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Genetic Variation of Northern Long-Eared Bat (Myotis septentrionalis) Populations in Nebraska

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UNIVERSITY OF NEBRASKA AT OMAHA

HONORS THESIS ABSTRACT

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Abstract:

The objective of this experiment was to determine genetic variation between Nebraska populations of Northern Long-eared Bats (*Myotis septentrionalis*) and to relate this variation to White-Nose Syndrome resistance. We hypothesized that these populations would show variation due to spatial separation and a lack of intermittent hibernacula. Wing tissue samples were collected from different areas in Nebraska including Ponca State Park, Fontenelle Forest, Elm Creek, and Larrabee Creek. A total of 31 specimens were caught between these locations. Mitochondrial DNA was extracted, amplified, and run on a gel using 6 samples from Ponca State Park. The gel from the first amplification (55 ºC annealing temperature) did not produce any bands. After this result, a new annealing temperature of 48 ºC was used. The gel from the second amplification produced bands of DNA but not those required for sequencing (~400 base pairs). The results of the first gel suggest the PCR was unsuccessful. With the alternate annealing temperature, the PCR was successful but produced undesired DNA fragments. This suggests that the primer used was not functioning correctly. We were unable to sequence DNA because of the lack of desired mitochondrial DNA fragments. As a result, genetic variation could not be determined.

Introduction:

The northern long-eared bat (*Myotis septentrionalis*) is a medium-sized species of bat (3 to 3.7 inches in length and a wingspan of 9 to 10 inches) that is distinguishable by its long ears (Figure 1) (U.S. Fish & Wildlife Service, n.d.). This species is distributed across a large portion of North America, including the eastern and central U.S (Figure 2, U.S. Fish & Wildlife Service, n.d.). This species exhibits seasonal movement patterns where individuals migrate between summering areas and winter hibernacula. Females form maternity colonies in the spring to give birth and to rear their offspring, while males and non-reproductive females roost individually or in small groups. In late summer, males and females recongregate (possibly for mating) before returning to their hibernation grounds, a behavior known as swarming. The northern long-eared bat has been known to travel up to 100 km between its summer and winter habitats (Johnson et al. 2015). In 2015, the northern long-eared bat was listed as a federally threatened species wherever it is found, mainly due to the harmful impacts of White-Nose Syndrome (WNS) (U.S. Fish & Wildlife Service, n.d.).

Figure 1. The northern-long eared bat (*Myotis septentrionalis*), shown here on a rock in Ponca State Park, Nebraska.

Figure 2. Range of the northern long-eared bat across the U.S. Retrieved from https://ecos.fws.gov/ecp0/profile/speciesProfile?spcode=A0JE#rangeInfo (U.S. Fish and Wildlife Service, n.d.).

In Nebraska, northern long-eared bats use caves, mines, and possibly rocky outcroppings as hibernacula. It is not completely known whether they use rock outcroppings to hibernate in Nebraska, although they have been detected during winter at exposed rock outcroppings. Additional support of this possible alternative hibernaculum comes from a correlation between the occurrence of northern long-eared bats in winter with the occurrence of exposed bedrock throughout the state (Figure 3). More support comes from the idea that this bat is a short-distance migrator (Lemen et al. 2016). Although the northern long-eared bat has been known to travel up to 100 km for its migration between summer and wintering grounds, reports on their movement and migration are limited (Lemen et al. 2016, Johnson et al. 2015). In Nebraska, there is a limited number of mines available and they are distant from another, suggesting that if northern

long-eared bats exclusively used these, they would have to travel further than their longest recorded distances to find suitable hibernacula. This suggests the need of alternative hibernacula, including rock crevices. Although they have been detected near areas of exposed bedrock, there are other possibilities of why they were detected, such as the presence of manmade structures or possible hibernacula in surrounding forests. The ratio of bats that use caves or mines to those that use these rocky outcroppings is largely unknown and requires more research to further understand where this species hibernates. These different hibernacula used by this bat may have large conservation management implications regarding the impact and spread of WNS in this species (Lemen et al. 2016).

