USING GENE SEQUENCING TO INVESTIGATE THE PHYLOGENETIC RELATIONSHIPS BETWEEN INTRADERMAL MITES IN ANURANS IN CENTRAL TEXAS

by

Monica Alexandra Argueta

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Thesis Supervisor:

David Rodriguez

Second Reader:

Ferris Zughaiyir

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LIST OF ABBREEVIATIONS

Bd- *Batrachochytrium dendrobatidis*

DNA- Deoxyribonucleic acid

dNTPs- deoxyribonucleotide triphosphates

ExoSAP- Exonuclease Shrimp Alkaline Phosphatase

MM- Master Mix

PCR- Polymerase Chain Reaction

Pfu- *Pyrococcus furiosus*

Taq- *Thermus aquaticus*

T_m- Melting temperature

ABSTRACT

Emerging infectious diseases have serious impacts on global amphibian populations and contribute to their decline worldwide. In Texas, mite larvae (commonly known as chiggers), have been found to parasitize amphibians, increasing their susceptibility to diseases such as chytridiomycosis and *ranavirus*. Previous studies have identified many of these pathogens using molecular techniques such as DNA sequencing; however, there is limited genetic information about the genera of endoparasites affecting anuran populations. This research focuses on investigating the genetic diversity of intradermal mites affecting amphibians in Texas using DNA sequencing. Mites were collected from various species of frogs and their DNA was sequenced to determine their phylogeny using existing data of genetic markers for acarids. The results obtained expand the knowledge available of the species of intradermal mites present in the state and can be compared to other species in North and South America.

I. Introduction

Amphibian populations have been declining worldwide due to habitat destruction, climate change, and the introduction of non-native species. Recently, emerging infectious diseases such as chytridiomycosis—caused by the parasitic fungus *Batrachochytrium dendrobatidis (Bd)*—and infections of *ranavirus* have had serious impacts on anuran populations (Lips et al., 2008; Marshall et al., 2019). However, these are not the only organisms that thrive in amphibian skin. Some species of mites have been found to develop ecological relationships with amphibians by inhabiting the epidermis or dermis of the host. These parasites belong to the families Hydrachnidae, Thermacaridae, Trombiculidae, and Leeuwehoekidae. The larval stage of Trombiculidae (Vercammenia and Nadchatram) and Leewenhoekiidae (*Hannemania*) only infest the connective tissue of the host (Quinzo & Goldber, 2015).

These amphibian specialists encapsulate in the skin of their host by using their chelicerae and feed until engorged. Once full, this organism will leave the host where the adult form is free-living and feed on arthropods in the water and organic matter (Fuente et al., 2016). Infestations are visible as red-orange lesions, swollen nodules, or pustules in areas where they have punctured the skin to form a capsule that is enclosed by secretions and connective tissue produced by the host (Bakkegard et al., 2019; Costa-Silveira et al., 2019; Westfall et al., 2008). Chigger infestations may cause structural damage such as inflammation, necrosis, and abscesses that can be visible to the naked eye. These infestations have been associated with decreased reproductive success, damage to the limbs and body, and increased susceptibility to disease (Sladky et al., 2000; Wohltmann et al., 2006).

In North and South America, the endoparasitic larvae of the genus *Hannemania* infest the same amphibian populations affected by *Bd*, and *Hannemania* infections might be an additional contributing factor to amphibian declines. Although *Hannemania* mites were first identified in 1910, their phylogenetics, morphology, and host dynamics are sorely understudied (Wohltmann et al., 2006). Recent amphibian research has largely focused on *Bd*, yet the impacts of concurrent parasites in the same populations remain unknown. Understanding the role of co-infections in populations already infected with *Bd* may reveal crucial morbidity and mortality patterns in the host.

In Texas, subdermal mites have previously been misidentified since their biodiversity in the area is not well described (Mertins et al., 2011). *Hannemania* spp. are restricted to humid microclimates, therefore we expect to find high prevalence within many ecoregions in Texas and tropical forests, where host declines have been attributed to *Bd*. The introduction of *Bd* has already been documented in the state of Texas (Marshall et al., 2019), and I have detected subdermal mites in two widespread host species, cricket frogs (*Acris crepitans*) and leopard frogs (*Lithobates berlandieri*). However, the morphological and genetic features of subdermal mites in Texas are understudied leading to a lack of information on the impact it may have in amphibian populations.

