

HIGH GENETIC DIVERSITY AND LACK OF STRUCTURE IN EASTERN RED BATS

LASIURUS BOREALIS

by

JENNIFER MICHELLE KORSTIAN

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Rice University
Houston, Texas

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Introduction

Bats provide a number of important ecosystem services from pollination and seed dispersal to pest control (Burland and Wilmer 2001, Boyles et al. 2011). Pest control services provided by insectivorous bats have been estimated at a value of 3.7 billion dollars per year (Boyles et al. 2011). Bat species worldwide are known or suspected to be in decline (Racey and Entwistle 2003, Winhold and Kurta 2006, Kunz et al. 2007). For example, a ten-fold decrease in abundance was documented in one local population of eastern red bats (*Lasiurus borealis*) over a 38-year period (Winhold et al. 2008). There are many threats facing bat species including, climate change, habitat loss and degradation as well as emerging pathogens. Unexpectedly, the development of wind energy has also been identified as a potential threat to the persistence some populations of bats.

Large numbers of migratory, tree-roosting bats are killed in turbine collisions at wind energy facilities worldwide (reviewed in Kunz et al. 2007, Arnett et al. 2008). The species composition of fatalities at wind farms varies regionally; however, three species including eastern red bats (*Lasiurus borealis*), hoary bats (*Lasiurus cinereus*), and silver-haired bats (*Lasionycteris noctivagans*) comprise almost 75% of casualties at wind farms across North America (Kunz et al. 2007). All three species are migratory, tree-roosting bats with expansive ranges that cover much of North America. Bat fatalities peak during the period from July to October, which corresponds with the southward journey of migratory bat species (reviewed in Arnett et al. 2008). The cause of these fatalities has not yet been determined, but studies have found that most of these collisions occur on low wind-speed nights,

particularly in the days following storm fronts (Arnett et al. 2008.) To fully understand bat fatalities at wind farms and minimize them in the future will require that we learn more about the details of bat migration.

Genetic studies can potentially provide information about the population structure and dispersal patterns of bats (Burland and Wilmer 2001, Holland and Wikelski 2009). Non-migratory species are expected to show more genetic differentiation between populations as a result of low levels of gene flow between sedentary populations (Burland and Wilmer 2001). Migratory bats that mate prior to migration would show a genetic signature closely resembling non-migratory bats. In migratory species that mate during migration, gene flow is expected to be high, resulting in low levels of population structure. If the migration corridors used by migratory bats can be identified, then the location of these corridors could be considered when planning new wind facilities building conservation plans.

Eastern red bats are migratory, tree roosting bats that are found over much of the United States and southern Canada east of the Rockies. Very little is known, however, about their migration strategies or the exact routes they follow during migration. Reliable estimates of population sizes are also lacking for this species owing to their non-colonial tree-roosting habits. Eastern red bats are believed to be migratory since their abundance in the southern part of their range (Figure 1) increases during the winter months (Shump and Shump 1982). Records of the extent of their winter range are largely based on samples from museum collections (Cryan 2003). Eastern red bats are known to roost in deciduous leaf litter during winter months, a trait that makes it difficult to ascertain their abundance during

winter (Moorman et al. 1999). Males have been found to be more common than females in the northern extremes of their summer ranges (LaVal and LaVal 1979; Cryan 2003; Mormann and Robbins 2006). In the southern part of their range where the winters are less severe (like Texas), eastern red bats may stay in the same region year round (Schmidly 1991). The extent of these non-migrant (i.e., permanent resident) populations is currently unknown.



Figure 1. Range of eastern red bats.
Modified after Cryan et al. 2003.

For this study we analyzed the genetics of eastern red bat carcasses that were collected at a wind farm in north-central Texas from 2009-2011. We used mitochondrial DNA (mtDNA) and nuclear microsatellite loci to determine levels of genetic diversity and test for potential genetic population structure in the bats that were killed at this facility during the fall migratory period.

