn snail, *Cerithidia pliculosa*, as their first intermediate host in Galveston Bay. We extracted the larvae of these two species from *C. pliculosa* collected in a Galveston marsh off Sportsman's Road, and through use of molecular genetic techniques and assessment of genetic diversity, we plan to reveal cryptic species complexes present in these trematodes. This study will help in quantifying the true trematode biodiversity of Galveston Bay by isolating multiple lineages of a single morphotype; and also hopefully help in understanding any implications these trematode lineages impose on their hosts. If successful, this study will reveal data, which will further expand on cryptic speciation within two of the trematodes known to infect humans.

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NOMENCLATURE

PCR	Polymerase Chain Reaction
COI	Cytochrome oxidase I
Miracidium	Free-living motile form of a trematode covered with cilia
Sporocyst	An elongated sac that produces rediae
Redia	A trematodes larval form containing an oral sucker
Cercaria	The infective stage and larval form of a trematode
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetic Analysis
K2P	Kimura 2 Parameter

CHAPTER I

INTRODUCTION

Cryptic speciation

Species which share the same morphological features but are distinct from each other in terms of their genetic compositions are called cryptic species. They are often classified as a single species despite presence of distinct lineages because of the lack of distinct morphological differences. They are common in the marine environment and often differ in their feeding modes, life cycles, and habitat preferences. Cryptic speciation has been reported to be common among parasites. Due to a limited range of morphological features among parasite taxa, many species exhibit similar or identical morphology; however, they may differ in host-parasite interactions (Bowels and McManus, 1995). Recent phylogenetic studies have detected cryptic speciation of digenean trematodes through the use of molecular genetic techniques (Detwiler et al. 2010; Leung et al. 2009; Miura et al. 2005). DNA barcoding is a method that utilizes relatively short sequence of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) to delineate species in a variety of animal phyla, because it tends to be conserved within species, while showing significant variation among species (Hebert et al. 2003). COI has recently been used to identify cryptic species of trematodes (Van Steenkiste et al. 2014).

Trematode life cycle

The complex life cycle of trematodes commonly follow these general stages. First, eggs are released from fecal matter and miracidium larvae are released into theenvironment. They then seek out a snail intermediate host and, once introduced to the snail, the trematode enters its

sporocyst stage. Asexual reproduction occurs in the sporocyst stage, typically during its time in the intermediate host. From there it becomes a free-living cercariae seeking out its next host. Depending on the type of trematode it can then either infect the human skin or enter an avian host. Once it enters its final host it becomes mature and is able to sexually reproduce. In the final host the cercaria migrate to portal blood in the liver and mature into adult worms. From there they migrate into the rectum where they begin to lay eggs, which will then repeat its life cycle (Lagrue and Poulin, 2007).

Parasitic digenean trematodes

Digenean trematodes are parasitic helminths which are important from a medical and veterinary perspective. They have complex life cycles and are sources of disease in amphibians, mammals, and birds. They go through several developmental stages in intermediate hosts before completing their life cycle in final mammalian or avian hosts. Mollusks such as gastropods usually serve as the first intermediate host, within which the larvae complete their miracidia, redia, and cercaria stages (Huspeni et al. 2005). In intermediary hosts such as the snail *Cerithidea pliculosa*, infection of trematodes are obtained through the ingestion of sediment. The trematodes can affect the host's reproductive system and in various occasions cause castration, as they are predominantly found within the gonads (Wardle 1987). Once the free-swimming cercaria larvae are released into the environment, they search for another host. Depending on the trematode species, second intermediate hosts can be crustaceans, polychaetes, and other mollusks. Definitive hosts in which the trematodes reach sexual maturity are primarily fish and birds . In Galveston Bay, Texas, at least 14 species of trematodes infect *C. pliculosa*, commonly known as the plicate horn snail, as their first intermediate host (Childs, 2009). In this study, genetic

analyses of two trematode morphospecies, *Austrobilharzia variglandis* and *Mesostephanus appendiculatus,* were performed to detect cryptic speciation. Both species are natural parasites of shore birds. *Austrobilharzia variglandis* belonging to the family Schistosomatidae is a medically important species, which can infect human skin and cause cercarial dermatitis, which is an inflammation and itchiness of the skin (Stunkard and Hinchcliffe, 1952). Cercarial dermatitis is a non-contagious rash caused by an allergic reaction to the infection of the parasite. The associated parasites burrow into the skin and due to lack of a suitable environment in the human host, slowly die within a few weeks. *Mesostephanus appendiculatus*, belonging to the family Cyathocotylidae, is a veterinary important species, which has been reported to infect the small intestines of canines (El-Gayar, 2007). Revealing cryptic speciation within these species will aid in recognizing their unidentified diversity, detecting multi-species infections, and possibly in understanding mechanisms of diseases.

