White-nose syndrome (WNS) is an emerging wildlife disease that has caused the most rapid wildlife population declines ever reported and is threatening all temperate bat species. Analysis of the growth from WNS inflicted bats has conclusively identified the causative agent of WNS, a fungus designated *Geomyces destructans*. Since the first report from New York in 2006, WNS has been detected in 16 additional states and implicated in over five million bat deaths in North America. The Gypsum caves found throughout the Red Hills of Kansas have the state’s most diverse and largest population of cave roosting bats, and are home to some Tier 1 ranking species as noted by the Kansas Comprehensive Wildlife Conservation Plan (KCWCP). Currently, WNS has not been detected in the Gypsum caves. However, the rapid westward movement of WNS from the Eastern United States, the likely occurrence of WNS in neighboring counties of Oklahoma, and the already fragile populations of bats in the Red Hills of Kansas dictate aggressive action to help aid the understanding of this impending epizootic disease. In this study, cave soil was obtained from the Red Hills. Using the polymerase chain
reaction, a 624 nucleotide DNA fragment specific to the Type1 Intron/ITS region of the 18S rRNA gene from Geomyces species was amplified. Subsequent DNA sequencing and direct comparison to the same genetic locus in *G. destructans* was performed. The data indicates that *G. destructans* DNA was detected, along with 26 Geomyces variants.

Continued surveillance to monitor trends of *G. destructans* distribution in the Red Hills of Kansas is critical to the management of a possible WNS outbreak should it occur.
DIVERSITY OF THE TYPE I INTRON/ITS REGION OF THE 18S rRNA GENE IN

**GEOMYCES SPECIES FROM THE RED HILLS OF KANSAS**

A Thesis

Presented to

The Department of Biological Sciences

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Xi Chen

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ACKNOWLEDGMENTS

I would like to give my thanks to my advisor, Dr. Scott Crupper, for his assistance and guidance in both the laboratory and the writing of this thesis. I would also like to thank my committee members, Dr. Yixin Eric Yang and Dr. John Richard Schrock, for their suggestions and assistance in the writing of this thesis. Additionally, I would like to thank my lab mates, Changjun Lin, Cailin Wilson and Younshim Park for their assistance in the lab as well as their kindness and understanding throughout my graduate studies at ESU. Lastly, I would like to thank my family in China and my friends in Emporia for their encouragement, support, and love. Without them I would not have been able to accomplish what I have.
PREFACE

This thesis was prepared following the publication style of the American Society for Microbiology.
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Introduction

White-nose syndrome (WNS) is an emerging wildlife disease that has caused the most rapid wildlife population declines ever reported and is threatening all temperate bat species (10, 25). WNS is characterized by visually apparent white fungal growth on exposed skin of ears, muzzle, tail, snout, and wing membranes of hibernating bats (4, 19). The fungus, designated as *Geomyces destructans*, has been conclusively identified as the causative agent of WNS (19, 27). This fungus causes bats to lose fat reserves needed to survive winter hibernation and causes them to prematurely emerge from hibernacula in mid-winter (3, 6).

Since its initial detection on 16 February 2006 at Howes Cave, Albany, NY, WNS has resulted in the death of more than five million bats in North America (10). This far exceeds the magnitude of any previously known mortality events in bats (3, 6). *Geomyces destructans* has been found in nine species of bats in North America, and population models even predict that little brown bats (*Myotis lucifugus*) will face regional extinction in the near future, which could cause a serious imbalance in the ecosystem with unforeseen consequences (4, 10). It is assumed that all the species of cave hibernating bats will be at risk as WNS spreads to new areas (6). Starting from New York, WNS has spread rapidly throughout seventeen U.S. states and four Canadian provinces (Figure 1). It is moving westward, as evidenced by its detection in Missouri and Oklahoma.

The Gypsum caves found throughout the Red Hills of Kansas have the state’s most
Figure 1. A map of WNS in North America. (www.tnbwg.org; March, 2013)
diverse and largest population of cave roosting bats (Figure 2). Currently, WNS has not been detected in the Gypsum caves. However, the rapid westward movement of WNS from the Eastern United States and the already fragile populations of bats in the Red Hills of Kansas dictate the scientific community must act aggressively to help aid the understanding of this impending epizootic disease.