Materials and Methods

Sample collection

All tissue samples used in this study were obtained from eastern red bat (*Lasiurus borealis*) carcasses that were recovered during post-construction mortality surveys of a wind farm in in Cooke County, Texas (Wolf Ridge Wind, LLC). Located just south of the red river (N 33° 43' 53.538" W 97° 24' 18.186"), the landscape in the wind resource area (48 square kilometers) includes a mix of agriculture and pasture and is located near a wooded ridge. The facility, which went online in 2008, operates 75 1.5 MW GE wind turbines.

Fatality surveys were conducted March-October in 2009, July-October in 2010 and April-October 2011. Although bat fatalities at this facility are highest from early July through early September, within this high-risk period there are 'pulses' of higher fatality with considerable daily variation within the pulses (Figure 2). Pulses were arbitrarily defined as a period of multiple days with two or more fatalities per day that are not interrupted by more than two days of lower (<2 bats per night) fatality levels (Figure 2). For eastern red bats in 2009, three pulses were detected; pulse 1: 17-July thru 8-August, n = 84, pulse 2: 10-August thru 16-August, n = 31, pulse 3: 19-August thru 30-September, n = 138. In 2010, only one pulse (9-Jul thru 30-July: n = 60) occurred for eastern reds. No pulse was detected in 2011 because mortality levels were very low. Bats located outside of pulses (i.e. 'non-pulse' bats) were treated as two groups in 2009: before pulse 1, n = 18 and after pulse 3, n = 10. In 2010, 'non-pulse' bats (n = 26) were only considered from after pulse 1 because the sample size prior to the pulse (n = 2) was too low for analysis. Sample sizes from

each of the years varied substantially (2009: n = 289 carcasses; 2010: n = 97 carcasses; 2011: n = 30 carcasses). A portion of the wing membrane (approximately 5mm²) was removed and placed into vials containing 20% DMSO/6M NaCl and stored at room temperature until DNA extraction.

