DIETARY ANALYSIS AND CONSERVATION GENETICS OF THE ENDANGERED OZARK BIG-EARED BAT *CORYNORHINUS TOWNSENDII INGENS*

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GENETICS OF THE ENDANGERED

OZARK BIG-EARED BAT,

CORYNORHINUS TOWNSENDII INGENS

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Abstract:

Populations of endangered Ozark big-eared bats (*Corynorhinus townsendii ingens*) are restricted to 19 essential maternity caves or hibernacula in Oklahoma and Arkansas and total only 1,600–1,800 individuals. Effective conservation and management should encompass many aspects of the species natural history and genetics.

This dissertation first presents a list of insect species consumed by Ozark big-eared bats which can be used to better understand habitat selection if influenced by prey availability. Traditionally, diet studies have relied on morphological analysis of prey body parts found in fecal material, but that method can overlook species because key features used in identification might be damaged by chewing, digestion, or decomposition after excretion. I used molecular analysis of cytochrome *c* oxidase gene from prey DNA found in bat fecal material to identify 20 insect species from nine families and two orders. Fifteen of these were unknown prey items for this taxon. Notodontids were found in the majority of samples (64.3%) and were consumed in greater proportion than their abundance in the environment.

The fragmented nature of populations of Ozark big-eared bats make them susceptible to reduced levels of genetic variation, inbreeding, or fixation of deleterious alleles from genetic drift. For my second chapter, I developed 15 polymorphic nuclear microsatellites to be used in the third chapter with mitochondrial D-loop DNA sequence to characterize the population genetics of these bats and determine conservation units. Molecular data suggested that genetic mixing is occurring at swarming sites among geographically close colonies within Oklahoma but does not include an Arkansas population. I recommend colonies in eastern Oklahoma and western Arkansas be managed as two separate units to protect unique alleles but with protection of surrounding habitat to facilitate low levels of gene flow. Additional genetic analysis of isolated populations in Marion Co., Arkansas is warranted because they may be a separate management unit. Finally, comparison of genetic characteristics of current populations to those 10 years earlier suggested considerable change in gene frequencies, likely as a result of genetic drift. Decreases in genetic variation could potentially affect popoulaitons of Ozark big-eared bats so I strongly recommend continued genetic monitoring.

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CHAPTER I

MOLECULAR ANALYSIS REVEALS NEW PREY ITEMS FOR ENDANGERED OZARK BIG-EARED BATS (*CORYNORHINUS TOWNSENDII INGENS*)

Abstract

Populations of the endangered Ozark big-eared bat (*Corynorhinus townsendii ingens*) are restricted to eastern Oklahoma and Arkansas and total only 1,600–1,800 individuals. Effective conservation of this species includes understanding habitat selection, which may be influenced by prey availability. Morphological analysis of prey items from culled body parts on inhabited cave floors have provided a list of insect species presumed to be prey items of Ozark big-eared bats, but this list is incomplete. Studies relying on morphological analysis can overlook certain species of soft-bodied prey or other species because key features used in identification might be damaged by chewing, digestion, or decomposition after excretion. Molecular techniques allow amplification of a mini-barcoding gene, cytochrome *c* oxidase (COI), of prey DNA found in bat fecal material that can be used to identify insect species. In this study, a portion of the COI gene from guano pellets of Ozark big-eared bats was cloned and sequenced. Twenty insect species from nine families and two orders were identified and resulted in

an additional 15 species of known prey items for this taxon. Notodontids were found in the majority of samples (64.3%) and were consumed in greater proportion than moth abundance data in similar habitat reported by Dodd et al. (2008).

Introduction

The Ozark big-eared bat (*Corynorhinus townsendii ingens*) is a federally listed endangered subspecies that historically occurred in eastern Oklahoma, southern Missouri, and northwestern Arkansas. The taxon apparently was extirpated in Missouri in the 1970s (Kunz and Martin 1982; U.S. Fish and Wildlife Service 1984, 1995; Graening et al. 2011). The total population size of Ozark big-eared bats throughout Arkansas and northeastern Oklahoma was estimated at 1,700 individuals in the 1980s and 1,600–2,300 during the 1990s (U.S. Fish and Wildlife Service 1995). Currently, the majority of the estimated 1,600–1,800 individuals occupy maternity caves and hibernacula in Oklahoma (12 caves) and Arkansas (7 caves; Graening et al. 2011).

The Ozark big-eared bat is geographically isolated from the other four subspecies of Townsend's big-eared bats (*C. t. australis, pallescens, townsendii*, and *virginianus*), and its highly localized distribution is thought to be the result of limited suitable habitat, particularly caves for roosting (Stark 2008; Graening et al. 2011). Ozark big-eared bats depend upon limestone caves and rarely use anthropogenic structures (Harvey and Barkley 1990; Clark et al. 1996a; Clark et al. 1996b; Wethington et al. 1996). Females exhibit philopatry to specific maternity caves (U.S. Fish and Wildlife Service 1984, 1995; Clark 1991; Clark et al. 1996a; Weyandt et al. 2005) and typically travel only 2–8 km from roosting sites to feed (Clark et al. 1993; Wethington et al. 1996). Identification of

characteristics critical to habitat selection by Ozark big-eared bats would help U.S. Fish and Wildlife personnel search more efficiently for additional caves used by Ozark big-eared bats for either maternity colonies or winter hibernacula; however, Clark et al. (1996b) and Wethington et al. (1997) did not find significant differences in habitat and land-use practices between areas surrounding used and unused caves. It has been suggested that the presence of specific prey items (Dodd and Lacki 2007; Dodd et al. 2008) could play a role in Ozark big-eared bat distribution; therefore, effective conservation management of this taxon should include thorough knowledge of its diet.

As direct observations of bats feeding are rare, a variety of other methods have been used to study their diets. Traditionally, researchers examined stomach contents, which resulted in sacrificing the animal. Searching for remains of prey in roosts or in collected fecal material served as a preferred alternative. Leslie and Clark (2002) collected guano from Ozark big-eared bat maternity colonies in Adair Co., Oklahoma, and identified prey items to insect order using key morphological characteristics of wings, legs, elytra, antennae, and other chitinous remains. They were not able to identify remains to species because Ozark big-eared bats often remove the wings and legs, and these appendages typically possess the diagnostic traits used to distinguish species of arthropods (Leslie and Clark 2002). Identifying species from fecal material is also difficult because key features can be damaged by digestion (Kunz and Whitaker 1983). Finally, relying on morphological characteristics recovered from feces underestimates soft-bodied prey (Rabinowitz and Tuttle 1982). Due to these potential shortcomings, Dodd and Lacki (2007) collected culled moth body parts from the floor of caves used by Ozark big-eared bats and identified 49 species from eight families of moths.