To further investigate the possible occurrence of WNS in the Red Hills caves, a bat survey was conducted during the winter months of 2011-2012 (Appendix 1). Even though no evidence of WNS was detected, soil samples were collected to determine if *G. destructans* was present. Previously, Lorch et al. (16) developed a PCR-based diagnostic approach to detect *G. destructans* based on the amplification of the Type1 Intron/ITS region of the 18S rRNA gene. This technique was subsequently employed by Lindner et al. (15) to detect *G. destructans* in bat hibernacula. Based on this established methodology, the goal of this study was to conduct a PCR-based survey of the Red Hills caves to detect the occurrence of *G. destructans*, parallel to the studies of Lindner et al. (15).
Figure 2. Map of Kansas.
Materials and Methods

Sample Collection

Soil samples used in this study were collected from caves located in the Red Hills of Kansas by Dr. William Jensen (Emporia State University) and his research team from December 2011-January 2012. Using sterile spatulas, soil samples were placed into sterile collection bags. In total, 12 samples were obtained from each cave (n=16). All samples were stored at 4 °C until used for DNA purification.

Bacterial Strains and Media Used in This Study

Escherichia coli TG1 was used as the host strain for transformation experiments. It was routinely propagated at 37 °C in Luria-Bertani (LB) media (10 g Bactotryptone, 5 g yeast extract, 10 g NaCl/L). Agar plates were prepared by adding agar (20 g/L) to liquid media. Ampicillin was used at 100 μg/ml.

DNA Isolation

Total DNA was isolated from soil samples using a ZR Soil Microbe DNA MicroPrep™ (Zymo Research; Irvine, CA) according to the manufacturer’s instructions. Plasmid DNA from recombinant E. coli TG1 was isolated using a QIA prep® Spin Miniprep Kit (Qiagen; Valencia, CA) according to the manufacturer’s instructions. All DNA was stored at 4 °C until used.
DNA Concentration Determination

DNA concentration was determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific™; St. Louis, MO). The final concentration of all DNA samples was adjusted to 50 μg/ml using H₂O.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to amplify gene specific regions from DNA templates. The amplification process was carried out as described in Table 1 using a Bio-Rad T100 thermocycler (Bio-Rad; Hercules, CA). Reactions typically consisted of a deoxynucleotide triphosphate mix (200 μM each dNTP), polymerase specific reaction buffer, 50 ng DNA, 1.5 mM MgCl₂ and 1 U Taq polymerase for the 16S rRNA amplification and screening for the 624 bp Type1 Intron/ ITS region of the 18S rRNA gene. Once an amplicon was obtained, PCR was repeated using 1U of High-Fidelity DNA Phusion Polymerase (Thermo Scientific™) to obtain the fragment for subcloning.

Agarose Gel Electrophoresis

To effectively separate DNA fragments of various sizes, agarose gel electrophoresis was employed according to standard conditions (21). Briefly, 30 ml of 1X TAE buffer prepared from a 50X TAE stock (242 g Tris, 57.1 ml acetic acid, and 4 ml 0.5 M EDTA in 1 L of H₂O), 0.3 g of agarose, and 2 μl of 10 mg/ml ethidium bromide (EtBr) were mixed
Table 1. PCR primers and amplification conditions.

