GENETIC DIVERSITY, POPULATION STRUCTURE, AND EFFECTIVE POPULATION SIZE IN TWO YELLOW BAT SPECIES FROM SOUTH TEXAS

by

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Bachelor of Arts, 2018
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Submitted to the Graduate Faculty of the College of Science and Engineering Texas Christian University in partial fulfillment of the requirements for the degree of

Master of Science

May 2020

ACKNOWLEDGEMENTS

I would like to first thank my advisors, Dr. Amanda Hale and Dr. Dean Williams for their patience and mentorship during my two years at TCU. I am appreciative of the opportunities they provided me in order to learn about the methods and concepts integral to my research. I'm grateful for Dr. Matt Hale and Dr. John Horner for serving as committee members and for providing insight to my project given their areas of expertise. Without Dr. Sara Weaver, this project would not have been possible, and I am appreciative of her willingness to share all of the samples that I used in this study. I would like to thank the TCU College of Science and Engineering and the Biology Department for providing funding in the form of grants and the Adkins Fellowship that made the completion of this project possible. If not for Dr. Russ Benedict at Central College, I would not be the biologist I am today. To my family and friends for their continued support: thank you.

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INTRODUCTION

Bats currently face a multitude of threats worldwide, making their study and conservation essential for the preservation of species and the important ecosystem services they provide (Boyles et al. 2011; Kunz et al. 2011; O'Shea et al. 2016; Frick et al. 2019). Many aspects of bat biology are poorly understood, and fear of bats has led to the extermination of entire colonies and roost destruction, which are major drivers of species decline (O'Shea et al. 2016; Tuttle 2017; Frick et al. 2019). Changes in land use, driven by increased agriculture and urbanization, have eliminated valuable bat habitat while also accelerating effects of climate change, which together threaten both bats and the ecosystem services they provide (Williams-Guillén et al. 2016). Efforts being made to mitigate the effects of climate change have altered the way we produce energy in favor of renewable processes, like wind energy. Although mitigating the impacts of climate change by reducing greenhouse gas emissions through an increase in electricity generation from renewable sources like wind power has the potential to benefit wildlife conservation (Kiesecker et al. 2011; Allison et al. 2019), bat mortality at wind energy facilities is an unanticipated consequence that unfortunately may simultaneously threaten the persistence of bat populations (Kunz et al. 2007; Erickson et al. 2016; Frick et al. 2019).

Several studies have suggested that wind energy development could cause declines in bat populations, yet few studies have provided quantitative estimates of wind-related fatality and the effect of wind energy on long-term population viability (Frick et al. 2017; Rodhouse et al. 2019). In the United States and Canada, most of the research investigating bat mortality at wind energy facilities has focused on three bat species: *Lasiurus borealis*, *L. cinereus*, and *Lasionycteris noctivigans*, as they comprise the majority of fatalities reported annually (Arnett and Baerwald 2013; Smallwood 2013; Zimmerling 2016). All three species are migratory, solitary, and tree-roosting, making it impossible to estimate measures such as population size or monitor

population trends using traditional mark-recapture methods. Given this challenge, studies of these three species have implemented population genetics methods to gain knowledge of genetic diversity and population connectivity (Luikart et al. 2010). From the studies published to date, all three species have high genetic diversity, large effective population sizes, and no evidence of population sub-structure (Korstian et al. 2015; Vonhof and Russell 2015; Pylant et al. 2016; Sovic et al. 2016), most likely due to individuals mating during annual migration resulting in high gene flow among populations.

Two of the three species, *L. borealis* and *L. cinereus*, make up the majority of fatalities at wind energy facilities annually, making periodic population genetic assessment a priority for their conservation (Frick et al. 2017). Given that population genetics methods can be implemented in repeated sampling efforts to detect evidence of decline over time, genetic data collected from these species at present are valued as a starting point for genetic monitoring in which the same estimates could be calculated repeatedly in the future (Schwartz et al. 2007; Antao et al. 2011). Thus far, these population genetic approaches have been limited to the three species mentioned above. Therefore, there is limited population genetic data for other North American bat species that are also impacted by wind energy mortality despite the annual abundance of carcasses available for DNA collection (Arnett and Baerwald 2013). The large quantity of bat carcasses salvaged from distinct geographic locations at known times of the year offers a chance to answer questions related to population status, cryptic species, geographic ranges, and seasonal movements – all aspects of bat biology that warrant additional investigation. With the population status of many bat species worldwide being unknown, fatalities at wind facilities in North America provide an opportunity to indirectly estimate the population-level effects of wind-related mortality if repeated genetic sampling efforts are carried out over time (Frick et al. 2019).

Recent wind energy expansion into the Rio Grande Valley of Texas (Starr and Hidalgo counties) has led to two additional bat species, the northern yellow bat (*Dasypterus intermedius*) and the Texas state-threatened southern yellow bat (*D. ega*), as being identified as collision fatalities at wind turbines (AWWI 2018; Texas Parks and Wildlife. 2019. Federal and State Listed Mammals in Texas. Available from https://tpwd.texas.gov [Accessed 11 November 2019]). These two yellow bat species are sister taxa to the *Lasiurus* bats (Baird et al. 2017), and the potential for impacts from wind energy development is high given the level of mortality seen annually in *L. borealis* and *L. cinereus*. Wind energy is a new source of mortality for *D. ega* and *D. intermedius*, and these species were prioritized for our study given the availability of genetic data from sister taxa allowing for comparison of the population genetic measures among genera.

As both *Dasypterus* species have a limited geographic range in Texas (Ammerman et al. 2012; Decker et al. 2020; Fig. 1), our objective was to use fatalities from far-south Texas to better understand aspects of basic biology such as seasonal movement, population connectivity, and life-history characteristics in both species. We sought to provide contemporary estimates of genetic diversity, effective population size, and population structure which can be used as a baseline in the long-term genetic monitoring of both *D. ega* and *D. intermedius*. The location of our study site allowed us to also consider the status of *D. intermedius* subspecies designations (*D. intermedius floridanus* and *D. i. intermedius*; Webster et al. 1980) to determine whether the groups are genetically distinct and if both putative subspecies are sympatric in south Texas.

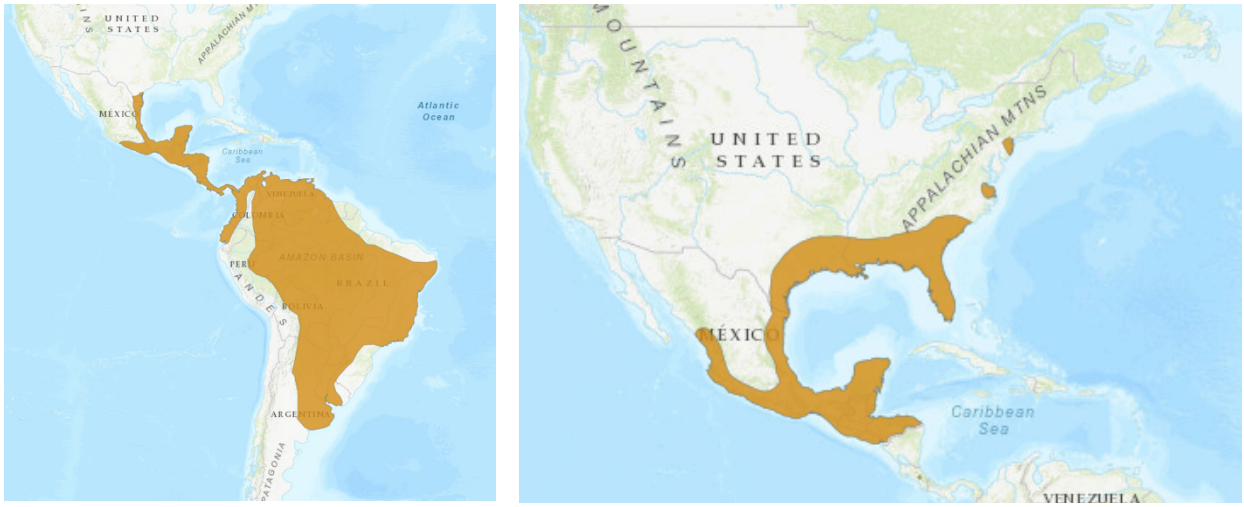

Fig. 1. Geographic range of *Dasypterus ega* (left) and *D. intermedius* (right) from the IUCN Red List www.iucnredlist.org (Barquez and Diaz 2016; Miller and Rodriguez 2016).

