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TRIATOMINAE (HEMIPTERA:REDUVIIDAE) IN TEXAS: MITOCHONDRIAL

GENOME ASSEMBLY, *TRYPANOSOMA CRUZI* DETECTION, AND BACTERIAL

COMMUNITY ANALYSIS

by

CHISSA-LOUISE RIVALDI

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master's of Science
Department of Biology

Srini Kambhampati, Ph.D., Department Chair

College of Arts and Sciences

The University of Texas at Tyler
May 2015

The University of Texas at Tyler,

Tyler, Texas

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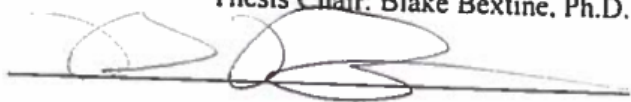
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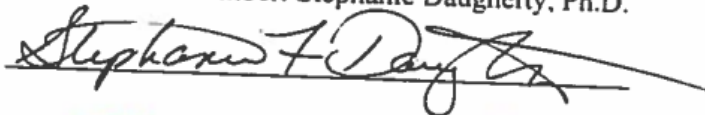
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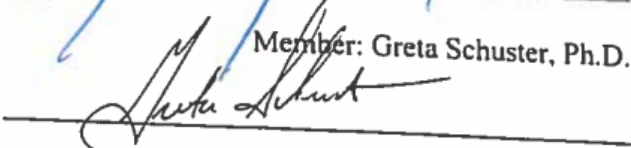
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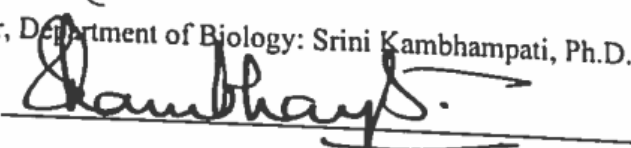
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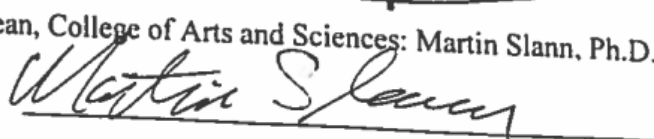
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Chissa-Louise Rivaldi

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May 2015

The genus *Triatoma* contains vectors of the protozoan parasite *Trypanosoma
cruzi*, the pathogen responsible for Chagas disease. The following research presents

several inductive studies to develop vector control approaches targeted to the genus or

species level. Two-hundred and eighty-two insects were collected, identified, and tested

for the presence of *T. cruzi*. Four species of the *Triatoma* genus were collected - the

overall incidence of *T. cruzi* was 52% (*T. gerstaeckeri* -51%, *T. lecticularia – 92%,* other,

29%). From these 282 insects, the bacterial communities of eight specimens of *T.

gerstaeckeri* were sequenced and analyzed using pyrosequencing technology. The

bacterial communities were assigned taxonomy *in silica*. Bacterial communities were

consistent with previous analyses conducted with differing methods, and had low alpha

and beta diversity, which makes this system ideal for a targeted vector control approach.

Whole genomic mitochondrial DNA was isolated from two species of *Triatoma*. This

mtDNA was then sequenced with a high-thoroughput sequencing platform. The resulting

sequence data was assembled de novo and referenced to an existing mitochondrial genome (*T. dimidiata*, the leading vector of *T. cruzi* in South America). The mitogenomes were similar, containing approximately 17 000 kbp, similar coding regions, and 30% GC content, suggesting little divergence between the species. A gene deletion in *T. lecticularia* was found when compared to both *T. dimidiata* and *T. gerstaeckeri,* which could be useful in vector control efforts. Results from this research should be used to develop and implement vector control strategies to inhibit the spread of *T. cruzi*.

Chapter One

Literature Review of the Triatominae in Texas

General

American Trypanosomiasis, commonly referred to as Chagas disease, is caused by a kinetoplastid protozoan parasite, *Trypanosoma cruzi*. This pathogen is transmitted by Hemipteran insects in the Reduviidae family, subfamily Triatominae - specifically the genera *Rhodnius, Triatoma* and *Panstrongylus*. The following study focuses on better understanding the biology of *Triatoma* in order to contribute to the pool of information concerning their role as vectors of *T. cruzi*.

Vectors

Members of the subfamily Triatominae are found almost exclusively in the North and South America (one species of the *Triatoma* genus can be found in Asia). The insects were identified as the vectors for *T. cruzi* in South America in 1909 by Carlos Chagas (Chagas, 1909). There are now approximately twenty *Triatomine* species that are recognized as capable of transmitting *T. cruzi* to humans (Figure 1.1) (Gourbiere, 2012), although some are associated with a higher epidemiological risk due to proximity to humans and domestication.

Figure 1.1. Distribution of some Triatomine vectors in the Americas. Gourbiere 2012.

In Texas, there are seven *Triatomine* species, all in the genus *Triatoma*. In this research, the species *Triatoma gerstaeckeri* and *Triatoma lecticularia* are examined; of the two, *T. gerstaeckeri* has a greater distribution and abundance (Figure 1.2)(Lent and Wygodzinsky, 1979). Both of these species are associated with human dwellings (Kjos, 2009). Habitats of *Triatoma* are located in warm climates and include large populations of mammals that serve as hosts for the insects (Stevens, 2011). The species distributions of *T. gerstaeckeri* and *T. lecticularia* include Texas, and models predicting the shift of the distributions estimate the insects' presence moving further north into Texas with no control of the vectors (Sarkar et al., 2010). The effects of climate

change are estimated to shift these distributions even further in the same direction (Garza et al.,
unpublished data), increasing the risk of transmission for reservoirs of *T. cruzi* in these areas, and
therefore increasing the risk of Chagas' disease in humans.
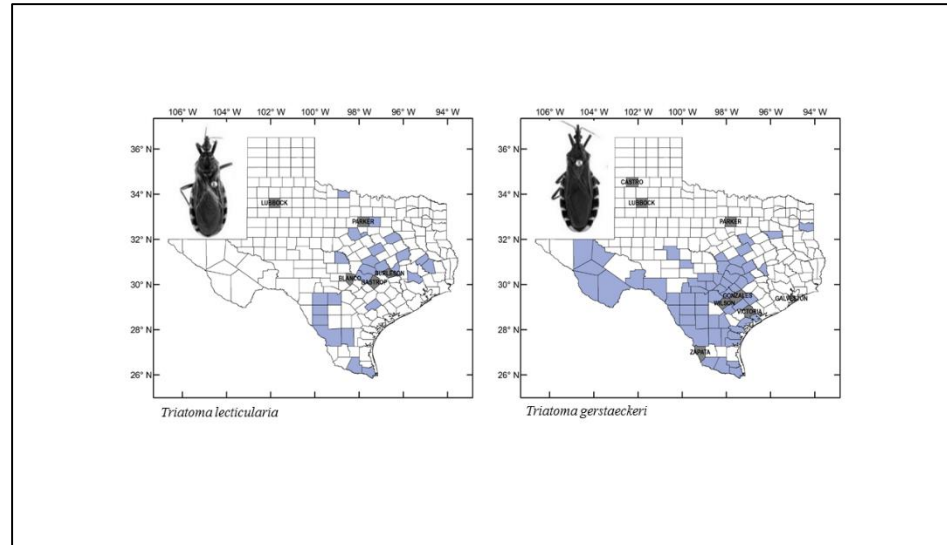
*Figure 1.2 . Two species of Triatoma in Texas and their respective distributions by
county (Left:Triatoma lecticularia, right:Triatoma gerstaeckeri). Map figures taken from Sarkar
et al.,. (2010). Counties were labeled grey to indicate new records of that species, while purple-
labeled counties were previously recorded locations. Insect pictures taken from Kjos et al.,.
2009.*

Thorough studies of the vector competence of these two species are not available, though
unpublished data and results in this study indicate greater parasite infection in *T. lecticularia*.
This species has been found much less than other species (particularly *T. gerstaeckeri*) – often as
many as ten times less – and so small sample size could contribute to distortion in numbers
representing parasite incidence.   Even so, many factors contribute to overall vector competence,
including abundance, dispersal and proximity to humans, aggressiveness when feeding, and time
between feeding and defecation (Lent and Wygodzinsky, 1979, Alirol. 2011). Many of these

elements vary between species for reasons not well understood, but interspecific variances are becoming more defined with genetic and phylogenetic studies (de la Rua, 2014). More than 50% of specimens collected in a large study in Texas in 2009 tested positive for the presence of *T. cruzi* (Kjos, 2009, Bern, 2011).