Due to the confirmed presence of both subdermal mites and *Bd* in Texas and in South America, it is imperative to determine which species of intradermal mites are present in amphibian populations infected with *Bd* and compare regions with low host diversity and high host diversity. To inform future conservation efforts, a better understanding of host, pathogen, and parasite dynamics is important. Thus, investigating the genetic diversity of the subdermal mites found in Texas can aid in comparing the

relationships between hosts and pathogen-parasite infections. In addition, the prevalence of chiggers among species of amphibians can differ depending on their environment (Wohltmann et al., 2006), therefore it is suspected that the co-infection rates between mites and *Bd* varies and affects populations at different intensities. These factors are expected to decrease host body condition index and increase susceptibility in amphibian populations affected.

PCR is a molecular diagnostic tool that has a broad range of clinical applications such as the identification of pathogens and novel emerging diseases. The principle of this technique is based on amplifying short regions of target DNA. PCR requires the use of a thermostable DNA polymerase enzyme to produce a large number of copies from nucleotides and a template strand of DNA. Examples of thermostable enzymes for this process are Taq polymerase and Pfu. Isolated from the bacteria *Thermus aquaticus* and *Pyrococcus furiosus*, these enzymes have been vastly used due to their remarkable tolerance to high heat, in contrast to other polymerases which may denature at high temperatures. With an optimum temperature between 70-80 $^{\circ}$C, these enzymes facilitate the denaturing of DNA required for PCR each heating cycle (Abramson, 1995).

Additionally, primers are used in PCR to begin the amplification and synthesis of a specific region of DNA, which can be accomplished with small amounts of DNA. Primers are short, single-stranded nucleic acid sequences (also known as oligonucleotides) generally 18 to 24 pairs in length of complementary DNA. A forward primer will attach to the start codon and the reverse primer will attach to the stop codon of the template DNA. These are also known as the anti-sense and sense strands respectively where the 5' end of each will bind to the 3' end of the template DNA. These

primers are added to the PCR reaction along with the DNA polymerase, dNTPs, and buffer which are needed to provide the polymerase with an optimal environment to function. The buffers may include $MgCl_2$ and KCl. The use of varying concentrations of magnesium aids in the lysis of the cell membrane during DNA extraction while salts support primer elongation during the annealing stage. Using cofactors in DNA amplification is essential and act as a catalyst for proper polymerase activity and the addition of deoxyribonucleotide triphosphates (dNTPs).

Different temperatures are required during DNA amplification to make multiple copies of the single region of interest of DNA. These temperatures can be differentiated in three different steps: denaturation, annealing, and elongation which complete a cycle. A repeated cycle of these steps is also known as thermal cycling and results in amplification of DNA with the potential to make millions of copies. Varying incubation times and temperatures are adjusted in this process depending on the concentrations of buffer and the nature of the DNA template itself. The high temperature in the first step leads to the denaturation of the DNA strand and results in two single strands allowing the primers to bind to the specific region. Generally, the denaturing temperatures in PCR are done at 94-98 °C for 1-3 minutes. Optimization of primers can be done by using different ranges of annealing temperatures; however, obtaining results can be limited by mispriming. Using touchdown (TD) PCR facilitates the optimization of primers as it is a method that increases specificity, sensitivity, and yield without having to design specific primers or calculating melting temperatures (T_m). TD-PCR uses an annealing temperature higher than the primer T_m and lowers during subsequent cycles to allow correct annealing at the required temperatures. When the temperature is raised again (elongation stage), the

extension of primers results in the new DNA strand made by the Taq polymerase enzyme.

Gel electrophoresis is technique that allows analyzing the obtained PCR results. DNA fragments are separated by size through a gel matrix (1-3% agarose gel) pulling the fragments apart by an electric current. Because of the negative charge of DNA, the samples will run to the positively charged side of the apparatus. TBE buffer is also added to the agarose medium to carry the current across the apparatus. The PCR products can be visualized under UV light as bands using fluorescent dyes. The amount of product obtained can be analyzed by observing the intensity of the bands in comparison to the ladder by molecular weight.

Mites infesting amphibian populations and potentially contributing to their decline are severely understudied. Dr. David Rodriguez conducts research in pathogens affecting amphibian and reptile populations in the New World. Further investigating co-infection rates between *Bd* and mite infestations is necessary to establish adequate conservation efforts. In addition, the presence and effects of parasitic mites in Texas have not been published; therefore, this study has the potential to increase the knowledge of intradermal amphibian parasites in the United States and the Americas. The objective of this project is to identify the species of parasitic mites present in amphibian populations in Texas and where they fall phylogenetically.

