DIET ANALYSIS OF THE TEXAS HORNED LIZARD

By

Kaitlyn Upton

Submitted in partial fulfillment of the

requirements for Departmental Honors

in the Department of Biology

Texas Christian University

8 May 2017

DIET ANALYSIS OF THE TEXAS HORNED LIZARD

Project Approved:

Supervising Professor: Dean Williams, Ph.D.

Department of Biology

Matthew Hale, Ph.D.

Department of Biology

F. Komla Aggor, Ph.D.

Department of Spanish & Hispanic Studies

Abstract

The Texas horned lizard (*Phrynosoma cornutum*) is a vulnerable species that is thought to eat almost exclusively harvester ants (*Pogonomyrmex spp*.). Previous research in our lab has indicated that there is an abundance of nonharvester ant prey in horned lizard scat samples collected in Karnes County, Texas including termites, other ant species, and beetles. This study aimed to create a primer pair for species level identification of prey, specifically focused on ant species, from horned lizard scat. Thus enabling the identification of the Texas horned lizard diet using molecular techniques. We collected insect samples from Karnes County and successfully sequenced 44 ant individuals as well as other insects from different orders. We built an alignment of ant sequences, both our own and some from the online database Genbank, and created neighbor-joining trees for a 560 base pair region of the mitochondrial cytochrome oxidase I gene as well as a 146 base pair region within the larger part. Both trees showed similar genetic relationships among samples and supported the claim that primers for the shorter region would be conserved enough to amplify a wide variety of ant species but also specific enough to still yield a species level identification.

Introduction

The Texas horned lizard (*Phrynosoma cornutum*) is a vulnerable species whose decline is thought be coupled with habitat disturbance and a decline in their preferred food source (Donaldson et al. 1994, Wolf et al. 2013). The invasion of fire ants (*Solenopsis invicta*) has also been linked to their decline by predation on eggs and young (Donaldson et al. 1994, Wolf et al. 2013). Many dietary studies point to ants as the staple diet for horned lizard species (Lemos-Espinal et al. 2004), specifically harvester ants (*Pogonomyrmex spp.*) for *P. cornutum* (Pianka and Parker 1975). These lizards are adapted to coat ants in mucus to prevent harmful stinging in the digestive system and are resistant to the venom produced by harvester ants (Schmidt et al 1989, Sherbrooke and Schwenk 2008). The use of pesticides in urban environments to control fire ants is thought to be another reason for the decline in Texas horned lizards because it also reduced the abundance of native ants that they eat (Donaldson et al. 1994).

In previous research conducted in our lab, we have a seen a large variety of prey items in Texas horned lizard scat samples and a minority of harvester ants (Alenius 2016). The most common prey items have been termites (thought to be *Gnathamitermes tubiformans*), small ant species, and Coleoptera (beetles) (Alenius 2016). 71% of the prey items morphologically identified by volume in the scat were termites while harvester ants (*Pogonomyrmex*) comprised only 7.6% of the volume of the scat (Alenius 2016). This does raise the question as to whether the Texas horned lizard is truly a harvester ant specialist. No previous studies have indicated a preference for termites in the horned lizard diet.

In addition to morphological methods, genetic analyses are important components of dietary studies. Sorting of insect parts into the correct genus or species groups requires knowledge of insect morphology and is nearly impossible within some genera. Because we are

working with scat, the digestive system of the horned lizard will typically destroy most soft-bodied prey items which means that morphological sorting can only identify species with hard exoskeletons (Zeale et al. 2011). Even though the chitin in the insects' exoskeleton remains mostly unaltered, many of the insects are separated into multiple pieces or are missing anatomical parts such as mandibles that are often crucial for species identification. Additionally, many species of ant, those in the genera *Solenopsis* for example, can only be distinguished via genetic identification. Genetic sequencing is a more reliable way for species identification from scat, because it does not rely on proper identification of anatomical features (Zeale et al. 2011).