Although these traditional methods have provided useful information on the diet of Ozark big-eared bats, the list is likely incomplete because prey body parts could have been chewed or digested (Zeale et al. 2011) or decayed when resting on the cave floor (Dodd and Lacki 2007), thereby either not providing diagnostic characters or limiting diagnosis to taxonomic levels above species. Fortunately, molecular methods offer an alternative approach for identifying prey items and can be used with highly degraded DNA typical of that found in fecal material (Deagle et al. 2006; King et al. 2008). DNA sequences can serve as a species-specific barcode and have been successful in identifying prey items from a variety of taxa including in the following bat species: eastern red bat (*Lasiurus borealis*; Clare et al. 2009), little free-tailed bat (*Chaerephon pumilus*) and Angolan free-tailed bat (*Mops condylurus*; Bohmann et al. 2011), little brown bat (*Myotis lucifugus*; Clare et al. 2011), northern long-eared bat (*M. septentrionalis*; Dodd et al. 2012), gray long-eared bat (*Plecotus austriacus*) and brown long-eared bat (*P. auritus*; Razgour et al. 2011), and mountain long-eared bat (*P. macrobullaris*; Alberdi et al. 2012). This method has also been shown to identify orders of insects missed by morphological analysis (Zeale et al. 2011). Therefore, this study used a DNA mini-barcode approach that has enough resolution to sufficiently identify insect species (Zeale et al. 2011) to address two objectives: 1) to identify insect species consumed by Ozark big-eared bats and compare these findings to previous studies and 2) to look for evidence suggesting selection of certain prey items by comparing consumed prey items with the reported abundance of prey items in habitats supporting Ozark big-eared bats. Ozark big-eared bats are known to select moths (Leslie and Clark 2002; Dodd and Lacki 2007; Dodd et al. 2008), specifically from the family Sphingidae (Dodd et al 2008). Data

presented in this study will provide insights into the diet of Ozark big-eared bats and provide useful information for managers to use as they work to promote the presence of specific insects eaten by this endangered subspecies.

Methods

Study site description

Samples were collected from a maternity colony of Ozark big-eared bats (AD-10) on the Ozark Plateau in Adair Co., Oklahoma. This forested area is dominated by oak (*Quercus* spp.), hickory (*Carya* spp.), and winged elm (*Ulmus alata*). The geology is dominated by Mississippian and Pennsylvanian carbonates (limestone), with lesser amounts of shale and sandstone. Years of erosion and weathering have dissected this uplifted region into ridges separated by steep-sided valleys. Subterranean dissolution of the carbonate rocks by water has created karst features such as springs and sinkholes and the limestone caves (Blair and Hubbell 1938) used by Ozark big-eared bats year round as either spring/summer maternity colonies or winter hibernacula (Humphrey and Kunz 1976; Stark 2008). Cave AD-10 has an average of 255 Ozark big-eared bats per year (Graening et al. 2011) and 234 were estimated using video recordings with infrared lighting during the summer of 2012 when the samples were collected (R. Stark, pers. comm.).

Sample collection and DNA isolation

U.S. Fish and Wildlife personnel collected guano pellets by placing mesh screen on a wood frame (2’ x 3’) in two locations of the cave, with each screen apparatus

positioned under a cluster of bats. The screens were raised slightly off the cave floor to allow water to drain, thereby keeping the feces relatively dry. Screens were placed in the cave on 4 April 2012 and not checked again until 30 July 2012 to avoid disturbing the bats while they were rearing young. Some bat species, including corynorhinids, are highly susceptible to human disturbance (Kunz et al. 1996; Graening et al. 2011) and sampling at maternity colonies can endanger young and adults and cause abandonment (Kunz et al. 1996). Individual pellets were gathered and placed collectively in 30 ml collection tubes and transferred to Oklahoma State University where lids were removed to allow guano pellets to air dry. Twenty individual pellets were selected for DNA isolation in December 2012. DNA extraction on individual pellets was performed using the QIAmp DNA Stool Mini Kit (Qiagen) following protocol modifications recommended by Puechmaille et al. (2007) and Boston et al. (2012).

Identification of bat species

Because fecal pellets might have been deposited by other bat species known to share the cave, such as northern long-eared myotis, tri-colored bats (*Perimyotis subflavus*), and big brown bats (*Eptesicus fuscus*) a 190-base pair (bp) fragment of the mitochondrial 16S ribosomal gene was sequenced to identify the bat species to which each of the 20 guano pellets originated. Amplification of the 16S ribosomal gene was done in nested polymerase chain reactions (PCRs). The first PCR reaction contained 2 μL DNA, 2 mM $MgCl_2$, 0.14 mM of each deoxynucleoside triphosphate (dNTP), 0.15 μM of primers Mysp1 and Mysp2 (Zinck et al. 2004), 0.8 mg/mL bovine serum albumin (BSA), 2X buffer, 1 unit Go*Taq* polymerase (Promega), and ddH_2O to a final volume of

30 μL. The thermal profile consisted of an initial denaturation step of 95°C for 5 min, followed by 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. A final elongation of 72°C for 7 min was used to ensure reactions ran to completion. The reaction mix from the first PCR was then diluted 1:50 with ddH_2O and used to provide the template DNA for a second PCR with the same conditions described above. An aliquot of these PCR amplifications were gel electrophoresed and positive PCR products were purified using the Wizard SV Gel PCR Prep DNA Purification System (Promega). Products of the appropriate size were subsequently sequenced with primer Mysp1 and Big Dye chain terminators using an ABI 3130 Genetic Analyzer (Applied Biosystems Inc.). DNA sequences were examined for variable nucleotide positions using Geneious v. 5.5.6 (Biomatters Ltd.) and compared with 16S ribosomal gene sequence of other potential bat species obtained from Genbank and sequences obtained in the lab from Ozark big-eared bat wing punch samples collected for another study.

Identification of prey species

To identify prey species consumed by Ozark big-eared bats, a 157-bp fragment of the cytochrome *c* oxidase gene (COI) was sequenced from DNA extracted from guano pellets determined to be from Ozark big-eared bats. PCR was done in 30 μL reactions containing 2 μL DNA, 2 mM $MgCl_2$, 0.14 mM of each dNTP, 0.15 μM of primers ZBJ-ArtF1c and ZBJ-ArtR2C (Zeale et al. 2011), 0.8 mg/mL BSA, 2X buffer, 1 unit Go*Taq* polymerase (Promega). The thermal profile included 3 min at 94°C, 16 cycles of 30 sec at 94°C, 30 sec at 61°C (which decreased by 0.5 °C each cycle) and 30 sec at 72°C, and then 24 cycles of 30 sec at 94°C, 30 sec at 53°C, and 30 sec at 72°C. A final step of

72°C for 7 min was used to ensure the reactions ran to completion. Reactions were gel electrophoresed in a 1.5% agarose gel, and PCR products of the appropriate size were purified using the Wizard SV Gel PCR Prep DNA Purification System (Promega).

PCR products were cloned using the pGEM-T Easy Vector System (Promega). Both a 2:1 and 4:1 insert to vector ratio were used in the ligation reactions following the manufacturer's recommended protocol. Reactions were stored overnight at 4°C. Transformations were completed using 16.5 µl of high-efficiency competent cells (≥ x 10^8 cfu μg^{-1}) and 0.67 µl of ligation product. Reactions were placed on ice for 20 min and then heat shocked at 42ºC for 45 sec. Recombinant colonies were identified using X-gal-mediated blue/white selection and individual recombinant colonies were selected and added to 5 µl of ddH_2O. Bacterial cells were lysed with a 95ºC incubation for 5 min and combined with 2 mM $MgCl_2$, 0.14 mM of each dNTP, 0.15 µM of primers T7 and SP6 (Promega), 0.8 mg/mL BSA, 2X buffer, 1 unit Go*Taq* polymerase (Promega), and ddH_2O to volume. The thermal profile consisted of 95°C for 5 min, 35 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 2 min. A final incubation at 72°C for 7 min was used to ensure the reactions ran to completion. Reactions were checked for inserts of the expected size using gel electrophoresis with a 1.5 % agarose gel, and clones containing an insert were sequenced with the forward primer ZBJ-ArtF1c only and Big Dye chain terminators on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc.). DNA sequences were aligned using Geneious v. 5.5.6 (Biomatters Ltd.) and imported into MacClade (Maddison and Maddison 2000) for visual inspection, removal of vector and initial primer sequences, and translation into amino acid residues to verify a COI open-reading frame.