II. Materials and Methods

Acris crepitans (Northern cricket frog) and *Eleutherodactylus marnockii* (Cliff chirping frog) with visibly encapsulated mites were collected in Lueders, Texas and San

Marcos, Texas, respectively. Mites were extracted from the host using a sterilized scalpel and preserved in 70% ethanol. A single mite was placed in a 1.5 mL microcentrifuge tube prior to DNA extraction using a dissecting microscope. The morphology of the mite was not meticulously observed or recorded. The tube was left to air-dry any excess ethanol.

DNA extraction was performed using the PrepMan Ultra Preparation Reagent (Applied Biosystems, Inc.). A total of 14 mites were sampled (**Table 1**) and 50 µL of PrepMan were added to each tube while macerating the mite with the pipette tip. Then the tubes were spun to move contents to the bottom of the tube. The tubes were placed at 99 °C for 10 minutes and placed at room temperature for 2 minutes. They were then spun at full speed using a microcentrifuge for 5 minutes and 15 µL aliquots were taken and placed in another clean 1.5 mL microcentrifuge tube.

Potential universal primers were selected based on previous literature targeting arachnid and mite genes; namely, nuclear 18S rRNA and nuclear 28S rRNA (Otto & Wilson, 2001; Burger et al., 2012). A 100-µM stock solution was made for the primers being tested by centrifuging the tubes for 3 seconds and resuspending the contents with 1 part Elution Buffer (GeneJet DNA Extraction Kit, Invitrogen) and 1 part nuclease-free H_2O accordingly (**Table 2**). The primers were vortexed for 15 seconds and left to rest at room temperature overnight. Then 10 µl working primer solutions were made from the 100 µM stock as 9 parts nuclease-free H_2O to 1 part 100 µM stock, and 90 µL of nuclease-free watered and 10 µL of stock were transferred to a new centrifuge tube for each primer and labeled accordingly (3 forward and 3 reverse primers) as listed in **Table 3**. Each tube was vortexed for 15 seconds and placed in a freezer for storage. The stock solution was stored in a freezer at -80 °C.

To carry out the Polymerase Chain Reaction (PCR), a master mix (MM) was made containing DreamTaq 2X MM (ThermoFisher Scientific), respective forward and reverse primers, $MgCl^2$, and nuclease-free H_2O. A MM was made for each set of primers and quantities adjusted according to sample size (**Table 4**). For the first primer test, 24-tube strips were labeled according to mite sample, primer used, and PCR protocol. Four mite samples were used for this test and 0.5 μL of each extracted mite tDNA were added to a different tube. Then, 12 μL of MM were added with each primer respectively for a total solution containing 12.5 μL per sample (**Table 5)**. Different temperature ranges were tested using touchdown PCR (TD-PCR). TD-PCR 55 and TD-PCR 60 were the programs used to test all three sets of primers. The same MM and tDNA procedures were followed to run the reactions for the 10 other mite samples. Only the 18S (**Figure 1**) and 28S gene target primers were used under TD-PCR 55 for the rest of the samples.

Gel electrophoresis was performed on the PCR product in a 2% agarose medium. The gel was made by adding 0.1 g of agarose to 50 mL of 1X TBE buffer and heated for approximately 30 seconds in a microwave until clear. The solution was placed in a water bath to cool down and poured in a gel casting tray with combs. The wells were filled in with 1X TBE buffer until the liquid was covering the gel. For the DNA samples, 3 μL of gel red were mixed with 4 μL of each PCR product. The ladder was loaded in the first well for comparison and the samples were placed in the subsequent wells according to mite sample, primers, and PCR protocol. A high voltage electric field between the two electrodes was conducted using an electrophoresis power supply. Gel imaging and analysis was performed, and PCR products were labeled accordingly as seen in **Figure 2** and **3**.

An ExoSAP PCR clean-up was performed to clean the amplified PCR product from excess primers and nucleotides. For this reaction, 2 μL of ExoSAP Master Mix were added to the samples which contained approximately 8 μL of template DNA for a total of a 10 μL reaction volume per sample. The ExoSAP MM can be made using nuclease-free H_2O, exonuclease I, and shrimp alkaline phosphatase (**Table 6**). The samples were incubated in a thermocycler at 37 °C for 15 minutes followed by another period of incubation at 80 °C for 15 minutes for inactivation of the enzymes. The cycle sequence reaction was carried out by making a MM containing nuclease-free H_2O, 5X buffer, respective primer, and BigDye 3.1 Ready Reaction Mix per sample (**Table 7**). The MM was combined with cleaned-up PCR products and placed in the thermocycler using the program CycSeq50 (**Table 8**). Following cycle sequence clean-up via Sephadex G-50 columns, the samples were sequenced on an Applied Biosystems 3500 Genetic Analyzer.