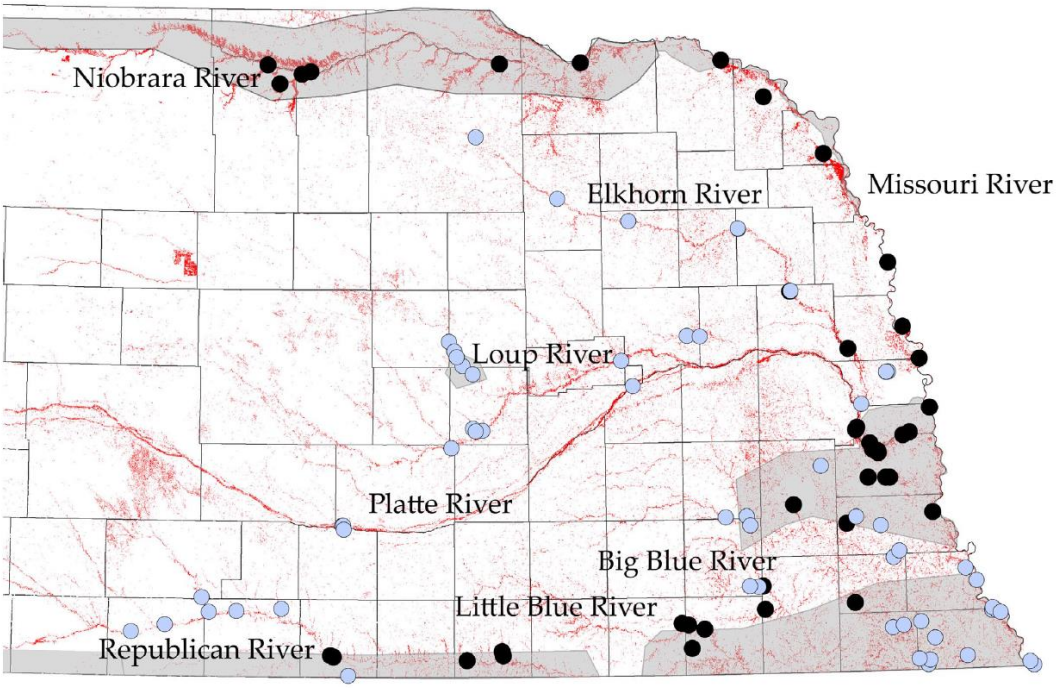

Figure 3. Known locations of northern long-eared bats based on acoustical survey. Black dots indicate where northern long-eared bats were detected and blue dots indicate areas that were surveyed but showed an absence of northern long-eared bats. Areas in gray are outcroppings of bedrock that reach the surface (suspected hibernacula) (Lemen et al. 2016).

WNS is a disease caused by the fungus *Pseuodgymnoascus destructans* (*P. destructans*) (Pikula et al. 2017). As of 2015, this disease has been confirmed in several species of bats including the big brown bat (*Eptesicus fuscus*), eastern small-footed bat (*Myotis leibii*), gray bat (*Myotis grisescens*), Indiana bat (*Myotis sodalis*), little brown bat (*Myotis lucifigus*), tri-colored bat (*Perimyotis subflavus*), and our study organism, the northern long-eared bat (Coleman 2014). Hibernating bats are largely at risk because their hibernacula provide the proper humidity and temperature for the growth of *P. destructans* (Marroqui et al. 2017). WNS is characterized by a skin infection (which often leads to white lesions on the nose, hence the name) that leads to damage in flight membranes of the bats due to skin lesions (Pikula et al. 2017). The disease can also cause early arousal from torpor, which is often lethal because of increased demands for food and water, which are not yet available, and because it decreases fat reserves (Micalizzi et al. 2017, Pikula et al. 2017). Furthermore, mortality can result from immune reconstitution syndrome, where the regain of immune function after hibernation-induced immunosuppression leads to severe and lethal immune responses (Micalizzi et al. 2017). *P. destructans* is believed to have been introduced to the U.S. from Europe. It was first detected in the U.S. in New York in February 2006 (Figure 4). Since its introduction, it has spread widely across the U.S. and Canada and is predicted to keep spreading, potentially threatening half of all North American bat species (Micalizzi et al. 2017). Bat populations have been devastated by this disease, and there have been millions of mortalities of bats across several species, including the northern long-eared bat (Marroqui et al. 2017). Some populations of the northern long eared bat have had declines up to 99% in certain hibernation sites in the Northeast (U.S. Fish and Wildlife Service, n.d.).