Triatoma insects are hematophagous and coprophagous. The mouthparts of *Triatoma* insects feature a segmented rostrum which is used to take a bloodmeal from the host, while simultaneously injecting an anesthetic and an anticoagulant that aids in evading detection from the host (Periera, 1998). The insects are commonly referred to as kissing bugs due to the feeding near the mouth area in humans. The insects are nocturnal, and use a variety of signals to detect potential hosts - often heat, humidity and carbon dioxide, though *Triatomine*s have been found to take bloodmeals from ectothermic reptiles and amphibians (Schofield, 1997, Guerenstein, 2009). *Triatoma* have been observed feeding on other arthropods, including *Triatoma* (Sandoval, 2010). All instars of *Triatoma* must take blood meals before molting and all instars are capable of hosting the parasite (Stevens, 2011). Upon bloodmeal intake, if the host is carrying *T. cruzi*, the parasite will enter the insect's digestive tract along with the blood. Once past the salivary glands and in the midgut, the trypomastigotes adavnce into epimastigotes. Subsequently, the trypanosomes are transported through the gut and attach to the tissue of the rectal sac, where trypomastigotes (the infectious stage of *Trypanosoma*) are released into the insect feces (Kjos, 2008). Transmission occurs when the infected feces of the insect enter the bloodstream of the host, often through the wound inflicted by the vector itself during feeding (Figure 1.3) (Stevens, 2011).
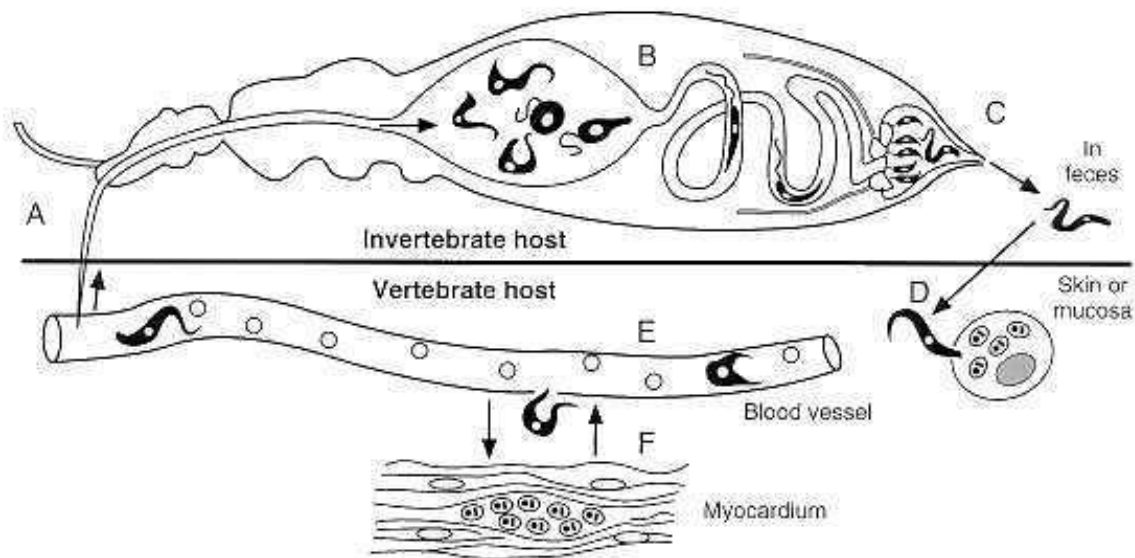
Figure 1.3. Life cycle of T cruzi in the intestine of a Triatomine bug and in the vertebrate host. After entering the bug in infected blood (A) the trypanosomes transform to epimastigotes in the stomach and midgut. B) Epimastigotes attach to the walls of the rectal sac and produce infective metacyclic trypomastigotes, which are eliminated with feces (C) and enter the vertebrate host through breaks in the skin. The parasites transform to amastigotes inside local cells (D), and multiply to release blood typanosomes, which invade other tissues (E & F) (Lent and Wygodzinsky, 1979, Rossi, 2010).

The species morphological features vary widely from one another. *T. lecticularia* is up to 23 mm in length and covered in thick setae, while *T. gerstaeckeri* has been recorded up to 35 mm in length and is glabrous (smooth, no setae). Other distinguishing morphological features of these species include dimensions of the head, the presence or absence of specific protrusions, or tubercles, on the body, and the shape, pattern, and color of the margin of the connexival plate of the abdomen (Lent and Wygodzinsky, 1979). The morphological features of the genus vary, and some polymorphisms within species can make identifying the insects with a dichotomous guide challenging. To identify species when morphological identification is inadequate, a technique

called genetic barcoding is performed. Extraction of DNA from chitinous tissue is followed by a polymerase chain reaction, in which a specific gene is targeted and exponentially replicated in a process that mimics cell machinery. For species identification, the mitochondrial Cytochrome oxidase 1 (CO1) gene is commonly used. This gene is sequenced and compared to other published CO1 sequences from other species in order to identify the species which contributed the DNA (Seifert, 2007, Park 2011).

Pathogen

Trypanosoma cruzi is a hemoflagellate kinetoplastid protozoan. In invertebrate hosts, such as *Triatoma*, the parasites take the the the non-infective stage, known as epimastigotic. When it is transferred to a mammal, i.e., it moves from the vector's feces and into the new host, it transforms into the infective metacyclic trypomastigotes. It is in this form that *T. cruzi* infects mammalian host cells. As the parasites' range of hosts is so wide, the specific mechanism of entry in to the cells is still unknown. Literature suggests an array of receptors with a common feature that the flagellate can exploit, such as electrical charge (Burleigh, 1995). Inside the host cell, the pathogen can begin to replicate. The replicating parasite is now called an amastigote, and will continue replicating until the mammalian host bursts, flooding the bloodstream with amastigotes that will search for and infect new host cells. It is during this stage that a feeding insect vector can become infected with the pathogen while taking a bloodmeal (Andrews, 1995) (Figure 3).

Triatoma become hosts for the parasite by feeding on a reservoir already infected with the protozoan. *T. cruzi* enters the insect's body via the digestive tract, and is maintained in the midgut until defecation, which occurs after the ingestion of a new blood meal. The likeliness of the

parasite entering the new host is determined by the amount of time between the bloodmeal and

defecation, as the parasite is transferred from the vector to the host through the feces of *Triatoma*

in its infective form (Figure 1.4) (Andrews, 1995).
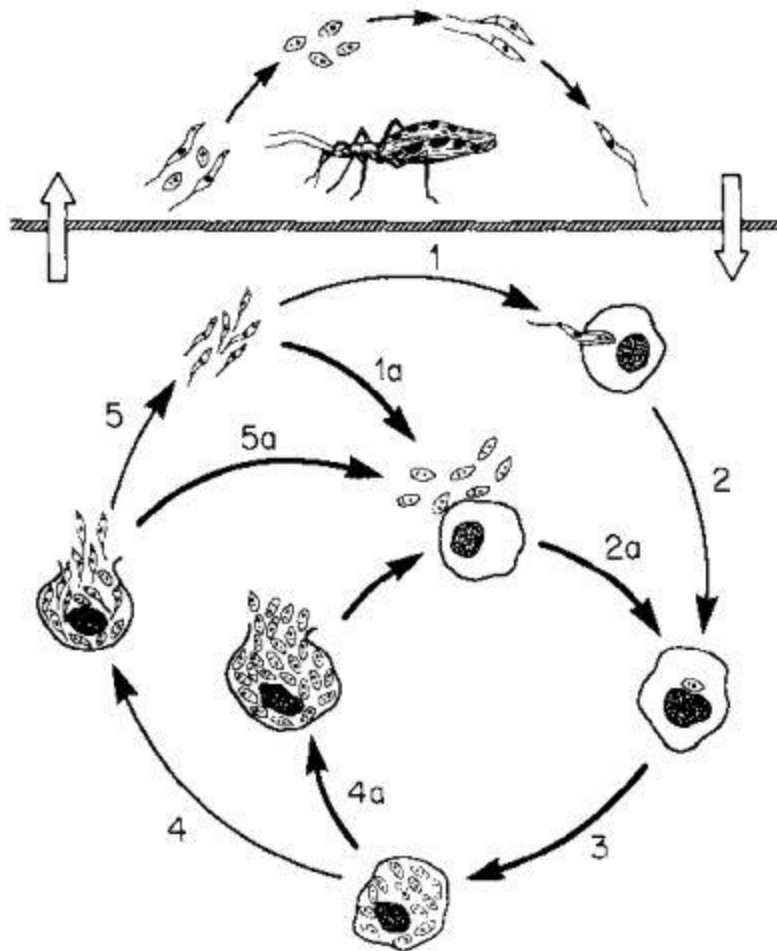
Figure 1.4. Life cycle of Trypanosoma cruzi. With a blood meal, the insect vector ingests bloodstream trypomastigotes from the host (left arrow, top), which, in the lumen of the insect digestive tract transform into replicative, non-infective epimastigotes. Epimastigotes differentiate into infective metacyclic trypomastigotes, which are released with the feces of the insect and enter the vertebrate host through the bite wound (right arrow, top). Metacyclic trypomastigotes invade vertebrate cells (step 1);escape from the vacuole and transform into amastigotes (step 2); replicate in the cytoplasm (step3); and differentiate into bloodstream trypomastigotes (step 4), which are released by rupture of the host cell (step 5), An alternative subcycle in the vertebrate can occur when amastigotes, either derived from premature rupture of host cells (steps 4a, Sa) or through extracellular differentiation of trypomastigotes (step la), are

ingested by macrophages, where they can survive and complete the intracellular cycle (Andrews, 1995).

A wide range of sylvatic mammals distributed throughout North and South America are capable of carrying the parasite. Reservoirs for *T. cruzi* include humans and non-human primates, canines, opossums, raccoons, skunks, armadillos, woodrats, and others (24 total wildlife reservoirs) (Bern, 2011). Reservoirs are mammals, though non mammal hosts (e.g., reptiles, amphibians) of *Triatoma* should be considered potential reservoirs for *T. cruzi* (Stevens, 1999). Transmission into humans, non-human primates and canines results in similar pathology (Kjos, 2009). There are six known strains of *T. cruzi* that are of epidemiological importance, named TcI-TcVI. Only two of these strains have been found in the United States in reservoirs and vectors (TcI and TcIV), and autochthonous human cases have been TcI infections (Bern, 2011, Dorn, 2007).

Disease

After the parasite is introduced into a human, there is an incubation period that ranges from one to two weeks, after which the acute phase begins. The acute phase of Chagas does not present symptoms in every human infected, and symptoms are generally mild when present (Rassi, 2010). Even in South and Central America, where the disease has a strong social presence, these symptoms are often disregarded, as the symptoms themselves are generally mild (fatigue and fever, for example), and medical care is not a viable option for all of those that do present them (Hotez, 2012). These events lead to an unknown amount of autochtonous cases going undiagnosed. During the acute phase, the protozoan is replicating throughout the host, and during this time can

be visible when serological tests are conducted (examination of tissue under microscope, culture). Absence of the parasite in microscopic exams marks the beginning of chronic Chagas disease, approximately 8-12 weeks after the initial infection (Rassi 2010). Though the defining characteristic of this phase of the pathology is an absence of the parasite from the blood, the host is still capable of transmitting the parasite (through the vector, blood donations or organ transplants). The parasite encysts in cardiac or digestive muscle tissue and enters the amastigote phase, which renders the parasite much less vulnerable to attack. Chronic Chagas disease can cause severe damage in the myocardial tissue, often leading to death (Figure 1.5) (Rossi, 2010). Digestive organs are also susceptible to damage from the parasite, including a wide range of manifestations of damage, from megaesophagus to colon failure (de Oliviera, 1998).