The nuclear 18S and 28S gene sequences were compared through a BLAST search using the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The percent identity to estimate the similarity between the query sequence and the target sequence was set above 95% for the 18S gene region and 92% for the 28S gene region. The GenBank complete aligned sequences were downloaded and transferred to the Geneious Bioinformatics Software for Sequence Data Analysis (Biomatters Ltd.). In Geneious, the sequences were first trimmed and then aligned using MUSCLE. A neighbor-joining tree was inferred for the 18S gene region to conduct phylogenetic analysis of the samples sequenced using 1,000 bootstrap replicates.

III. Results and Discussion

Mite Morphology

The mites collected appeared as red pustules on the host's skin and upon extraction appeared as red mite larvae (**Figure 4**). The morphological features of the parasite that were visible to the naked eye and under a dissecting microscope concurred with literature attributing red pustules to mite larvae from the genus *Hannemania*. This genus of mite was expected in the Texas region due to the visible parasite encapsulation on amphibian hosts and the climates the hosts are found in which overlap with the microclimates these parasites are known to thrive in.

PCR Results

The primer test resulted in only 18S and 28S gene regions showing amplicons after analyzing the gel, therefore the set of primers NS7 and NS8 were not used in the PCR reactions that followed as seen in **Figure 2**. However, the mite DNA samples used for primers NS7 and NS8 displayed slight bands which could be due to possible contamination. Additionally, another possibility is that tissue and blood from the host remained in the digestive system of the parasite which resulted in bands owing to the universality of the primers. Furthermore, both TD-PCR 55 and TD-PCR 60 were tested. TD-PCR 55 was used for the rest of the reactions since it displayed more well-defined bands when testing the primers. The next PCR reaction with both sets of primers and using the TD-PCR 55 program displayed well-defined bands of similar sizes in all samples (**Figure 3**).

Nucleotide Sequence Analysis

The sequence obtained showed a high percentage of similarity in the 18S gene region to other organisms in the class Arachnida in the web BLAST search. In addition, this region was found to have above 95 % similarity to several organisms from the subclass Acari. In

contrast, the 28S gene showed to be hypervariable in some regions. A phylogenetic tree for this region was not generated due to the low similarities between different populations. Although a phylogenetic tree for the 28S region would not be ideal due to poor alignment of sequences, this gene region can be used to analyze mites within the same population (**Figure 5**).

Phylogenetic Analysis

In the neighbor-joining tree, the mites collected in San Marcos clustered with sequences from *Hannemania hepatica* (**Figure 6**). Contrastingly, the mite collected in Lueders, TX appears in the same clade as *Hannemania* species, however it did not show to have a high enough percentage of similarity to conclude that it belongs to one of the species already in the database. Therefore, we hypothesized that while it most likely belongs within *Hannemania,* but this species of mite has never been sequenced before (**Figure 7**).

Conclusion

Amphibian populations continue to decline globally at an alarming rate; therefore, it is critical to investigate other factors that are contributing to declines. *Hannemania* species, known to infect amphibians in both Texas and South America, remain understudied. Cataloging genetic diversity of *Hannemania* mites will elucidate yet another factor that may contribute to amphibian declines. Thus, by describing their genetic diversity, parasite biodiversity in addition to identifying host morbidity and mortality rates due to co-infections can be accounted for. Because *Bd* virulence and parasite load can be affected by temperature, sampling in Texas and countries with high amphibian biodiversity, such as South America, can facilitate developing predictions about co-infection impacts in different climates across the world.

Future Directions

Researching new emerging diseases is crucial to support species conservation efforts. Many vertebrate diseases are introduced, which increases disease susceptibility of already vulnerable populations. Continuing to genetically identify these pathogens will expand the knowledge of how potential co-infections of mites and *Bd* may affect amphibian populations. In the future, infestation prevalence in specific amphibian populations of Texas can be taken into consideration. Additionally, specific primers can be designed to further inquire the relationships between mites found in the United States and other acarids found globally.