One of the challenges our lab has faced with genetic sequencing methods is finding a primer that can be utilized for the wide variety of insect species found in the lizards' scat samples. The primers we have used previously were the universal ZBJ primers that amplify a 157 bp region of the mitochondrial cytochrome oxidase I (Cox I) gene and were originally developed to study the diets of insectivorous bats (Zeale et al. 2011). These primers, however, did not amplify ant species in our samples. We then attempted to develop ant specific primers (Alenius 2016) and we were able to amplify some ant species, however, these primers did not amplify harvester ants and some other ant species which are known to be present via morphological identification. This study was designed to expand our knowledge of the diet of the Texas horned lizard through genetic diet analysis by creating primers for amplification and identification of all ant species in scat samples.

Methods

Field Collection

All insects were collected in Kenedy and Karnes City, Texas, two small towns located in Karnes County (Figure 1). Since 2013, 15 transect sites across the two towns have been used for Texas horned lizard research through the TCU Horny Toad Project. We collected insect specimens periodically from May through August 2016 with a variety of methods. We used 10 pitfall traps for 16 days in May, June, July, and August seven transect sites. These traps consisted of two 16-ounce plastic cups that were buried flush to the ground approximately 30 to 45 cm apart (Figure 2). Between the two cups ran a 5 cm piece of reinforced plastic that was partially buried in the ground to stop insects and direct them to fall into the cups. Each cup was filled with propylene glycol to preserve insects that were collected. Traps were emptied once per day over an aquarium net and propylene glycol was re-filled when necessary. In addition, baited traps were also placed in close proximity to each pitfall trap (in order to be more easily found). These consisted of clear, plastic cylinders, approximately thumb-sized that were baited with either hot dog or honey (Figure 3).

Figure 1. Karnes County, Texas


Figure 2. Pitfall trap set at a transect site in Karnes County

Approximately 30 minutes after they were set, baited traps were checked and between 3 and 5 individual ants were removed from each trap. Other collection methods utilized were ad hoc hand catching and a handheld vacuum. All collected insects were preserved in 100% ethanol.

Figure 3. Baited trap set at a transect site in Karnes County

Lab Work

Preserved insect samples were sorted morphologically by order and then Hymenoptera (ant species) were sorted by genus or species when possible. At least one individual from each genus or species was sampled for a total of 80 ants. Additionally, 4 termites and 7 beetles were also sampled for this study.

Insects were placed in 300 µl of lysis buffer with 15 µl Proteinase K (20 mg/ml). For some larger individuals, legs were removed for DNA extraction rather than using the whole body. A drill and pestle was then used to grind up each specimen and then the samples were incubated overnight on a heating block at 55°C. After cooling to room temperature, a half volume of 7.5 M Ammonium Acetate was added to each sample and then each sample was vortexed and placed in the freezer for at least 10 minutes. Samples were then centrifuged to pellet proteins and these pellets were discarded after decantation of the supernatant containing the DNA into tubes of 0.7 volumes of Isopropanol. These were thoroughly mixed and then centrifuged to pellet the DNA. Each pellet was washed with 400 µl of 70% ethanol and then resuspended in 75 µl of 10 mM Tris.

For each sample, a portion of the Cox I gene was amplified using polymerase chain reaction (PCR) using universal primers dgLCO1490 (5’

GGTCAACAAATCATAAAGAYATYGG 3') and dgHCO2198 (5' TAAACTTCAGGGTGACCAAARAAYCA 3') (Meyer 2003). Polymerase chain reactions (PCR) (10µl) contained 1 µl DNA (20-50 ng/µl), 0.5 µM of each primer, 2X BSA, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, and 1X Multiplex PCR buffer with 3mM $MgCl_2$ pH 8.7, and dNTPs. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were one cycle at 95°C for 15 min, followed by 40 cycles of 30s at 94°C, 90s at 50°C , 90s at 72°C, and then a final extension at 72°C for 5 minutes. A negative control was run in every group of samples. Agarose gel electrophoresis was used to confirm the success of each amplification. Samples were then cleaned enzymatically using *Exo I* and *rSap* as per manufactures instructions. PCR products were then sequenced in both forward and reverse directions using BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems) and electrophoresed on an ABI 3130XL Genetic Analyzer. Next, all sequences were imported into Sequencher v. 5.0 where the ends were trimmed and contigs for each sample were created. In total, I obtained 44 high quality sequences.