<table>
<thead>
<tr>
<th>Primer Sequence</th>
<th>Reaction Conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC[F] ---5’- CCA GAC TCC TAC</td>
<td>94 °C/5min</td>
<td>(20)</td>
<td>Amplification of 16S rRNA gene</td>
</tr>
<tr>
<td>GGG AGG CAG C- 3’</td>
<td>94 °C/1min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD[R] --- 5’- CTT GTG CGG</td>
<td>55 °C/1min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCC CCC GTC AAT TC- 3’</td>
<td>72 °C/45sec</td>
<td>Repeat cycle, 29X, 72 °C/5min, 12 °C/∞</td>
<td></td>
</tr>
<tr>
<td>Gd enrichment [F] --- 5’-GGG</td>
<td>94 °C/5min</td>
<td>(16)</td>
<td>Amplification of 624bp Type 1 Intron/ITS Region of the 18S rRNA gene of Geomyces species</td>
</tr>
<tr>
<td>GAC GTC CTA AAG CCT- 3’</td>
<td>94 °C/1min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gd enrichment[R] --- 5’-TTG TAA</td>
<td>52 °C/1min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGA CGC TCG GAC- 3’</td>
<td>72 °C/1min</td>
<td>Repeat cycle, 29X, 72 °C/5min, 12 °C/∞</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Reaction conditions described by Lorch et al., 2010 were slightly modified in this study.
and heated in the microwave until the agarose dissolved. The mixture was poured into a gel casting tray containing a gel comb. After solidification, the comb was removed and 1X TAE buffer was added to completely cover the gel. Subsequently, DNA samples were mixed with loading buffer, loaded into the wells, and electrophoresed using a Bio-Rad model 250/2.5 power supply (Bio-Rad), at ~100 volts for 30-40 min. DNA was visualized after electrophoresis using a UV Intensity Transilluminator (Fisher Scientific™; St. Louis, MO).

**DNA Purification from an Agarose Gel**

DNA was excised from agarose gels using a razor blade. The slice of agarose containing the DNA was placed in a 1.5 ml microcentrifuge tube and purification accomplished using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research) according to the manufacturer’s instructions.

**DNA Ligation**

DNA was ligated into pJET 1.2 (Thermo Scientific) using a Fast-Link™ DNA ligation kit (Fisher Scientific) according to the manufacturer’s instructions. Reactions consisted of 1.5 μl 10X Fast-Link Ligation Buffer, 0.75 μl of 10 mM ATP, 1 μl of linearized pJET1.2, 1 μl of insert DNA, 2 U of DNA ligase, and 9.75 μl of distilled water to reach a total volume of 15 μl. Reaction mixtures were incubated at room temperature for 15 min.
Preparation of Competent Cells

*Escherichia coli* TG1 was grown in 2X LB medium at 30 °C overnight with shaking at 250 rpm. Subsequently, 0.5 ml of an overnight culture was used to inoculate 200 ml of fresh 2X LB in a flask at 30 °C with shaking. When the OD_{600} of the culture reached 0.3, 4 ml of 1M MgCl\(_2\) was added and incubation continued until an OD_{600} of 0.45-0.55 was obtained. Subsequently, the culture was placed on ice for 2 hrs prior to centrifugation at 3000 rpm at 4 °C for 5 min in a J2-HS centrifuge (Beckham Coulter Inc; San Diego, CA). The cell pellet was resuspended in ice-cold 100 mM CaCl\(_2\) media (0.05 M CaCl\(_2\), 0.04 M MnCl\(_2\), and 0.02 M CH\(_3\)COON, pH=7.5). After incubation on ice for 40 min, cells were pelleted by centrifugation and resuspended in fresh ice-cold 100 mM CaCl\(_2\) media containing 15% glycerol. The competent cells were stored in 100 μl aliquots at -80 °C.

Transformation

Ligation reactions were transformed into *E. coli* TG1 competent cells by mixing 5 μl of each ligation mixture with 100 μl of cells. After incubation on ice for 15 min, reactions were heat shocked at 42 °C for 90 sec. After a 1 min recovery on ice, 900 μl of LB medium was added and the transformation mixture incubated at 37 °C for 1 hr. Subsequently, 100 μl of the transformation mixture was spread onto LB/ampicillin (100 μg/ml) plates followed by incubation at 37 °C overnight. Recombinants were selected for further analysis.
DNA sequencing

DNA sequencing was carried out by the DNA Sequencing Facility at the University of Arkansas for Medical Science, Little Rock, Arkansas.