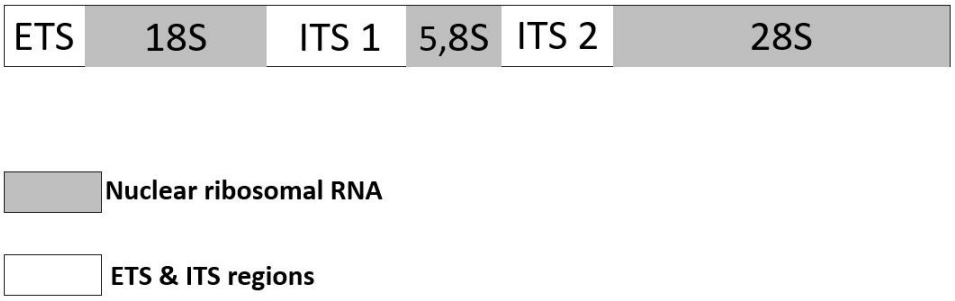
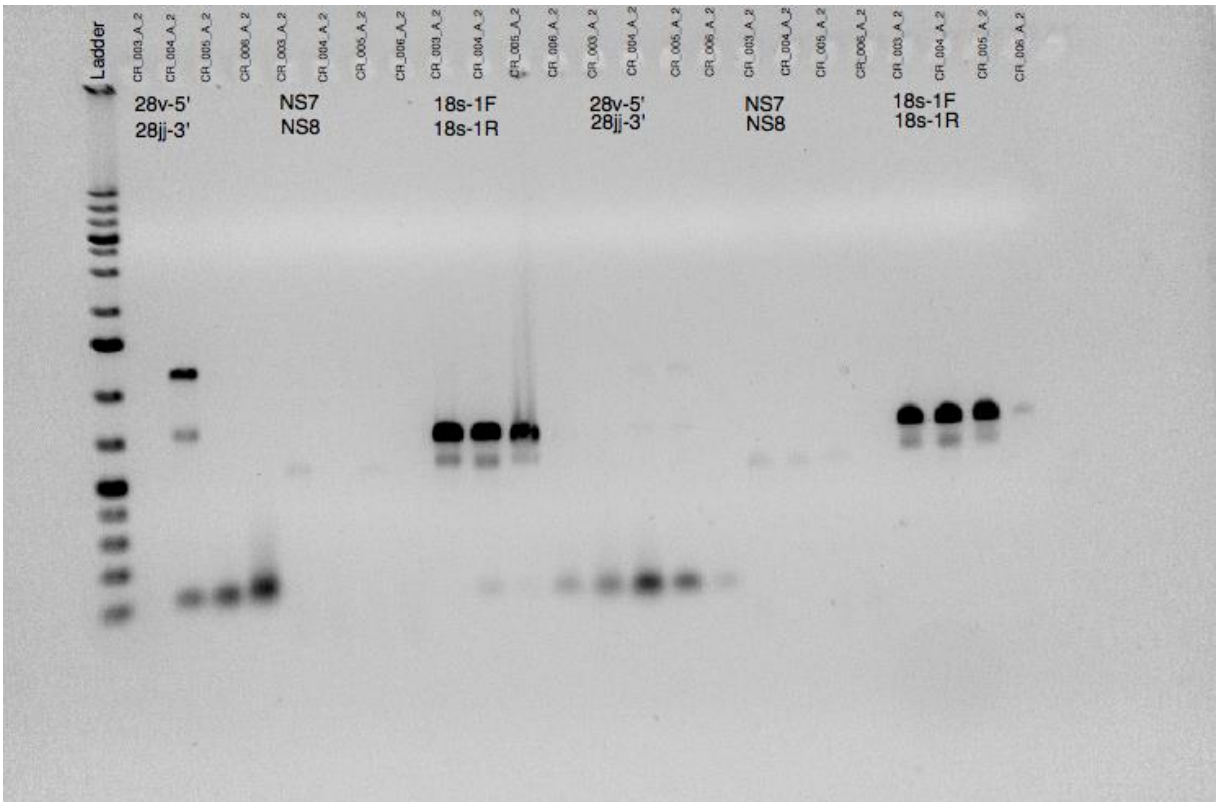
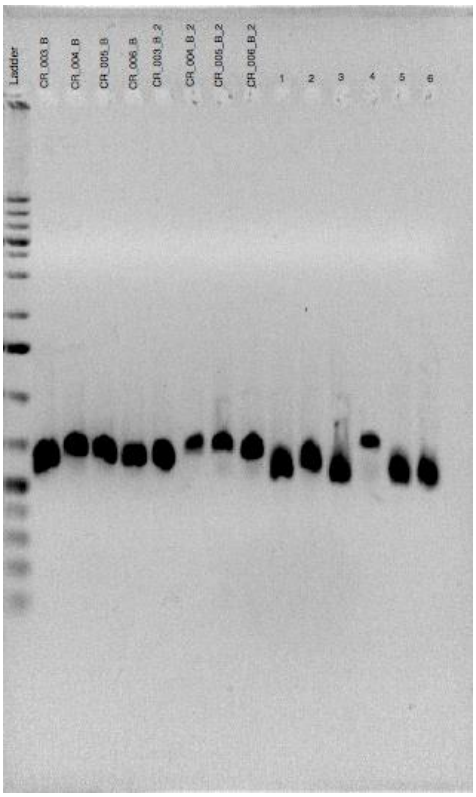