MATERIALS AND METHODS

Sample Collection

Wing tissue samples were obtained from bat carcasses collected during post-construction fatality surveys at wind energy facilities in Starr and Hidalgo Counties (Texas) from March through November of 2017 and 2018 (n = 439 carcasses; Fig. 2). Carcasses were identified to species (*D. ega* or *D. intermedius*) and sex (male, female, or unknown) based on external morphology. Time since death (< 1 day, 2-3 days, ≥ 4 days) was estimated based on state of decomposition and decay as well as body tissues present (Weaver 2019). Wing tissue samples were taken from carcasses and stored in vials containing 20% DMSO/6M NaCl. We extracted DNA from the preserved tissue samples following the methods detailed in Korstian et al. (2013).

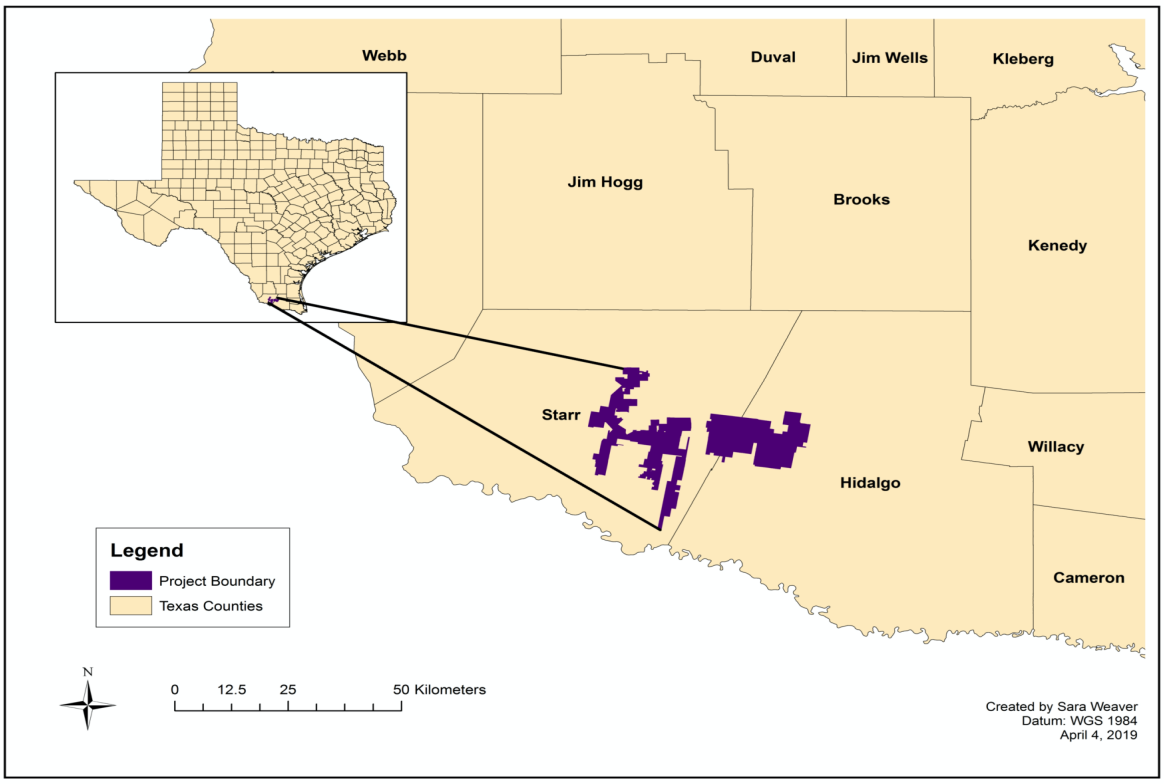

Fig. 2. Location of the wind energy facilities surveyed in Starr and Hidalgo Counties, Texas in 2017 and 2018. Study site map courtesy of Sara Weaver.

Mitochondrial DNA Sequencing

We sequenced DNA extracted from all wing tissue samples at a 550bp section of the mitochondrial cytochrome *c* oxidase I (COI) gene. To amplify the COI gene using a polymerase chain reaction (PCR), we used an M13-tailed primer cocktail (cocktail 2 in Clare et al. 2007). PCR reactions (10uL) contained 10-50 ng DNA, 0.2 uM of the primer cocktail, 1X BSA, and 1X AccuStart™ II PCR SuperMix. PCR reactions were completed using an ABI 2720 thermal cycler with parameters: one cycle at 94 °C for 15 min, followed by 30 cycles of 30 s at 94 °C, 90 s at 57 °C, 90 s at 72 °C, and then a final extension of 5 min at 72 °C. Products were sequenced using ABI Big Dye Terminator Cycle Sequencing v3.1 Chemistry (Applied Biosystems, USA) with the

PCR primers. DNA sequences were analyzed on an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA); trimmed, edited and assembled into contigs using Sequencher v5.1 (Gene Codes, USA); and then aligned in MEGA v10 (Kumar et al. 2018). Aligned sequences were translated to verify the absence of stop codons, after which they were compared to GenBank voucher sequences to generate a species ID. Only sequences > 400 bp in length were used and our criterion to accept a molecular species identification required an identity value > 98% in BLAST. Unique sequence haplotypes were detected using GenAlEx v6.5 (Peakall and Smouse 2006). We used chi-square contingency tests to determine if correct species identification was independent of estimated time since death (<1 day, 2-3 days, ≥ 4 days; $\alpha = 0.05$).

Molecular Sex Determination

Using PCR, we amplified the *zfx* and *zfy* introns found in the sex chromosomes using primers in Korstian et al. (2013). PCR reactions deviated from the mitochondrial sequencing protocol, as 0.5 uM of X-primer and 0.35 uM of Y-primer were used in the 10uL reaction. PCR cycling parameters were: one cycle at 95 °C for 15 min, followed by 30 cycles of 30 s at 94 °C, 15 s at 57 °C, 30 s at 72 °C. PCR products were fluorescently stained with Gel Red, electrophoresed at 200 volts in a 1% agarose gel for 20 minutes, and visualized using UV light. Sex was determined by the presence of one band in females corresponding to the X-chromosome intron (245 bp), whereas males had two bands, one for the X-chromosome intron and one for the Y-chromosome intron (80 bp). We used Fisher's exact tests to compare the proportions of males to females using both molecular and field sex identification ($\alpha = 0.05$). We used chi-square contingency tests to determine if correct sex determination was independent of estimated time since death (<1 day, 2-3 days, ≥ 4 days; $\alpha = 0.05$).

Nuclear Microsatellite Loci Amplification

We amplified 118 *D. ega* and 262 *D. intermedius* samples at 13 microsatellite loci in three groups: multiplex A with primers: Coto_G12, LAS7468, LAS8830, LAS9555 and LAS9618; multiplex B with primers: Cora_F11, LAS2547, LAS8425, LAS9151 and LbT; and multiplex C with primers: LAS7831, LcM, LcU. Primers were previously developed for use in *L. borealis* and *L. cinereus* by Keller et al. (2013) and Korstian et al. (2013). PCR reactions were performed using the same ratios of reagents as mitochondrial sequencing, but had cycling parameters of: one cycle at 94° C for 15 minutes, followed by 30 cycles of 30 seconds at 94° C, 90 seconds at 60° C, 90 seconds at 72° C, and then a final extension of 30 minutes at 60° C. The PCR products were diluted with 200 µL dH_20. For all samples, 0.5 µL of diluted product was loaded in 15 µL HIDI formamide with 0.1 µL LIZ-500 size standard (ThermoFisher Scientific, Waltham, MA, USA) and electrophoresed using an ABI 3130XL Genetic Analyzer (ThermoFisher Scientific, Waltham, MA, USA). We scored and binned genotypes using Genemapper v5.0 (ThermoFisher Scientific, Waltham, MA, USA).

Genetic Diversity Analyses

Microsatellite Loci - We used GenAlEx v6.5 to determine the number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_E), and F_{IS} at each locus in each taxon separately (Peakall and Smouse 2006; Peakall and Smouse 2012). Microsatellite loci were tested for deviations from Hardy-Weinberg Equilibrium (HWE) with heterozygote excess, as well as genotypic linkage equilibrium using GENEPOP v4.7 (Rousset 2008). We used a sequential Bonferroni correction to account for multiple comparisons in these tests. Null alleles were identified using MICROCHECKER v2.2.3 (Van Oosterhout et al. 2004), and then loci with null alleles and significant deviations from HWE were removed from further analyses. HP-RARE

was used to calculate allelic richness (A_r) using rarefaction to take into account the differences in sample sizes between taxa (Kalinowski 2005).