BLAST analysis

The Basic Local Alignment Search Tool (BLAST) (1) was performed to compare DNA sequences of cloned samples to the 624 bp *G. destructans* Type 1 Intron/ ITS Region of the 18S rRNA gene (GenBank: EU884921.1).
Results

Total DNA was isolated from all 189 soil samples and standardized to a concentration of 50 μg/ml. Amplification of 16S rRNA gene from each DNA sample was successfully performed, indicating the DNA was of sufficient quality for PCR (Figure 3). Using *Geomyces* specific primers for the 624 bp Type 1 Intron/ITS Region of the 18S rRNA gene, the expected amplicon of 624 bp was identified in 12 samples from 6 different caves (Figure 4). A heterogeneous 624 bp fragment was eluted from the agarose gel representing each positive cave and cloned into pJET1.2. A total of 302 recombinant clones (~50/cave) were selected and subjected to DNA sequencing. BLAST analysis demonstrated the 624 bp *G. destructans* sequence could be identified in soil samples from 4 caves along with 26 different *Geomyces* variants (Table 2). Appendix 1 contains the complete 624 bp DNA sequence for *G. destructans* and all variants described in this study.
Figure 3. Amplification of 16S rRNA gene. This is a representative agarose gel of all 189 samples. Lane M – DNA ladders; Lane 1-38 – PCR amplification of the 603 bp 16S rRNA gene.
Figure 4. Amplification of the Type1/ITS region of the 18S rRNA gene from the genus *Geomyces*. This is a representative agarose gel of all 189 samples. Lane C – 624 bp amplicon from *Geomyces destructans*; Lane M – DNA ladders; Lane 1-29 and 31-37 – negative amplifications from soil samples; Lane 30 – positive amplification of the 624 bp fragment.
Table 2. *Geomyces destructans* and type variants of the *Geomyces* Type 1 Intron/ITS Region of the 18S rRNA gene identified in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Number of Samples</th>
<th>Caves&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Closest GenBank Accession Number Match/(bp Homology)</th>
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<tr>
<td><em>G. destructans</em></td>
<td>n=62</td>
<td>CAC DCC DAC GEC</td>
<td>EU884921.1/(624/624)</td>
</tr>
<tr>
<td>Type1</td>
<td>n=178</td>
<td>BSC CAC DCC DAC GEC SBC</td>
<td>JX270511.1/(624/624)</td>
</tr>
<tr>
<td>Type2</td>
<td>n=2</td>
<td>BSC CAC</td>
<td>JX270511.1/(623/624)</td>
</tr>
<tr>
<td>Type3</td>
<td>n=2</td>
<td>BSC GEC</td>
<td>JX270511.1/(623/624)</td>
</tr>
<tr>
<td>Type4</td>
<td>n=7</td>
<td>BSC CAC DAC</td>
<td>JX270511.1/(623/624)</td>
</tr>
<tr>
<td>Type5</td>
<td>n=1</td>
<td>CAC</td>
<td>JX270511.1/(623/624)</td>
</tr>
<tr>
<td>Type6</td>
<td>n=1</td>
<td>CAC</td>
<td>JX270511.1/(623/624)</td>
</tr>
<tr>
<td>Type7</td>
<td>n=1</td>
<td>CAC</td>
<td>HM848977.1/(623/624)</td>
</tr>
<tr>
<td>Type8</td>
<td>n=1</td>
<td>CAC</td>
<td>JX270621.1/(618/625)</td>
</tr>
<tr>
<td>Type9</td>
<td>n=7</td>
<td>CAC DAC SBC</td>
<td>JX270626.1/(621/624)</td>
</tr>
<tr>
<td>Type10</td>
<td>n=1</td>
<td>CAC</td>
<td>JX270344.1/(623/624)</td>
</tr>
<tr>
<td>Type11</td>
<td>n=1</td>
<td>CAC</td>
<td>JX270511.1/(623/624)</td>
</tr>
<tr>
<td>Type12</td>
<td>n=1</td>
<td>DCC</td>
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</tr>
<tr>
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<td>n=2</td>
<td>DCC SBC</td>
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<td>JX270626.1/(621/624)</td>
</tr>
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<td>n=16</td>
<td>DAC GEC SBC</td>
<td>JX270626.1/(621/624)</td>
</tr>
<tr>
<td>Type</td>
<td>n</td>
<td>Site</td>
<td>Code</td>
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<td>----</td>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>Type16</td>
<td>2</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Type20</td>
<td>1</td>
<td>SBC</td>
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</tr>
<tr>
<td>Type21</td>
<td>1</td>
<td>SBC</td>
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</tr>
<tr>
<td>Type22</td>
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</tr>
<tr>
<td>Type23</td>
<td>2</td>
<td>DAC SBC</td>
<td>JX270626.1/(620/624)</td>
</tr>
<tr>
<td>Type24</td>
<td>1</td>
<td>SBC</td>
<td>JX270626.1/(620/624)</td>
</tr>
<tr>
<td>Type25</td>
<td>1</td>
<td>SBC</td>
<td>JX270511.1/(623/624)</td>
</tr>
<tr>
<td>Type26</td>
<td>1</td>
<td>DCC</td>
<td>JX270511.1/(623/624)</td>
</tr>
</tbody>
</table>