Each COI sequence was compared with existing DNA databases of Genbank sequences using basic local alignment and search tool (BLAST; Altschul et al. 1990) and the Barcode of Life Data System (BOLD; Ratnasingham and Hebert 2007). Both databases were used because they have unequal representation of arthropod COI sequences. For example, Lepidoptera are better represented in BOLD, but Genbank has more deposited sequences for other insect orders (Dodd et al. 2012). All identifications were made with a similarity value of greater than 97% and met one of the following criteria:

1. Match to both databases: If the species with the highest similarity value was the same in both databases and occurs in eastern Oklahoma with flying stages usually during the month of fecal collection, it was the identification chosen.
2. Different match in each database: If the species with the highest similarity value was different in each database, the species that occurs in Oklahoma and is in flight during the month of fecal collection was chosen. If both species occur in Oklahoma and are in usually in flight during the month of fecal collection, the identification with the highest similarity value was chosen.
3. Multiple matches in a single database: If the sequence matched with equal similarity values to more than one species in a single database, the species that occurs in eastern Oklahoma with aerial stages at the time of fecal collection was chosen. If the sequence matched to multiple species co-occurring in eastern Oklahoma, the species that also matched to the other database was chosen.

4. No species match: If the sequence matched to multiple species with the same similarity value in both databases and all occur in eastern Oklahoma, a genus level or higher identification was made.

Flight information for insects was found using bugguide.net and butterfliesandmoths.org.

Selection of families

Selection of moth families was determined by comparing the percentage of guano pellets containing DNA sequence from each moth family to the percentage of moth traps containing each moth family in the Dodd et al. (2008) study. They collected insect abundance data from two counties in Arkansas (Crawford Co. and Marion Co). The Crawford Co. sites occur in the Ozark National Forest, and the sites in Marion Co. occur in a fragmented landscape. Only the data from Crawford Co. were used to compare in this study because those caves (Devil's Hollow and Whitzen Hollow) have similar woodland vegetation such as oak (*Quercus* spp.) and hickory (*Carya* spp.; Dodd et al. 2008) and were expected to have similar moth abundance. Additionally, Whitzen Hollow and Devil's Hollow are 17 and 56 km, respectively, from the cave used in the current study.

Results

Bat DNA was successfully isolated from 17 (85%) of 20 guano pellets, and all had 16S DNA sequence matching to Ozark big-eared bat. COI gene sequence was amplified in 14 (82.4%) of the 17 samples. Cloning of 14 purified PCR products resulted in 108 bacterial colonies with inserts of the predicted size. Useable COI sequence was

obtained from 102 of 108 (94.4%) colonies. There were 1–24 colonies sequenced per fecal sample with an average of seven per fecal sample, resulting in 36 unique COI sequences.

In total, 20 insect species from two orders were identified from the guano samples (Table 1.1). Lepidoptera occurred in 13 (92.8%) of 14 pellets and Diptera in two (14.3%) of 14 pellets. Eight families of Lepidoptera and one family of Diptera were detected. Among the Lepidoptera, Notodontidae was found in the majority of samples (9, 64.3%); the families Pyralidae, Crambidae, and Noctuidae each occurred in four (28.6%) pellets. The number of species per pellet was 1–5 and averaged 2.5. The white-dotted prominent moth (*Nadata gibbosa*) was the most consumed species occurring in seven (50%) of the samples (Table 1.1). Most of the other species were found in 1–2 pellets only. Fifteen species detected in this study were not previously reported in the Ozark big-eared bat diet (Table 1.1).

Three lepidopteran families (Crambidae, Erebidae, and Glyphidoceridae) were found in fecal material but not in moth traps used by Dodd et al. (2008), and one family (Arctiidae) was caught in moth traps but not detected with molecular DNA analysis (Fig. 1.1). Most notable was the disproportionately high frequency of consumption compared with the percentage of moths per trap for species of the Notodontidae (Fig. 1.1). No data were reported on families of Diptera recovered by Dodd et al. (2008), so selection of Sphaeroceridae could not be evaluated.

Discussion

Ozark big-eared bats are known moth specialists (Leslie and Clark 2002; Dodd and Lacki 2007; Dodd et al. 2008), and this study provided added confirmation. Nineteen of the 20 species identified by COI DNA sequences in guano pellets were moths (Table 1.1). The frequencies of Lepidoptera recovered in this study (92.8%) and in the morphological analysis of Leslie and Clark (2002; 91.4%) were very similar. The frequency of Diptera recovered followed the same pattern occurring in 14.3% of the fecal pellets in this study and 18.4% of those in Leslie and Clark (2002). However, dipteran species represented only 0.9% of the culled insect remains detected by Dodd and Lacki (2007). The small size of flies might make the wings less likely to be culled before ingestion and more likely to be found whole in feces (Leslie and Clark 2002).

Five families of moths (Geometridae, Noctuidae, Notodontidae, Pyralidae, and Sphingidae) were common to this study and that of Dodd and Lacki (2007). However, the relative frequencies of these families differed between these two studies. For example, Notodontidae was found in the majority of the fecal samples in this study (64.3%) but was only 7.4% of the culled body parts, and Noctuidae was found in 28.6% of the fecal samples in this study but was 58.4% of the culled body parts (Dodd and Lacki 2007). Some families also were unique to only one of the two studies. These differences could be explained by seasonal variation in their diet or inherent differences in detection methods. The guano pellets would likely have DNA from insect species flying from April–July but the culled body parts were collected in July and August by Dodd and Lacki (2007), and it is not known how long they could have been on the cave floor.

Together these data suggest multiple species identification methods and collecting during different time periods are necessary to fully determine the diet of bats.

Results from this study compared with abundance data from Dodd et al. (2008) suggest notodontid moths are important for Ozark big-eared bats because they were consumed in greater proportion than their abundance in forested habitat (Fig. 1.1). Species in this family are called the prominent moths because they have an upward projecting tuft on their forewing (Miller 1991). They are typically medium size, and most are drably colored with shades of brown and gray (Scoble 1995). Most species are nocturnal, except for neotropical species in Dioptinae, and they fly while bats are feeding (Fullard et al. 2000); defense mechanisms include tympanal organs in the metathoracic area that facilitate detection of insectivorous bat echolocation calls (Scoble 1995; Yack et al 1999). This form of sensory detection allows eared moths to fly at higher forest strata where bats feed (Lewis et al. 1993). Eared moths are also more likely to become airborne (Morrill and Fullard 1992). They likely are chosen as prey by Ozark big-eared bats because species in this family do not fly erratically possibly making them more easily detected by a narrow echolocation signal from a searching bat (Lewis et al. 1993). In Kentucky, notodontids avoid clearings in habitats with Virginia big-eared bats (*C. t. virginianus*) and are captured more frequently in moderate-aged saw-timber (Burford et al. 1999).

Diptera were consumed in proportion to abundance in Leslie and Clark (2002), but results from this study could not be compared with abundance data because Dodd et al. (2008) only reported information on Lepidoptera. Flies do not form a large portion of the Ozark big-eared bat diet but may be an important prey source. Specifically, the cave

dung fly (*Spelobia tenebrarum*) is a common species found in caves of south-central United States including the Ozarks. They are often found on bat guano, and although small, they make up a large percentage of the total biomass in a cave (Barnes et al. 2009). Because of their close proximity to the bats, they might be used opportunistically as a food source when other insect species are in low abundance. Although doubtful, it is possible the cave dung fly DNA recovered in two guano pellets could be contamination from fly body parts on the guano instead of being consumed by the bats.