Mitochondrial DNA - We calculated haplotype diversity (h) in GenAlEx v6.5 and nucleotide diversity of mitochondrial haplotypes (π) using DNASP (Librado and Rozas 2009). Mitochondrial DNA structure was examined by constructing a maximum-likelihood tree in MEGA v10 (Kumar et al. 2018). We compiled haplotypes from this study, vouchered samples downloaded from GenBank, and haplotypes from Korstian et al. (2016) to construct a maximum-likelihood tree. The tree was inferred using the HKY + G model of base substitution with the highest value of the Bayesian information criterion (BIC) and the pairwise deletion option for missing data. Bootstrap values for the branching pattern were calculated using 1000 replicates. Mitochondrial COI sequences from putative *D. intermedius* subspecies sampled in this study and downloaded from GenBank were used to create a Minimum Spanning Network in PopArt (Leigh and Bryant 2015).

Population Structure

We tested for evidence of population structure for each taxon individually, for *D. i. floridanus* and *D. i. intermedius* together, and for *D. ega* with *D. intermedius* combined using STRUCTURE v2.3.4 which clusters multilocus microsatellite genotypes based on the number of genetically distinct populations (Pritchard et al. 2000). We assumed admixture, correlated allele frequencies, and omitted prior taxon designation. We used the Monte Carlo Markov Chain for 10^6 iterations after a burn-in period of 10^4 iterations for 10 replicates of K = 1 – 5 clusters. Parameters were set to allow α, the degree of admixture, to vary between clusters, and we decreased the initial value of α from 1.0 to 0.2 (Wang 2017). The most likely K was estimated using the method from Evanno et al. (2005) and by determining the highest LnP(D) before values plateaued (Pritchard et al. 2000). We used CLUMPP v1.1.1 to average the most likely K

across ten replicate runs (Jakobsson and Rosenberg 2007). To be considered admixed, individuals were required to have ancestry of (q) $\geq$ 0.10 in two or more clusters (Johnson et al. 2015).

Population Expansion and Effective Population Size

We tested for neutrality using DNASP v5 in each taxon and used the COI sequences to calculate Fu's F and Tajima's D (Fu 1997; Tajima 1998; Librado and Rozas 2009). Values showing significant negative deviations from the null model of a stable population indicate historical population growth. Historic female effective population size (N_{Ef}) was estimated by first calculating Watterson's estimator of COI sequence diversity (θ) in Arlequin v3.5, and then by using the equation: $\theta = 2N_e u$, where u is the mutation rate per sequence per generation (Excoffier and Lischer 2010; Shenekar and Weiss 2011). As the mutation rate of the COI gene is not known for either yellow bat species, we used mutation rates of the cytochrome b gene from other bat species of Vespertilionidae (Nabholz et al. 2008). The high and low mutation rates were 9.115 x 10^{-5} and 6.751 x 10^{-6} per sequence per year, respectively. Contemporary effective population size (N_e) was estimated from the microsatellite genotypes using NeEstimator v2.1, and a minimum allele frequency of 0.05 was used to calculate upper and lower limits of N_e with the linkage disequilibrium model assuming random mating (Do et al. 2014).

RESULTS

DNA Barcoding

Of the 439 tissue samples collected during surveys, 83% of carcasses (n = 366) were correctly identified to species based on DNA barcoding analysis. DNA barcoding improved the overall proportion of carcasses with accurate species identification to 97% (n = 426). Correct species identification in the field was independent of time since death, suggesting decomposition and

scavenging did not interfere with species identification ($\chi^2 = 1.45$, df = 2, P = 0.484). DNA barcoding revealed the presence of two additional species in the dataset: *D. xanthinus* (n = 36) and *Lasiurus blossevillii* (n = 3). Of the 295 carcasses that were field-identified as *D. intermedius*, 88% (n = 261) were correctly identified as *D. intermedius*, whereas 5% were *D. ega* (n = 14), and 4% were *D. xanthinus* (n = 11). Of the 144 carcasses that were field-identified as *D. ega*, 73% (n = 105) were correctly identified as *D. ega*, whereas 17% were *D. xanthinus* (n = 25), 4% were *D. intermedius* (n = 6), and 2% were *L. blossevillii* (n = 3; Fig. 3). Due to DNA degradation, we were unable to obtain DNA barcodes for 3% (n = 9) of field-identified *D. intermedius* and 4% (n = 5) of field-identified *D. ega*.

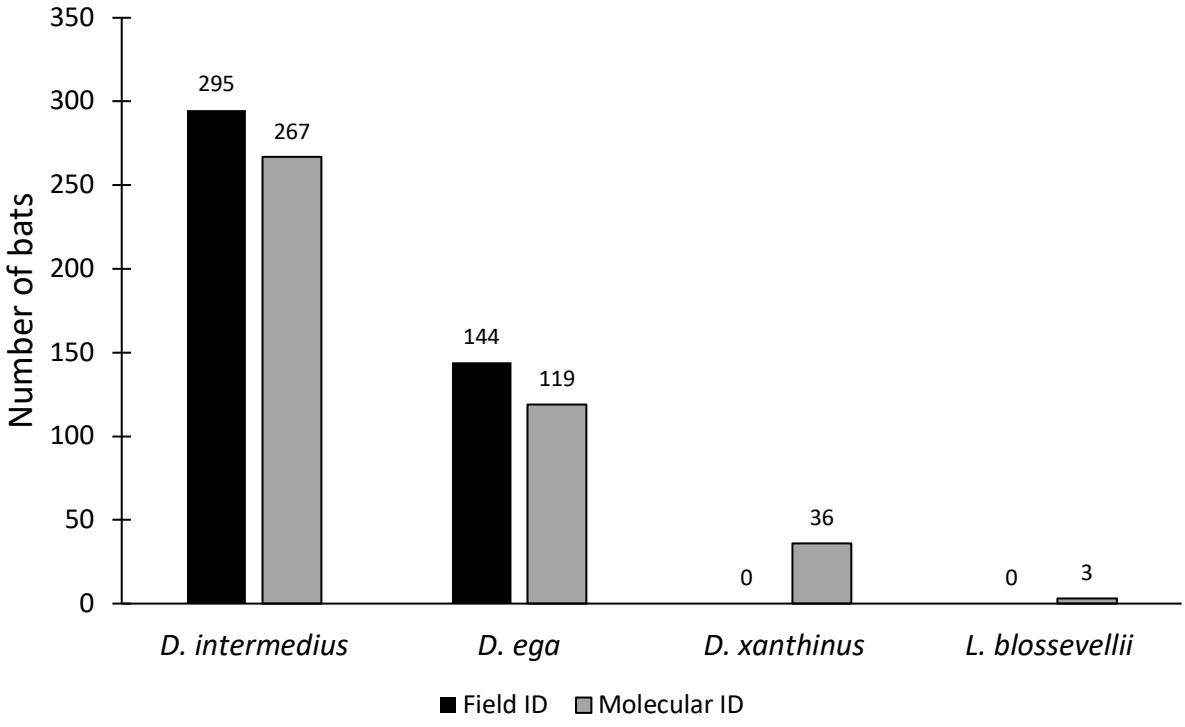

Fig. 3. Number of individual bats that were identified to each species based on either external morphology in the field or DNA barcoding.

Molecular Sex Determination

Survey efforts in the field assigned sex to just 35% (n = 153) of the total carcasses, of which 94% had a correct sex assignment that was confirmed using molecular methods (Fig. 4). Of carcasses identified as either male or female based on external morphology, there was no significant deviation from a 50:50 male:female sex ratio (Fisher's exact test: P = 0.87). Using molecular methods, 94% (n = 411) of the DNA samples amplified at the sex chromosome introns. With all species pooled together, 56% of bats were female (n = 230), with a significant deviation from a 50:50 sex ratio (Fisher's exact test: P = 0.016; Fig. 4) when using molecular sex determination. All 3 *L. blossevillii* bats were females. Of the 112 *D. ega* with successful sex chromosome intron amplification, 62% (n = 69) were female representing a significant deviation from a 50:50 sex ratio (Fisher's exact test: P = 0.018; Fig. 5). Of the 254 *D. intermedius* with successful sex chromosome intron amplification, 53% (n = 134) were female, which was not significantly different from a 50:50 sex ratio (Fisher's exact test: P = 0.415; Fig. 5). Of the 34 *D. xanthinus* with successful sex chromosome intron amplification, 62% (n = 21) were female, which also was not significantly different from a 50:50 sex ratio (Fisher's exact test: P = 0.229; Fig. 5).

In contrast to species identification, correct sex determination based on external morphology was dependent upon time since death ($\chi^2 = 162.1$, df = 2, $P < 0.001$); fresh carcasses (< 1 day) were significantly more likely to be correctly identified as male or female compared to carcasses with longer estimated times since death. There were only a small number of samples (n = 8 bats) for which we were able to determine sex but were unable to obtain a molecular species ID. Samples for which we were able to obtain a molecular species ID, but not determine sex were somewhat more common (n = 22 bats). Twenty-two tissue samples were lacking sex data

from field surveys and their molecular sex determination was included in the counts of males and females.

