

USE OF DNA BARCODING TO DISTINGUISH
BETWEEN MORPHOLOGICALLY
SIMILAR RED BATS

by

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ABSTRACT

Across North America, bats are being killed in large numbers at wind energy facilities and there is concern that this level of mortality threatens bat populations. Currently three species of migratory tree bats, including two *Lasiurus* species, comprise ~75% of all known fatalities; however, as wind energy development expands into new areas (e.g., the southwestern U.S.) there is the potential for new species to be impacted. Ongoing work in our labs has indicated that our current understanding of the distribution of *Lasiurus* species across North America is limited, at best, and that more species are impacted by wind energy development than previously thought. Accurate knowledge about which species are being impacted where, and to what extent, will greatly improve the implementation of effective mitigation strategies. We obtained 19 bat fecal samples from wild-caught *Lasiurus* bats from a study being conducted at Texas State University to improve the species-specific effectiveness of an ultrasonic acoustic deterrent (UAD) at deterring bats from approaching operational wind turbines. Based on morphology, these wild-caught bats were identified as eastern red bats (*L. borealis*), but it is possible that some of the individuals were western red bats (*L. blossevillii*). I extracted DNA from the bat fecal samples and amplified the COI mitochondrial gene to determine the correct species identification for each sample. I obtained good sequence data from 13 of the samples, of which 12 were western red bats and one was a northern yellow bat. Not one of the samples was from an eastern red bat. Confirming species identification with DNA barcoding will be essential for these types of studies going forward. These data will improve the accuracy of the results from the flight cage study at Texas State University and will contribute to improving strategies to reduce bat fatalities at wind energy facilities.

INTRODUCTION

Laisurus blossevillii, the western red bat, was once thought to be a subspecies of *Laisurus borealis*, the eastern red bat (Geluso and Valdez, 2019). However, with recent genetic analysis, it has been shown that both of these bats are separate species and thus have differentiating characteristics. Eastern red bats are known to have larger skulls in males, western red bats are known to have a more pronounced skull angle, and eastern red bats have lighter color ventral fur and western red bats have a darker color of ventral fur. Western red bats' fur is described as having a "color of rusty red to brownish without frosted appearance." Conversely, eastern red bats' fur is described as having "color reddish with frosted appearance resulting from white-tipped hairs." (Geluso and Valdez, 2019).



L. borealis

L. blossevillii

Figure 1. Images of the eastern red bat (*L. borealis*) and the western red bat (*L. blossevillii*) showing differences in ventral fur coloration (from Geluso and Valez 2019).

The known habitat distribution for *L. blossevillii* includes the northwestern United States extending all the way down to South America, not including south-central Texas or north-central Texas (see Figure 1). *L. borealis* is known to have a habitat range that includes south-central Texas and north-central Texas (see Figure 2). However, new findings from a TCU study using

DNA barcoding have revealed the presence of 2 bat species, the *western red bat Lasiurus blossevillii* and the western yellow bat *Lasiurus xanthinus*, that were previously unknown to occur in far-south Texas and were therefore not expected to be impacted by wind energy development in this region (Chipps et al. 2020a). These sampling locations are well outside of the known ranges for these two bat species, revealing that our ability to predict potential impacts from wind energy development on bats is limited by our lack of knowledge about their population ranges.