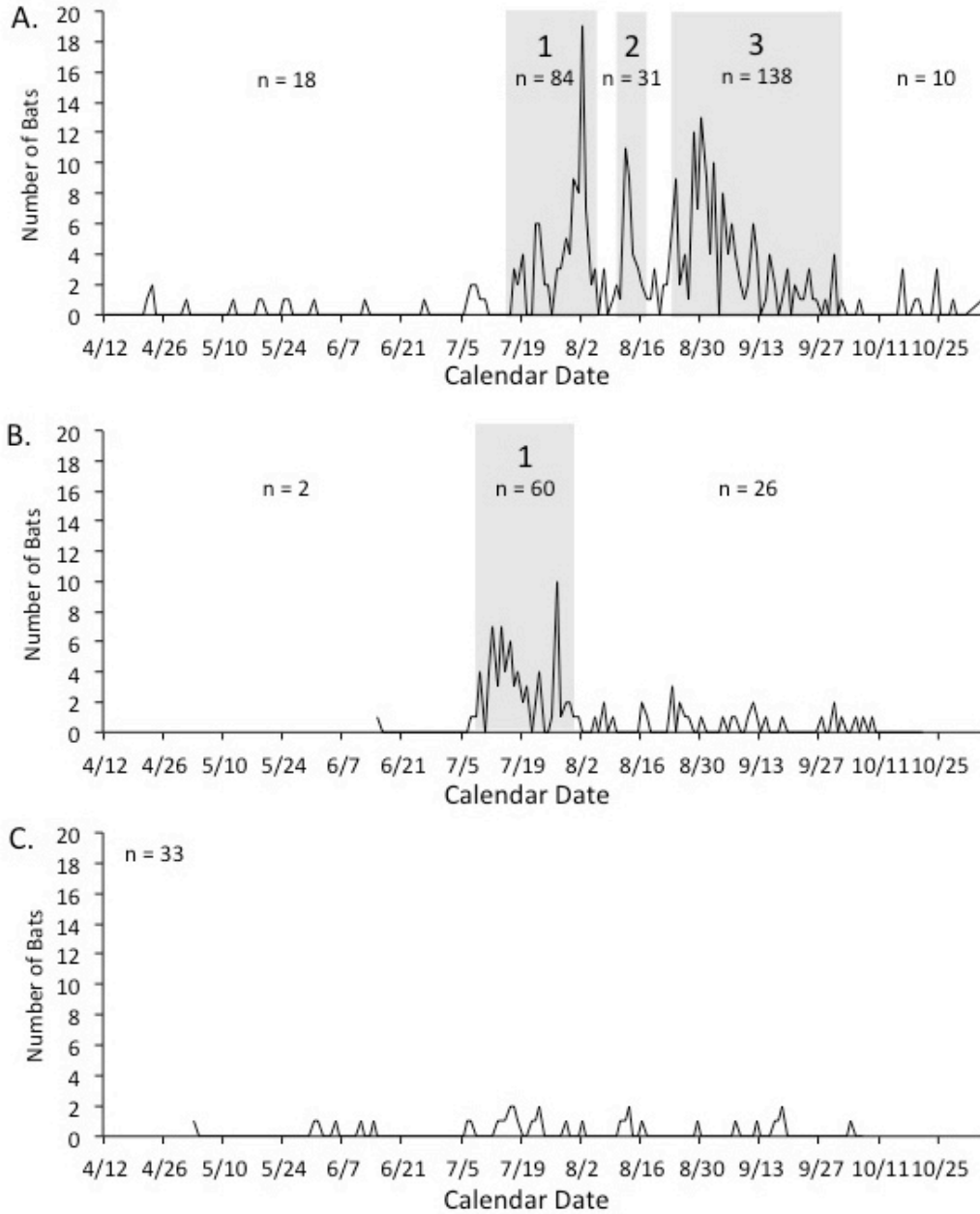


Figure 2. Red bat (*Lasiurus borealis*) mortality at Wolf Ridge wind farm 2009-2011. A: 2009, B: 2010, C: 2011. 'Pulse' periods of higher mortality are shaded in grey, see text for dates of pulses.

DNA Extraction

Wing membrane tissue samples were placed in 300 μ L lysis buffer (75mM NaCl, 25mM EDTA, 1%SDS) with 7 μ L Proteinase K (20 mg/ml), and then incubated at 55°C overnight. Ammonium acetate (1.5 volumes of 7.5 M) was added to each tube and the samples were placed at -20°C for 1.5 hours to precipitate proteins, which were then pelleted by centrifugation for 15 minutes. DNA was precipitated by adding 0.7 volumes of isopropanol to the supernatant and centrifuging for 15 minutes. The DNA pellet was washed with 70% ethanol and allowed to dry before resuspending in 50 μ L 10mM Tris-HCl pH8.5. The resulting DNA solutions were diluted to approximately 20ng/ μ L.

Microsatellite Development

Eastern red bat microsatellite loci were isolated using the protocol of Glenn and Schable (2005). DNA was digested with *RsaI* and *XmnI* and the resulting fragments were linked to SuperSNX linkers. Fragments were then hybridized to biotinylated oligos (AC₁₂, GA₁₂) and captured with streptavidin coated magnetic beads (Promega USA). The enriched fragments were cloned with the pGEM-T Easy Vector System (Promega USA). We sequenced ~150 clones using pGEM primers. Sequences were electrophoresed on an ABI 3130 Genetic Analyzer (Applied Biosystems USA) using ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Applied Biosystems USA). We screened all sequences with MSATCOMMANDER (Faircloth 2008) for dinucleotide loci that had >10 repeats and found 23 loci. We designed primers for 19 clones that contained sufficient flanking regions using Primer3 (Rozen and

Skaletsky 2000). From these loci, two were polymorphic, amplified consistently, and had scorable profiles (Table 1). Of the remaining loci, three failed to amplify and 14 did not produce a specific product.

Table 1. Polymorphic microsatellite loci developed for *Lasiurus borealis*.

Locus	Primer sequence (5'-3')	Repeat in original clone	Size in original clone (bp)	Size Range (bp)	Number of Alleles
Labo08	F: CGATAGATTCCCGCAGACTC R: CGGGGTAAACAGAGAACCA	GT ₁₄ GA ₂₅	205	163-281	51
Labo10	F: TTGGCTCCAGAGTCCACATT R: GCAGCCATTTCTGTCTGGAT	GT ₁₁ GA ₂₂	200	194-202	43

Additional Microsatellites

In addition to the two loci developed for eastern red bats, we tested 29 loci developed in other closely related bat species (Burland et al. 1998, Piaggio et al. 2009). From these loci, five were polymorphic, amplified consistently, and had scorable profiles (Table 2).