After all samples were sequenced, an alignment was built in Mega v6.0 (Tamura et al. 2013) for the 44 extracted sequences. This alignment was entered into both Genbank and BOLD (Barcode of Life Database) online databases for species identification. To be identified to the species level, a sequence must match the database sequence to at least 99%, 94.9% for genus level identification, 91% for family level identification, and 85.9% for order level identification. Additional sequences (n = 31) which were taken from GenBank were added to this alignment for species of ants that are known to be found in Karnes County compiled by Rachel Alenius from AntWeb (https://www.antweb.org/) as well as the Texas A&M 'Common Ant Genera of Texas'

ID guide. This alignment was then used to create a consensus neighbor-joining tree in Mega with Kimura-2 parameter distances and 500 bootstrap iterations to test for branch support.

Using the previously identified ZBJ region as a guide, we selected a 146 bp region of the 560 base-pair Cox I gene alignment to use as a target for the primers we were creating. We used Primer3 (Untergasser et al. 2012) to identify the best sequences for creation of our primers adding in degenerate bases at nucleotide sites that varied across ant genera. We also created another consensus neighbor-joining tree based off of this smaller region. This tree had 70 sequences because 5 of the sequences used in the original tree did not hit the 146 bp region.

Results

We were able to identify 25 of the samples from our alignment to the species level representing 11 different ant species: *Solenopsis invicta*, *Dorymyrmex flavus*, *Pheidole sitiens*, *Nylanderia vividula*, *Neivamyrmex swainsonii*, *Pheidole diversipilosa*, *Pheidole dentata*, *Pheidole pelor*, *Pogonomyrmex barbatus*, *Camponotus sayi*, and *Cyphomyrmex rimosus*. We identified 9 samples to the genus level, 8 samples to the family level, and 2 to the order level. Based on the created alignment and neighbor-joining trees, we then used a 3% sequence difference cut-off to determine different species. For example, our sample A31 in Figure 4 is 7% different than both A1 and A2 in Fig. 4 and so A31 is a different species in the genus *Solenopsis* than A1 and A2 which were both identified as *Solenopsis invicta*. In contrast, A48, A49, A51, A53, A5, and A6 in Figure 4 are only 0.2% different and so they are all classified as the same species. Using this method, we were able to determine 9 other unidentified species which we were previously unable to identify through sequence similarity on the online databases. This brought us to a total of 20 different species identified. Additionally, using sequences from extracted termite DNA, we were able to determine that the species of termite that is part of the

horned lizard diet in Karnes County is not the desert termite *Gnathamitermes tubiformans* as we previously thought (Alenius 2016) but rather appears to be *Nasutiform cinereus*.

The 146 base-pair region eliminated two species from our tree (A71 Hymenoptera and A26 *Neivamyrmex swainsonii*) because the sequences did not include this region. Because this tree still demonstrated similar relationships between species, we believe that this region is conserved enough to be used with a variety of ant genera but is still specific enough to be able to identify ants at the species level. The degenerate primers we created for this region are:

Left: 5' WARRAARTTWCCRAAYCCYCCAA 3'

Right: 5' ATTYTWTAYTTYWTYTTWGCWATYTGAKC 3'

Mixed bases were used in order to make the primers better able to pick up a variety of ant species (R= A,G; W= A,T; Y= C,T; K= G,T).