Although fecal DNA analysis revealed 15 new species of prey for Ozark big-eared bats, the list is likely still incomplete. Leslie and Clark (2002) identified prey remains in Ozark big-eared bat fecal matter from Coleoptera, Homoptera, Hymenoptera, Neuroptera, and Trichoptera, but no COI DNA sequences from these orders were detected. Dodd and Lacki (2007) added members of Blattoidea, Odonata, and Orthoptera to the list of prey species based on culled body parts from Ozark big-eared bat maternity caves, but these were also not represented in the present study. Each of the approaches used to study food habits of Ozark big-eared bats has limitations. Leslie and Clark (2002) examined prey remains in fecal pellets but Ozark big-eared bats often remove the wings and legs, and these appendages typically possess the diagnosable traits used to distinguish species of arthropods (Leslie and Clark 2002). Key morphological features can also be damaged through chewing and digestion making identification difficult (Kunz and Whitaker 1983). Additionally, relying on morphological characteristics recovered from feces likely under represents soft-bodied prey (Rabinowitz and Tuttle 1982). Finally, to collect feces, Leslie and Clark (2002) placed a cloth sheet under the flyway inside a maternity cave. Although they removed pellets smaller than the typical feces of

Ozark big-eared bats to avoid sampling tricolored bats, northern long-eared myotis also use this cave. It is possible they unknowingly used fecal material from northern long-eared myotis in their study. Dodd et al. (2012) showed that in addition to Lepidoptera and Diptera, northern long-eared bats also consume Coleoptera, Hymenoptera, and Trichoptera. These are orders detected by Leslie and Clark (2002) but not recorded in this study. Dodd and Lacki (2007) collected culled moth body parts from the floor of caves used by Ozark big-eared bats, but this approach assumes that any culled body parts are the result of Ozark big-eared bat foraging. Again, big brown bats, northern long-eared myotis, and tricolor bats also utilize the same caves as Ozark big-eared bats, and it is unclear how frequently these other non-targeted species contributed culled moth parts.

Although a more costly method, an advantage of using DNA sequences is that all fecal pellets included in this analysis were first identified to species of origin based on the mitochondrial 16S ribosomal gene. This gene unequivocally distinguishes Ozark big-eared from big brown, northern long-eared, and tricolor bats, and I am confident that all fecal pellets included in this study were from Ozark big-eared bats. Given this, what explains the discrepancies from results of other studies of the Ozark big-eared bats? Each approach to dietary analysis has inherent strengths and weaknesses as highlighted by the study of Dodd et al. (2012) on food habits of northern long-eared bats. Dodd et al. (2012) identified volumes of prey identified from fecal samples and detected Lepidoptera most frequently followed by Coleoptera, Diptera, Hemiptera, and Trichoptera. When Dodd et al. (2012) used a DNA barcoding approach similar to this study, they detected Lepidoptera, followed by Diptera, Coleoptera, Hemiptera, Hymenoptera, and Arachnida.

Thus, their four most abundant taxa were the same using both approaches, but differences were detected between the two approaches for the less frequent dietary items.

Clearly, the total number of pellets examined should have an effect on ability to detect prey items that are chosen less frequently. Clare et al. (2009) in their dietary analysis of eastern red bats produced a species accumulation curve and showed that species detection continued to increase with increasing number of fecal pellets examined. Moreover, the number of clones sequenced per fecal pellet will influence species detection ability. Alberdi et al. (2012) performed a rarefaction analysis for their study on mountain long-eared bats and revealed that by sequencing 20 clones per fecal pellet, 22 of 29 samples reached the asymptote. They suggested that, at least for their study, the time and cost associated with sequencing additional clones would not result in a substantial number of added species. In the case of this study, the one pellet with the most clones sequenced (24) only had DNA from two insect species. Five species was the most detected in a single guano sample and this number was reached by sequencing 10 clones. Most species were detected in only one guano pellet, therefore I recommend sequencing less clones per fecal sample but including multiple pellets.

In the present study on Ozark big-eared bats, the COI gene was amplified, cloned, and sequenced from 14 fecal pellets. An average of seven colonies was sequenced from each fecal pellet. Inconsistency in cloning success among guano pellets resulted in a range of 1–24 sequenced colonies. Relatively small numbers of clones sequenced per pellet might help explain some of the discrepancies between the results of this study and those studies by Leslie and Clark (2002) and Dodd and Lacki (2007). However, this is likely only to underestimate those rare or less frequently captured prey items and does not

account for the observation that taxa were detected in this study that were not reported by either Leslie and Clark (2002) or Dodd and Lacki (2007). In their analysis of the feeding habits of mountain long-eared bats, Alberdi et al. (2012) compared results from morphological analysis of insect parts and COI sequences and also detected differences between the two approaches even when examining the same fecal pellets.

In addition to identifying 15 new species of arthropods consumed by Ozark big-eared bats, this study demonstrated useable DNA from prey items could be harvested from guano even after months of exposure and storage. It has been suggested that the longer the samples remain in the field, the more difficult it would be to retrieve good quality DNA because of sensitivity to environmental conditions such as high temperature or humidity (Taberlet and Luikart 1999; Nsubuga et al. 2004; Piggott 2004; Panasci et al. 2011). Since Ozark big-eared bats are sensitive to researchers in their caves (Graening et al. 2011), the collecting apparatus had to be placed in the cave before parturition and not collected until the juveniles were flying. The samples in this study were collected at the end of a four-month period and then not processed in the lab for an additional four months. Amplification and PCR were still successful, indicating guano samples serve as a useful source of both predator and prey DNA for at least eight months. Although this study utilized DNA from older pellets, there is no reason to believe that the DNA from some arthropods would be more sensitive to decay. Furthermore, DNA found in guano can now be used with high-throughput sequencing to determine the diet of bat species (Bohmann et al. 2011; Razgour et al. 2011), which has the advantage of detecting less abundant prey items typically missed with cloning methods (Alberdi et al. 2012).

Future work should include sequencing DNA from more guano pellets because many species identified in this study were only found in one fecal pellet. Samples should also be collected in other seasons to identify what Ozark big-eared bats are eating year round. It would be possible to determine if the bats select different prey items to correspond with varying energy demands through the seasons. Pond bats (*Myotis dasycneme*) in Poland consume larger more profitable prey during lactation although it is unclear if the bats make opportunistic use of the resource or have preference for larger prey (Ciechanowski and Zapart 2012). A seasonal study would also reveal the winter diet of Ozark big-eared bats as they do arouse in winter (Clark et al. 2002). It is this aspect of their life history that may protect Ozark big-eared bats form White Nose Syndrome. White Nose Syndrome is caused by the cold adapted fungus *Geomyces destructans*, and is responsible for killing over five million bats in the northeastern U.S because it causes premature arousals during hibernation and a resulting loss of fat reserves (Reeder et al. 2012). It is possible White Nose Syndrome would not be as detrimental to Ozark big-eared bat populations because this species is adapted to winter arousal and has a food source available.

It is clear Ozark big-eared bats eat a variety of insects, specifically moths, therefore conservation management for this species should aim to promote insect diversity in the landscape surrounding occupied or potentially occupied caves. Optimally there should be an effort to maintain or restore forest habitat supporting the preferred groups of prey. Knowing which insects are preferred will require a more comprehensive list of prey species and a more thorough analysis of food selectivity. Finally, the distributions of insect species consumed by Ozark big-eared bats could be used as a

component in building an ecological niche model for this species. Such models have been used to identify suitable habitat, recommend rarely surveyed sights with high potential for occurrence, and suggest how a species might be affected by climate change (Guisan and Thuiller 2005).