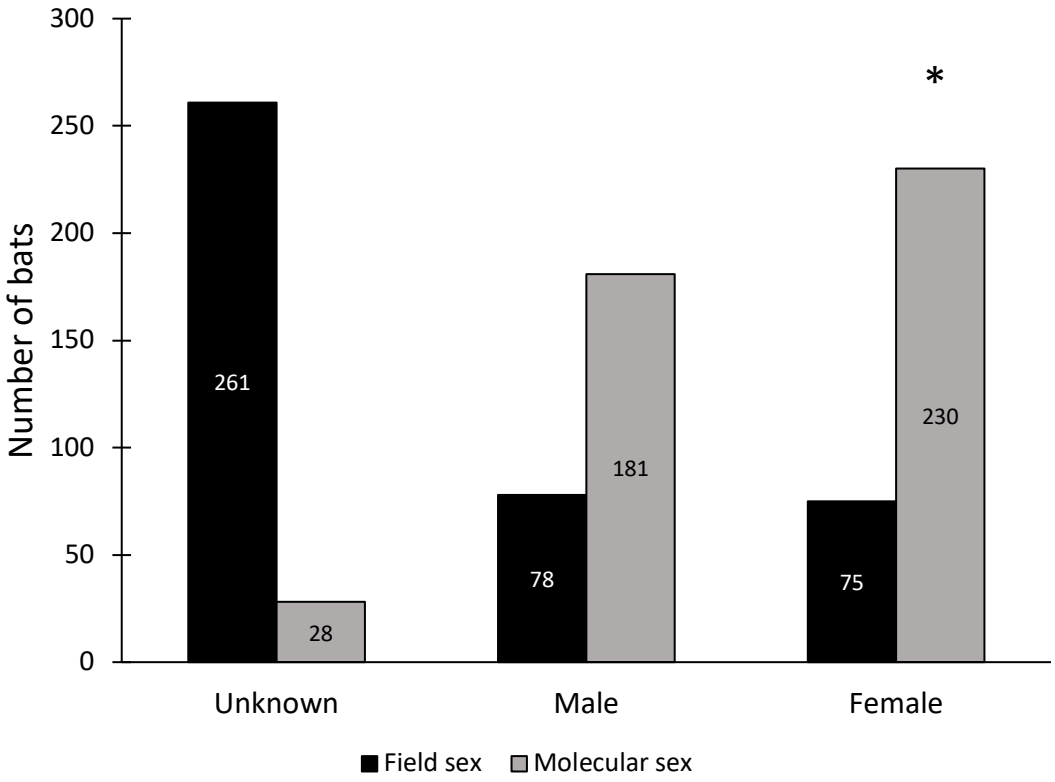

Fig. 4. Number of individual bats from all species combined that were assigned the sex of unknown, male, or female based on external morphology in the field (n = 417) or using molecular methods (n = 439). The star above the female category indicates a significant deviation from a 50:50 sex ratio (Fisher's exact test: P = 0.016). Twenty-two samples were missing sex data from field surveys.

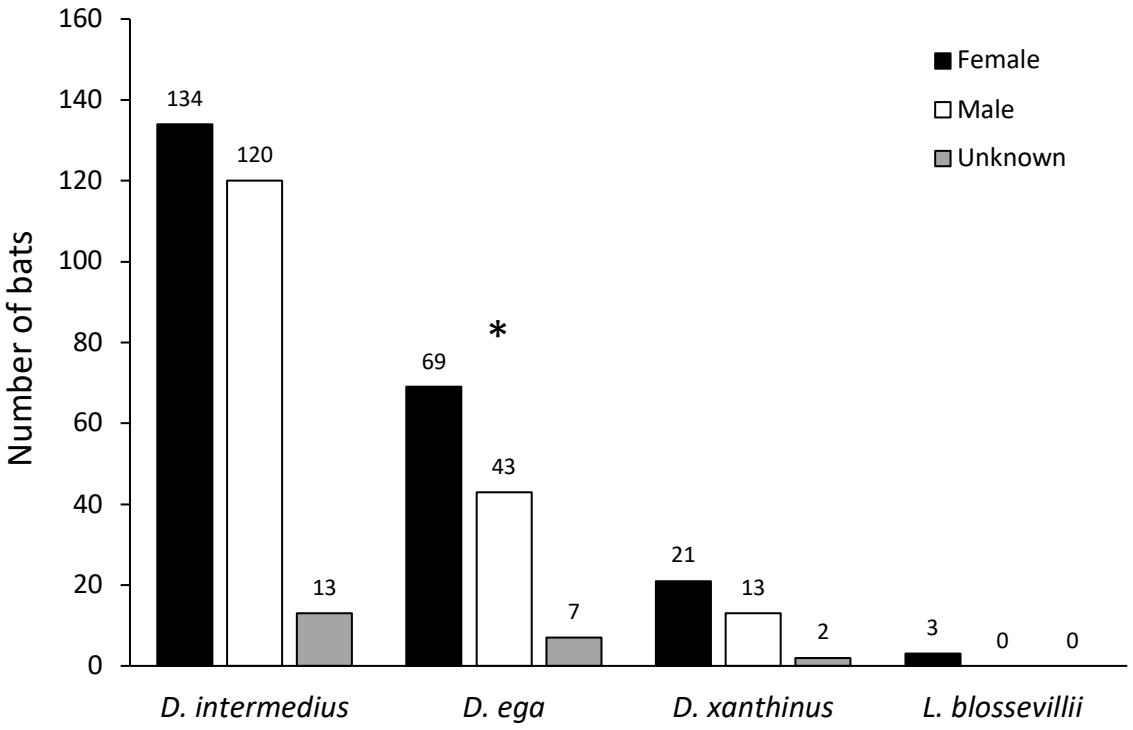

Fig. 5. Number of individual bats from each species identified as female, male, or sex unknown using molecular methods. The star above *D. ega* indicates a significant deviation from a 50:50 sex ratio (Fisher's exact test: P = 0.018).

Dasypterus ega - Microsatellite Genetic Diversity

After genotyping 119 *D. ega* individuals at 13 microsatellite loci, 4 loci were removed due to either null alleles or deviations from HWE. None of the remaining nine loci exhibited a heterozygote deficit or genotypic linkage disequilibrium. One sample was removed after failing to amplify at > 50% of the loci. One hundred fifteen individuals amplified successfully at all loci (Table 1). Observed heterozygosity (H_o) ranged from 0.513 to 0.974 across loci, with the number of alleles ranging from 2 to 40. Allelic richness (A_r) ranged from 3.14 to 4.61.

***Dasypterus intermedius* - Microsatellite Genetic Diversity**

A total of 267 individuals identified as either of the two *D. intermedius* subspecies (50 *D. i. floridanus* and 217 *D. i. intermedius*) were genotyped at 13 microsatellite loci. For *D. i. floridanus*, 5 loci were removed due to null alleles or deviations from HWE. Forty-nine individuals amplified at all loci (Table 1). Observed heterozygosity (H_o) ranged from 0.300 to 0.980 across loci, with the number of alleles ranging from 4 to 20. Allelic richness (A_r) ranged from 2 to 4.41. For *D. i. intermedius*, 6 loci were removed due to null alleles or deviations from HWE. One hundred eighty-four individuals amplified at all loci, and we removed 5 samples after failing to amplify at > 50% of loci (Table 1). Observed heterozygosity (H_o) ranged from 0.209 to 0.938, with the number of alleles ranging from 5 to 24. Allelic richness (A_r) ranged from 2.1 to 4.37. Combined *D. intermedius* was analyzed at 6 shared loci, and 255 individuals amplified at all loci (Table 1). Observed heterozygosity (H_o) ranged from 0.226 to 0.946, with the number of alleles ranging from 5 to 37. Allelic richness (A_r) ranged from 2.08 to 4.52.

Table 1. Characterization of microsatellite loci for each taxon examined. Each locus has the number of individuals that amplified (n), allele size range, number of alleles (N_a), allelic richness (A_r), observed heterozygosity (H_o), expected heterozygosity (H_e), and F_{IS}. A_r was only calculated using loci shared among all taxa.