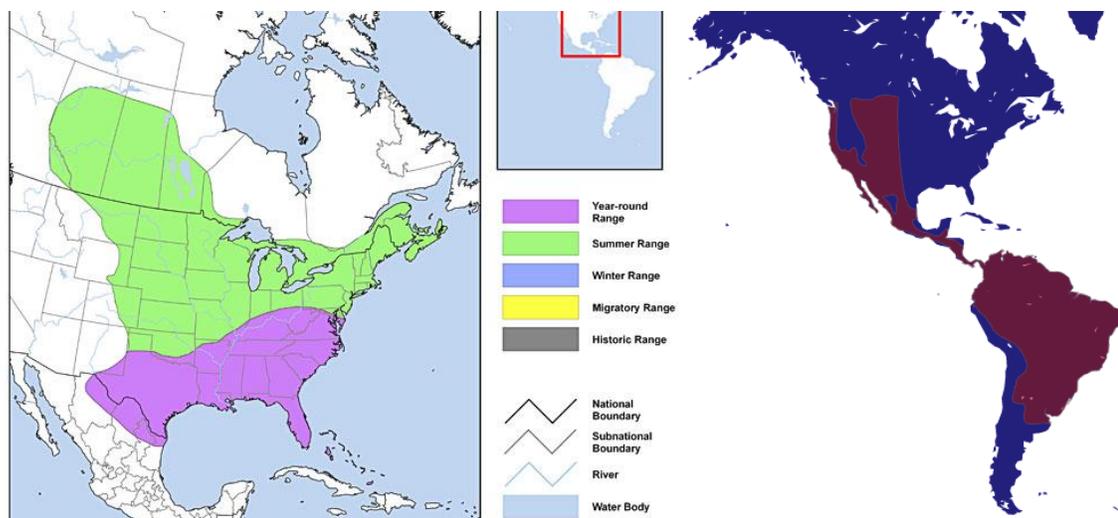


Figure 2. Geographic range of the eastern red bat (left) and western red bat (right) (Montana National Heritage Program, Bat Conservation International)

Wind energy is a growing source of clean energy in the United States. Nationwide, about 8.4% of all electricity is produced by wind facilities (US EIA, 2021). The advantages of this energy source for the environment focus on reducing greenhouse gas emissions, lowering water usage, and reducing the effects of global warming. As a result, the wind energy production in the United States is expected to reach close to 20% within the next decade (Wind Vision 2015). Nonetheless, wind energy development has been linked to an increase in number of bat fatalities

at these facilities, especially in the state of Texas, which utilizes wind energy more than any other state in America. We know that *Lasiurus borealis*, comprises 22% of bat fatalities at wind turbines across the nation (Arnett and Baerwald 2013, AWWI 2020). Bats are a large subset of mammal, making up about one fifth of all mammalian diversity and providing many important ecological functions such as consuming certain agricultural pests (Boyles et al. 2011, Kunz et al. 2011). Little data is available for estimating bat mortality at Texas wind farms. As a result, little is known about the genetic diversity of bats that are killed at these locations and the environmental impact that results from these fatalities. Previous research revealed that four species of *Lasiurus* bats were killed at wind turbines in the south Texas region: *L. borealis*, *L. cinereus*, *L. ega*, and *L. intermedius* (Chipps et al. 2020a). Previously, there had been no evidence of *L. blossevillii* being found at wind facilities in South Texas before the aforementioned TCU study (Chipps et al. 2020a).

This discovery of *L. blossevillii* in a range outside their suspected habitat calls for further study. If this species is indeed expanding its range, then the implications for wind energy development in these regions need to be adjusted to reflect the proper species composition of the area, including the western red bat. As a result, more genetic testing is needed to truly confirm if eastern and western red bats are inhabiting these regions, and are, in fact, being killed at these wind energy facilities.

Further study could provide new insights into the potential impacts of wind energy development on bats across North America. For this project, we obtained 19 bat fecal samples from wild-caught *Lasiurus* bats from a study being conducted at Texas State University to improve the species-specific effectiveness of an ultrasonic acoustic deterrent (UAD) at deterring bats from approaching operational wind turbines. These bats were captured in Hays County,

Texas – near the Freeman Center where the flight cage for the UAD trials is located. Based on the study site location and morphology, these wild-caught bats were identified as eastern red bats (*L. borealis*), but it is possible that some of the individuals were western red bats (*L. blossevillii*). The fecal samples were collected from the cloth bags that were used to house the bats after they were captured and before and after the flight cage experiments. By extracting DNA from the fecal samples using the methods of Korstian et al. (2015), I could then use DNA barcoding techniques to correctly identify each bat to species (Korstian et al. 2016). Given that field tests of the effectiveness of UADs at reducing bat mortality have shown varying results among species (e.g., Romano et al. 2019, Weaver et al. 2020), it is imperative that the Texas State University study have the correct species identification for the bats used in their trials. This is especially so for eastern red bats, as current UAD technology does not appear effective at reducing mortality for this species (Romano et al. 2019). The data generated in my project will improve the accuracy of the results from the flight cage study at Texas State University and will contribute to improving strategies to reduce bat fatalities at wind energy facilities.