CHAPTER II

METHODS

Collection and extraction

Cerithidia pliculosa were collected from the start of August of 2014 to March of 2015 from two separate locations in Galveston: East Beach (29.334340, -94.751792) and Sportsman's Road (29.255851, -94.916578). The snails were collected in plastic containers along with marsh sediment and water to keep them alive. For each snail the length of the shell was measured from the operculum to its apex and the number of whorls were recorded prior to dissection. The shell was gently cracked with a hammer, and the contents were transferred into a petri dish with 5mm of filtered seawater. Under a dissecting microscope, the gonadal region of the snail was gently teased apart with forceps to extract the cercariae and redia stages of the trematode. Cercariae were identified under a dissecting microscope and a compound microscope, using an unpublished dichotomous key by Wardle (1987) and photographic guides by former students working on related projects (Childs, 2009; Oldiges, 2014).

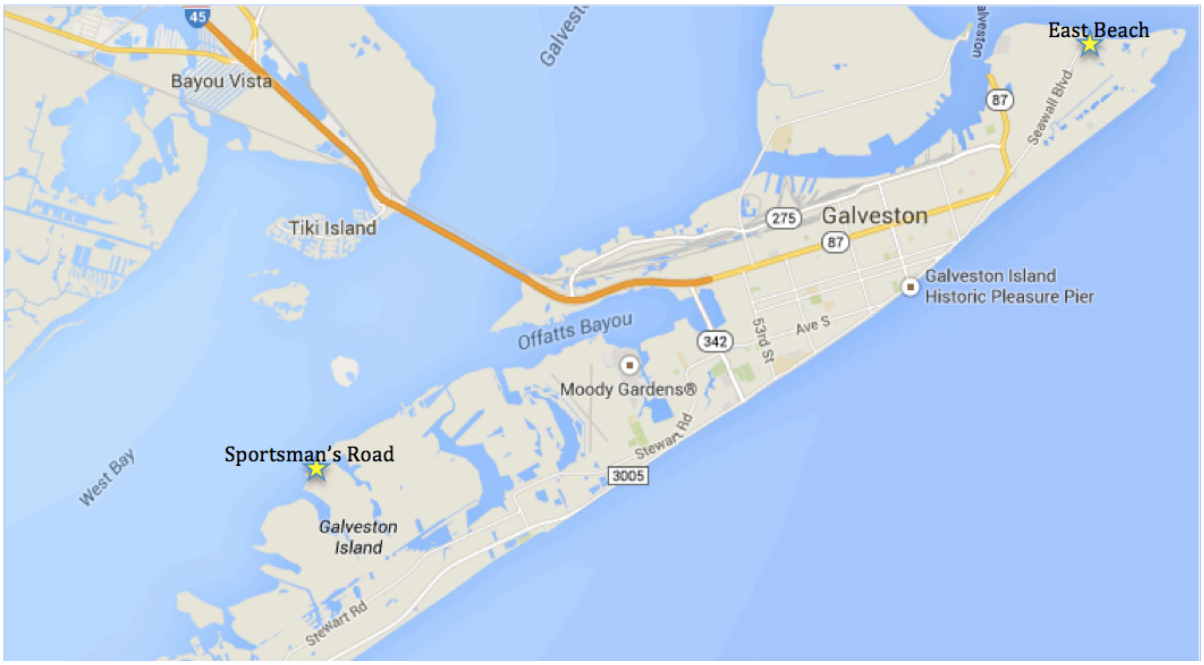

Figure 1: Galveston island map including two study sites, East Beach and Sportsman's Road.