Species	*Locus*	*n*	*Size range (bp)*	N_a	A_r	H_o	H_e	F_{IS}
D. ega	Coto_G12	118	211 - 231	10	-	0.839	0.808	-0.038
	LAS7468	115	311 - 419	40	4.61	0.974	0.954	-0.021
	LAS8830	117	254 - 284	13	3.59	0.821	0.822	0.002
	LAS9555	116	441 - 489	16	-	0.793	0.836	0.051
	Cora_F11	117	146 - 155	2	-	0.513	0.498	-0.029
	LAS9151	118	262 - 306	11	2.64	0.551	0.588	0.063
	LAS7831	116	401 - 423	11	3.76	0.871	0.849	-0.026
	LcM	116	195 - 241	20	-	0.690	0.712	0.031
	LcU	116	220 - 253	7	3.14	0.793	0.750	-0.057
D. i. floridanus	Coto_G12	50	211 - 231	10	-	0.800	0.839	0.047
	LAS7468	49	325 - 376	20	4.41	0.980	0.927	-0.057
	LAS8830	50	262 - 276	8	3.19	0.760	0.751	-0.012
	LAS8425	50	166 - 172	4	-	0.300	0.269	-0.117
	LAS9151	50	272 - 290	4	2.00	0.520	0.491	-0.059
	LAS7831	50	399 - 418	10	3.82	0.800	0.854	0.063
	LcM	50	203 - 245	13	-	0.740	0.803	0.078
	LcU	50	228 - 253	7	2.99	0.640	0.662	0.033
D. i. intermedius	LAS7468	208	325 - 384	24	4.37	0.938	0.930	0.932
	LAS8830	211	264 - 278	8	3.23	0.716	0.764	0.766
	LAS2547	184	428 - 461	15	-	0.842	0.827	0.829
	LAS8425	211	166 - 188	5	-	0.209	0.202	0.202
	LAS9151	211	264 - 293	9	2.10	0.507	0.519	0.520
	LAS7831	205	397 - 417	11	3.94	0.873	0.876	0.878
	LcU	211	224 - 228	7	2.99	0.720	0.715	0.717
D. intermedius	LAS7468	257	325 - 384	37	4.52	0.946	0.946	0.001
	LAS8830	261	262 - 278	9	3.22	0.724	0.762	0.050
	LAS8425	261	166 - 188	5	-	0.226	0.215	-0.051
	LAS9151	261	264 - 293	9	2.08	0.510	0.513	0.008
	LAS7831	255	397 - 418	12	3.93	0.859	0.876	0.019
	LcU	261	224 - 253	9	2.96	0.705	0.708	0.004

Mitochondrial Genetic Diversity

Two unique haplotypes were identified from 112 *D. ega* individuals (Table 2). The most common haplotype had a frequency of 0.991 and nucleotide diversity (π) was 0.00003. Six unique haplotypes were found in 50 *D. i. floridanus* individuals, with the most common haplotype having a frequency (*h*) of 0.580 (Table 2). Seven unique haplotypes from 212 individuals were found in *D. i. intermedius*. The most common haplotype had a frequency of 0.527. In *D. i. floridanus* nucleotide diversity was 0.00225, whereas in *D. i. intermedius* it was 0.00177.

Table 2. Number of individuals (n) of each taxon analyzed for mtDNA diversity using number of unique haplotypes (H), haplotype diversity (h), and nucleotide diversity (π). mtDNA sequences were also used to identify population expansion by calculating Tajima's D, Fu's F_s, and Watterson's estimator (θ).

Taxon	***n***	***H***	***h***	***π***	***Tajima's D***	***Fu's F_s***	***θ***
D. ega	112	2	0.018	0.00003	-1.011	-2.344	0.188
D. i. floridanus	50	6	0.588	0.00225	-0.821	-0.369	1.786
D. i. intermedius	203	7	0.542	0.00177	-0.825	-0.663	1.522

A maximum-likelihood tree using 20 unique haplotypes from samples in this study, from Korstian et al. (2016), and from GenBank revealed 2 clades supporting the distinctiveness of each *Dasypterus* taxon in this study (Fig. 6). Bootstrap values at the nodes delimiting species or subspecies were in excess of 95, and *D. i. floridanus* and *D. i. intermedius* each represented a

monophyletic clade. The minimum spanning haplotype network of *D. i. floridanus* and *D. i. intermedius* indicated complete differentiation of haplotypes between the two taxa (Fig. 7).

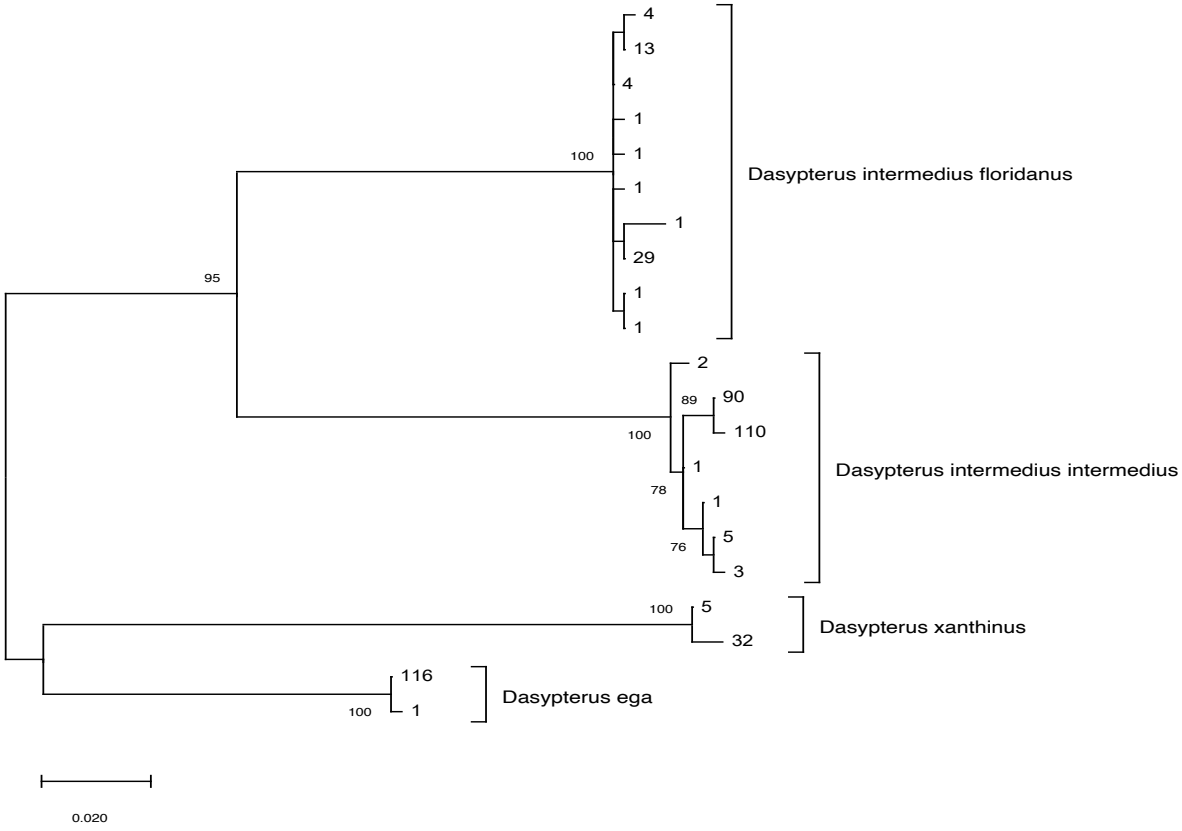

Fig. 6. Maximum likelihood tree of unique COI haplotypes from samples in this study, as well as samples obtained from Korstian et al. (2016) and from GenBank. Numbers at the nodes indicate bootstrap support following 1000 replicates, and branch length represents genetic distance. Numbers at branch tips represent the number of individuals with that unique haplotype.