Table 2. Polymorphic microsatellite loci developed in other bat species

Locus	Size Range (bp)	N _a	Species	Reference
CoraE10	281-309	10	<i>C. rafinesquii</i>	Piaggio et al. 2009
CoraF11	151-201	23	<i>C. rafinesquii</i>	Piaggio et al. 2009
CoraH07	274-342	27	<i>C. rafinesquii</i>	Piaggio et al. 2009
CotoG12	218-254	19	<i>C. townsendii</i>	Piaggio et al. 2009
Paur02	212-314	5	<i>P. auritus</i>	Burland et al. 1998

Microsatellite Amplification

We amplified the seven loci in two multiplexes (A: Paur02, Labo08, CoraH07, and B: CoraF11, CotoG12, CoraE10, Labo10) using Qiagen's Multiplex Reaction Kit.

Polymerase chain reactions (PCR) (10µL) contained 10-50 ng DNA, 0.2 µM of each

primer, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3mM MgCl₂ pH8.7, 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 30 cycles of 30s at 94°C, 90s at 60°C, 90s at 70°C, then a final extension at 60°C for 30 minutes. The resulting PCR products were diluted 20X with dH₂O. For each sample, 0.5µL of diluted product was loaded in 10µL HIDI formamide with 0.1 µL LIZ-500 size standard (Applied Biosystems USA). Multiplexes were electrophoresed on an ABI 3130XL Genetic Analyzer (Applied Biosystems USA). Alleles were sized using Genemapper v4.0 (Applied Biosystems USA) and binned using TANDEM (Matschiner and Salzburger 2009).

Mitochondrial Sequencing

We amplified a 550 bp section of the mitochondrial cytochrome *c* oxidase I gene (COI) using universal primers LCO and HCO from (Folmer et al. 1994). Polymerase chain reactions (10µL) contained 10-50 ng DNA, 0.2 µM of each primer, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3mM MgCl₂ pH8.7, 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 30 cycles of 30s at 94°C, 90s at 50°C, 90s at 70°C, then a final extension at 60°C for 30 minutes. Products were sequenced using ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Applied Biosystems USA) using the PCR primers. Sequences were electrophoresed on an ABI 3130XL Genetic Analyzer (Applied Biosystems USA).

Sequences were trimmed, edited, and contiged using Sequencher v4.8 (Gene Codes USA) then aligned in Bioedit using Clustal X v2.0 (Larkin et al. 2007).

Genetic Diversity

We tested microsatellite loci for deviation from Hardy-Weinberg equilibrium (HWE) and genotypic linkage equilibrium using Genepop v 4.0 (Rousset 2008). We estimated null allele frequencies using Genepop v 4.0. We used the program GenAlEx 6.3 to calculate the number of alleles, observed heterozygosity (H_o), and expected heterozygosity (H_E) for each microsatellite locus (Peakall and Smouse 2006). We tested for genotyping errors with Micro-checker (Oosterhout et al. 2004).

For the mitochondrial haplotypes, we used GenAlEx 6.3 to calculate the frequency of haplotypes and haplotype diversity (h). Relationships among haplotypes were visualized as a statistical parsimony network using the program TCS (Clement et al. 2000). Kimura-2 genetic distances were calculated for all sequences using MEGA 5 (Tamura et al. 2011).

Genetic Differentiation

Multilocus microsatellite genotypes were clustered using STRUCTURE (Pritchard et al. 2000). We ran the Monte Carlo Markov Chain (MCMC) for 10^6 iterations following a burn-in period of 10^5 iterations for $K = 1$ to 10 using the correlated allele frequencies model and assuming admixture (the default values). The K with the

highest log-likelihood was considered to be the most likely number of genetic clusters in the data.