METHODS

We extracted DNA from 19 fecal samples from red bats captured in Hays County, TX using QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions (see Korstian et al. 2015 for more details). Amplifications were performed in 20 μ L, with 4 μ L DNA, 1 μ L primers, 5 μ L deionized water, and 10 μ L Accutaq Supermix containing AccuTaq DNA polymerase, dNTPS and PCR buffer. PCR was then performed on 4 μ L of DNA with 16 μ L of master mix on each sample to elongate the mitochondrial genes using the gyaa059 COI primers for fecal matter (i.e., primer cocktail), containing the primer pairs COX1Bat-1F/COX1Bat-1R,

COX1Bat-3F/COX1Bat-3R, and COX1Bat-4F/COX1Bat-4R in 2:1:1 ratio. PCR cycling was completed with an ABI 2720 thermal cycler, and parameters included 1 cycle at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55°C for 40 s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min (Guan et al. 2020). Following this step, gel electrophoresis using GelGreen and Blue/Green LED light was performed on approximately 8 microliters of the PCR product to check that amplification was successful.

Following elongation confirmation, products were sequenced using ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Applied Biosystems, USA) with M13 primers. DNA sequences were analyzed on an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA). Sequences were trimmed and put into contigs using Sequencher v. 5. We submitted all sequences to GenBank (nucleotide Blast) to identify the samples to species. Samples that had a 98% or greater match were identified to species.

RESULTS

Thirteen out of 19 DNA samples were successfully amplified and produced good sequence data, although not every sequence was matched at 98% or higher. Twelve of the 13 successfully sequenced samples were identified as *L. blossevillii* (Table 1). Only five of the 12 were identified at 98% match or higher. Seven of the 12 were identified between 91-98% match. Six of the 19 samples were successfully identified at 98% match or higher when compared to GenBank. The results showed that 5 out of the 6 samples that found matches were indeed *L. blossevillii* and the remaining fecal sample was identified as *L. intermedius*, the northern yellow bat, and was identified at 100% match when compared to GenBank (Table 1).

Table 1. Summary of the bat species identifications from DNA barcoding.

Species Identification	Number of Samples (91% match or higher)
<i>L. blossevillii</i>	12
<i>L. intermedius</i>	1

DISCUSSION

The confirmed presence of the western red bat in the Hays County region calls for a re-evaluation of past bat wind-turbine fatality data. It appears that this species is indeed increasing its habitat range and is therefore at risk of being killed at wind energy facilities. This means that there is a chance that previously collected bats, once identified in the field as *L. borealis*, were actually *L. blossevillii*. Because of the discovered presence of the western red bat in this region, this species of bat needs to be studied and factored into future and current studies involved with bat mortality at wind energy facilities and possible prevention and conservation measures. The identification of *L. intermedius* in this data set confirms the high bat diversity in the Hays County region. These results show the need for genetic identification of these bat species or heightened awareness that Hays County is also within the geographic range of the western red bat. If red bats are being studied in this area, researchers should know that both species are present and so more care should be taken with species identification, either using morphological features or though DNA barcoding.

There is little knowledge available currently on the relationship between bat populations and wind energy facilities in south Texas. Less so, there is a lack of information available for proper fatality mitigation strategies for eastern and western red bats. Currently, there are studies being conducted in Hays County using a flight cage on the effectiveness on ultrasonic acoustic

deterrents (UADs). This study is aimed at understanding a bat's ability to navigate in the presence of a UAD that is emitting ultrasonic noise across six different frequencies: 20, 26, 32, 38, 44, and 50 kHz. The bats are placed in this cage after being safely captured in the wild and fly in the presence of a UAD to see if they avoid using the airspace in close proximity to the UAD. After the study is concluded, the bat is released safely back at its capture location. Figure 3 illustrates the experimental design of this study.