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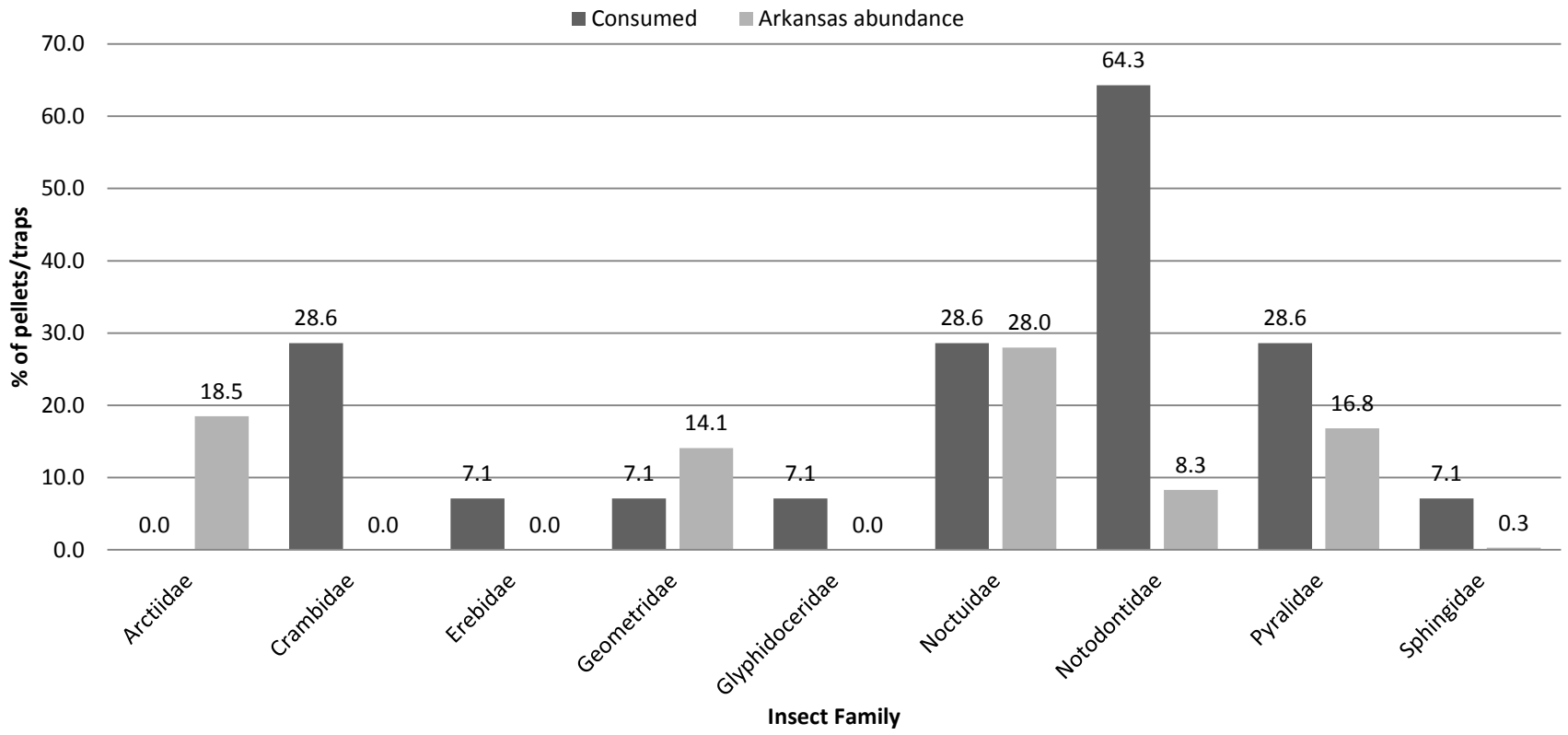
Figure Caption

Figure 1.1 Frequency of insect families consumed by Ozark big-eared bats (*Corynorhinus townsendii ingens*) based on DNA sequences recovered in guano pellets in Adair Co., Oklahoma compared with frequency of insect families caught in blacklight traps in Crawford Co., Arkansas from Dodd et al. (2008).

Table 1.1 Prey taxa detected in Ozark big-eared bat (*Corynorhinus townsendii ingens*) guano pellets based on DNA sequences recovered. * indicates a taxon previously undocumented as prey of Ozark big-eared bats.

Order	Family	Genus / Species	Frequency per pellet
Diptera	Sphaeroceridae*	*Spelobia tenebrarum**	14.3%
Lepidoptera	Crambidae*	*Lineodes integra**	7.1%
		*Parapediasia teterrellus**	21.4%
	Erebidae*	*Palthis angulalis**	7.1%
	Geometridae	*Prochoerodes lineola**	7.1%
	Glyphidoceridae*	*Glyphidocera* meyrickella or septentrionella*	7.1%
	Noctuidae	*Bleptina caradrinalis**	15%
		*Galgula partita**	7.1%
		*Spodoptera frugiperda**	7.1%
	Notodontidae	*Hyperaeschra georgica**	7.1%
		Lochomaeus bilineata	7.1%
		Lochomaeus manteo	7.1%
		Lochomaeus bilineata or manteo	21.4%
		*Misogada unicolor**	7.1%
		Nadata gibbosa	50%
		*Schizura leptinoides**	7.1%
	Pyralidae	*Arta olivalis**	7.1%
		*Canarsia ulmiarrosorella**	7.1%
		*Peoria opacella**	7.1%
		*Peoria** sp.	7.1%
	Pyralidae sp		7.1%
	Sphingidae	*Amorpha juglandis*	7.1%
Lepidoptera sp.			7.1%

Figure 1.1


Consumed
Arkansas abundance
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
% of pellets/traps
0.0
18.5
28.6
0.0
7.1
0.0
7.1
14.1
7.1
0.0
28.6
28.0
64.3
8.3
28.6
16.8
7.1
0.3
Arctiidae
Crambidae
Erebidae
Geometridae
Glyphidoceridae
Noctuidae
Notodontidae
Pyralidae
Sphingidae
Insect Family

CHAPTER II

DEVELOPMENT AND CHARACTERIZATION OF 15 POLYMORPHIC TETRANUCLEOTIDE MICROSATELLITE LOCI FOR TOWNSEND'S BIG-EARED BAT (*CORYNORHINUS TOWNSENDII*) AND CROSS AMPLIFICATION IN RAFINESQUE'S BIG-EARED BAT (*CORYNORHINUS REFINESQUII*)

Abstract

Townsend's big-eared bat, *Corynorhinus townsendii*, is a species of special conservation concern with two subspecies (*C. t. ingens*, *C. t. virginianus*) listed as federally endangered. To properly manage populations of this species, monitoring changes in abundance and genetic connectivity among populations is critical. Fifteen tetranucleotide microsatellite loci were developed and characterized across four of the five subspecies (*C. t. australis*, *C. t. ingens*, *C. t. pallescens*, and *C. t. virginianus*) and Rafinesque's big-eared bat, *C. rafinesquii*, which is also a species of conservation concern. Overall, the majority of loci were highly polymorphic (polymorphic information content > 0.5) in all *C. townsendii* subspecies analyzed. As microsatellites continue to be a useful source of genetic data for addressing a wide range of ecological

questions, the markers presented in this study will be useful to those managing populations of *C. townsendii* and *C. rafinesquii*.