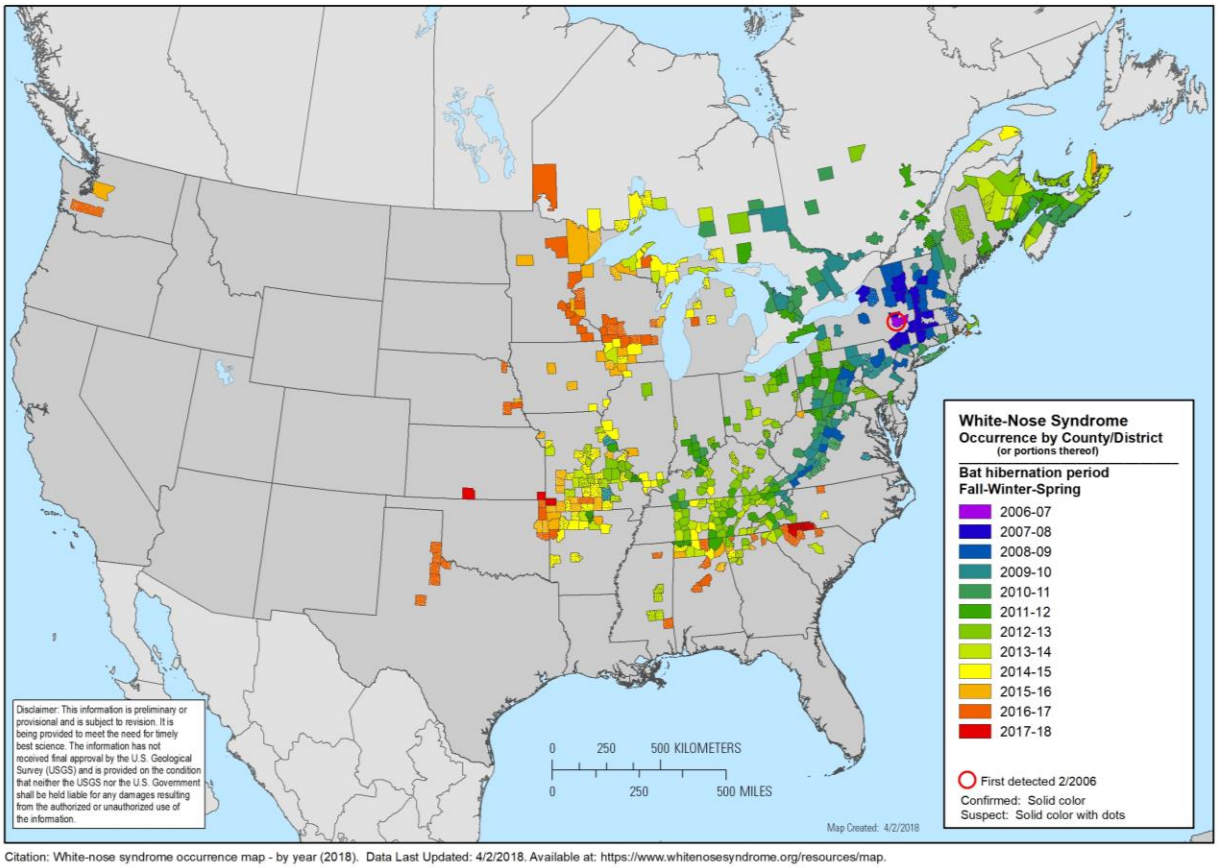

Figure 4. U.S. Fish and Wildlife Service (2018) graphical representation of the occurrence and spread of White-Nose Syndrome across North America since its first detection in 2006. WNS syndrome was first detected in Nebraska in 2015-2016. Retrieved from https://www.whitenosesyndrome.org/resources/map