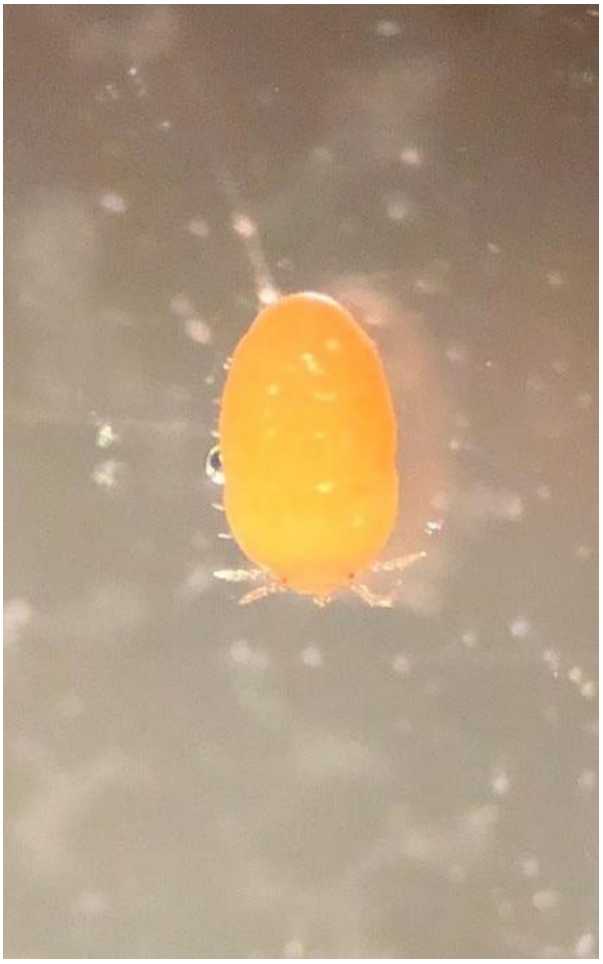
Figure 1:Location of nuclear ribosomal RNA along with the external transcribed spacer (ETS) and internal trancribed spacers (ITS). Nuclear small subunit 18S and nuclear large subunit 28S regions are shown.



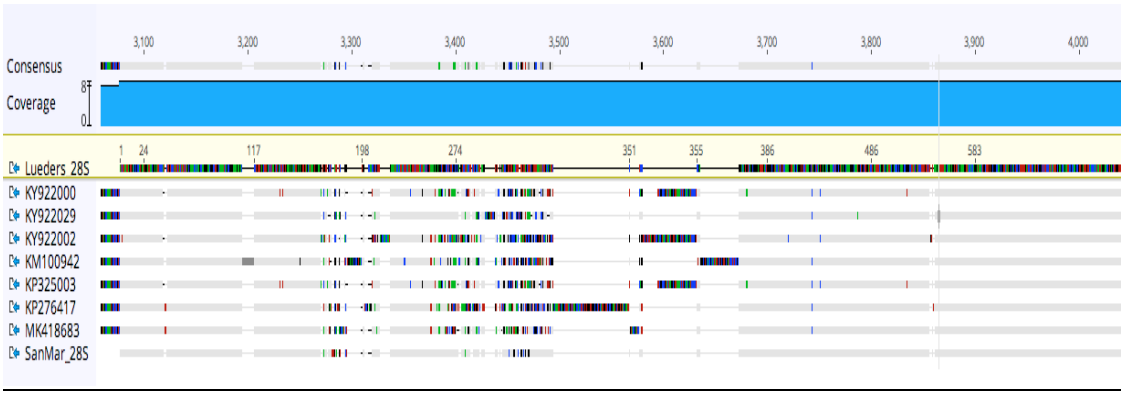
<u>Figure 2</u>: Gel electrophoresis analysis of PCR products using TD-PCR 55 and TD-PCR 60. The samples in wells 1-12 are using the temperatures in the program TD-PCR 55 and samples 13-24 are using the temperatures in TD-PCR 60. This figure shows all three sets of primers being tested with under both TD-PCR programmed temperatures.