Introduction

Townsend's big-eared bat, *Corynorhinus townsendii*, is comprised of three subspecies in western North America and two in isolated regions in the central and eastern U.S. (Handley 1959). Two subspecies are listed as special concern (*C. t. pallescens* and *C. t. townsendii*; U.S. Fish and Wildlife Service 1994) and two as federally endangered (*C. t. ingens* and *C. t. virginianus*; U.S. Fish and Wildlife Service 1979). Many state agencies have management plans for monitoring changes in abundance of declining populations; however, effective management should also include maintenance of natural genetic connections within and among populations (Crandall et al. 2000). Currently, some populations of bats are monitored through emergence counts, but this method can be difficult due to inaccessible locations of some caves. Moreover, these surveys do not provide data on the individual composition of the colonies. Proper management of threatened or endangered bat populations should include a combination of methods (Kunz et al. 1996), and genetic monitoring would provide additional information for agencies to use in conjunction with cave counts for effective management of *C. townsendii* populations.

Microsatellites have rapidly become the most popular genetic marker to address many ecological questions. There are currently eight microsatellite loci for *C. townsendii* (Piaggio et al. 2009), but several consist of imperfect dinucleotide repeats making scoring difficult and violating mutation models used in population genetic analyses (Guichoux et

al. 2011). Therefore, the objective of this study was to develop polymorphic tetranucleotide microsatellite loci for *C. townsendii* that could be unambiguously scored with little genotyping error. These loci were also amplified in another species at risk, Rafinesque's big-eared bat, *C. rafinesquii* (U. S. Fish and Wildlife Service 1994).

Methods

From microsatellite libraries generated by Genetic Identification Services (GIS; www.genetic-id-services.com) with *C. t. australis* DNA, I screened 24 polymorphic loci to examine levels of variation in *C. t. australis* (*N* = 16) and *C. rafinesquii* (*N* = 16) to determine the most polymorphic loci to amplify in the other subspecies. PCR reactions included 2–4 ng of template DNA, 9.0 µl True Allele PCR mix (Applied Biosystems, Inc.), 0.5 µl of each primer, and ddH_2O to a final volume of 15.0 µl. The thermal profile consisted of 95°C for 12 min, 35 cycles of 94°C for 40 sec, 57°C for 40 sec, 72°C for 30 sec, and 72°C for 4 min. Primers successful in individual amplification were then used in 30 µl multiplex reactions containing 14–28 ng of DNA, 20.0 µl True Allele PCR mix (Applied Biosystems, Inc.), 0.5 µl of each primer and ddH_2O. PCR product (1.0µl) was added to 9.0 µl of formamide (Applied Biosystems, Inc.) and 0.5 µl of ROX size standard (Applied Biosystems, Inc.). An ABI 3130 Genetic Analyzer was used to visualize PCR products and GeneMapper 4.0 (Applied Biosystems, Inc.) was used to genotype individuals.

Loci were tested for null alleles and heterozygote deficiencies in Microchecker (Van Oosterhout 2004). GenAlEx v. 6 (Peakall and Smouse 2006) was used to calculate number of alleles per locus (*A*) and observed /expected heterozygosity (H_o/H_e). Tests of

Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium were performed in GENEPOP v. 4.0 (http://genepop.curtain.edu.au/; Raymond and Rousset 1995; Rousset 2008) with 100,000 Markov Chain Monte Carlo simulations and sequential Bonferroni correction to test for statistical significance (Rice 1989). Because many of the samples were obtained from maternity colonies or a single locality, probability of identity (P_{ID}; Waits et al. 2001) may be insufficiently discriminating. Therefore probability of identity for siblings ($P_{ID\ SIB}$) was calculated using Cervus v. 3.0.3 (Kalinowski et al. 2007). The loci were then ranked based on their polymorphic information content (*PIC*; Botstein et al. 1980). The 15 most polymorphic loci in *C. t. australis* were amplified in the other *C. townsendii* subspecies (*C. t. ingens*, $N = 47$; *C. t. pallescens*, $N = 19$; *C. t. virginianus*, $N = 16$). The 15 loci were divided into five multiplex groups of three loci (Group 1: B106, D7, and D5; Group 2: D107, D109, and D6; Group 3: B107, D110, and D108; Group 4: B7, C5, and B6; Group 5: D1, B105, and D123). After all individuals were genotyped for the 15 loci, some DNA remained for *C. t. ingens* and *C. t. pallescens.* This allowed these individuals to be genotyped for additional loci. Multiplex PCR, genotyping, and analysis of the remaining subspecies followed methods described above.

Results

Results from the 24 potentially polymorphic loci tested in *C. t. australis* and *C. rafinesquii* are in Table 2.1, and results from the 15 most polymorphic loci tested in *C. t. ingens*, *C. t. pallescens*, and *C. t. virginianus* are in Table 2.2–Table 2.4. No loci deviated from HWE, had evidence of null alleles or were in linkage disequilibrium in more than one population. Evaluation of the 15 loci common to each data set revealed

the majority of loci had PIC values > 0.5. While samples from only four of the five *C. townsendii* subspecies were included, all 15 loci were polymorphic across each subspecies suggesting they would also be polymorphic in *C. t. townsendii*.

Discussion

As microsatellites continue to be a useful source of genetic data for addressing a wide range of ecological questions, the markers presented in this study will be beneficial to management of threatened and endangered populations of *C. townsendii* and *C. rafinesquii*. For example, these 15 loci can discriminate 1 in 30,581 *C. t. ingens* and 1 in 41,841 *C. t. virginianus*. Given that there are only approximately 1,600–1,800 *C. t. ingens* (Graening et al. 2011) and 11,500 *C. t. virginianus* (Douglas 2008) remaining, these loci are adequate to address small-scale population genetic questions. These microsatellites can also be used for long term genetic monitoring, providing an opportunity to evaluate the efficacy of management programs and identify early when a population may require a change in management action (Schwartz et al. 2007). Additionally, microsatellites are suitable to estimate population size (Puechmaille and Petit 2007) from DNA isolated from guano (Puechmaille et al. 2007). As some bat species are highly susceptible to human disturbance, sampling at maternity colonies can endanger young and adults and cause abandonment (Kunz et al. 1996). Genetic monitoring of big-eared bats without handling would greatly reduce the stress to these bats while at the same time provide information critical for long-term management of the species.

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Table 2.1 Microsatellite markers characterized in *Corynorhinus townsendii australis* and *Corynorhinus rafinesquii* (bold and italicized). Loci are ranked by decreasing PIC values for *C. t. australis*. Number of individuals genotyped (*N*), number of alleles observed per locus (*A*), observed heterozygosity (H_o), expected heterozygosity (H_e), probability of identity corrected for sampling of siblings ($P_{ID\ SIB}$), and polymorphic information content (*PIC*).