Diagnostic tests during acute Chagas disease can be effective, but the chronic stage often relies on more tests and still yields uncertain results (Rassi, 2010). Many people that are at risk for Chagas do not have easy access to healthcare, and treatment, when available, is expensive. Only two treatments are available, neither of which are approved by the FDA and both of which have high toxicity and low efficacy (benznidazole and nifurtimox) (Coura, 2002). Furthermore, in the United States, there is a social stigma that associates Chagas with immigration, so individuals that might be infected could intentionally avoid what diagnosis or treatment is available (Hotez, 2012). Blood donor screening for *T. cruzi* is becoming a more important diagnostic tool, but is not mandatory for collection facilities (Garcia, 2014). In the years 2008 to 2012, the blood collected from about 1/6500 donors in Texas tested positive for *T. cruzi* (Figure 1.5)(Garcia, 2014).
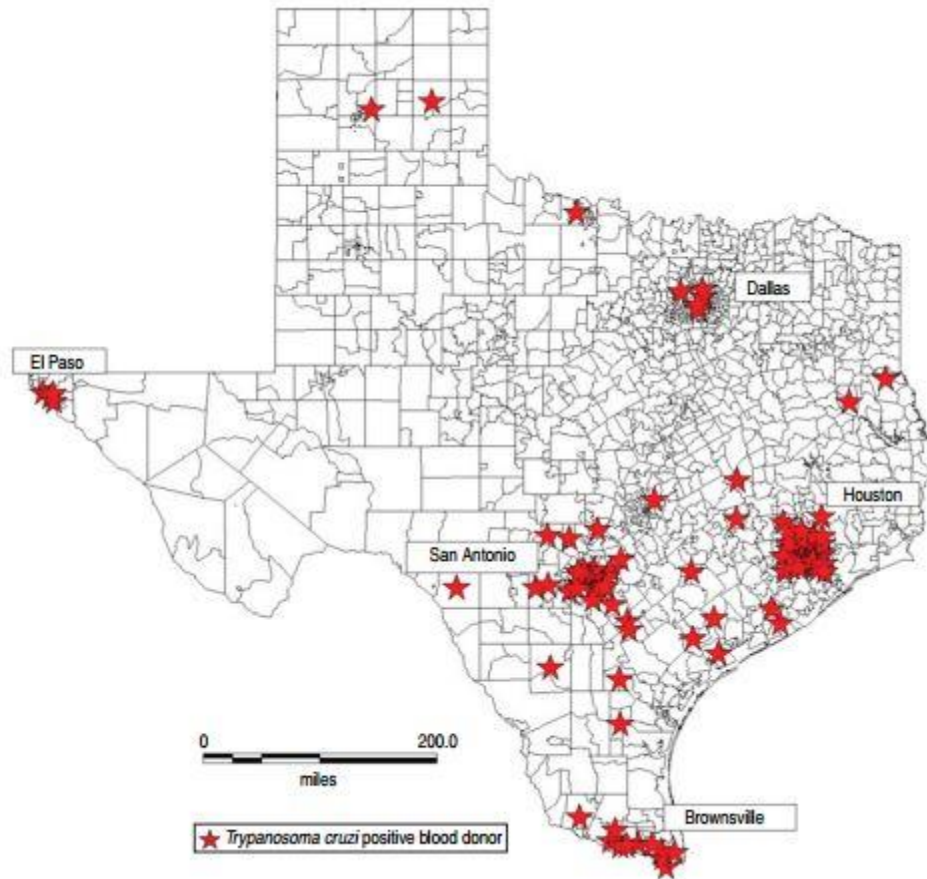
Figure 1.5. Distribution of T.cruzi *infected blood donors in Texas. (Garcia 2014).*

The epidemiological challenges presented by Chagas disease have placed importance on vector control in affected areas. The Southern Cone Initiative (South America) was successful in reducing the incidence of Chagas disease by implementing vector control through a combination of pesticide use and public education (Dias, 2007). Anthropogenic methods have been implemented as well, such as moving woodpiles, which house mammalian reservoirs, further away from human dwellings and replacing thatch roofing with tin or concrete roofs. Other approaches, such as paratransgenic methods and manipulation of symbiotic bacteria, are being explored in order to further combat this disease (Schofield, 1997). While these methods have been effective

where circumstances allow full implementation, any additional negative impact on the effectiveness of the vector could help control the spread of the disease (Schofield, 1997).

Bacterial community analysis:

The symbiotic bacteria found in many arthropods, specifically insects, increase fitness of the arthropods by conferring reproductive or physiological advantages, such as microbial metabolites supplementing an arthropod specialized diet. Bacteria required for the insects to live are categorized as primary endosymbionts; other facultative bacteria which may have more recently developed symbioses with the insect are secondary endosymbionts. Primary endosymbionts are vertically transmitted and have co-evolved with the insect hosts; their presence is essential for the survival of the arthropod host and elimination of the organism has a deleterious effect on the host (Nogge, 1976). They reside in specialized cells called bacteriocytes or mycetocytes that can be part of larger structures such as the bacteriome or mycetome (Moran, 2001). The isolation and protection of primary endosymbionts has resulted in their genomes being degraded and losing genes required for independent life (Thao, 2000, Moran, 2001, Oliver, 2003, McLean, 2010). The bacteria *Wolbachia* (Alphaprotiobacteria) control phenotypic aspects of invertebrates that have only recently been studied (Werren, 2008). Some of these effects are behavioral & interfere with reproduction and have been found in hemipteran insects (Negri, 2006). In one example, the leafhopper *Zyginidia pullula* displays a femininizing effect in the presence of *Wolbachia,* removal of the bacteria allows the male phenotype to reappear (Negri, 2006).

Manipulating the effects of endosymbionts, as well as the endosymbionts themselves, and targeting proteins such as endoglucanases (Arthrobacter luteus β-1,3-glucanase, as well as lyticases, purified from Anterobacter) are effective tools to decrease *T. cruzi*'s ability to take hold in the *Triatomine* gut (Jose, 2013). These endoglucanases disrupt the linkages in the surface

glycoproteins found on the membrane of *T. cruzi*. More research on these glycoproteins is needed, possibly directly targeting their role in *T. cruzi*'s ability to form cysts in cardiac muscle tisxue, as well as esophageal or colon muscles. Another potential method of vector control is to manipulate the bacteria that are required for an insect to molt to the next instar. Nymph *Triatomine* insects receive obligate symbiotic bacteria through coprophagic activity of adult *Triatoma*. Studies that limit or completely inhibit the population of these bacteria by prohibiting coprophagic activity report nymph insects that fail to molt and become adults (Hill, 1976, Beard, 1998).

Previous methods of surveying arthropod bacterial communities have relied on culture dependent assays involving cultivation (Beard, 1998, Da Mota, 2012). While effective for many types of bacteria, these methods prevent detection and identification of bacteria that cannot be cultivated in vitro due to the limitations of their physiology. These bacteria cannot survive outside of the host, and therefore have evaded past analyses. Missing key components of a bacterial community could be identified through deductive reasoning, but the variation of possible bacteria renders this methodology inefficient in terms of both time and cost. More recently, PCR amplifications have been utilized with a universal primer set to amplify the barcoding 16S ribosomal gene, which is ideal in bacteria classification due to its conservation and the large existing database of published sequences (RDP,SILVA,GreenGenes) (Da Mota, 2012). The PCR products are separated using a denaturing gradient electrophoresis gel (DGGE) after which they are removed, purified, and sequenced (Muyzer et al., 1993). This methodology is useful in obtaining an overall view of the composition of the bacterial community, and has the advantage of not requiring the successful culture of bacteria in order to obtain accurate results. There are disadvantages to this approach, including difficulty in replication, time consumption, and

decreased accuracy due to the nature of gels (multiple bands may look like smear of one) imposes a degree of subjectivity on results.

A more recent approach involves next-generation 454 pyrosequencing (Schuster 2008). This technique records sequences of DNA through the detection of light created by one of four reactions that occur when a specific nucleotide is added to a growing DNA strand. High thoroughput sequencing is performed on the product of a 16s PCR amplification similar to the DGGE analysis previously described. The advantages of 454 sequencing include increased accuracy, parallel sequencing, reduced time and automation, particularly when compared with traditional Sanger sequencing (Schendure, 2008).

Analyses of bacterial communities use many bacterial sequences as input (sequences from 454 reads number in the thousands), and cluster them together based on similarities in their sequences – many parameters may be set, but commonly a 97% sequence similarity is chosen that will separate sequences into species. Sequences from these clusters are then compared to a database of 16S sequences and assigned taxonomy by comparison to an existing 16S database. Highly accurate fingerprints of the bacterial community in a specific environment can be acquired in this way and used to understand relationships between vectors and pathogens.

Mitochondrial analysis

The practice of using mitochondrial DNA to identify species is well established, particularly in barcoding analyses, which taxonomically identify a specimen by comparing a marker gene from the specimen to a database of sequences of the same gene (Hebert, 2003). In arthropods, a common mitochondrial gene used is the Cytochrome Oxidase I gene (COI) – this gene has been used to identify hemipterans and their phylogenetic relationships with success (Park,

2011). In *Triatoma,* morphological identification of species can be ambiguous, and molecular methods can be employed to decrease uncertainty (Lent and Wygodzindky, 1979). The use of genomic mitochondrial DNA has numerous advantages over nuclear DNA for identification and phylogenetic analyses, as well as population genetics and molecular evolution. The high mutation rate in non-coding regions, the lack of sexual recombination, and the simple genetic structure are all advantageous properties of using genomic mtDNA for the identification and analysis of such closely related species as the members of *Triatoma* in Texas (Aviles, 1987, Kjos, 2009). The comparison of the mitochondrial genomes of the *Triatoma* species in Texas has not been made, though all species have varying infection rates (Kjos, 2009). This study aims to identify differences in the genomic mtDNA of *T. gerstaeckeri* and *T. lecticularia,* as the most abundant and infectious species in Texas, respectively, and therefore the most logical choice for vector control.