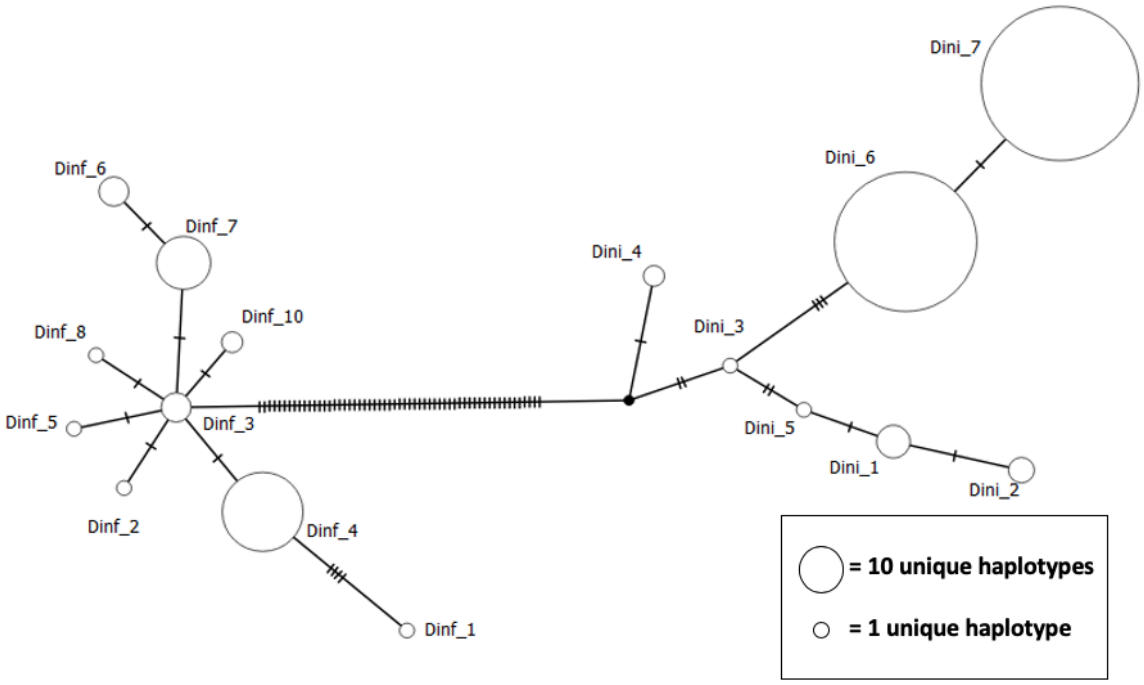

Fig. 7. Minimum spanning haplotype network of unique COI sequences from *D. i. floridanus* (Dinf) and *D. i. intermedius* (Dini) individuals from this study and from GenBank. Circles indicate haplotypes and the size of each circle corresponds to the number of individuals having that haplotype. Vertical hatch marks represent the number of nucleotide substitutions between haplotypes.

Population Structure

F_{ST}, between *D. i. floridanus* and *D. i. intermedius* was 0.057 and unbiased Nei's genetic distance was 0.046. F_{ST}, and unbiased Nei's genetic distance between *D. ega* and *D. intermedius* was 0.610 and 0.788, respectively. There was no evidence of population substructure within either yellow bat taxon. When *D. i. floridanus* and *D. i. intermedius* were subclustered and analyzed together, there was no evidence of genetic differentiation between the two subspecies. With *D. ega* and *D. intermedius* analyzed together, the Evanno et al. (2005) method identified *D.*

ega and *D. intermedius* as being separate genetic clusters with K = 2 being the best-supported number of clusters (Fig. 8a). The Lnp(D) method provided evidence of K = 3 as the number of clusters with the highest support, which suggests genetic differentiation between *D. ega*, *D. i. intermedius*, and *D. i. floridanus* (Fig. 8b). The STRUCTURE analysis further provided evidence of hybridization between *D. ega* and *D. i. intermedius* as 5 D. ega and 2 D. *intermedius* had proportions of ancestry (Q) > 0.10 in the other species' cluster. One individual *D. ega* had Q of 0.051 for *D. ega* and 0.9459 for *D. intermedius* when K = 2 (Fig. 9). When K = 3, the same individual clustered in *D. ega* had Q of 0.0692 in *D. ega*, 0.3321 in *D. i. floridanus*, and 0.5987 in *D. i. intermedius* (Fig. 10). At K = 3 many individuals had mixed ancestry between *D. i. floridanus* and *D. i. intermedius*.

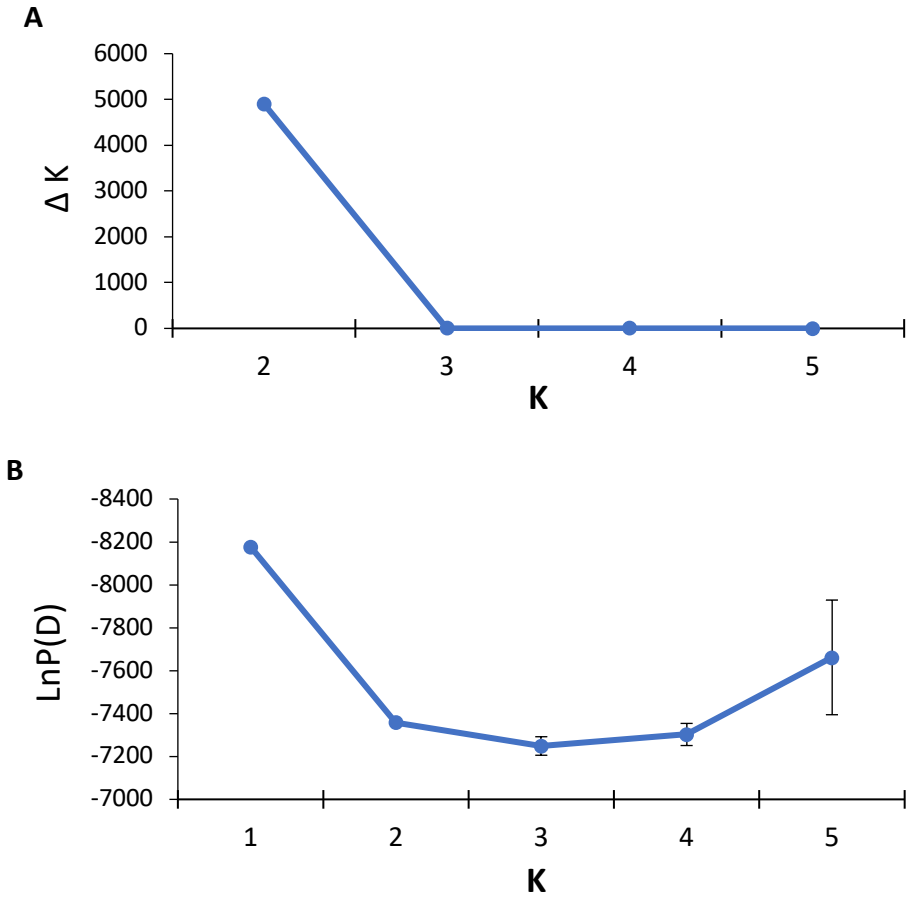

Fig. 8. (A) The Evanno et al. (2015) method indicated K = 2 as the most likely number of populations, which correspond to *D. ega* and *D. intermedius*. (**B)** The LnP(D) method showed K = 3 as the number of populations with the highest support, with populations corresponding to *D. ega*, *D. i. floridanus*, and *D. i. intermedius*. The error bars represent one standard deviation.

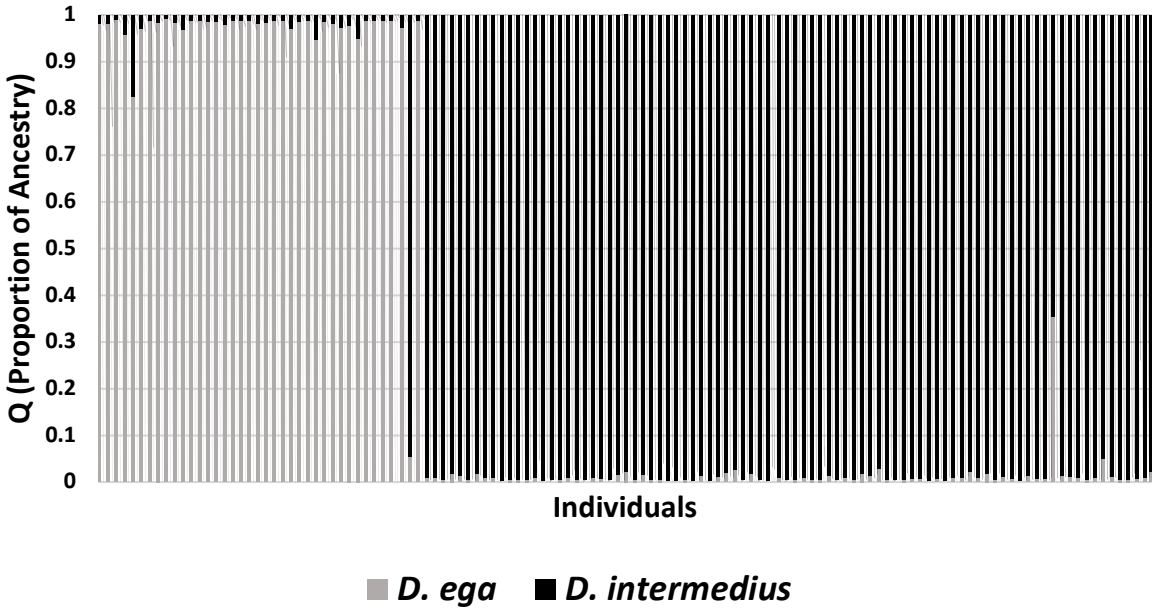

Fig. 9. Structure plot when K = 2. Vertical bars with both colors indicate individuals of mixed ancestry.