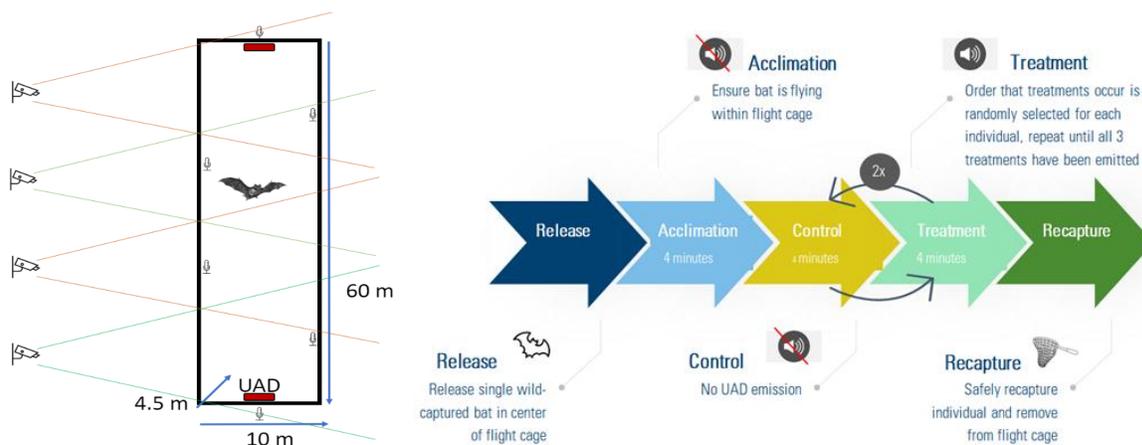


Figure 3. The flight cage set up (left) and study method (right). Courtesy Texas State University

When mounted on operational wind turbines, UADs are thought to interfere with different bat species' ability to echolocate by sending out ultrasonic waves into the surrounding airspace, causing the bats to avoid the zone the highest risk (aka the rotor-swept zone) and travel safely around the wind energy facility. These UADs have proven successful for reducing wind turbine fatalities in two species of bats: *L. cinereus* and *T. brasiliensis* (Weaver et al. 2020b). There are currently 6 species undergoing testing in the flight cage study in Hays County, TX. One of these species is considered to be *L. borealis*, the eastern red bat. However, since the discovery of *L. blossevillii* in the south Texas region (Chipps et al. 2020a), further testing was needed to confirm the species identity of the red bats tested in the flight cage experiments..

The addition of *L. blossevillii* to the south Texas region would call for a reexamination of previous data on the conservation of different migratory bat species in this area. As a result, these ongoing UAD experiments would need to be adjusted to include the western red bat species to see if their response differs from the eastern red bat. As this data set continues to grow and be studied, the implications for conservation of these bat species will be adjusted as a result. This calls for collaboration between different governing institutions that regulate wind energy in the United States, which is currently the fastest growing form of renewable energy.

As wind energy continues to grow, there will be continued studies on the implications that wind energy facilities have on wildlife in the United States. Currently, wind turbine projects are governed by state and local planning commissions, zoning boards, and city councils, often who lack the proper knowledge of the ecological impacts that wind energy might bring with the construction of these facilities (Arnett et al. 2016). Most of the time, bat mortality will not be addressed when these wind turbines are built, and federal law does not protect most species of bats, most likely due to a lack of information about these species. The U.S. Fish and Wildlife Service provides guidelines to help reduce the impact of wind energy facilities on wildlife for developers, however, these are only voluntary and do not result in penalty when not followed (Arnett et al. 2016). Bat populations are currently threatened worldwide (O'Shea et al. 2016, Frick et al. 2019), and there are increasing concerns that wind turbine fatalities may threaten the persistence of the hoary bat (Frick et al. 2017). More research is needed to develop and test the effectiveness of strategies to reduce risk to bats (Allison et al. 2019, Hayes et al. 2019, Hein and Hale 2019).

Studies such as this one will allow for better regulation and improved solutions to bat-wind turbine conflicts because they increase our knowledge of species' ranges and allow for

testing of potential mitigation strategies on individual species. This will lead to greater investigation into the effectiveness of UADs and more conservation efforts. Ultimately, this research allows for the connection between Texas scientists, Texas Parks and Wildlife Department, and wind energy companies to grow. As a result, more wind energy will be able to be produced, but with sufficient information on protecting these wildlife species that mitigates fatalities at these facilities that provide a very useful and necessary form of renewable energy.

This data can help bridge the gap between wind farm development and bat mortality. This would allow conservation biologists, wind energy developers, and regulatory agencies to use information to guide future wind energy development. This research paves the way for future collaboration with Texas scientists, TPWD, and wind energy companies to conserve these species of bats in Texas while still pushing forward with this impactful form of clean energy.

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