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Chapter Two

Survey of Trypanosoma Incidence in Texan Triatominae

Introduction

Seven species of the genus *Triatoma* have been recorded in Texas to date (Lent and

Wygodzinsky, 1979). The most abundant is *T. gerstaeckeri*, followed (by most estimates) by *T.*

sanguisuga and *T. lecticularia*. *Triatoma indictiva*, *neotoma*, *recurva*, *protracta*, and *rubida* are

species that are found less often and with lesser distribution than the three major species (Sarkar

et al., 2010).

The *Triatoma* genus is of epidemiological importance because it belongs to the subfamily

Triatominae. Members of this subfamily feed on blood and are vectors for the hemoflagellate

kinetoplastid protozoan *Trypanosoma cruzi*. This pathogen causes American Trypanosomiasis,

or Chagas Disease in humans, which is recognized by cardiomyopathy and digestive organ

failure, ultimately leading to death (Laranja, 1956). Approximately 70% of those infected

become asymptotic carriers, which makes estimates of people affected by the disease difficult to

accurately obtain (Rassi, 2010). Non-human primates and canines are also at risk, canines

particularly so in Texas (Kjos, 2008) (Figure 2.1).

Though the pathogen and its effects on humans have been well studied, there is no cure

for the disease, and, if diagnosed, medication is invasive and expensive (Bern, 2011). Climate

change has been predicted to shift the distributions of *Triatomine* vectors northward further into

in United States (Garza, 2014). These factors combine to make Chagas a potentially devastating

disease. Vector control, such as increased and targeted pesticide use, alongside public education,

has been the most successful approach in combating further spread and reducing transmission in

endemic areas (Dias, 2007). More targeted vector control is important as a method of

elimination as well as prevention for continuing this work in current and projected geographic

locations.
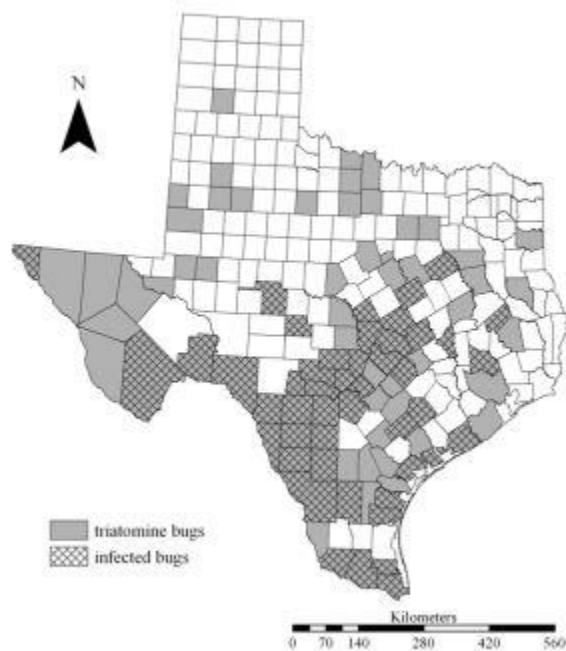
Figure 2.1. Distribution of insects in Texas counties. Counties where T. cruzi positive insects were found are shown in patterned counties. Kjos 2007.

Methods and Materials:

Adult insects were collected in a variety of locations in Kleburg County, Texas and

stored in 95% ethanol. Morphological identification of the insects was performed by two

researchers according to Lent and Wygodzinsky, *Triatoma* of the United States (Lent and

Wygodzinsky, 1979). Insects were dissected using a sterile scalpel to remove the caudal part of

the abdomen. Post dissection, DNA extraction was performed using either a 2% cetyltrimethyl

ammonium bromide (CTAB) extraction protocol described by Crosslin et al. (2011) or with the

Qiagen DNeasy Blood and Tissue extraction kit (Qiagen, Valencia, CA), following the

manufacturer's instructions for animal cells. Concentration of extracted DNA was measured

using a NanoDrop 1000 (Thermo Scientific). Extracted DNA was suspended in molecular grade

water.

Four sets of PCR primers were used for a subset of the samples – primers and

thermocycler programs are listed in Table 2.1. Each 25.0 µL reaction included the following:

12.5 µL of AmpliTaq Gold 360 master mix (Applied Biosystems, Foster City, CA), 1.0 µL GC

enhancer (Applied Biosystems, Foster City, CA), 2.5 µL primer (forward and reverse), 5.5 µL

H_2O, and 1.0 µL template DNA. For sequencing reactions, all *T. cruzi* incidence was determined

by detection of the amplification of the 18SSU ribosomal gene. All specimens were tested for

the presence of *T. cruzi* using PCR detection of the 18SSU ribosomal gene. For the 18SSU

amplification, a nested PCR protocol was used. After PCR was conducted using the 18S

external primer set, a 10:1 dilution step was performed on the PCR product from the first round

of amplification and used as the template DNA for the second round. The 18S internal set of

primers was used in the same protocol as the first round. (Noyes et al.,. 1999).   A negative

control was used in all PCRs (water as template), as well as all DNA extractions.

The positive control for all PCR amplifications was DNA extracted from a *Triatomine*

specimen from one field site. In preliminary trials, the specimen tested positive for *T. cruzi* in

PCRs for all primers used in this study. To verify, the PCR product from a reaction using 18S

primer was sequenced (Sanger sequencing, Yale Sequencing Core) and compared to published

sequences in BLAST (ncbi.gov).

Detection of *T. cruzi* was determined by gel electrophoresis of the amplicon of the 18SSU internal gene. A 1% agarose gel stained with 5.0 µL of Ethidium Bromide was used for electrophoresis. Bands were detected visually on a UV imager.

Target Gene	Sequence	Amplicon Size(bp)	Reference
Glutathione Peroxidase	F-5'-CGT GGC ACT CTC TCC AAT TAC A-3' R-5'-AAT TTA ACC AGC GGG ATG C-3'	360	Lauthier et al.,. 2012
Kinetoplastid Minicircle 67/34	F-5'-ACA CCA ACC CCA ATC GAA CC-3' R-5'-TGG TTT TGG GAG GGG SSK TC-3'	122	Sturm, 1989, Ramsey et al.,. 2012
18SSU External	F-5' CAG AAA CGA AAC ACG GGA G-3' R-5'-CCT ACT GGG CAG CTT GGA-3'	927	Noyes et al.,. 1999
18SSU Internal	F-5'-TGG GAT AAC AAA GGA GCA-3' R-5'-CTG AGA CTG TAA CCT CAA AGC-3'	700	Noyes et al.,. 1999

Table 2.1. Primers used for the identification of T. cruzi in collected Triatoma specimens

Results:

Species collected in this study were as follows: *T. gerstaeckeri*-254, *T. lecticularia*-13, *T. indictiva*-6, other/unknown-13 (specimens were either mutilated or damaged as to be unidentifiable by morphological characteristics, while others did not yield a unanimous result from researchers using the Lent and Wygodzinsky key). Out of 282 adult specimens tested for the presence of *T. cruzi* (4 were not tested due to extraction difficulties), 147, or 52%, returned positive results. Males had a 52.8% positive incidence (Tc$^+$) and females had a 52.4% incidence (Figure 2.2).   When incidence was compared by location, the highest percentage of Tc$^+$ insects were obtained from the Texas A&M University-Kingsville (TAMUK) rabbit facility – an area of the TAMUK property in which a large concentration of rabbits are bred and housed, although it is not known how far away from the rabbits the insect specimens were collected (Figure 2.3).