To prevent the spread of this disease and the decimation of North America bat populations, it is important to undertake conservation management actions to control this outbreak. Before making management decisions for at-risk species, it is important to understand the structure and population dynamics of the species. Population genetics can be used in conservation efforts to delineate management units, manage populations in decline, and to reintroduce species. The first step in doing all of this, and as a result the first step in the conservation of a species, is to determine genetic variation patterns between populations (Burns et al. 2014). The purpose of our experiment was to determine the genetic variation patterns

between Nebraska northern long-eared bat populations by sequencing and analyzing mitochondrial DNA. This data was intended to aid in the understanding of the population structure and dynamics of this species to be used in future conservation management decisions. We hypothesized that there would be significant genetic variation between each population because of the large spatial separation between the sample locations and a lack of preferred hibernacula between them.

Materials and Methods:

Tissue Sample Collection: Bats were collected from four different locations across Nebraska, including Elm Creek, Ponca State Park, Larrabee Creek, and Fontenelle Forest (Figure 5). Bats were caught using mist nets and 2 mm wing biopsies were taken in duplicate from each specimen (this does not cause disruption to their flight). From Elm Creek (N 40.1183, W -98.4450), 4 bats were caught and sampled on June 20th and June 30th, 2017. At Ponca State Park (N 42.6022, W -96.7154), 18 bats were caught and sampled on July 5th and 6th, 2017. At Larrabee Creek (N 42.8546, W -102.4455), 3 bats were caught and sampled on July 7th and 8th, 2017. Lastly, at Fontenelle Forest (N 41.17625, W -96.7154), 6 bats were caught and sampled on July 14th, 2017. Between all sites, a total of 31 wing tissue samples were collected in duplicate. More than half of the samples were taken from the Ponca State Park population. All tissue samples collected were dried and stored at -80 °C until DNA extraction.