Molecular data

DNA was isolated from 10-15 redia from each snail using the Qiagen DNeasy Blood and Tissue Kit®. DNA was extracted from cercariae in the case of double-species infection due to difficulties associated with rediae identification. PCR amplification of cytochrome oxidase I (COI) for *M. appediculatus* was conducted using JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') of Bowels and McManus (1995) as a forward primer and COI-Trema (5'-CAACAAATCATGATGCAAAAGG-3') of Miura (2005) as a reverse primer. For *A. variglandis*, two different sets of primers described in Van Steenkiste (2014) were used for PCR amplification and genetic sequencing. For PCR, Dice1F (5'-ATTAACCCTCACTAAATTWCNTTRGATCATAAG-3') was used as a forward primer, and Dice11 (5'-RTAATACGACTCACTATAGCWGWACHAAATTTHCGATC-3') was used as a reverse primer. All PCR reactions were run in a 25µL reaction mixture containing 2µL of template, 1µL of $MgCl_2$ (2.5mM), 1µL of each primer (0.4x mM), 12.5µL of GoTaq® Green

Master Mix (Promega), and 7.5μL of autoclaved Milli-Q® (Millipore) water. PCR reactions ran under the following settings: initial delay for 2 minutes at 94C°, and 35 cycles of denaturing at 45 C° for 30 seconds, annealing at 45 C° for 30 seconds, and extension at 72 C° for 45 seconds, followed by a final delay 72 C° for 7 minutes and an indefinite soak at 11 C°. PCR products were visualized with a 1% agarose gel containing ethidium bromide. PCR products were diluted 1:10 with water, and then cycle sequencing reactions were run using the BigDye® Terminator Kit v3.1 Cycle Sequencing Kit (Applied Biosystems). For cycle sequencing, T3 forward primer (5'-ATTAACCCTCACTAAA-3') and T7 reverse primer (5'-TAATACGACTCACTATA-3') were used. Products were purified with the ZR DNA Sequencing Clean-Up Kit™ (Zymo Research). Both forward and reverse sequences were sequenced on the Applied Biosystems 3130 Genetic Analyzer in the Marine Genomics at Texas A&M at Galveston.

Data analyses

Forward and reverse fragments for the obtained sequences were combined and edited, and consensus sequences were generated with Sequencher 4.2 (Gencodes). GenBank's Basic Local Alignment Search Tool (BLAST) was used to verify that the obtained sequences were indeed trematodes. Consensus sequences were aligned in BioEdit Sequence Alignment Editor. The alignments were uploaded onto MEGA Ver. 4.0 (Tamura et al. 2007), and a maximum likelihood bootstrap consensus tree (700 bootstrap replicates) was generated. Kimura 2-Parameter (K2P) distances within the ingroup and between the ingroup and the outgroup were also calculated. Outgroups for both, *A.variglandis* and *M. appendiculatus*, were selected based on their close similarities as well as information availability provided through GenBank. Trees were then edited through use of FigTree Ver. 1.4.2.

CHAPTER III

RESULTS

There was a wide range of infection rate of trematodes in *Cerithidia pliculosa* found in Galveston at locations Sportsman's Road and East Beach (Figure 2). Species *Mesostephanus appendiculatus* and *Austrobilharzia variglandis* were among the trematodes found to infect *C.pliculosa* (Figure 3). Of 61 snails collected, 14 (23%) were uninfected by trematodes. The highest rates of infection found in the study were *Mesostephanus appendiculatus* (20%), *Euhaplorchis californiensis* (19%), and *Levinseniella* sp. (16%). Double infections, or more than one trematode species, in *C. pliculosa* were rarely detected (6.5%). 10 of the 14 trematode morphospecies previously reported from Galveston Bay, TX were observed. A fragment of COI of approximately 800bp was successfully sequenced from 10 individuals of *M. appendiculatus* infecting 2 host snails and 9 individuals of *A. variglandis* infecting 1 snail. After trimming of the terminal regions, the resulting alignments were 520bp in length. *Mesostephanus appendiculatus* samples showed a K2P distance of 0.6% among them from the same snail host (Figure 4). The K2P distance between *M. appendiculatus* and *E. canadensis* was 0.587. Similarly, a low genetic variation was observed between *A. variglandis* samples as shown by 0.6% K2P distance (Figure 5). Finally the K2P distance between *A. variglandis* and *A. terrigalensis* was 26%.