Two other collection sites have names which suggest high concentrations of mammal (TAMUK ungulate facility and deer pens), but, as with the case of the rabbit field site, it is unknown whether or not the insects were collected in close proximity to the animals. The species with the highest percentage of Tc$^+$ insects was *T. lecticularia* (92%) (Figure 2.4), while *T. gerstaeckeri* Tc$^+$ was 51%. None of the six specimens of *T. indictiva* tested postitive for *T. cruzi*.
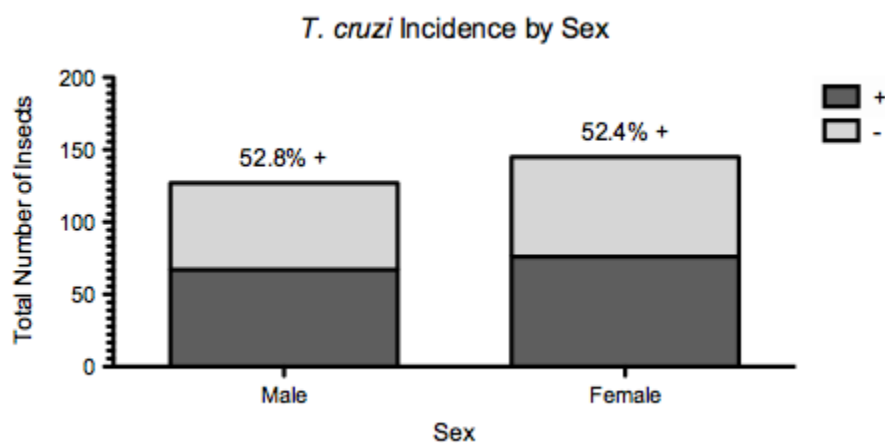
Figure 2.2. Stacked barplot showing percentages of infected Triatoma compared by sex.
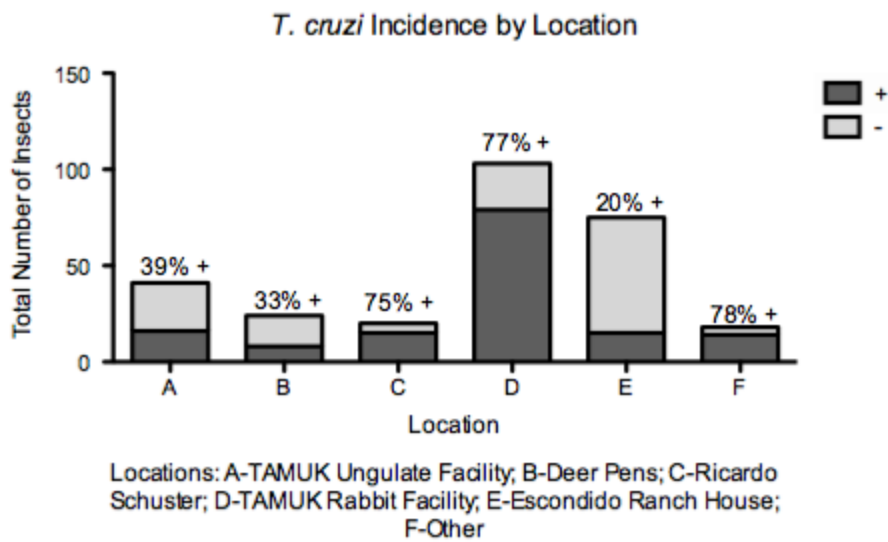
Figure 2.3 Stacked barplot showing percentages of infected Triatoma compared by location.
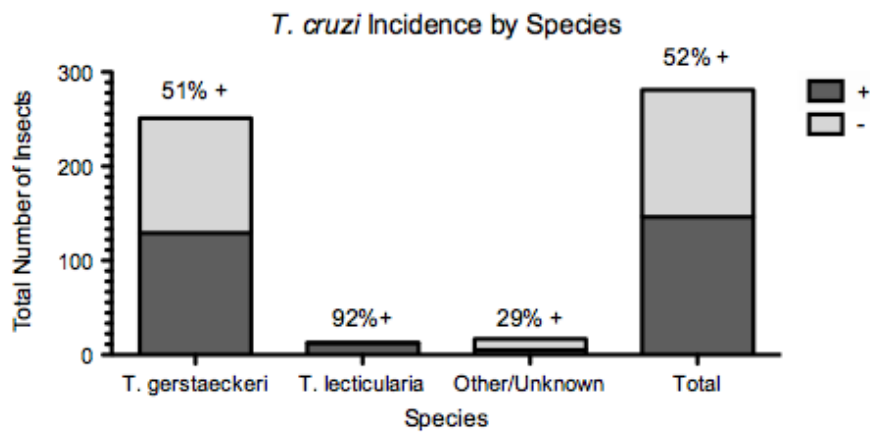
Figure 2.4. Stacked barplot showing percentages of infected Triatoma compared by species.

Discussion:

The overall incidence of *T. cruzi* in *Triatoma* populations is reflective of recent similar studies, but geographic location should be taken into consideration (Kjos, 2009, Bern 2011). Previous surveys tested insects from a more expansive study area (though still in Texas), and thus specific pathogen incidence for the area tested in this study cannot be directly compared. All locations in this study were contained roughly within the same area (all within Kleberg County, collection sites included rabbit cages, deer pens and personal properties). Replicating this study in the future would provide information regarding distribution of the pathogen and the insect and could provide real world data to supplement published distribution models (Sarkar et al., 2010, Garza et al., 2014).

Analysis of location-based variance of Tc$^+$ insects from identification of the feeding hosts from the insects (CO1 genotyping of blood meals). The higher incidence of *T. cruzi* near rabbit populations versus populations of ungulates/deer may be explained by the knowledge of the source of the bloodmeal. Sequencing insect and pathogen genes and comparing genotypes to host preferences could identify correlations and relationships between bloodmeal preference and vector competency. Exhibiting a preference towards a host that comes into more contact with humans would increase vector competency, such as rabbits often handled by humans (as opposed to deer, which generally have little human contact). Links between host preference and vector competence have been found in mosquitos, which would make further study of these systems in Triatominae a logical step in targeted vector control (Severson, 1994).

The higher incidence rate of *T. cruzi* in *T. lecticularia,* if not a result of a biased sample size, may be attributed to physiological differences between the species (e.g., bacteria hosting, host preference). The low abundance of this species may offset this high capacity for pathogen hosting. *Triatoma indictiva* showed no presence of *T. cruzi* in this study, though such a small sample size likely does not indicate much regarding the species's ability to carry *T. cruzi*. Though the complete phylogeny of the Texas genera has not been agreed upon, it is more or less accepted that *T. sanguisuga* and *T. indictiva* are closely related, and *T. sanguisuga* is considered one of the more important epidemiological vectors in the United States (De la Rua, 2014). Species have been studied in regards to their ability to transmit pathogens in a laboratory environment previously, but many things, including season of activity and preference for urban versus sylvatic environments factor into a species overall vector competence (Turell, 2001). For example, bedbugs (family Cimicidae) have been reported to transmit *T. cruzi* in a laboratory setting, but anthropogenic factors, low chance of feeding on reservoirs, and behavior diminish their competence to act as vectors. Collection and analysis of the *Triatoma* species found in the United States should be continued to better understand their epidemiological importance as vectors of *T. cruzi*.

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Chapter Three

Bacterial Community Analysis of Triatoma gerstaeckeri

Introduction:

 Triatominae, the subfamily of the hemipteran family Reduviidae, are commonly referred to as kissing bugs, and are found in North and South America, though the only genus found in Texas is *Triatoma* (Lent & Wygodzinsky, 1979). Seven species of *Triatoma* have been recorded in Texas, of which *Triatoma gerstaeckeri* is the most abundant (Kjos et al., 2009). *Triatoma* are obligate hematophagous insects for which mammals are common hosts, although reptiles and other insects can be used as a food sources. Coprophagy is also employed in the feeding cycles of *Triatomine*s, and serves as a mode of transmission of symbotic bacteria between insects of different generations (Beard et al., 2001). These insects are of medical and veterinary importance, as they are a primary vector for the kinetoplastid protozoan *T. cruzi* to mammalian hosts (Chagas, 1909). The close proximity of mammals to many humans (particularly those living in rural areas due to rodents in wood piles and thatch roofing, for example) increases the probability of transmission of *T. cruzi* by attracting the presence of *Triatoma* insects. This flagellate parasite reproduces inside the digestive tract of the *Triatomine* insect and is deposited onto the surface of the host during defecation. The protozoan moves into the body of the host through the wound made by the feeding *Triatoma*, and circulates through the body of the host until it is picked up by another feeding *Triatoma*. Reservoirs for *T. cruzi* include woodrats (*Neotoma*), skunks, raccoons, squirrels, and others (Charles et al., 2013). In humans, this pathogen is the etiological agent for American trypanosomiasis, or Chagas disease. Nearly 10% of humans that become infected with

T. cruzi experience symptoms in the acute stage. *Trypanosoma cruzi* replicates in the bloodstream of the host, and in, patients diagnosed with chronic Chagas disease, can accumulate in the cardiac muscle or the smooth muscle of the esophagus or colon. The resulting myopathy culminates in organ failure and/or death after a varying amount of time (20-30 years is commonly cited) (Rassi, 2010). Death from infection of the parasite is also widely recorded in dogs and non-human primates (Kjos et al., 2008). No vaccination, immunization, or permanent treatment for Chagas disease exists and, due to the asymptomatic nature of the disease, infections often go undiagnosed (Bern and Montromery, 2009). It is unknown how many deaths are the result of an undiagnosed trypanosomiasis infection. Treatment is often too expensive and difficult to undergo for the people that are most at risk for *T. cruzi* infection (Castillo-Riquelme, 2008, Bern and Montgomery, 2009, Hotez, 2012). The disease affects an estimated seven to eight million people, and causes an estimated 10,000 deaths annually (WHO 2014).

Vector control is an effective approach to decrease parasite exposure. Programs are enacted by governments to reduce the amount of thatch roofing and increase the distance between residences and woodpiles (both are ideal habitats for *Triatoma* colonies), and pesticides are being used more frequently and correctly (Diaz, 2007). The Southern Cone Initiative has been successful in reducing transmission by controlling populations of *Triatoma infestans* and screening blood donors in Argentina, Brazil, Bolivia, Chile, Paraguay and Uruguay, though resistance to pesticides is increasing (Diaz, 2007). Paratransgenic control of *Triatoma infestans*, in which an obligate endosymbiont is introduced for the purpose of preventing the maturation of the vector, is being explored currently, which could aid ongoing vector control mechanisms (Hurwitz et al., 2011, Durvasula, 2007).

Another approach is to decrease the competency of the vector to transmit the pathogen. This can potentially be accomplished by way of making the vector gut hostile to *T. cruzi*. Manipulation of the bacterial community in the *Triatomine* insects has been demonstrated as a means of achieving this goal (Beard et al., 2001). Previous studies have explored and presented these mechanisms in detail, including the genetic modification of obligate endosymbiotic bacteria found in some members of the *Triatominae* subfamily (Beard, 2007).

Though bacterial communities are increasingly being explored and exploited for this purpose, much of the prokaryotic contents of the gut of North American members of *Triatominae* remain unstudied. Until relatively recently, identification of bacterial communities was dependent on cultivation. Since only a small percentage of bacteria are identifiable through these methods, accurate views of these systems were limited by the methods used to obtain them (Pace, 1997). Cultivation-independent methods, such as polymerase chain reaction analysis (PCR) are increasingly more affordable and reliable, and eliminate problems of neglecting the identification and cataloging of bacteria that cannot be cultured. PCR of the 16S ribosomal subunit gene has been used to survey the bacterial community in *Triatoma* previously; results were determined using denaturing gel gradient electrophoresis (DGGE) (Da mota, 2012). The same gene is used in this analysis combined with next-generation sequencing techniques in order to identify bacteria present in smaller numbers. In similar pathogen transmission systems, bacteria have been initially identified using this same methodology, allowing greater insight of the relationships between bacteria and host (Hail et al., 2012). These methods are employed here, for the purpose of the exploration of the symbiotic bacteria of *T. gerstaeckeri* and have been used to gain a better understanding of other related taxa (Arp et al., 2014, Hail et al., 2011, Powell 2014). This study is designed to produce data that may be used to generate future hypotheses.