Locus	Repeat	Primer 5'-3'	*N*	*A*	Range	H_o	H_e	$P_{ID\ SIB}$	*PIC*
D5	TAGA	F:NED/CCTGGTCTCCTGGTTGAAC	16	10	282-318	0.875	0.846	0.337	0.829
		R:CCTGCGAACTGAAGAGTCC	***8***	***1***	***202***	***0.000***	***0.000***	***1.000***	***0.000***
B106	CATC	F:FAM/ATGCACCCTTTTGTAAATGATG	16	8	100-128	0.875	0.840	0.341	0.822
		R:ATCCCACCTCACCTTAACTTG	***15***	***6***	***104-140***	***0.533***	***0.622***	***0.484***	***0.584***
D7	TAGA	F:HEX/TGCCAAATAAGCAGCGAG	14	8	208-236	0.786	0.842	0.340	0.822
		R:TTGCCCCTGAACACAGAC	***15***	***7***	***204-232***	***0.667***	***0.720***	***0.417***	***0.690***
D108	TAGA	F:NED/CCTCCCCCTTCCTCTATG	15	7	284-316	0.933	0.778	0.382	0.745
		R:TTGGAACCTAGTGGGCATAC	***16***	***5***	***292-308***	***0.750***	***0.758***	***0.396***	***0.717***
D6	TAGA	F:NED/CCAGGGAGAGCATTCACC	15	7	236-264	0.733	0.773	0.384	0.743
		R:CGTCAGGGCACACACCTA	***16***	***8***	***236-280***	***0.875***	***0.824***	***0.351***	***0.802***
D107	TAGA	F:FAM/AATGGGAAAATAACCTTGGGT	16	6	106-126	0.875	0.773	0.385	0.738
		R:TGTTGTGCTGGGTGTTGTC	***13***	***3***	***90-110***	***0.077***	***0.269***	***0.754***	***0.248***
B107	CATC	F:FAM/CCTGAGACCTTCTTGGTGTGT	16	6	106-126	0.750	0.758	0.395	0.719
		R:GACGAATGAATGGGTGGATAG	***12***	***4***	***106-142***	***0.333***	***0.413***	***0.636***	***0.386***
B7	CATC	F:FAM/CCTGGCACACAGTAGGAGTT	16	7	140-168	0.750	0.746	0.403	0.708
		R:ACCACACAGAGTCCCATTTC	***16***	***6***	***128-160***	***0.688***	***0.756***	***0.397***	***0.717***
D109	TAGA	F:HEX/TGCCCAAGAGGTGAGGATA	9	5	194-210	0.778	0.747	0.403	0.704
		R:GTTCAATCCCTGCCCTAATC	***0***	***0***	***0***	***0.000***	***0.000***	***1.000***	***0.000***
D110	TAGA	F:HEX/AGCCTCCATGATTACATAAGC	13	5	188-204	0.846	0.731	0.414	0.687
		R:ACGATGCTTTTAACCTCTGAG	***16***	***6***	***184-204***	***0.938***	***0.744***	***0.404***	***0.704***

B6	CATC	F:NED/GCCTCCTTCAGGTTGAGTATG	15	5	254-274	0.733	0.718	0.422	0.672
		R:AGTGCCGATTCAATATCCTTG	***16***	***1***	***250***	***0.000***	***0.000***	***1.000***	***0.000***
D1	TAGA	F:FAM/CTCTCCCTCTCCCTTCCTTTAT	16	6	110-134	0.750	0.602	0.497	0.568
		R:CAACCTGGTGCCATCAAAC	***16***	***4***	***122-134***	***0.563***	***0.682***	***0.451***	***0.616***
C5	TACA	F:HEX/CTTGGCTATTCTCTTTCTCTGC	16	5	216-232	0.750	0.633	0.485	0.562
		R:ACCAACAGGAATTGAACTGTG	***16***	***1***	***216***	***0.000***	***0.000***	***1.000***	***0.000***
B105	CATC	F:HEX/CTCCTGCTCTGCCTCACAG	16	4	158-170	0.750	0.613	0.497	0.55
		R:GATGCCCTCGGCTCTAAAG	***16***	***6***	***138-170***	***0.750***	***0.766***	***0.390***	***0.730***
D123	TAGA	F:NED/TTTACCGTGTGGAAAGAAGTT	13	5	218-234	0.615	0.568	0.523	0.526
		R:AGGTGTGCTCACTGCTATTG	***16***	***6***	***210-234***	***0.813***	***0.799***	***0.368***	***0.769***
B114	CATC	F:FAM/TGGAATGACTGTTCTTTC	16	4	174-186	0.688	0.580	0.526	0.493
		R:GGAAGTTGGTAAGGGAATGTG	***15***	***2***	***170-178***	***1.000***	***0.500***	***0.594***	***0.375***
A118	AAAC	F:FAM/CCCTCTCTCTTCCACTCTCTC	13	4	146-158	0.538	0.482	0.585	0.445
		R:CTCCACCTACTGAGCATTCAC	***16***	***2***	***146-150***	***0.188***	***0.170***	***0.841***	***0.155***
B104	CATC	F:NED/TCCATTGTTATACCAGCATCTG	16	3	224-240	0.438	0.525	0.565	0.438
		R:GGTCCATGTAAGTGAAGGTAGG	***16***	***3***	***228-272***	***0.188***	***0.174***	***0.836***	***0.166***
C102	TACA	F:FAM/CAGTTACTCGTCCCCTCATA	15	4	142-154	0.600	0.473	0.592	0.437
		R:GGTCAAAAGGCATCTATTAGC	***16***	***4***	***146-158***	***0.500***	***0.697***	***0.439***	***0.638***
A110	AAAC	F:HEX/AGGCTCTGTAAAGACCTACTG	16	3	180-188	0.625	0.490	0.593	0.397
		R:AGGGACTAACTCAGCGATAAG	***16***	***3***	***176-184***	***0.500***	***0.490***	***0.593***	***0.397***
C4	TACA	F:HEX/CACTTTTACCACCTCTCAG	15	3	186-194	0.467	0.371	0.675	0.323
		R:GCTCCAAGGATTCAAACAC	***16***	***3***	***190-198***	***0.313***	***0.471***	***0.606***	***0.386***
C110	TACA	F:HEX/CCCTCCTTTCCACTCTCTCTA	16	2	206-210	0.188	0.170	0.841	0.155
		R:GGTGGCTTTCTCATTACATTG	***16***	***2***	***206-210***	***0.375***	***0.430***	***0.640***	***0.337***
C6	TACA	F:HEX/CCAGCCAGTAGAGAGTTTCTG	16	2	230-234	0.188	0.170	0.841	0.155
		R:GGTTTGTTGGGAGTCAGC	***16***	***1***	***210***	***0.000***	***0.000***	***1.000***	***0.000***
C101	TACA	F:FAM/CAGCGAACCTCACACAGT	15	1	154	0.000	0.000	1.000	0
		R:TTCTTGATGCTTCTCCTTCTC	***16***	***2***	***158-162***	***0.125***	***0.219***	***0.799***	***0.195***

Mean	5	0.647	0.593	0.518	0.5516
	4	***0.424***	***0.438***	***0.642***	***0.401***

Table 2.2 Microsatellite markers characterized in *Corynorhinus townsendii ingens*. Abbreviation descriptions follow Table 2.1. Dashes indicate loci were not tried.

Locus	*N*	*A*	Range	H_o	H_e	$P_{ID\ SIB}$	*PIC*
D5	40	6	298-318	0.700	0.662	0.455	0.632
B106	46	6	100-124	0.674	0.622	0.491	0.556
D7	31	4	216-228	0.548	0.493	0.579	0.449
D108	31	6	288-312	0.710	0.772	0.386	0.735
D6	31	6	248-268	0.613	0.739	0.408	0.698
D107	32	6	102-122	0.656	0.710	0.426	0.668
B107	46	3	110-118	0.500	0.581	0.523	0.501
B7	32	3	140-148	0.563	0.617	0.496	0.547
D109	45	4	202-214	0.489	0.431	0.628	0.380
D110	32	3	192-200	0.688	0.601	0.511	0.516
B6	32	3	250-270	0.625	0.585	0.522	0.500
D1	47	7	114-142	0.830	0.809	0.362	0.781
C5	32	2	220-224	0.313	0.305	0.73	0.258
B105	45	5	150-170	0.244	0.294	0.731	0.279
D123	31	4	222-234	0.806	0.682	0.450	0.621
B114	—	—	—	—	—	—	—
A118	—	—	—	—	—	—	—
B104	13	2	236-240	0.385	0.453	0.624	0.350
C102	15	2	138-150	0.267	0.231	0.789	0.204
A110	13	3	176-184	0.692	0.500	0.586	0.408
C4	14	2	194-198	0.286	0.337	0.706	0.280
Mean	32	4.05		0.557	0.549	0.548	0.493

Table 2.3 Microsatellite markers characterized in *Corynorhinus townsendii pallescens*. Abbreviation descriptions follow Table 2.1. Dashes indicate loci were not tried.