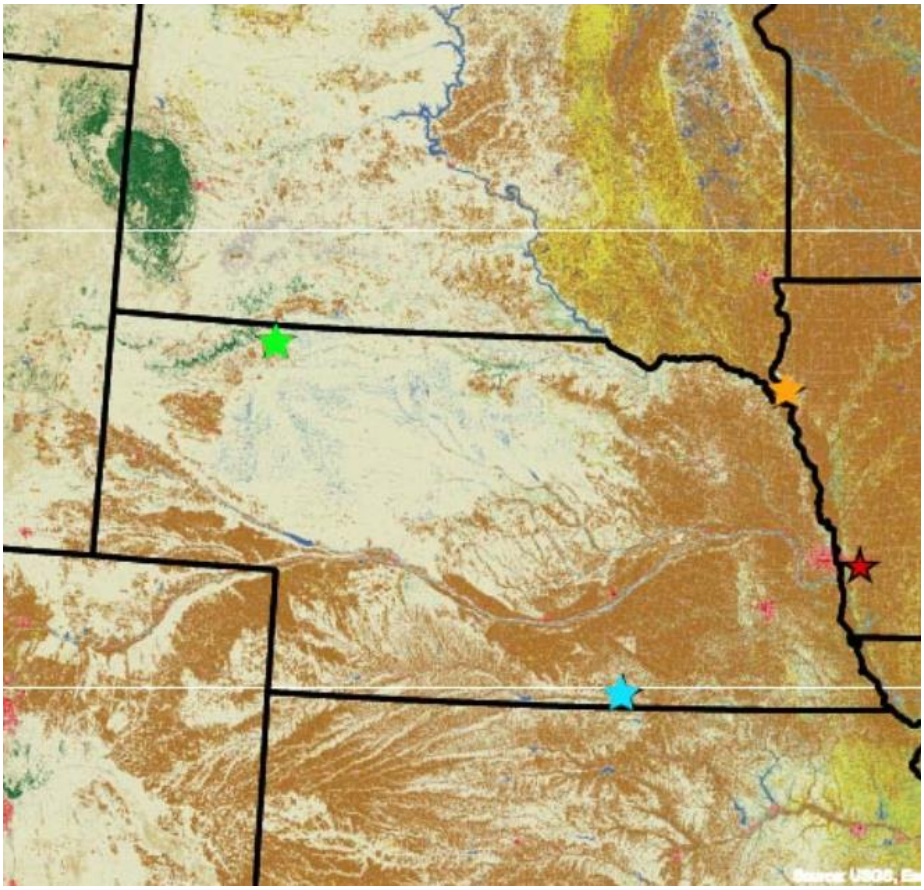

Figure 5. Topographical map showing the sites in Nebraska where northern long-eared bats were caught and wing membrane tissue samples were collected. Samples were collected in Summer of 2017. Green: Larrabee Creek (N 42.8546, W -102.4455); Orange: Ponca State Park (42.6022, W -96.7154); Red: Fontenelle Forest (N 41.17625, W -95.8916); Blue: Elm Creek (N 40.1183, W -98.4450).

DNA Isolation: We used 6 samples from Ponca State Park for DNA isolation because this was the site where we acquired the most samples. To our knowledge, this protocol has not been performed using this size of dried tissue. Isolation was performed using the DNeasy Blood & Tissue Kit by Qiagen as performed by Bailey et al. (2016), using the "Purification of Total DNA from Animal Tissues" protocol. Samples were first equilibrated to room temperature and placed in ATL buffer. They were not cut due to their small size. Proteinase K was added to lyse the cells while they were heated to 56 °C for approximately 3 hours. We then followed the remainder of the spin-column protocol. Samples were all checked for DNA concentration using a NanoDrop

2000 (ThermoFisher Scientific) spectrophotometer. Concentrations were as follows: Sample 1: 3.4 ng/µL; Sample 2: 3.7 ng/µL; Sample 3: 5.2 ng/µL; Sample 4: 5.3 ng/µL; Sample 5: 6.1 ng/µL; Sample 6: 5.8 ng/µL. The spectrophotometry showed successful isolation of DNA.

DNA Amplification: Next, we amplified a fragment of mtDNA using polymerase chain reaction (PCR). The region of mtDNA amplified was an approximately 400 base pair fragment of the hypervariable II (HVII) section of the control region, as previously described in Johnson et al. (2015). The primers used were from Invitrogen Custom Primers. The first primer was L16517 (5' to 3' sequence: (DNA) – GGTCATAAAGCCTAAATAGCC). The second primer was KAHVII (5' to 3' sequence: (DNA) – GTAGCGTGAATATGTCCTG). DNA concentrations were standardized in each sample to 50 ng of DNA per sample. The PCR cycles for the first amplification were as follows: Initial denaturation: 3 minutes at 98 °C; Denaturation: 10 seconds at 98 °C; Annealing: 30 seconds at 55 °C; Extension: 5 minutes at 72 °C. We performed a second amplification due to the lack of results from the first amplification where we kept the cycles the same except for changing the annealing temperature to 48 °C as performed by Johnson et al. (2015).

Visualization through Agarose Gel Electrophoresis: Once the samples had been amplified, they were run on a DNA gel along with a size standard (GeneRuler 1 kb from Fisher Scientific) to determine if the desired fragment was present. If the PCR was successful, a bright band would be present near approximately 400 base pairs. An agarose gel was made using ethidium bromide for visualization. The gels were run at 120 volts for approximately 45 minutes (Figure 6).