Methods and Materials:

Preparation of samples:

Insects were collected from Kingsville, Kleberg County, TX, US, from the Texas A&M-Kingsville campus (rabbit enclosures) and nearby residences. Upon receipt, insects were stored in 95% EtOH at -20° C. Insects were morphologically identified following Lent & Wygodzinsky (1979), and molecular identification was performed by PCR of the mitochondrial cytochrome oxidase 1 (CO1) gene followed by Sanger sequencing (Yale) (Park et al., 2011). Information regarding the sex of the insect and presence of a bloodmeal was recorded before and during dissection. Samples were chosen for microbiome analysis based on overall integrity of sample (e.g., freshness). Dissection was performed by removing a posterior segment of the abdomen with a sterile scalpel blade – this posterior tissue was used for extraction and downstream analyses. A blood meal was determined present if the abdomen of the insect contained visible blood when the dissection was performed. A total of eight insects were selected for bacterial analysis.

Molecular preparation of samples:

Post dissection, DNA extraction was performed using a 2% cetyltrimethyl ammonium bromide (CTAB) extraction protocol described by Crosslin et al.,. (2011). Concentration of extracted DNA was measured using a NanoDrop 1000 (Thermo Scientific). Samples were suspended in molecular grade water. The CO1 region of each insect was amplified by PCR consisting of 1.25μL of 10x PCR Buffer, 0.0625μL of 10mM dNTP, 0.625μL of 50mM MgCl, 0.125μL of 10μM forward primer (LepF1 5'-ATT CAA CCA ATC ATA AAG ATA TTG G-3'), 0.125μL of 10μM reverse primer (LepR1 5'-TAA ACT TCT GGA TGT CCA AAA AAT CA-3') 0.06μL of Platinum® Taq DNA Polymerase (Invitrogen, CA), >10ng <100ng of template, and

raised to 12.5μL total volume with ddH$_2$O. Thermal cycling was performed as followed; 2 min at 95°C; 5 cycles of 40 sec at 94°C, 40 sec at 45°C, 1 min at 72°C; 35 cycles of 40 sec at 94°C, 40 sec at 51°C, 1 min at 72°C; 5 min at 72°C; held at 4°C (Park et al.,. 2011). Amplicon sequencing was completed by the Yale sequencing facility on Science Hill (New Haven, CT). Sequences were trimmed for quality and aligned to generate a consensus sequence, then screened against BlastN (Madden, 2002).

Sequencing and Analysis**:**

Sequencing: Four hundred nanograms of purified DNA from each sample were sent to the sequencing facility (Research and Testing Laboratories, Lubbock, TX) where pyrosequencing took place. Concentrations were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and diluted with sterile nuclease free water. Pyrosequencing of the 16S gene was performed as described by Powell (Powell, 2014). Primers used for 16S pyrosequencing were Gray28F 5'GAG TTT GAT CNT GGC TCA G 3' and Gray519R 5' GTN TTA CNG CGG CKG CTG 3' (Hail et al.,. 2010, Hail et al.,. 2012). One specimen (T8) could not be amplified and is hereafter excluded from the following analyses.

Data analysis: Quality trimming, denoising and chimera detection/elimination were performed by the sequencing facility. Quality trimming eliminates data in the form of sequence reads based on quality scores of the base pairs of the sequences. Chimeras, which can occur in DNA amplification and sequencing reactions, must be removed to avoid confusion with true sequences. Chimeras were detected using a *de novo* method of UCHIIME and subsequently removed by the sequencing facility (Edgar, 2011). The remaining data were then analyzed using macQIIME (1.8) (Caporaso, 2010). Sequences from each sample were clustered into operational taxonomic units (OTUs) using UCLUST at a 97% similarity (Edgar, 2010). Clustering was

performed using an open reference OTU picking method to recover optimal taxonomical identification. Sequences were first clustered and assigned to the GreenGenes database; OTUs from sequences that did not cluster were picked *de novo* (DeSantis et al., 2006). Ninety-seven percent similarity is optimal to assign bacteria to taxonomic classification as specific as the species level. Genus classification can be achieved at 95%, family classification can be achieved at 90%, and order classification can be achieved at 80% similarity. Representative sequences assigned to OTUs were selected from each cluster based on abundance per cluster. The closed-reference OTUs were then assigned taxonomy from alignment to the greengenes reference database using PyNAST and assembled into a table, from which all further analyses were conducted (Da Mota et al., 2010, DeSantis et al., 2006. Alpha diversity was measured using the Chao1 metric with a 2,000 random sequence sample size. This number of random sequences was chosen in order to get the greatest representation of diversity while avoiding replication in samples. At this point, one specimen was removed from subsequent analyses due to a limiting number of quality reads (<1000 reads). All further analyses are conducted with six samples. Beta diversity was measured in order to compare diversity of samples to each other. UniFrac measurements were calculated both considering abundances (weighted) and not (unweighted) to calculate distance between samples for construction of phylogenetic trees (also weighted and unweighted). To test the strength of the comparison for tree construction, jackknifing was performed. OTU networks were constructed using Cytoscape 3.1 (Cline, 2007).

Results:
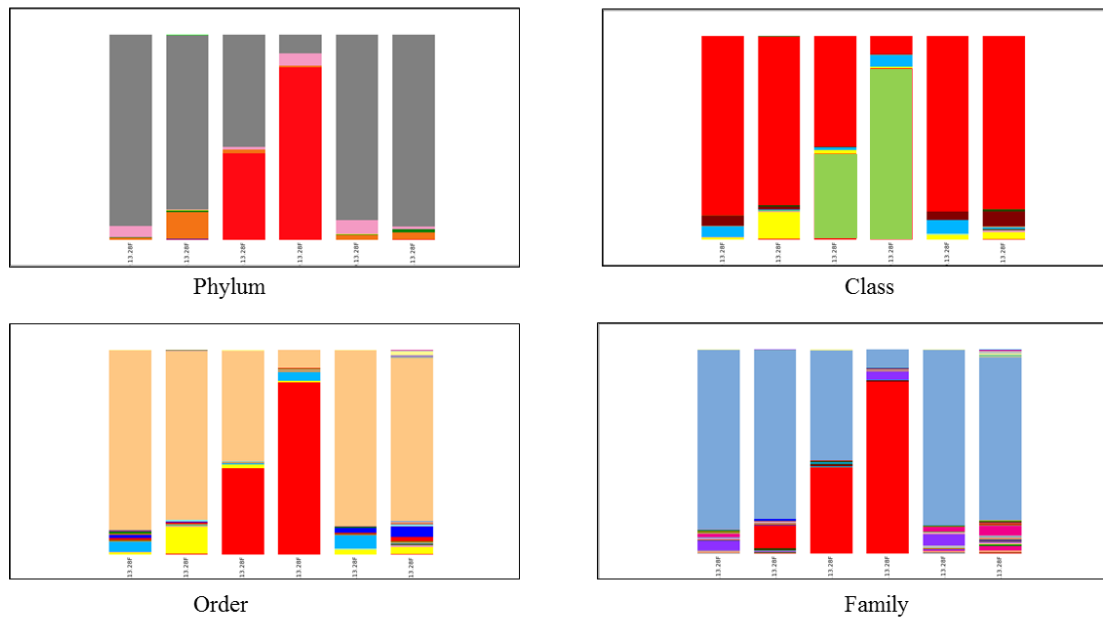
Figure 3.1. Bar plots of bacteria found in Triatoma gerstaeckeri *across taxonomic levels. Consistency between specimens can be advantageous for vector control methods.*

CO1 identification: CO1 sequences obtained from samples were submitted to nucleotide blast (blastn) non-redundant nucleotide database and Barcode of Life Database (BOLD). All specimens submitted returned as *Triatoma gerstaeckeri*, which verified the morphological identification.

In all specimens, the phylum that was most abundant was Proteobacteria, accounting for over half (63.8%) of all represented and identified phyla (Figure 1). One OTU did not match any OTUs in the Greengenes 16S database and accounted for 84% of one specimen's sequences and 42% of another's (overall presence 20.0%). The same OTU was present in less than 1% of the other samples. The dominating OTU was classified as specific as the family level and assigned to the Enteribacteriaceae family – there was no further assignment information in the Greengenes database. This overall distribution and diversity of bacteria was consistent through taxonomy levels. Other taxa worth mentioning were the genus *Enterobacter,* which was present at four and

two percent in two specimens, but absent in others. An OTU for *Corynebacterium* was only detected in half of the specimens, and in trace amounts (<1.5%), though past studies have identified this genus as obligate for other Triatomine species. The order Enterobacteriales, and therefore the family Enterobacteriaceae, was present in all specimens, and represented over half of all identified taxa in more than half of the insects, which is consistent with previous studies that did not use high-thoroughput sequencing methods.

Further assignment of OTUs to known genera was unsuccessful in many sequences of this analysis, but some biologically interesting findings follow: genera classification did not further identify than Enterobacteriaceae, as neither of the two OTUs that made up this group were assigned any further taxa. *Corynebacterium* was present in small amounts (<1.5%) in three specimens. *Leucobacter* accounted for 26.4% of one female specimen, but was present in only one other specimen, at 1.1%. *Brevibacterium* was also present at 2.4% in the same specimen, but was not detected or negligible in the other specimens.