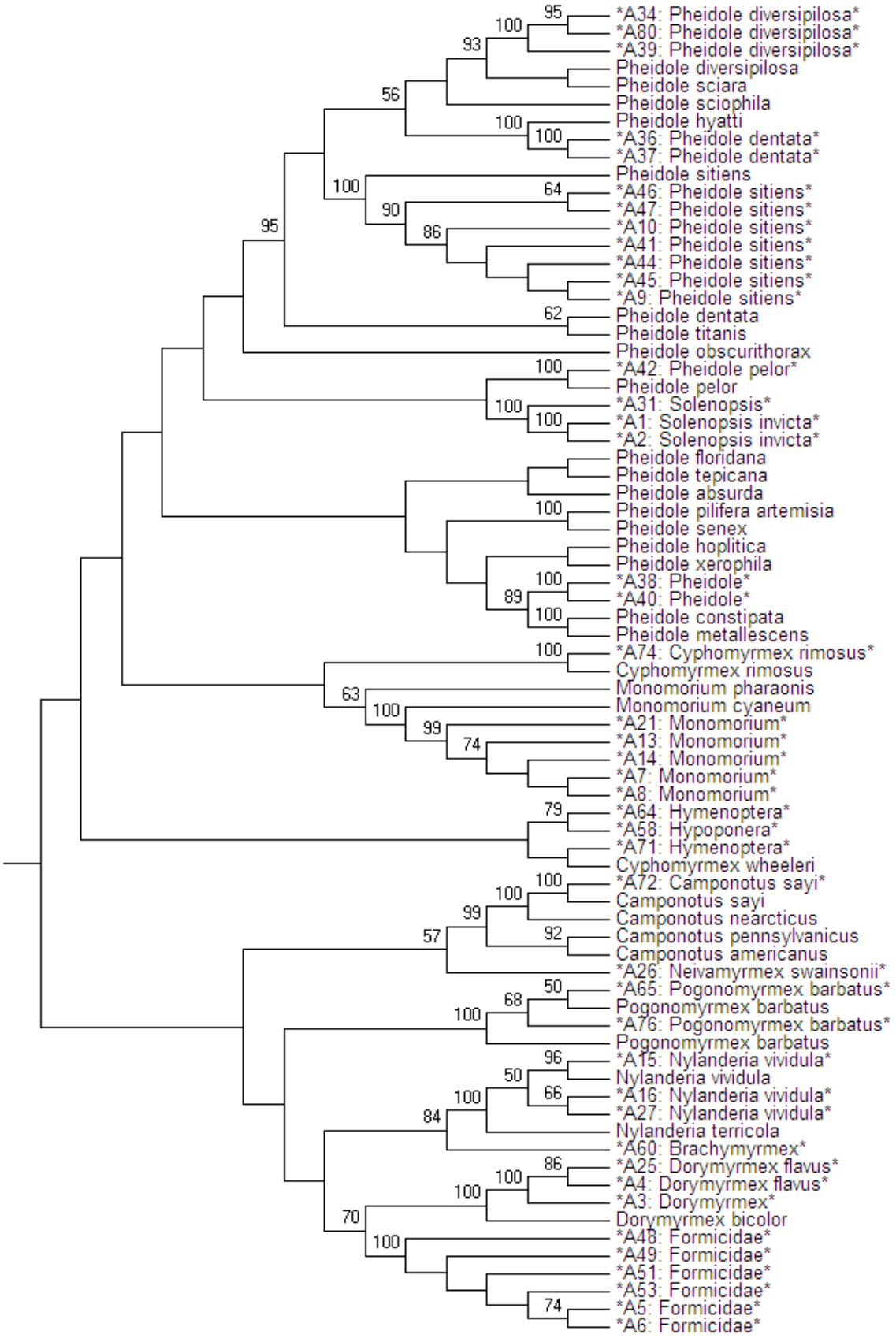

Figure 4. Consensus neighbor-joining tree for ant species in Karnes, Texas from a 560 bp sequence of the Cox I gene and Kimura-2 parameter distances. Bootstrap values >50 are labeled. Branch length is not proportional to amount of genetic variation.
*-sequences from this study (n = 44), 31 additional sequences from GenBank