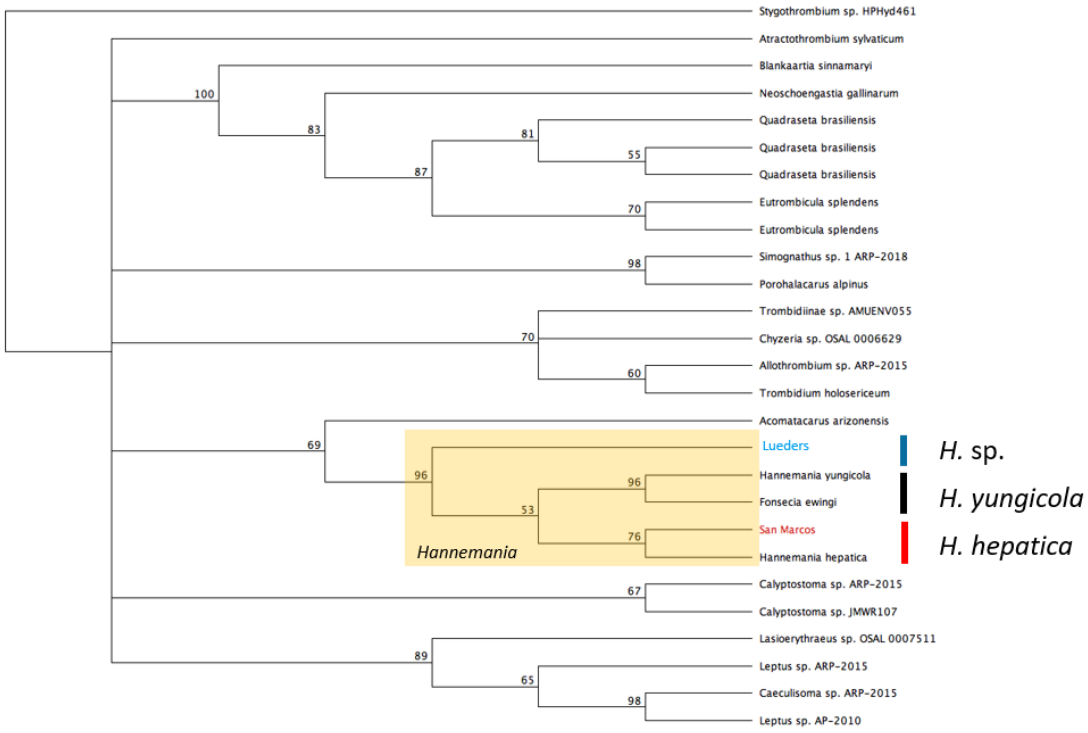
<u>Figure 3</u>: This figure shows the mite samples using the forward and reverse primers targeting the 18S and 28S gene regions. Samples labeled 1-6 come from the host Eleutherodactylus marnockii.



<u>Figure 4</u>: Image showing a mite larva extracted from the skin of a frog.



<u>Figure 5:</u> Aligned sequence showing the hypervariable regions of the nuclear 28S ribosomal RNA for the Lueders and San Marcos mites.



<u>Figure 6</u>: Neighbor-joining phylogenetic tree showing the relationship between other organisms with >95% nucleotide similarity to the San Marcos and Lueders Mites sequenced at the 18S gene.

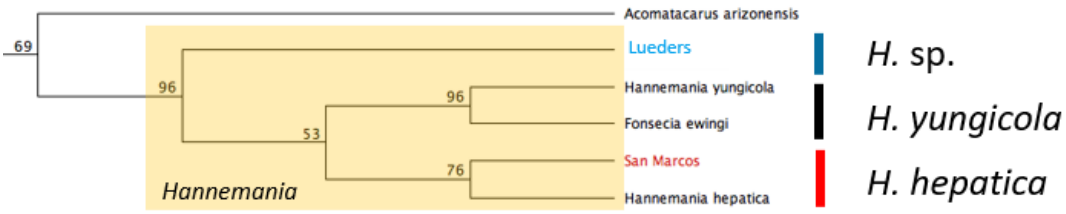


Figure 7: Clade displaying the San Marcos and Lueders mites possibly belonging to the genus Hannemania. The San Marcos mite appears to be associated with H. hepatica and the Lueders mite of a different species that has not been sequenced before.