We grouped bats into categories that might reveal genetic differentiation and conducted an analysis of molecular variance (AMOVA) for these categories in GenAlEx. We conducted AMOVAs between years and between ‘pulses’ with 1000 permutations. We tested for differentiation between pulses since these may represent bats arriving to the wind farm from different geographic localities.

Results

Microsatellite Genetic Diversity

Of the 416 bat samples, 387 amplified at all loci, 17 individuals amplified at 5-6 loci, and the 12 individuals that failed to amplify at \geq four loci were excluded from the analysis (Table 3).

Table 3. Characterization of microsatellite loci for eastern red bats. N: number of individuals successfully amplified at that locus, N_a : number of alleles, H_o : observed heterozygosity, H_e : expected heterozygosity. *indicates significant heterozygote deficits ($p < 0.0002$). †indicates possible scoring errors due to stutter.

Locus	N	Size Range (bp)	N_a	H_o	H_e	F_{IS}	Estimated Null Allele Frequency
CoraE10†	404	281-309	10	0.319*	0.366	0.129	0.0634
CoraF11	404	151-201	23	0.936	0.931	-0.004	0
CoraH07†	402	274-342	27	0.398*	0.904	0.560	0.271
CotoG12	404	218-254	19	0.901	0.918	0.019	0
Paur02†	401	212-314	5	0.778*	0.960	0.191	0.0933
Labo08	391	163-281	51	0.491*	0.962	0.491	0.2411
Labo10	404	194-202	43	0.106*	0.166	0.361	0.1019

Observed heterozygosity (H_o) ranged from 0.106-0.936, mean = 0.561 ± 0.119 and the number of alleles ranged from 5-51 ($n = 404$; Table 3). Five of the

seven loci tested exhibited heterozygote deficits and deviated significantly ($p < 0.0002$) from expectations under Hardy-Weinberg Equilibrium. Null alleles were likely at these same five loci (estimated null allele frequency in Table 3). Two loci pairs showed evidence of genotypic linkage disequilibrium (Labo10 & Labo08: $p < 0.00001$, CoraF11 & LB10: $p = 0.000447$). Three loci showed potential scoring errors due to stutter (Table 3).

Mitochondrial Genetic Diversity

We identified 177 unique mitochondrial haplotypes from 405 individuals (Figure 3). Sequence diversity averaged 0.0071 ± 0.004 across these haplotypes. Pairwise differences between haplotypes averaged 3.9 ± 1.95 . The haplotype network has a star shape with many haplotypes radiating out of one very common haplotype in the center of the network (Figure 3). Most haplotypes are only differentiated by one to two base-pairs. The most common haplotype was found in 107 individuals and all other haplotypes were found in fewer than 20 individuals (Table 4). Haplotype diversity (mean = 0.928 ± 0.009 , 2009 = 0.911, 2010 = 0.943, 2011 = 0.929) was high for each of the three years sampled. Kimura-2 genetic distances calculated between unique haplotypes were low (mean = 1%, range = 0.2-3.6%).