*a Big Surprise Cave, BSC; Can Cave, CAC; Dancers Cave, DAC; Dead Coyote Cave, DCC; Gentry Cave, GEC; Swartz Bat Cave, SBC.*
Discussion

The genus *Geomyces* is composed of psychrophilic fungi which aid perennial plants in adapting to low-nutrient environments (7). They are also keratinophilic fungi which can degrade hairs and nails and have been studied for possible biodecompositional use (22). To date, nine *Geomyces* species have been identified, including *Geomyces asperulatus* (23), *Geomyces auratus* (24), *Geomyces cretaceous* (24), *Geomyces destructans* (11), *Geomyces laevis* (14), *Geomyces pannorum* (23), *Geomyces pulvereus* (13), *Geomyces sulphureus* (24) and *Geomyces vinaceus* (26). Among these species, *Geomyces pannorum* is reported to have the ability to cause skin infections in humans and animals (9, 12). The most notable member of this group, however, is *Geomyces destructans*, the causative agent of WNS.

Detection of the *Geomyces* genus in this study employed amplification of the Type1 Intron/ITS region of the 18S rRNA gene of *Geomyces*. The 18S ribosomal RNA (rRNA) genes are one of the most frequently used genes in eukaryotic phylogenetic studies because they are highly conserved throughout evolution and can be readily amplified with universal primers (18). Previous studies have shown that 18S rRNA genes are useful in detection and functional investigation of fungi (2, 8). Other highly conserved genes, such as 16S rRNA genes, are also commonly amplified with universal primers in the identification of bacteria (5).

In this study, PCR amplification was accomplished following the established
protocol of Lorch et al. (16) using conserved primers to amplify the Type1 Intron/ITS region of the 18S rRNA gene. Unfortunately, the primer pair employed could not discriminate specifically *G. destructans* but rather *Geomyces* species. To separate the heterogeneous 624 bp 18S rRNA amplicon, purified DNA was cloned into the cloning vector pJET1.2. Cloning with pJET1.2 provides for a high percentage of recombinants since recircularized pJET1.2 vector will not propagate due to the production of a lethal restriction enzyme. The DNA sequence of ~50 recombinants from each putative positive cave was determined and compared to the 624 bp *G. destructans* Type1 Intron/ITS region of the 18S rRNA gene (GenBank: EU884921.1).

Results of this study confirmed the occurrence of the *G. destructans* gene in Red Hills cave soil, suggesting the possible presence of the fungus. However, no attempt was made to isolate viable *G. destructans* and no evidence of WNS-infected bats was observed in the caves (Appendix 2). Possible interpretations for the amplification of *G. destructans* specific DNA in the absence of WNS is that the fungus was once actively growing in the caves, but now only spore forms of the organism exist. It is also possible that *G. destructans* is a relatively new inhabitant in the cave. The occurrence of WNS in neighboring counties in Oklahoma provide a plausible explanation for its occurrence in the Red Hills of Kansas.