Results:

The first samples amplified were done using the previously stated protocol, with the 55 °C annealing temperature. When the gel was visualized, only one band was produced outside of the size standards. The fragment was not near 400 base pairs so it was not the fragment required for sequencing (Figure 6A). On the second attempt, a different annealing temperature of 48 °C was used while keeping all other steps of the protocol the same as suggested by Johnson et al. (2015). This new annealing temperature produced more bands for all samples. At 400 base pairs, however, there were no bands present for any of the samples. The large number of different bands present showed that there was amplification of other sized fragments, generally fragments larger than the desired fragment (Figure 6B). Because incorrect fragments were produced through amplification, we hypothesized that the primers used were not specifically amplifying the target sequence, most likely due to manufacturer error.

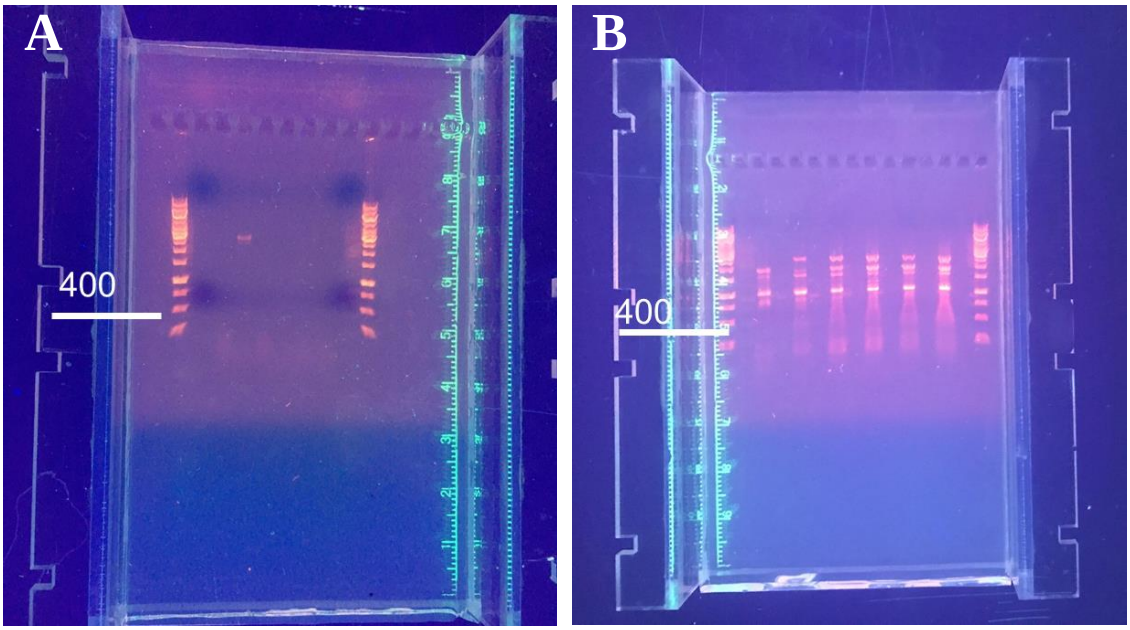

Figure 6. Agarose gels of the PCR products run with an A: 55 °C annealing temperature and a B: 48 °C annealing temperature. Both gels were run at 120 volts for approximately 45 minutes. On both gels, from left to right, the wells are: 1kb DNA ladder, sample 1, 2, 3, 4, 5, 6, and 1kb DNA Ladder. Desired band sizes are marked on the sides of the gel in base pairs.

Discussion and Future Directions:

We were unable to meet our original objective of sequencing the DNA of the northern long-eared bat because of the inability to amplify the necessary region of mitochondrial DNA. We suspect the issue in amplification was due to a malfunctioning primer which was not specifically replicating the HVII region of the mtDNA. Without DNA available to be sequenced, we were unable to answer our initial question or test our hypothesis about the genetic variation between these 4 populations. As a result, we could not address any factors relating to WNS. However, several modifications and improvements were determined for the future directions of this experiment.

The first adjustment to this experiment would be to order either a different primer or another sample of the original primer that would still allow the amplification of the HVII region of the mtDNA. Other primers may produce better results, or the primer that was originally used may have just needed to be replaced. If we had more time and funds to continue experimentation, other primers would have been tested.