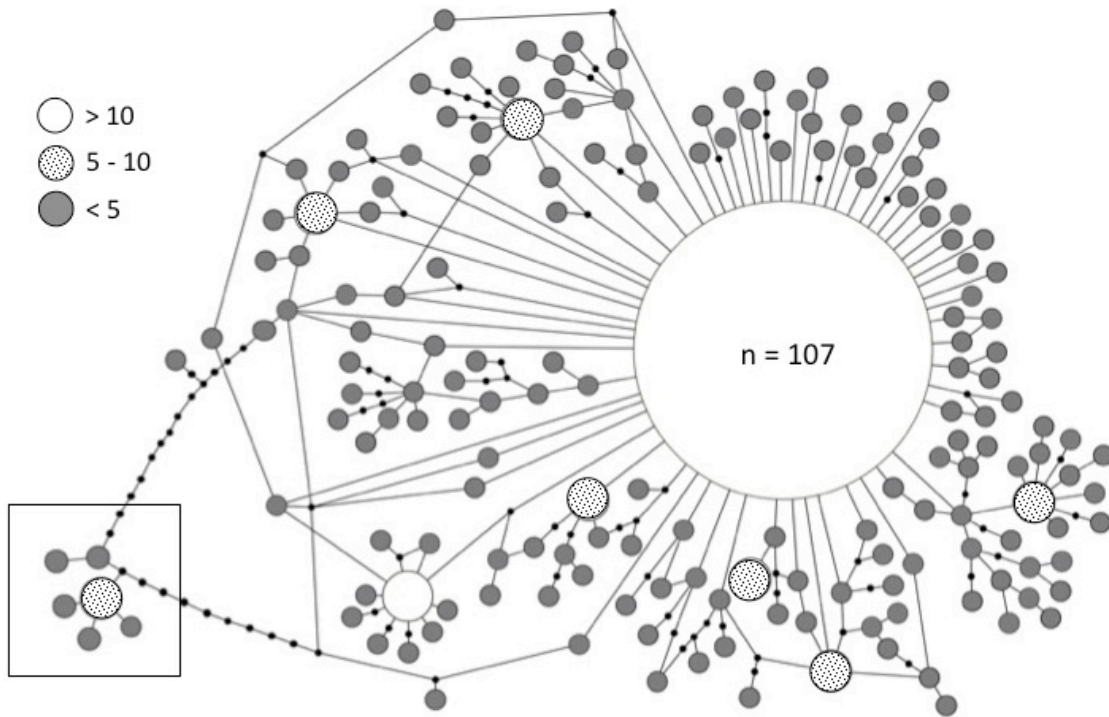


Figure 3. Haplotype network constructed from 177 unique mitochondrial haplotypes of the CO1 region of 405 eastern red bats. Each colored circle represents a unique haplotype. Each small black circle represents a theoretical intermediate haplotype. Each line between circles represents a single base-pair change between the two haplotypes. Size of circles is scaled to approximate relative frequency of that haplotype in the population. The square indicates a small group of divergent haplotypes (n=15, Kimura-2 distances 3-3.5% from most common haplotype).

Table 4. Frequency of 11 most common mitochondrial haplotypes from 177 unique haplotypes obtained from 405 individuals. All other haplotypes occurred at a frequency less than 0.01.

Haplotype	# Individuals	Frequency
A	107	0.262
B	18	0.044
C	10	0.025
D	9	0.022
E	7	0.017
F	6	0.015
G	6	0.015
H	6	0.015
I	6	0.015
J	5	0.012
K	5	0.012

Table 5. AMOVAs for microsatellite genotypes and mitochondrial haplotypes.

Group	Microsatellite Genotypes				Mitochondrial Haplotypes			
	Variance between groups	Variance within groups	phiPT	p-value	Variance between groups	Variance within groups	phiPT	p-value
By year	0%	100%	0.000	0.346	0%	100%	0.001	0.289
By 'pulse'	0%	100%	0.001	0.423	0%	100%	0.001	0.350

Genetic Structure

There was no evidence for strong genetic structure or clear groupings of individuals among our samples using either the microsatellite loci or mitochondrial haplotypes. STRUCTURE indicated our samples likely represent a single population with the highest log-likelihood at $K = 1$ (one population) (Figure 4). AMOVA analysis indicated that 100% of the genetic variance occurred within categories and 0% of the variance occurred between categories (Table 5).



Figure 4. Log-likelihood of number of 1-10 populations as calculated by STRUCTURE.

Discussion

Analysis of Population Structure

The samples used in this study afforded us a unique opportunity to sample individuals collected over a three-year period from a single study site. The STRUCTURE analysis, which was based solely on the microsatellite data, revealed no evidence of genetic substructure in our sample of eastern red bats. Similarly, none of our post-hoc groupings detected any genetic differentiation.

The star shaped haplotype network was composed of one very common haplotype that is assumed to be the ancestral haplotype, plus many unique or rare haplotypes connected to the most common haplotype by short branches. A 'star-shaped' network haplotype network suggests one large population that has expanded since the last ice age from a modest founding population to a large current population occupying a large geographic area (Avice 2000). Species with 'star-shaped' haplotype networks typically show shallow levels of genetic differentiation due to high levels of gene flow, usually as a result of high dispersal, coupled with a lack of long-term biogeographic barriers (Avice 2000). High levels of gene flow in eastern red bats are likely facilitated by migration of individuals across North America. Genetic diversity was also high across years for both microsatellite loci and mitochondrial haplotypes, which is consistent with a large, well connected population.