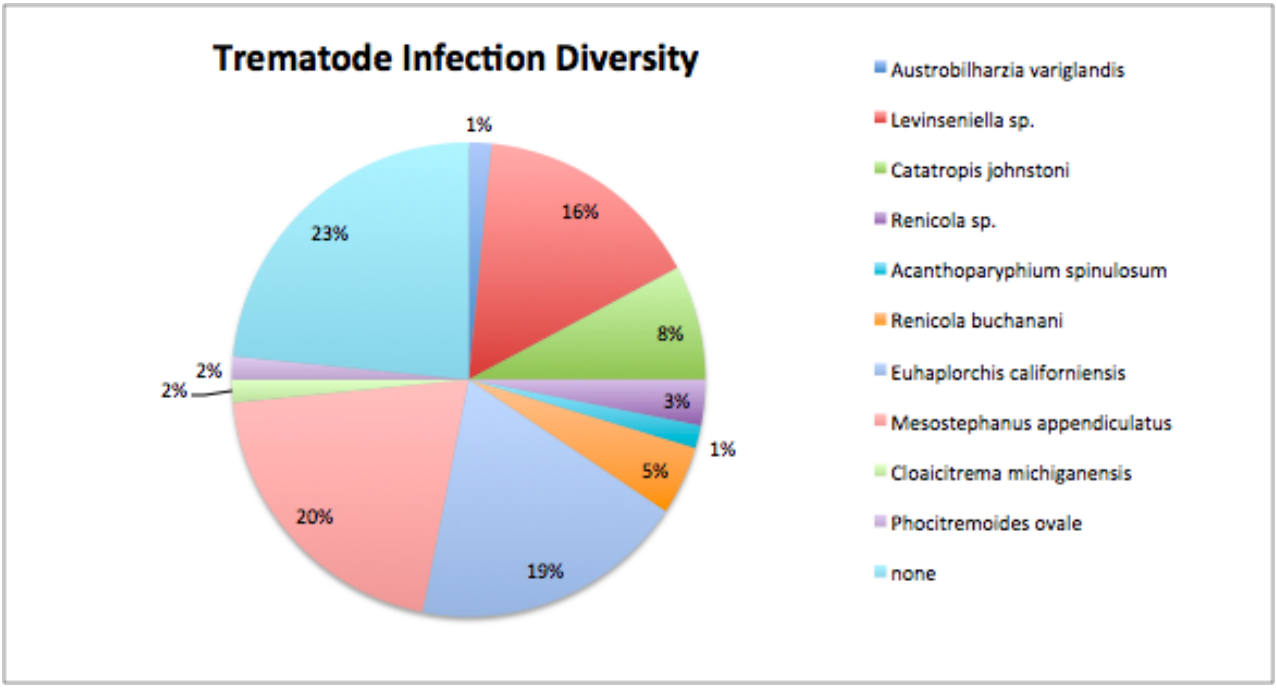

Figure 2: Trematode infection rate and diversity found within the plicate horn snail, *Cerithidia* pliculosa, in Galveston bay (n=10). Double infection not taken into account in figure.5