The second adjustment would be to use different protocols to isolate mtDNA that could eliminate the need for a primer. A different protocol was tested using very small samples of more abundantly available fish tissue to determine if the protocol could isolate DNA from our small samples. The protocol tested was the abcam Mitochondrial DNA Isolation Kit, which is used to isolate the entire mitochondrial genome. The protocol is briefly described here. Cells are homogenized in Cytosol Extraction Buffer, then centrifuged several times, removing the supernatant between centrifugations. After centrifuging, the remaining pellet consists of isolated mitochondria from the cells. This is lysed and mixed with enzyme to degrade any proteins or DNases. The final remaining pellet is mitochondrial DNA. When we tested this method on the fish samples, they yielded promising results after being sent off for concentration testing and

sequencing. Currently, this protocol is being used on the same bat tissues used in this experiment to hopefully produce mtDNA that can be sequenced.

Another adjustment that could be made to improve this experiment is to change the method of tissue collection. Although wing biopsies are considered an acceptable way to collect tissue samples, they are difficult to work with and to process once they have dried. Other sources of DNA, such as hair or blood, may be worth collecting as long as their collection does not cause harm to the bat. Another option could be to acquire tissue samples from bats that are preserved, such as from museum specimens. This was one protocol we considered, largely because we had a small sample size from most sites. These specimens could provide additional samples for sequencing from the sample locations and minimize any harm to threatened bats. Additionally, depending on when and where they were caught, these samples could give insight into the genetic changes the species and its populations have undergone over time. Due to our inability to process our tissue samples, we were unable to pursue this method. As far as we know, the isolation and sequencing of DNA from museum bat samples using dried specimens has not been done yet, although studies have extracted bat DNA from tissues that were flash frozen or stored in ethanol or dimethyl sulphoxide (Bailey et al. 2016). There are several valid protocols used to obtain and amplify DNA sequences from museum specimens (Moraes-Barros and Morgante 2007). Moraes-Barros and Morgante (2007) collected sloth skin samples from museums and successfully isolated and amplified nuclear and mitochondrial DNA. This protocol is another possibility for future experimentation, especially when the study organism is threatened or endangered (Moraes-Barros and Morgante 2007).

Conclusion:

In conclusion, we were unable to sequence mtDNA from Nebraska northern long-eared bat populations because of an incorrectly functioning primer. We were, however, able to determine future directions for the experiment, including the use of new primers, new methods of DNA isolation, the use of different samples for analysis, and isolation of DNA from preserved museum specimens. The alternative protocol for isolating mtDNA is currently being used to attempt to obtain mtDNA that can be successfully sequenced from the wing tissue samples.

Bat populations across North America, including populations of the northern long-eared bat, have been devastated by WNS. This decrease in population sizes and the increased need for conservation action is why it is important to understand the population genetics of this species (Marroqui et al. 2017). In addition to understanding the genetic structure of various populations, it is also important to mitigate the spread and effects of WNS. One way of doing this is to inhibit the growth of *P. destructans* in known bat hibernacula. Micalizzi et al. (2017) found over 100 microbes that inhibit the growth of *P. destructans*. This was done in a controlled lab study and still needs to be implemented in a natural setting, but it is still a promising method to inhibit the spread and occurrence of WNS. Several of these inhibitory microbes are naturally occurring in areas that have WNS, so they are less likely to be invasive. Several of the microbes also inhibited *P. destructans* growth at a temperature that is common in bat hibernacula (Micalizzi et al 2017). All of this together suggests that microbe introduction into bat hibernacula may be a relatively low-risk and effective method in preventing WNS.

This experiment is important to pursue and improve upon as it will provide essential data and knowledge that are needed for the conservation and protection of the northern long-eared bat. Before conservation actions can be implemented, more needs to be understood about the

northern long-eared bat, including the genetic variation between its populations. As more research is performed, more can be done to prevent any further declines in this species.

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