While much of the genetic evidence from our sample is consistent with one large panmictic population, it does not preclude the possibility of low substructure or a pattern of isolation by distance across the range of this population. The

Bayesian clustering method in STRUCTURE can potentially detect differentiation at an F_{ST} level of 0.05 but is often not capable of detecting lower levels of differentiation or isolation by distance. Three of seven microsatellite loci were in linkage disequilibrium and five of the seven loci had heterozygote deficits, which could be an indication of a potential Wahlund effect (Frankham et al. 2009). A heterozygote deficit can also result from null alleles, which appear to be at least partially, if not completely, responsible for the deficit in several of our markers. There is a small group ($n= 15$ in square on figure 3) of bats that, based on mitochondrial sequences, are differentiated from the other bats. These bats differ from the most common haplotype by 3% (the mean divergence for the entire population was 0.7%). Intraspecific variation can be high in bats, western red bats (*L. blossevillii*, a species closely related to eastern red bats), for example, had a mean sequence divergence of 8.2% (max of 13.96%) in samples from South America (Clare et al. 2011). Bats found in this divergent cluster on our haplotype network were found in all three years and shared no other obvious characteristics to explain their similarities. It is possible that these bats are part of some unique sub-population, but without further information, it will not be possible to identify the reasons behind their divergence.

If additional information on the geographic origins of these bats can be gathered through the use of stable isotopes, further investigations for subtle population structure can continue. With stable isotopes, bat researchers have used the isotopic signature of a hair sample to determine where that hair was grown (Cryan et al. 2004). Cryan et al. found that in some cases, the isotopic signature of

the hair differed significantly from the location where the bat was captured and were able to determine that in this situation, the bats are likely long-distance migrants. If these methods are applied to the bats used in this study (many of which also had hair samples taken), it may be possible to determine the approximate origin of these bats. Once the origins of these bats are known, the genetics data can be re-examined to look for low levels of population structure across the entire range of the species.

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VITA

Personal Background

Jennifer Michelle Korstian
Born in Fort Worth, Texas
Daughter of John Decker and Lilly Mary Korstian
Sister of Zachary Decker Korstian

Education

Western Hills High School, Fort Worth, Texas, 2002
B.A. Biological Sciences, Rice University, Houston, Texas, 2006
M.S. Biology, Texas Christian University, Fort Worth, Texas,
May 2012

Experience

Teaching Assistant, Texas Christian University, Fort Worth,
Texas, 2010-2012
Inventory Coordinator, The Container Store, Fort Worth, Texas,
2006-2010

Awards

Teaching Assistant of the Year, Texas Christian University,
2012
Adkins Fellowship, Texas Christian University, Department of
Biology, 2011

ABSTRACT

HIGH GENETIC DIVERSITY AND LACK OF STRUCTURE IN EASTERN RED BATS *LASIURUS BOREALIS*

by Jennifer Michelle Korstian
Department of Biology
Texas Christian University

Thesis Advisor: Amanda Hale, Assistant Professor of Biology

The purpose of our study was to characterize the genetic diversity and population genetic structure of eastern red bats (*Lasiurus borealis*) killed during fall migration at a wind farm in north-central Texas over a three-year period (2009-2011).

Microsatellite loci and mitochondrial haplotypes showed high levels of genetic diversity in this species. The pattern of diversity observed in the haplotype network is consistent with what is seen in species that have undergone rapid population and range expansions since the last ice age. Both clustering analysis (i.e. grouping individuals by genetics alone) and partitioning analysis (i.e. grouping by specific characteristics such as year and fatality pulse) indicated that our samples represent a single population without genetically distinct subunits. The eastern red bats sampled from our study site, which includes a mix of migratory and resident individuals, likely represents one, large population of bats with high levels of gene flow among local populations.