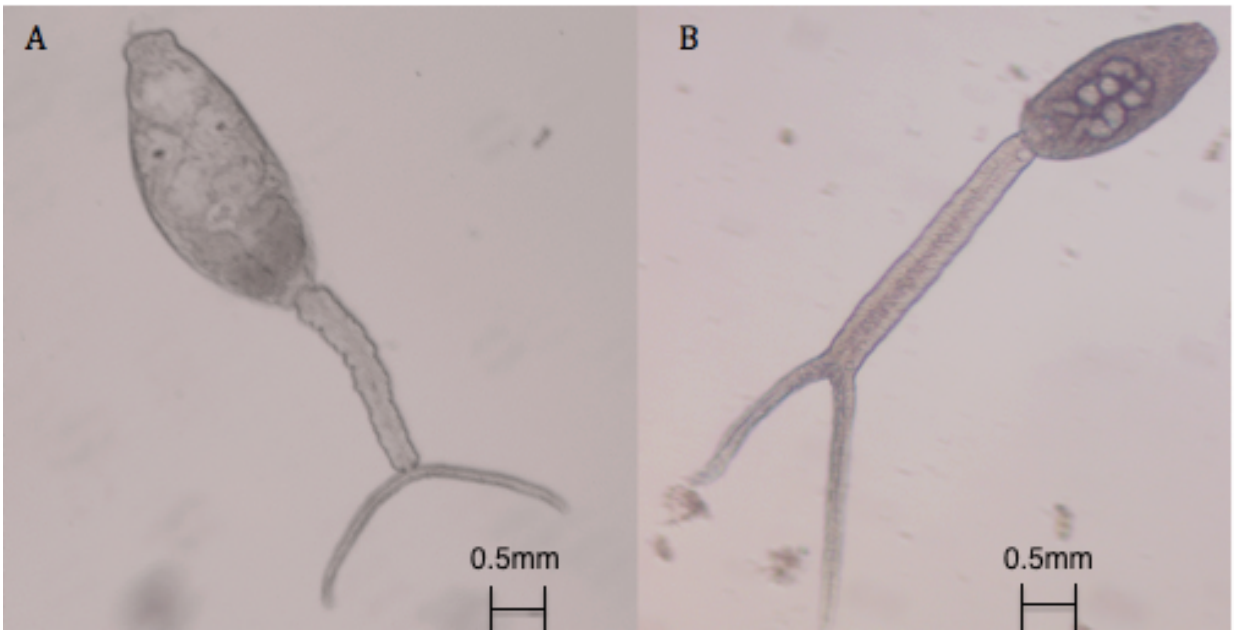

Figure 3: The two focal species of this study A) *Austrobilharzia variglandis* B) *Mesostephanus appendiculatus*.

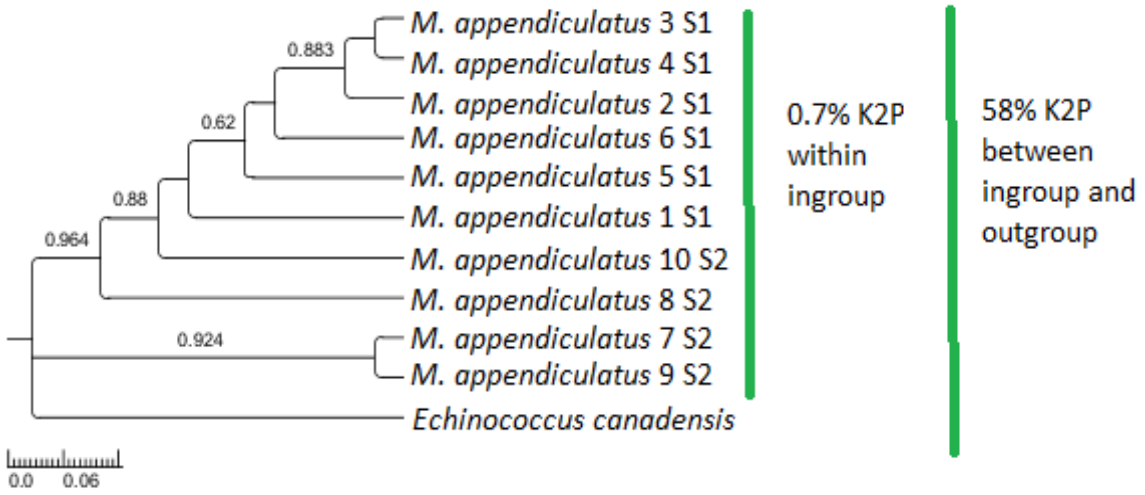

Figure 4. Maximum likelihood tree of the 10 *M. appendiculatus* sequences from two snail hosts (S1 & S2) with *Echinococcus canadensis* as an outgroup based on mitochondrial cytochrome *c* oxidase subunit I (800bp) and relevant K2P values. Numbers above nodes represent bootstrap support (<0.5 not shown).