Table 1: *Labels of the 14 individual mite samples extracted from frogs in Texas for DNA extraction.*

Sample	Mite ID	Host	Locality	Latitude	Longitude
1	CR_003_A_2	*Acris crepitans*	*Lueders, TX*	32.808648	-99.604088
2	CR_004_A_2	*Acris crepitans*	Lueders, TX	32.808648	-99.604088
3	CR_005_A_2	*Acris crepitans*	Lueders, TX	32.808648	-99.604088
4	CR_006_A_2	*Acris crepitans*	Lueders, TX	32.808648	-99.604088
5	CR_003_B	*Acris crepitans*	Lueders, TX	32.808648	-99.604088
6	CR_004_B	*Acris crepitans*	Lueders, TX	32.808648	-99.604088
7	CR_005_B	*Acris crepitans*	Lueders, TX	32.808648	-99.604088
8	CR_006_B	*Acris crepitans*	Lueders, TX	32.808648	-99.604088
9	Emar_1	*Eleutherodactylus marnockii*	San Marcos, TX	29.887773	-97.946607
10	Emar_2	*Eleutherodactylus marnockii*	San Marcos, TX	29.887773	-97.946607
11	Emar_3	*Eleutherodactylus marnockii*	San Marcos, TX	29.887773	-97.946607
12	Emar_4	*Eleutherodactylus marnockii*	San Marcos, TX	29.887773	-97.946607
13	Emar_5	*Eleutherodactylus marnockii*	San Marcos, TX	29.887773	-97.946607
14	Emar_6	*Eleutherodactylus marnockii*	San Marcos, TX	29.887773	-97.946607

Table 2: This table shows the volume of primer (nmol) and primer suspension solution to make 100 μL stock primer solution. Each forward and reverse primer was resuspended.

100 μL Stock Primer Solution
28 nmol (solid)
280 μL of primer resuspension solution
280 μL of 100 μM stock primer

Table 3: Forward and reverse primers tested and their corresponding sequence. An F was placed to indicate the forward primer and an R for reverse primer.

Primer	**Sequence**	**Source**
Mite 18S-IF	ATATTGGAGGGCAAGTCTGG	Otto & Wilson, 2001
Mite 18S-IR	TGGCATCGTTTATGGTTAG	Otto & Wilson, 2001
28v-5' F	AAGGTAGCCAAATGCCTCATC	Burger et al., 2012
28jj-3' R	AGTAGGGTAAAACTAACCT	Burger et al., 2012
NS7 F	GAG GCA ATA ACA GGT CTG TGA TGC	Kaliszewski et al., 1992
NS8 R	TCC GCA GGT TCA CCT ACG GA	Kaliszewski et al., 1992

Table 4: Volumes used to make a Master mix (MM) for each sample with the corresponding primers.

Contents (µL)	**Master Mix-1**	**Master Mix-2**	**Master Mix-3**
DreamTaq 2X	6.25	6.25	6.25
Primer F (10 mM)	18S IF 0.125	28v-5’ 0.125	NS7 0.125
Primer R (10 mM)	18S IR 0.125	28jj-3’ 0.125	NS8 0.125
$MgCl^2$ (25 mM)	25	25	25
Nuclease-Free H_2O	5.0	5.0	5.0
Total per sample	**12.0**	**12.0**	**12.0**

Table 5: Total volume of tDNA and MM used for each sample in the PCR reaction.

PCR Reaction Volumes (µL)	
Master Mix	12.0
Mite tDNA	0.5
Total Volume	**12.5 µL per sample**

Table 6: Reagents and volumes used to make an ExoSAP Master Mix.

ExoSAP Master Mix Volumes (µL)	
Nuclease-free H_2O	1.56
Exonuclease I	0.04
Shrimp Alkaline Phosphatase	0.40
Total Volume	**2 µL per sample**

Table 7: Reagents and volumes used to make a Master Mix for cycle sequencing.

Cycle Sequencing Master Mix Volumes (µL)	
Nuclease-free H_2O	1.68
5X Buffer	1.20
Primer	10 (µM)
BigDye 3.1 Ready Reaction Mix	1.00
Total Volume	**4 µL per sample**

Table 8: Volumes of Master Mix and template used in the cycle sequencing reaction.

Cycle Sequencing Reaction Volumes (µL)	
Master Mix	4.0
Template (Cleaned PCR Product)	2.0
Total Volume	**6.0 µL per sample**

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