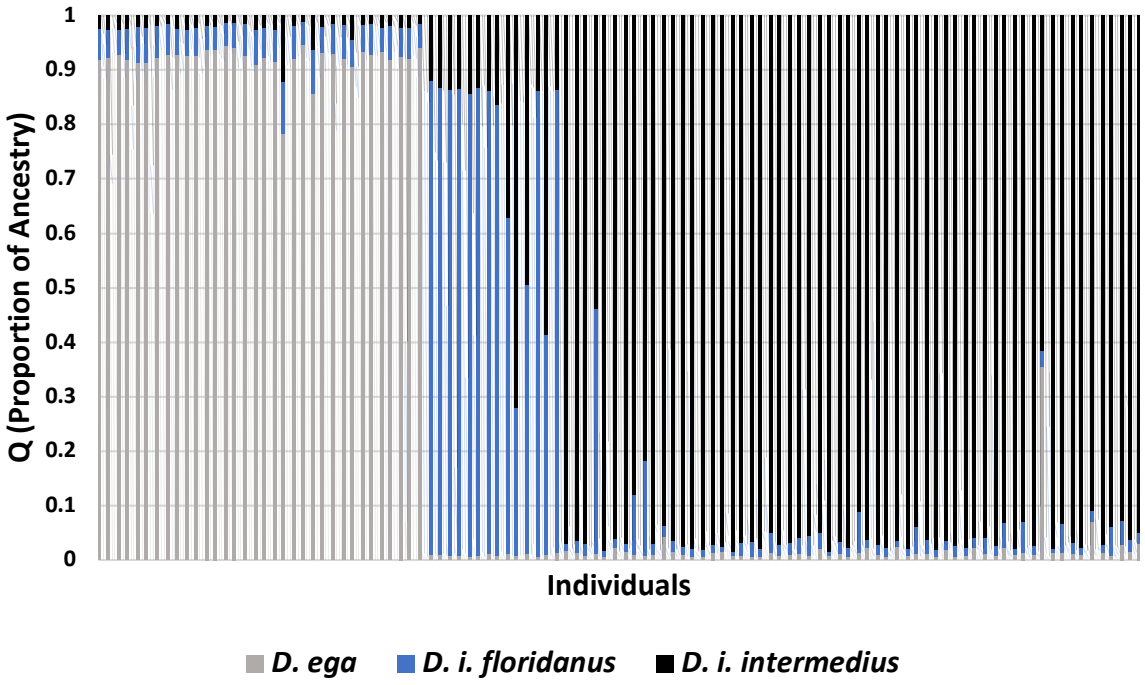

Fig. 10. Structure plot when K = 3. Vertical bars with multiple colors indicate individuals of mixed ancestry.

Population Expansion and Effective Population Size

Tajima's *s* D and Fu's F were both negative in all three taxa, but values were not significantly different from zero. Negative values of Tajima's D would suggest an excess of low frequency polymorphisms, and negative values of Fu's F would suggest an excess number of alleles. Together, negative values of each statistic would indicate historic population expansion. Estimates of N_{Ef} were lower for *D. ega* than *D. intermedius*, whereas estimates for the two subspecies were similar (Table 3). Contemporary estimates of N_e all had upper bounds of infinity. Similar to estimates of N_{Ef}, contemporary estimates of N_e and the lower 95% CI of N_e was lower for *D. ega* than *D. intermedius*.

Table 3. Estimated effective population size (N_e) with 95% confidence intervals and estimated historic female effective population size (N_{Ef}) for each taxon in this study.

Species	***n***	***N_e***	***Lower 95% CI***	***Upper 95% CI***	***N_{Ef}Low***	***N_{Ef}High***
D. ega	118	423	169	∞	1,031	13,924
D. i. floridanus	50	∞	551	∞	9,797	132,277
D. i. intermedius	212	1,032	325	∞	8,349	112,724
D. intermedius	262	2,880	396	∞	-	-

DISCUSSION

DNA Barcoding and Molecular Sex Determination – Implications for Wind Energy Development

Large numbers of bats are killed at wind energy facilities world-wide and there is a pressing need to improve our understanding of why this is happening and develop effective strategies to

minimize impacts (Kunz et al. 2007; Hein and Hale 2019). To accomplish this goal, however, we need accurate assessments of which individuals from which bat species are being killed, both at the wind project-level as well as at regional or range-wide scales. The error rate for species field identification of yellow bats (14% misidentified overall) included in this study was higher than what was reported for *L. borealis* (2.6% misidentified) and *L. cinereus* (0.4% misidentified) killed at a north-central Texas wind energy facility (Korstian et al. 2016). Species misidentifications in this study likely occurred at a higher rate due to the morphological similarity between the two focal species and the presence of two additional bat species that were outside their known geographic ranges (*L. blossevilli* and *D. xanthinus*; Ammerman et al. 2012). Field personnel were unaware of the potential presence of these additional species. Of the *D. ega* samples for which we obtained a DNA barcode, 24% of the bats had been misidentified in the field, perhaps due to the presence of *D. xanthinus* which is morphologically very similar to *D. ega* (Ammerman et al. 2012) and comprised 74% of *D. ega* misidentifications.

A recent study by Decker et al. (2020) provides updated geographic ranges, including range extensions at the county level, for all three yellow bat species in Texas; but it does not, however, differentiate between the two *D. intermedius* subspecies. The DNA barcoding results of this study clearly indicate that both *D. i. floridanus* and *D. i. intermedius* are present at our study site, although only *D. i. intermedius* was suspected to occur in this region of south Texas (Ammerman et al. 2012). This finding in combination with the abundance of *D. xanthinus* samples in our dataset, recent evidence of *D. xanthinus* range expansion in New Mexico (Zabriskie et al. 2019), and close proximity of our study site to a new county record for *D. xanthinus* in Texas (Decker et al. 2020), suggests that more research is needed to fully understand the distributions of bat species, especially in light of on-going land use modification and climate change. Furthermore, we will likely underestimate the number of bat species and

subspecies that may be impacted by wind energy as development continues to expand into new areas if we rely solely on historic range maps and country-level records to make inferences regarding potential impacts.

In addition to errors with species identification, errors or missing data for sex assignment also introduce uncertainty regarding the impacts of wind energy development on bats. Variation in post-construction fatality monitoring protocols (e.g., search interval length, carcass persistence times) as well as how field technicians are trained introduce variation in the quantity and quality of sex assignment data that can be obtained in the field. For example, in this study, personnel only assigned sex to 35% of the yellow bat carcasses they collected in the field, meaning that any inferences related to a potential sex-bias or a lack of sex-bias in mortality would be limited to a small subset of carcasses. By applying molecular methods, we were able to increase both the quantity and quality of the sex data for yellow bats killed at these wind energy facilities: the percentage of bat carcasses with sex information was increased to 94% (n = 411 of 429 bats), and we improved accuracy by identifying errors in sex assignment of 6% of the bats that were assigned sex in the field. In a similar study from north-central Texas, Korstian et al. (2013) showed that sex determination based on external morphology had a 10% error rate, and that by using molecular methods they increased the number of bats with sex data from 554 to 867, representing a 56% increase in available data.

Some previous studies have reported male-biased sex ratios for migratory tree bats killed at wind energy facilities (e.g. Johnson et al. 2003; Arnett et al. 2008); however, it is unknown if this observed bias is real or if it reflects the greater uncertainty of assigning sex to female bat carcasses, especially when they may have been partially scavenged and decomposing. Using molecular methods, sex ratios for *L borealis* killed at wind energy facilities did not differ from 50:50 (Korstian et al. 2013; Nelson et al. 2018), whereas sex ratios for *L. cinereus* either did not

differ from 50:50 (Korstian et al. 2013) or were female-biased (Nelson et al. 2018). In this study, sex identification based on external morphology suggested a 50:50 sex ratio, whereas molecular methods revealed a female-biased sex ratio across all species combined and for *D. ega* analyzed separately. We do not know if the female-biased sex ratio we observed is due to an overall female bias in yellow bat populations or if the behavior of female yellow bats increases their collision risk relative to males. Sex-specific conservation strategies for female yellow bats may be warranted as females are responsible for providing all offspring care and their mortality could lead to population declines over time (Grüebler et al. 2008). Moving forward, there is a need to gather more accurate data on sex ratios of bat-wind turbine fatalities to determine if sex-biases are consistent across species and geographic areas before making range-wide management decisions.