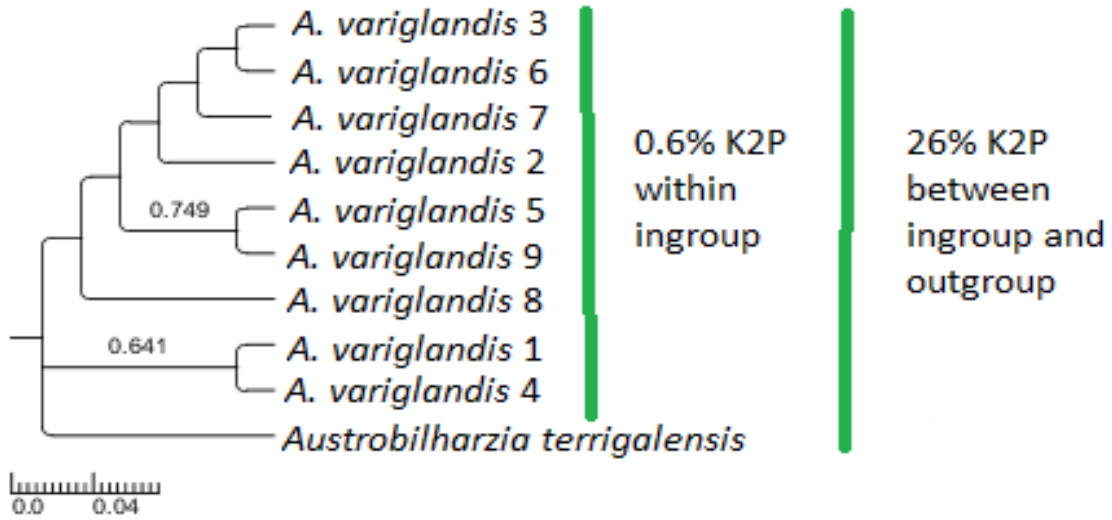

Figure 5. Maximum likelihood tree of the 9 *Austrobilharzia variglandis* sequences from one snail host with *Austrobilharzia terrigalensis* as an outgroup based on mitochondrial *cytochrome oxidase subunit I* (500bp) and relevant K2P values. Numbers above nodes represent bootstrap support (<0.5 not shown).

CHAPTER IV

CONCLUSION

Digenean trematodes are natural parasites of birds, fish, and mammals. Though species-rich, many species exhibit similar or identical morphology due to a limited range of morphological features, making them prone to misidentification (Bowels and McManus, 1995). Several studies taking place in Japan and North America in the past decade detected unique lineages within trematode populations that were once thought to be genetically homogeneous. These lineages possess unique biological and behavioral characteristics such as different life span lengths and host specificity (Miura et al. 2005; Detweiler et al. 2010). Our study did not detect cryptic speciation in either of our focal species, *Austrobilharzia variglandis* and *Mesostephanus apendiculatus,* in Galveston Bay, TX, possibly due to the small sample size; however, we detected low genetic variation among our samples collected from a single *Cerithidia pliculosa* host. In a related study conducted by Childs (2009), two haplotypes of *Phocitremaoides ovale* were detected from a single host. Traditionally, individuals of trematode infecting the first intermediate host are thought to be clones because it is the site of asexual reproduction (Miura et al. 2005; Detwiler et al. 2010); however, our results suggest that multiple miracidia belonging to one species can infect the same snail host. Therefore, trematode larvae infecting one snail host should not be assumed to be genetically identical.

The previous studies from undergraduate research scholars at Texas A&M University at Galveston yielded similar results of low success with sequencing DNA, low rates of double infection, and slight K2P distances between trematode samples (Childs, 2009; Oldiges, 2014).

These related studies found high infection rates of *Renicola* sp., *E. californiensis*, and *A. variglandis* within *C. pliculosa* in Galveston bay, which partially coincided with our preliminary data. Variation of highest infection rates could be attributed to different study sites in Galveston, small sample sizes, or possible biotic factors. Overall these two previous studies on trematodes in Galveston Bay resembled our acquired data on trematode diversity in *C. pliculosa* and results on cryptic speciation. Difficulties with this study resulted in minimal data, as the majority of our time was spent on protocol optimization. Trematodes in particular are a challenging species to work with. From the beginning, this project encountered several setbacks preventing us from achieving viable data. First, difficulties with DNA extraction had us working to fix issues with the trematode extraction protocol. It was noted that certain trematode species cooperated better with the Qiagen DNeasy Blood and Tissue Kit® protocol compared to others. PCR amplification was also slightly problematic and needed modifications. Issues with the Applied Biosystems 3130 Genetic Analyzer also showed delays in acquiring data. Inspite of these challenging setbacks, we were able to learn how to develop and customize protocols to specific trematode species.

Our study provides an initial overlook into trematodes in Galveston Bay as a foundation for future studies, on their cryptic speciation. As detailed sequences of the COI gene of most trematodes are not present in GenBank, it is important for further research to persist. DNA barcoding of trematode species would aid in facilitating the identification of these species in the future. Comprehending the diversity of these parasitic species is important in order to determine whether or not cryptic speciation of these trematodes pose further implications to their hosts as well as other impacts towards their habitat.

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