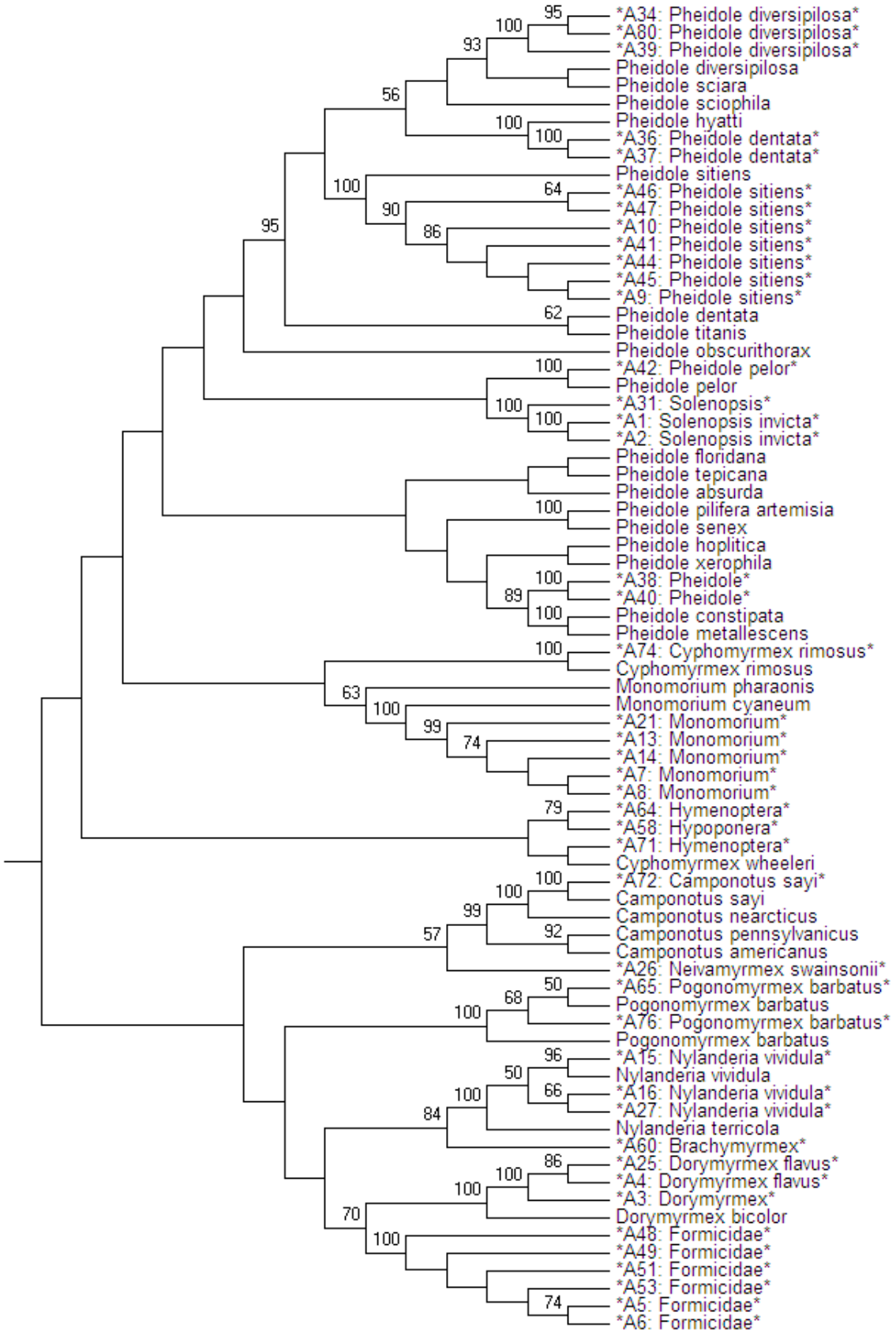

Figure 5. Consensus neighbor-joining tree for ant species in Karnes, Texas from a 146 bp sequence of the Cox I gene and Kimura-2 parameter distances. Bootstrap values >50 are labeled. Branch length is not proportional to amount of genetic variation.
*-sequences from this study (n = 42), 28 additional sequences from GenBank