In addition to detecting the *G. destructans* Type1 Intron/ITS region of the 18S rRNA gene, twenty-six variants at this genetic locus were also detected, many of which
have not been reported in GenBank. Since 100% of all WNS cases are caused by *G. destructans*, the role of these variants in WNS, if any, remains to be determined. The most common variant identified in this study, designated as Type1, matched 100% to a previously studied *Geomyces* species (17). However, *Geomyces* variants found in this study were not cultured in laboratory, making it impossible to determine the role of the variants. According to the ecological niches occupied by *Geomyces*, the variants might be helping plants survive low-nutrient conditions and/or be involved in biodegradation.

In conclusion, this study provides the first analysis and detection of the environmental occurrence of *G. destructans* in Kansas. It also demonstrates the vast diversity of this genus based on one genetic locus. Even though WNS has not been detected in Kansas, evidence of *G. destructans* occurrence still highlights the possibility of fungal translocation and transmission by animal and human activities. Continued surveillance will be needed to monitor trends in *G. destructans* distribution in the Red Hills of Kansas.
References


Appendix 1. DNA sequences of *Geomyces* variants in this study

*Geomyces destructans* (100% homology to GenBank EU884921.1)

TTGTAATGACGCTCGACAGAGCATGCCCATGCCCCCAGAAATCAGGGGGGCAGAATGT
GCGTTCAAGATTCTGATGATCTGAATCTGAAATTACATATTTCAGATCGCAT
TTCGCTGGTCTTCTCTATCGAGTACAGACAGAAGATCCGTTGTTGGAAGTTTT
AACTATTATATAGTACTGACAGATAGCAGACAAAAACAGAGTTAGTTACCCGAGCCGCAACC
CGGGCGACTACTGTAATGATCCTCTCCGAGGTTCACCTAGAATCAGGGGTTGTTGGA
GTCGCAGCTATACGACCGTTCAGCTCAGGTAGGCTGAGGACTACTGTAATGATCCTTCCGAGCCGCAACC

Type1 (100% homology to GenBank JX270511.1)

GGGGACGTCCTAAAGCCTACAACACCAACCAACTTACACCGGGAAAACCGAGGTAGGG
GCCCGTGCTAATACAGCAGGATTGTAAGAATAAGTATTGGAATATCTCCCTCCGAGCCG
GAACATATTAGATAATCGGACAGCCGACATACCATATAGCTACTATATGCTTAA
CTGTCAGCTAGTACTACGTCAGCAGACTACAGGACTAGCTAGCTACGTTTGGTGTAAG
CCTGCCCTCGGGTGCTACCGGGACGGAGGCAGGTTCACCGGCAGAGCCATCCGGCAAG
GCCTCCGCCGTAACCCACCCACTTTTTATTACACTTTGGTTGGTACG

Type2 (99% homology to GenBank JX270511.1)

GGGGACGTCCTAAAGCCTACAACACCAACCAACTTACACCGGGAAAACCGAGGTAGGG
GCCCGTGCTAATACAGCAGGATTGTAAGAATAAGTATTGGAATATCTCCCTCCGAGCCG
GAACATATTAGATAATCGGACAGCCGACATACCATATAGCTACTATATGCTTAA
CTGTCAGCTAGTACTACGTCAGCAGACTACAGGACTAGCTAGCTACGTTTGGTGTAAG
CCTGCCCTCGGGTGCTACCGGGACGGAGGCAGGTTCACCGGCAGAGCCATCCGGCAAG
GCCTCCGCCGTAACCCACCCACTTTTTATTACACTTTGGTTGGTACG
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTACAA

Type3 (99% homology to GenBank JX270511.1)

GGGGACGTCCTAAAGCCTACAACACCCAACCTACCCGGGAAACCGAGGTAGGGGCCCGTGTCAACTACACGGGGTGTTAAAGAATATGATAGATACTCCCTCCGGGGAACATGGATAATTACCCCGGCTCAAGTGGATACTACGGCTAGCTAGCTATATGGTAACGTTCCAGAACACTACGGGGTGTTAAAAGAATGTATGGATACTCCCTCCGGG

Type4 (99% homology to GenBank JX270511.1)