Genetic Diversity and Effective Population Size

Both mitochondrial and nuclear microsatellite genetic diversity were lower in *D. ega* and *D. intermedius* compared to what has been shown for two closely related species, *L. borealis* and *L. cinereus* (Vonhof and Russell 2015; Korstian et al. 2015; Pylant et al. 2016). Estimates of contemporary effective population size (N_e) and historic female effective population size (N_{Ef}) were also lower in *D. ega* and *D. intermedius* than for *L. borealis* and *L. cinereus*, suggesting the higher diversity in these two species is perhaps due to larger population sizes. Both *L. borealis* and *L. cinereus* mate while undertaking seasonal migration, leading to gene flow between geographically separated populations which would also maintain high nuclear and matrilineal genetic diversity (Cryan et al. 2012; Vonhof and Russell 2015; Korstian et al. 2015; Pylant et al. 2016). Although we are currently lacking information regarding seasonal movement patterns in *Dasypterus* spp., our estimates of genetic diversity would suggest that populations of both species at our study site have limited gene flow with populations elsewhere in their respective

geographic ranges. The lower genetic diversity found in samples from our study could also be reflective of genetic drift. This is, in part, supported by the populations being at a geographic range-edge (Decker et al. 2020), and the samples collected at our study sight may only represent a subset of the genetic diversity found at the range-center (Eckert et al. 2008). The microsatellite loci used in this study were developed for use in *L. borealis* and *L. cinereus*, and the lower diversity we observed in yellow bats compared to what was found for *L. borealis* and *L. cinereus* could be due to ascertainment bias (Li and Kimmel 2013). When amplifying microsatellite loci cross-species, it is possible to lose variability across loci which would lead to an underestimate of nuclear genetic diversity. Nevertheless, ascertainment bias seems an unlikely reason for the lower observed genetic diversity in *Dasypterus* spp. given the concordance we found between mitochondrial and nuclear markers.

Like *L. borealis* and *L. cinereus*, point estimates of contemporary effective population size (N_e) were lower than estimates of historic female effective population size (N_{Ef}) in both *D. ega* and *D. intermedius*, indicating a possible decline in population size over time. Estimates of N_{Ef} across yellow bat taxa in this study are consistent with large populations from this region historically.

Estimating contemporary N_e is difficult for species with N_e >1000 using the LD method (Waples and Do 2010). The upper limits to the 95% confidence intervals were infinite for all taxa. Our dataset included individuals of different generations and cohorts, which could in part explain the large variance associated with N_e estimates. To achieve greater confidence in estimates of N_e, future studies will require the use of additional genetic markers (Waples and Do 2010). The lower bound to the 95% confidence interval may still be informative as to the occurrence of bottlenecks if N_e is repeatedly estimated as part of a genetic monitoring protocol for yellow bats over time (Waples and Do 2010; Korstian et al. 2015). We recommend future

population-genetic studies of bats use next generation sequencing methods with many more markers to increase the power of conclusions inferred from genetic data.

Population Structure and Expansion

We found no evidence of population substructure in any of the yellow bat taxa, as all taxa had one cluster of individuals with nearly identical proportions of ancestry (Q). Individuals of *D. ega* in our study had only two unique haplotypes. The northern range boundary of *D. ega* is in southern Texas (Decker et al. 2020), and so haplotype diversity at our study sites may be low due to genetic drift at the edge of the species' range (Eckert et al. 2008; Cahill and Levinton 2015). Furthermore, *D. ega* appears to be expanding northward following plantings of ornamental palm trees, their preferred roost, and so they may be a recent colonist of south Texas (Spencer et al. 1988; Demere et al. 2012).

Both *D. i. floridanus* and *D. i. intermedius* had similar haplotype diversity even though southern Texas is believed to be at the northern range limit of *D. i. intermedius* (Webster et al. 1980). Both *D. ega* and *D. intermedius* have geographic ranges that extend along the Gulf Coast and south beyond the border with Mexico into Central and South America (Barquez and Diaz 2016; Miller and Rodriguez 2016). During the Pleistocene, both species may have existed in refugia along the Gulf Coast and south into Central America and later came into contact in Texas. *D. i. floridanus* has a star-shaped minimum-spanning haplotype network, which is expected under a scenario of past population expansion, and this would be corroborated by the negative values of Tajima's D and Fu's F. However, Tajima's D and Fu's F were not significantly different from zero, providing no statistical evidence of past population expansion. Future studies should use additional markers to increase the statistical power of each test. Furthermore, both subspecies will need to be sampled across their respective ranges to provide

additional haplotypes and more genetic variability to increase the accuracy of population expansion estimates.

Taxonomy

Interestingly, when only *D. i. floridanus* and *D. i. intermedius* were analyzed for evidence of genotypic clustering, STRUCTURE identified one genetic cluster, grouping the putative subspecies together. When *D. ega* was added and all three taxa were analyzed together, STRUCTURE could differentiate between *D. ega* and *D. intermedius* as well as *D. i. floridanus* and *D. i. intermedius*, depending on the analysis method. The Lnp(D) method used by STRUCTURE is less sensitive to admixture than the method described by Evanno et al. (2015). The difference between the results of the two STRUCTURE analyses methods suggest that hybridization sometimes occurs between *D. ega* and *D. intermedius* and that extensive hybridization occurs between the *D. intermedius* subspecies.

Previous work has separated *D. intermedius* into two subspecies based on morphology (mainly size differences; Webster et al. 1980); yet to date, no published study has evaluated the status of the subspecies using both mitochondrial and nuclear markers. The mitochondrial data support the existence of two subspecies, but the nuclear microsatellite markers indicate the two taxa have extensively hybridized in this region of Texas. F_{ST} is low between the subspecies and Bayesian clustering indicates they belong to either a single cluster or to two clusters with high levels of admixture.

With its predominately southern geographic range, *D. ega* could have colonized southern Texas towards the end of the Pleistocene with few individuals from refugia in Mexico (Seal et al. 2015). Alternatively, genetic differentiation among the putative subspecies would suggest that *D. intermedius* existed as isolated populations in refugia along the current day Gulf Coast during the Pleistocene. Organisms such as barred owls, southern *Pinus* spp., and salamanders exhibit a

similar genetic pattern attributed to geographic isolation during the Pleistocene (Schmidtling 2003; Zamudio and Savage 2003; Barrowclough et al. 2011). At the end of the Pleistocene, when climate in the United States began to warm, *D. i. floridanus* and *D. i. intermedius* would have dispersed from refugia leading to an area of overlap in south Texas. The occurrence of gene flow between *D. i. floridanus* and *D. i. intermedius* suggest that the current degree of genetic differentiation is not a barrier to gene flow. Both putative subspecies need to be studied across their ranges in order to determine whether they readily hybridize and if they are sympatric throughout their geographic ranges.

Conclusions

Our study is the first to provide population-genetic data for two species of yellow bats in south Texas. We recommend similar studies be performed on additional North American bat species in areas with recent wind energy development in order to identify bat species or populations as a priority for conservation action. Without baseline genetic data or knowledge of basic bat biology, it will be difficult to understand the long-term effects of wind-related mortality on this important and diverse vertebrate group.

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VITA

Austin Chipps was born September 21st, 1995, in Des Moines, Iowa. A 2014 graduate of Ankeny Centennial High School in Ankeny, Iowa, he received a Bachelor of Arts degree with a major in Biology and minors in Chemistry and French from Central College, Iowa, in 2018. He enrolled in graduate study at Texas Christian University, where he would go on to pursue a Master of Science degree in Biology. While working towards his master's degree, Austin served as a teaching assistant for Introductory Biology I and II labs and he received the Outstanding Graduate Student in Biology Award in 2020. After TCU, he will go on to pursue a PhD in Biology from Louisiana State University beginning in the fall of 2020.

ABSTRACT

GENETIC DIVERSITY, POPULATION STRUCTURE, AND EFFECTIVE POPULATION SIZE IN TWO YELLOW BAT SPECIES FROM SOUTH TEXAS

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There are increasing concerns regarding bat mortality at wind energy facilities, especially as installed capacity continues to grow. Wind energy development has expanded into south Texas, where bat species have not previously been exposed to wind turbines. Our study sought to characterize genetic diversity, population structure, and effective population size in *Dasypterus ega* and *D. intermedius*, two tree-roosting yellow bats native to this region and for which little is known about their population biology and seasonal movements. Using intrinsic DNA markers, we identified two additional bat species in our wind energy fatality dataset, as well as an overall female bias in mortality. Genetic diversity and effective population sizes were lower than what was found in previously studied migratory tree bat species. We found evidence of genetic differentiation between putative subspecies of *D. intermedius*, and recommend our data serve as a starting point for the long-term genetic monitoring of these species.