Discussion

Based on our neighbor-joining trees built from the sequence alignments of both the 560 base-pair region and the 146 base-pair region of the Cox I gene, it appears that the smaller region is conserved enough to match different ant species yet specific enough to help in species-level differentiation. This is ideal in order to use the primers we created to identify the species of ants that are found in scat samples. Moving forward, we will use these primers with the scat samples collected from the summer of 2016 as well as additional scat samples we collect in the future to identify ants at the species level in the horned lizard diet. This method will be used alongside morphological sorting in order to confirm and potentially improve morphological accuracy. In addition, these primers could be used to help in genetic diet analysis of other species aside from horned lizards that also eat ants. There is a high diversity of ant species and they are often difficult to differentiate morphologically to the species level. This means that a specific ant primer like the one we have created could be highly beneficial to other research groups as well.

Previously morphological sorting conducted in our lab has identified large numbers of insects other than ants that are present in scat samples with beetles and termites being seen in the highest abundance. We would like to create separate primers: one specific to beetles and one specific to termites that can also be used with scat samples in a similar fashion to the ant primer we have created. Though termite and beetle samples were sequenced in this project, there is a high degree of genetic difference between beetles, termites, and ants in the Cox I gene which made it not possible to fit all three into one alignment. This means that it is unlikely that one universal primer would be able to identify all three types of insects at the species level.

One drawback to insect genetic research is that there is a lack of available information in public databases. Many ant species, for example, simply do not have available genetic

information on GenBank or BOLD for us to match our sequences. There is work being done on ant genetics at various universities including Purdue University and Georgia Institute of Technology. This is why we used our neighbor-joining trees to identify species that were not identified at the species level through these online databases. As more research continues in this area, we hope that more sequences will become publically available and will improve our species identification. We will be submitting the sequences of species we have that are not currently available on GenBank.

References

Alenius, RA. 2016. Diet analysis of Texas horned lizards (*Phyrnosoma cornutum*) in two small Texas towns using morphological and genetic methods. Bachelors of Science Thesis. Texas Christian University.

Donaldson, W, AH Price, and J Morse. 1994. The current status and future prospects of the Texas horned lizard (*Phrynosoma cornutum*) in Texas. *Texas Journal of Science* 46:97-113.

Lemos-Espinal, JA, GR Smith, and RE Ballinger. 2004. Diets of four species of horned lizard (genus *Phrynosoma*). *Herpetological Review* 35(2): 131-134.

Meyer, CP. 2003. Molecular systematics of cowries (Gastropoda: Cypraeidae) and diversification patterns in the tropics. *Biological Journal of the Linnean Society* 79: 401–459.

Pianka, ER, and WS Parker. 1975. Ecology of horned lizards: a review with special reference to *Phrynosoma platyrhinos*. *Copeia* 1975:141-162.

Schmidt, PJ, WC Sherbrooke, and JO Schmidt. 1989. The detoxification of ant (*Pogonomyrmex*) venom by a blood factor in horned lizards (*Phrynosoma*). *Copeia* 1989(3):603-607.

Sherbrooke, WC and K Schwenk. 2008. Horned lizards (*Phrynosoma*) incapacitate dangerous ant prey with mucus. *Journal of Experimental Zoology* 309A: 447-459.

Tamura, K, G Stecher, D Peterson, S Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30(12):2725-2729.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3 - new capabilities and interfaces. *Nucleic Acids Research* 40(15):e115

Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3 *Bioinformatics* 23(10):1289-91

Wolf, AJ, EC Hellgren, V Bogosian III, and RW Moody. 2013. Effects of habitat disturbance on Texas horned lizards: an urban case study. *Herpetologica* 69(3):265-281.

Zeale, MRK, RK Butlin, GLA Barker, DC LEES, and G Jones. 2011. Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources* 11:236-244.