At the phylum level, Proteobacteria accounted for >80% in three out of the four specimens that had a bloodmeal present at the time of extraction (neither specimen removed from the analysis had a bloodmeal at the time of dissection). In the fourth, the unassigned OTU was present at 42%. Further taxonomic identification did not reveal further differences in bacterial profile between specimens with or without blood meals.

Discussion:

A previous culture-independent study performed on *Triatoma* and some other genera belonging to *Triatominae* had similar findings, although specimens from the genus *Triatoma* used in this study were colonies of a different species and cultivated for approximately 20 generations,

feeding on chicken blood (da Mota, 2012). The findings similar to these indicated that the microbiome in *Triatoma* are dominated by a few types of bacteria, particularly Enterobacteraceae. This does not differ from the findings of culture dependent based studies. The unclassified Enterobacter OTUs should be explored further, as *Serratia marcescens*, another Enterobacter, has been found in the gut of *T. infestans* and *Rhodnius prolixus*, both of which are highly effective vectors for *T. cruzi* in South and Central America (Da Mota, 2012). *Serratia marcescens* is able to negatively impact the presence of *T. cruzi* by lysing the cell wall of the parasite and holds potential as an important factor for vector control-proper identification could be highly beneficial in paratransgenic control research (Azambuja, 2004). In a more recent study, a protein purified from the microbiota of *R. prolixus* directly affected the presence of *T. cruzi* in the anterior midgut in infected *R. prolixus* specimens (Soares, 2015). Initially, sex was a variable used to explore differences in the *T. gerstaeckeri* bacterial community. This was explored due to the sample of specimens analyzed and suggestions in the literature that sexual dimorphism could influence vector capacity (Guameri, 2003).

The presence or absence of *T. cruzi* is a variable that should be used in order to further explore these data, in this set of specimens as well as greater sample sizes – in these specimens, only one negative result for *T. cruzi* was obtained when the set was tested with PCR primers to amplify the 18SSU ribosomal gene in *T. cruzi*. Paratransgenic control of *T. cruzi* has been proposed with the use of Actinomycete bacteria as well as Corynebacterium found in *R. prolixus*, *Rhodnius rhodnii (*Dasch, 1984, Dotson, 2003, Durvasula, 2008*)*. Further exploration of the unclassified Actinomycetales in this study is warranted to determine its importance.

Bloodmeal host identification could present another basis for comparison, although controlled food sources in colony cultivation over several generations would be the most ideal

experimental design, as corprophagy, or the ingestion of feces, is partially responsible for obligate bacteria found in the gut of *Triatominae*. This has been exploited in other studies; bacteria were successfully introduced to the insects in a substance resembling insect feces and then recovered in the F1 progeny (Beard et al., 1998). This substance itself is a possible means of vector control as a pesticide, but has not been used on a large scale to ascertain efficiency and impact.

Bacterial communities could also be affected by the geographic location of the insect. On a large scale, rainfall, altitude, and other climatic variables could have an observable effect on the bacterial communities. On a smaller scale, and related to host preference of the insect, specific collection information, such as whether the insect was found in a rabbit enclosure or a human household as opposed to in a sylvatic area or away from mammal concentrations. Insects collected from these varied environments may have differing microbiota that could affect their capacity as vectors (this added to the physical distance acting as a variable for infection).

Further study and exploration of the bacterial community of these insects is important for the development of vector control. Developing a pesticide that can exploit the relationship of the bacteria associated with *Triatoma* or *T. cruzi* is an area of research that can save lives in conjunction with methods of control that are already being utilized, though much of the currently existing control initiatives depend on methods that are often most difficult to implement by those at the greatest risk. The controlled use of pesticides, reconstructed residences, and management of wildlife are examples of control techniques that are often implemented, but as the spatial distribution of several species of *Triatoma* are predicted to shift northward and further into the United States, more humans and canines are threatened to be exposed, requiring new communication and implementation strategies for the application and maintenance of this control (Sarkar, 2010, Garza, 2014). The effectiveness of these methods is only enhanced by the addition

of techniques gained from studying the vector itself, in this case, focusing on its role as a host for *T. cruzi*. It should be noted that although specimens were treated uniformly and sterilized upon receipt, it is not known how they were collected and stored before shipment.

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Chapter Four

Mitochondrial Genome Sequencing of *Triatoma gerstaeckeri* and *Triatoma lecticularia*

Introduction:

The kissing bug, also commonly referred to as the conenose bug, belongs to the subfamily Triatominae (Hemiptera: Reduviidae). These insects act as vectors for the kinetoplastid protozoan parasite *Trypanosoma cruzi*. They are collectively hematophagous as well as coprophagous, and occupy a range of distributions in North and South America (Lent and Wygodzinsky, 1979). The insect will take a bloodmeal from a host by piercing the skin with its rostrum and subsequently defecate near the resulting microlesion. Parasites are deposited along with the feces, and enter the host body if the feces are rubbed or scratched into the wound. In a percentage of humans, non-human primates and domestic canines, infection from *T. cruzi* can result in Chagas disease, which is characterized by cardiac or digestive organ failure and eventually death (Rossi, 2010). Diagnostic difficulties result in approximations of the number of patients affected, but the World Health Organization estimates 7-9 million people are hosts to *T. cruzi (*WHO, 2014, Kirchoff, 2003). When diagnoses are successful, a cure is not available and treatment is invasive and expensive. Research on treatment, while progressing, is still in developmental stages, and makes continuing research on improving vector control necessary (Hotez, 2012).

Interspecific transmission methods exhibit some variance - much of which is poorly understood. Times between bloodmeal uptake and defecation, for example, vary between species, which has an appreciable impact on transmission and vector competence (Goddard,

2002, Pippin, 2015). Competition for hosts and blood meals, determining gene flow between

sylvatic (wooded) and domestic habitats, and humoral immune response to the presence of *T.*

cruzi in the mid-gut or the hemocoel are potential factors that may affect vector competency in

the Triatominae subfamily (Pippin, 2015). In addition, the interactions between the vector

species, the parasite, and the microbiome of the insect are promising directions in studies that

aim to improve vector control. A preference for the vector midgut by *T. cruzi* has been seen, but

this is not well understood – bacterial presence, vector immune response and stress response in

the parasite could all contribute to this behavior (Schaub, 1997).

The phylogeny of the *Triatomine* insects has been difficult to map for statistical and

morphological reasons (de la Rua, 2014). Current phylogenies based on genetic data have been

constructed using a combination of nuclear and mitochondrial sequences but these contradict

each other and lack statistical power (de la Rua, 2014). Not all species, including those of

epidemiological importance in North America, have been studied and included in these analyses.

Using whole mitochondrial genome information to continue the existing phylogenetic work can

take advantage of certain properties of mitochondrial DNA (mtDNA). Due to no sexual

recombination of mtDNA, all interspecific polymorphisms are assumed to be the result of

mutation and therefore can be directly compared to determine rates of mutation (Boore, 1995).

Highly conserved coding regions are interspersed with highly mutating non-coding regions,

which makes genomic mtDNA ideal for studying closely related species (Boore, 2015). The

reduced cost and increased quality of high-thoroughput sequencing make genomic studies more

available and easier to perform for researchers. This in turn allows mtDNA to be explored in

depth to identify species' relationships and identify more easily variance that may otherwise go

unnoticed (Loman, 2012). Combining mitochondrial genomic data with bacterial community

data from the previous chapter of this study could, as more data is collected on more species, be powerful in deciphering the taxonomic classification of this genus, and subsequently additional intraspecific polymorphism.

The current study sequences and assembles the mtDNA genome of two *Triatomine* specimens in Texas: *Triatoma gerstaeckeri* and *Triatoma lecticularia*. These species are competent vectors and abundant in Texas, where new cases of human-acquired trypanosomiasis have been recently reported. Surveys of vectors of *T. cruzi* find parasite presence (PCR detection) at an estimated 40% and 50% in *T. gerstaeckeri* and *T. lecticularia*, respectively (Stevens, 2007, Kjos, 2009).

Methods and Materials:

Specimens were collected in Kingsville, Kleburg County, Texas and stored in 95% ethanol. Morphological identification was performed following the key of United States species of Lent and Wygodzinsky (1979). Two specimens were selected, one *T. gerstaeckeri* and one *T. lecticularia*. Samples were chosen by visually identifying integrity of sample and determining most recently collected. Mitochondrial isolation was performed using the Qiagen Qproteome Mitochondria Isolation Kit on soft tissue from insect thoraxes. Following mitochondrial isolation, DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen). Extracted DNA was suspended in molecular grade water and sent to Research and Testing Laboratory for sequencing. To verify extraction, PCR amplification was performed on the mitochondrial Cytochrome Oxidase I gene.

The Illumina MiSeq high thoroughput platform was used to sequence whole genome mtDNA. Benefits of this platform include reduced costs and run times and high quality paired-

end reads (fragments are sequenced in both directions). Sequence data was analyzed with Geneious v8. Received sequences were assembled into contigs and annotated to the previously published mitogenome of *Triatoma* dimidiata (Dotson, 2001), as well as assembled de novo to compare results.