GGGGACGTCCTAAAGCCTACAACACCCAACCTACCCGGGAAACCGAGGTAGGGGCCCGTGTCAACTACACGGGGTGTTAAAAGAATGTATGGATACTCCCTCCGGG

Type5 (99% homology to GenBank JX270511.1)

GGGGACGTCCTAAAGCCTACAACACCCAACCTACCCGGGAAACCGAGGTAGGGGCCCGTGTCAACTACACGGGGTGTTAAAAGAATGTATGGATACTCCCTCCGGG
TGCCCTCGGCTCGCTCGGCAGGCTCCGGCGAGCGCTTGCCAGAGGACCTAAAC
TCTGTTTGTGTATAGTCTGAGTACTATATAATAGTTAAAAACTTTCAACAACGG
ATCTCTTGGTTCGTGAGATGAGAAAGAGCAGGAAATGGCATAGTATATAGT
GAATTCGAGAATTCAGTAATCATCGAATCTTTGAACGACATTTGCGCCCCCT
GTATTCGCCGGGCGCATGCCCTGTCGAGCTCATTACAA

**Type 6 (99% homology to GenBank JX270511.1 623/624)**

GGGGGACGTGCTCAAAGCCTACAAACAAACCAACCTACCCCGGGAAACCGAGGTTAGGG
GCGCGTGCTAAACTACACGGGGTGGTAAAGAAATGTAGTGTGGGACTCTTGTGGCAAG
GAACTATAGGATAATCCGCAGAAGCCACCACTACCCCGGGAAACCGAGGTTAGGG
GCGGGCCTCGTGAGGAGGCTGCTTGCAGTCGCTAGCTAGCTAGCCATCTAAGATTTAAGAGAGGCTCTCC
CTGTTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTACAA

**Type 7 (99% homology to GenBank HM848977.1)**

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**Type 8 (99% homology to GenBank JX270621.1)**

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ACGGATCTCTTGGCTATTGCAACCAATTCAGTAATCATCGAATCTTTGAACGCACATTGCAGGCC
CTGGTATTTCCGCGGGGCGATAGCTTGCCGAGCGTGACTTACAA

**Type 9 (99% homology to GenBank JX270626.1)**

GGGGACGTCCTAAGGCCTACAACACAAACCCCAGCAGGAGGAGGATAGGG
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GACACTGGAATTACGAGGACTAGATAGTACGCTAGCTATATATAGTAAAAACTTTCAACA
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CTGGTATTTCCGCGGGGCGATAGCTTGCCGAGCGTGACTTACAA

**Type 10 (99% homology to GenBank JX270344.1)**

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**Type 11 (99% homology to GenBank JX270511.1)**

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Type12 (99% homology to GenBank JX270511.1)

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Type13 (99% homology to GenBank JX270511.1)

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Type14 (99% homology to GenBank JX270626.1)

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Type 18 (99% homology to GenBank JX270511.1)

Type 19 (99% homology to GenBank JX270511.1)
Type20 (99% homology to GenBank JX270511.1)

GGGGGACGTCTCCTAAGACCTACAAACCAACCATACCCGGGAAACCCGAGGTAGGG
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Type21 (99% homology to GenBank JX270626.1)

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**Type 23 (99% homology to GenBank JX270626.1)**

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**Type 24 (99% homology to GenBank JX270626.1)**

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**Type 25 (99% homology to GenBank JX270511.1)**

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Appendix 2. Bat survey results

Number of individuals counted per four bat species, across 17 caves or bluff crevices, in the Red Hills region of Kansas and Oklahoma in December 2011 and January 2012. For this report, habitats that served as potential hibernacula consisted of “caves” (underground passages of sufficient size for human visitation) or a “crevice” (one site; a crack in a bluff face approximately 3 cm wide and no more than 2 m in height).

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<th>Bat species</th>
<th>Cave Code</th>
<th>Habitat</th>
<th>County</th>
<th>State</th>
<th>Day-Month</th>
<th>Year</th>
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*(No bats found)*

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*Data compiled by Dr. William Jensen, Department of Biological Sciences, Emporia State University, Emporia, KS.*
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Date

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Title of Thesis

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Signature of Graduate Office Staff

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Date Received