Results:

The two mitochondrial genomes of these species were both found to be approximately 17 000 base pairs in length. The top two contigs for *T. gerstaeckeri* were 17 039 bp and 17 038, while for *T. lecticularia* the lengths were 17 038 and 17 027 bp. Thirteen coding regions were found to exist in *T. gerstaeckeri* and twelve coding regions were found in *T. lecticularia* (Figure 4.1a, Figure 4.1b). The percentage of guanine-cytosine base pairs (GC content) was 30.3% in *T. gerstaeckeri* and 30.4% in *T. lecticularia (*Figure 4.2). This metric is important when designing primers for use in future PCR reactions, but also in determining information about sequencing reactions (e.g. higher GC content is correlated with lower sequencing accuracy).
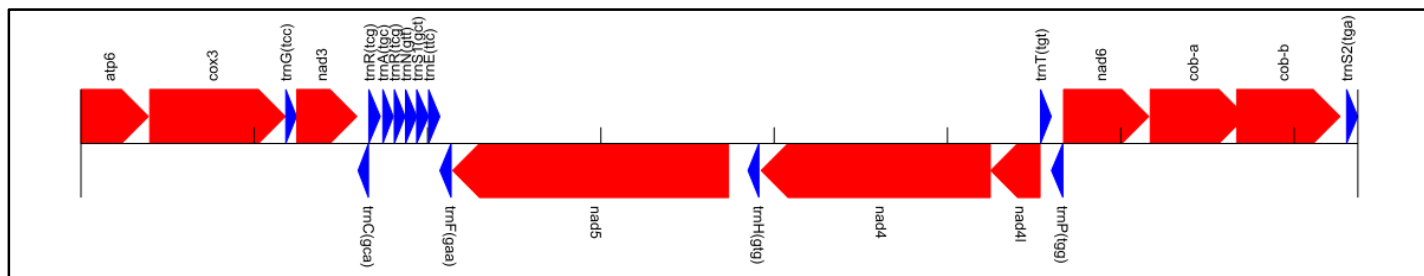
(a)
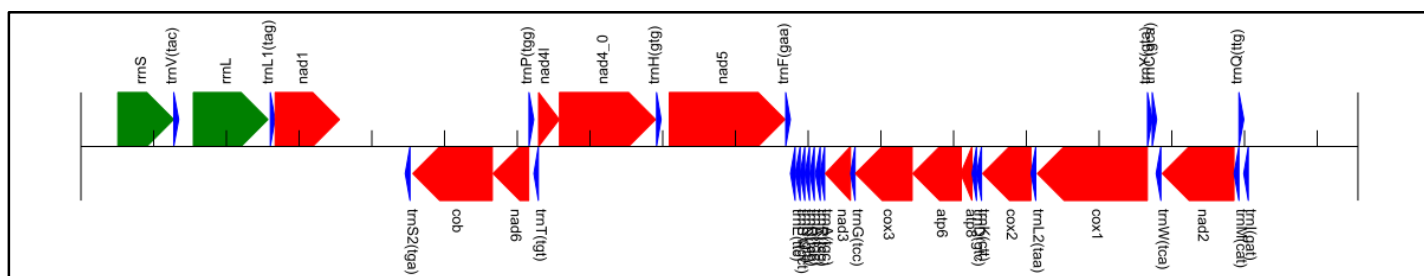
(b)

Figure 4.1- De novo assembled mitogenomes of Triatoma gerstaeckeri (a) and Triatoma lecticularia (b). coding regions are shown in red, non coding regions shown in green, tRNA regions shown in blue. Arrows indicate direction of transcription.
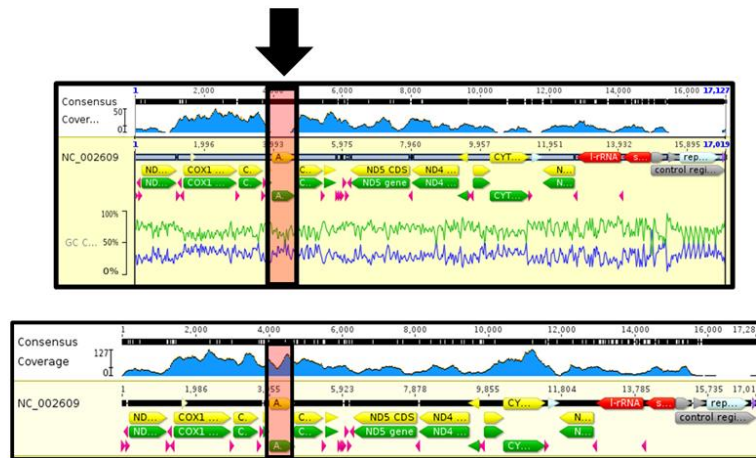
Figure 4.2. Comparison of genes of T. gerstaeckeri (bottom) and T. lecticularia(top) to T. dimidiata (both). Referenced assembly - arrow on highlighted boxes indicates ATP-synthase 6 gene position. T. lecticularia shows GC content of genome (lower blue line, GC, higher green line, AT content). Blue areas show coverage of sequenced mtDNA to compare to referenced assembly.

Discussion:

The sequencing of the mitochondrial DNA of *T. gerstaeckeri* and *T. lecticularia* provide insight into the phylogeny of the genus *Triatoma.* Mitochondrial DNA is ideal for analyzing closely related species, and including non-coding regions in analysis especially makes examining phylogenetic relationships meaningful. As technologies like MiSeq become more efficient in terms of cost and time, data similar to what was produced in this study can be analyzed more often and used to better understand the differences in vector biology and host preferences. This in turn can produce more targeted vector control strategies, but can be applied to other systems as well.

The deleted coding region in *T. lecticularia* is unexpected when compared to other arthropod mitogenomes and should be examined further. If this deletion stands under replication, PCR of this gene could be used as a more reliable method of identification between

species, as morphological identification often relies on delicate features that, when absent, render the specimen unidentifiable without a barcoding analysis (or similar). The coding region is for the ATP-synthase-6 gene in *T. dimidiata*, and its absence is unusual. Primers can be designed to look for the gene in other specimens of the same species to explore possible mutations. The possibility that the lack of coverage is a result of a sequencing error is remote, as all contigs from the *T. lecticularia* speciemn that aligned to *T. dimidiata* were missing coverage in the same region. The full coverage of this same gene in *T. gerstaeckeri* eliminates the possibility of the Geneious software being unable to read or recognize the gene. Amplification and sequencing of the gene in both species (as well as any other closely related species) should be performed in order to determine fully the reason for its absence in this specimen and further explore the possibility of using this deletion as a method of species identification. The specimen in question was preserved appropriately (all tissue intact, not dessicated), and DNA extracted yielded high quality when tested with a NanoSpec 1000 (Berkeley, CA).

Much of the sequence data returned with the mitochondrial reads were bacterial sequences. The tissue from which mitochondria were isolated was dissected from the thorax, which may have had contamination from the gut. This was necessary as isolation of mitochondria from chitinous tissue and hemolymph failed to yield enough DNA for sequencing. These bacteria should be studied and compared to existing knowledge, particularly from this study, as the specimens were collected from the same geographic region.

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Chapter Five

Conclusions and Future Research

Current and past studies largely focus on South American and Mexican taxa of the subfamily Triatominae, as these insects' geographic ranges cover areas of great risk of Chagas disease and *T. cruzi* transmission. This research focuses on the taxa found in Texas, particularly two of epidemiological importance, *Triatoma gerstaeckeri* and *Triatoma lecticularia*.

Currently, pesticide use and education of human populations have been most effective in slowing the transmission of Chagas Disease. Targeted and controlled pesticide use has been especially effective, though some *Triatomine* insects have shown resistance to these methods (Vassena, 2000). Preventative methods implemented by people in endemic areas has also been successful – moving woodpiles from residences, patching holes in thatch roofing, and implementing more concrete in structures contribute to the reduction in new cases in South America (Schofield, 1999). The screening of blood by blood donation centers is also extremely important, but not yet mandatory. Chagas is now a reportable disease in Texas, but without surveillance of the blood supply, it is extremely difficult to know how many people are carrying the parasite and are therefore at risk for chronic Chagas disease (Garcia, 2014).

Phylogenetic approaches are being implemented more often as high-thoroughput sequencing becomes more available (decreasing cost, reduced time) and more precise. A

cohesive phylogeny of the subfamily Triatominae has not yet been reached, but continued

analysis combined with increasing accessibility of genomic data place it well within reach.

Mitochondrial genome sequencing contributes to this goal, particularly when differentiating

between closely related taxa.

Bacterial communities, especially in vector-pathogen systems, are only recently

becoming more understood. The relationships between pathogen presence and bacterial ecology

that have the potential to be uncovered could be integral components of vaccines,

immunizations, and pesticides. Further analysis and manipulation of these systems and

relationships can reveal answers not only important for Chagas but for other pathologies that

occur as a result of insect vectors. By utilizing an interdisciplinary approach, conclusions can be

made that can implement highly effective vector control for the *Triatoma* genus.

In this study, several means of analyzing the biology and physiology of the genus

Triatoma are explored. Location of insects, whether on a large scale (geographic) or a small

scale (habitat) should be compared based on bacterial communities to determine how this

integral part of physiology has real world impacts. Paratransgenic control has been carried out &

shown to be in itself an effective form of vector control in a Corynebacterial symbiont (Beard,

2001). Using bacteria to translate proteins that negatively affect the presence of *T. cruzi* is also

known to be a possible means of control (Durvasula, 2008). Combining these effects with a

spatial element, that is, using the location of an insect, host or parasite to manipulate the presence

of the bacteria, could be an extremely powerful measure of control. One obstacle in applying

these methods as a form of vector control currently is to find an efficient means of introducing it

to the vector. A relationship between the location of an insect and the bacteria to which it is

either naturally exposed or biologically obligated to carry would greatly facilitate the implementation of any vector control program that takes advantage of these two variables.

In the mitochondrial genome analysis of this study, a deleted gene in one species may have phenotypic effect yet unrealized. One way to figure this out would be to, as previously mentioned, attempt amplification of the deleted gene. Failing amplification, the next step would be to see if the gene has been moved to the nuclear DNA. Given that the gene codes for ATP synthase-subunit 6, it is unlikely that is has been completely deleted. The genetic variation between species, however, could have some effect on the ability of the insect to host or transmit the parasite. Phenotypic differences resulting from this particular gene may have observable effect on vector competency, and can be found easily by comparing infection rates with varying genotypes.

Combining the location, bacterial community, and mitochondrial genome variation may reveal a relationship between vector and parasite that has previously remained unobserved. These approaches to vector control are powerful applications of analytical tools and sequencing power that will allow the understanding of host-vector systems in way previously unavailable and hold many possibilities to control the spread of a serious epidemiological concern.

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