Locus	*N*	*A*	Range	H_o	H_e	$P_{ID\ SIB}$	*PIC*
D5	19	8	286-314	0.789	0.830	0.347	0.810
B106	18	7	100-124	0.778	0.818	0.356	0.792
D7	18	7	208-236	0.611	0.748	0.399	0.717
D108	18	7	280-304	0.667	0.796	0.369	0.770
D6	18	6	244-264	0.722	0.796	0.370	0.765
D107	17	7	106-130	0.529	0.740	0.407	0.700
B107	19	5	106-122	0.737	0.780	0.381	0.745
B7	19	5	140-156	0.842	0.752	0.399	0.712
D109	19	6	194-214	0.789	0.683	0.443	0.647
D110	19	7	172-204	0.789	0.776	0.384	0.740
B6	18	6	242-274	0.667	0.765	0.390	0.728
D1	19	5	122-138	0.526	0.607	0.504	0.533
C5	18	5	220-236	0.667	0.611	0.500	0.539
B105	17	4	158-170	0.882	0.644	0.477	0.574
D123	18	7	214-238	0.667	0.522	0.554	0.491
B114	17	4	174-186	0.529	0.59	0.509	0.544
A118	19	2	146-154	0.158	0.145	0.863	0.135
B104	16	3	232-240	0.063	0.0007	0.689	0.309
C102	—	—	—	—	—	—	—
A110	—	—	—	—	—	—	—
C4	—	—	—	—	—	—	—
Mean	18.1	5.61		0.634	0.645	0.463	0.625

Table 2.4 Microsatellite markers characterized in *Corynorhinus townsendii virginianus*. Abbreviation descriptions follow Table 2.1. Dashes indicate loci were not tried.

Locus	*N*	*A*	Range	H_o	H_e	$P_{ID\ SIB}$	*PIC*
D5	16	6	298-318	0.625	0.775	0.384	0.740
B106	16	6	100-124	0.750	0.555	0.533	0.511
D7	16	5	208-224	0.625	0.732	0.412	0.692
D108	16	4	288-304	0.625	0.740	0.409	0.692
D6	14	5	248-264	0.643	0.691	0.441	0.641
D107	15	5	106-122	0.600	0.724	0.416	0.686
B107	16	3	106-118	0.500	0.447	0.622	0.371
B7	15	5	128-148	0.800	0.649	0.466	0.608
D109	16	3	202-210	0.375	0.461	0.608	0.398
D110	16	5	180-196	0.688	0.725	0.418	0.679
B6	14	3	250-270	0.571	0.622	0.492	0.551
D1	15	6	114-134	0.533	0.778	0.382	0.745
C5	15	3	180-224	0.067	0.127	0.878	0.123
B105	15	5	146-166	0.400	0.396	0.648	0.376
D123	15	5	222-238	0.600	0.640	0.480	0.571
B114	—	—	—	—	—	—	—
A118	—	—	—	—	—	—	—
B104	—	—	—	—	—	—	—
C102	—	—	—	—	—	—	—
A110	—	—	—	—	—	—	—
C4	—	—	—	—	—	—	—
Mean	15.3	4.6		0.56	0.604	0.506	0.559

CHAPTER III

IDENTIFICATION OF CONSERVATION UNITS AND TEMPORAL VARIATION IN ENDANGERED OZARK BIG-EARED BATS (*CORYNORHINUS TOWNSENDII INGENS*)

Abstract

The endangered Ozark big-eared bat (*Corynorhinus townsendii ingens*) is restricted to eastern Oklahoma and Arkansas, where populations are susceptible to losses of genetic variation because of potentially small effective population sizes. Therefore, the first objective of this study was to use mitochondrial D-loop DNA sequence and 15 nuclear microsatellites to determine conservation units of Ozark big-eared bat colonies. Assessment of seven maternity colonies revealed a haplotype not detected in 2002–2003, and there was evidence of reduced gene flow between colonies in eastern Oklahoma and western Arkansas. It is possible genetic mixing is occurring at swarming sites among colonies within Oklahoma, therefore I recommend colonies in eastern Oklahoma and western Arkansas be managed as two management units to protect unique alleles. Populations in north-central Arkansas (Marion Co.) need to be surveyed to establish their status as a separate management unit because they are geographically isolated from other

populations. The second objective was to compare levels of genetic variation of current populations with that of the same colonies examined 10 years earlier. All caves showed a decline in allelic richness with no decline in observed heterozygosity. When data were partitioned by sampling period, *F*-statistics, analysis of molecular variance, and clustering programs indicate divergence in allele frequencies over the 10-year interval. Decreases in genetic variation could potentially affect the long-term survival of Ozark big-eared bats so I strongly recommend continued monitoring of populations for changes in genetic diversity and effective population size.

Introduction

Since the late 1980s, conservation geneticists have emphasized the importance of delineating Conservation Units (CUs) for several threatened and endangered species (Ryder 1986; Waples 1991; Moritz 1994). CUs are population units identified within species with the most common being Evolutionary Significant Units (ESUs) and Management Units (MUs). Identification of CUs is an important first step in conservation and management so that mangers and policy makers know the boundaries of the population units they are trying to conserve (Funk et al. 2012). Since 1986, at least nine definitions have been applied to ESUs (Ryder 1986; Waples 1991; Dizon et al. 1992; Avise 1994; Moritz 1994; Volger and DeSalle 1994; U.S. Fish and Wildlife Service and National Fisheries Service 1996; Crandall et al. 2000; Fraser and Bernatchez 2001), but generally speaking ESUs are a population or group of populations that have an evolutionary trajectory independent of similar units within the species and therefore warrant separate management or priority for conservation (Funk et al. 2012). In fact,

ESUs are granted legal protection in the U.S., Canada, and Australia (Funk et al. 2012). An ESU can include multiple MUs, defined as demographically independent populations whose population dynamics (growth rate) depend on local birth and death rates rather than immigration (Palsbøll et al. 2007; Funk et al. 2012). Identification of MUs is essential for short-term management and conservation, and these units are typically used for monitoring and regulating effects of anthropogenic factors on the abundance of the population (Palsbøll et al. 2007).

The Ozark big-eared bat (*Corynorhinus townsendii ingens*) is a federally listed endangered subspecies that now occurs only in eastern Oklahoma and Arkansas (Fig. 3.1). Although never believed to be very numerous, populations of Ozark big-eared bats declined in the 1970s due to human disturbances, and the taxon has been extirpated from Missouri (Kunz and Martin 1982; U.S. Fish and Wildlife Service 1984, 1995; Fig. 3.1). The subspecies is geographically isolated from other subspecies of Townsend's big-eared bats (*C. t. australis*, *C. t. pallescens*, *C. t. townsendii*, and *C. t. virginianus*), and has been isolated from its sister taxon, Virginia big-eared bats (*C. t. virginianus*), for approximately 1.03 million years (Lack and Van Den Bussche 2009). The highly localized distribution of Ozark big-eared bats, is thought to reflect the limited number of limestone caves and talus slopes used as roost sites (Harvey and Barkley 1990; Clark et al. 1996a; Clark et al. 1996b; Wethington et al. 1996). Although recovery plans (U. S. Fish and Wildlife Service 1984, 1995) and a five-year review (Stark 2008) are available for Ozark big-eared bats, little is known regarding CUs for this species. The one genetic study focused on the fine-scale genetic structure among five caves in a single county in eastern Oklahoma (Weyandt et al. 2005).

Female Ozark big-eared bats typically travel only 2–8 km from roosting sites (Clark et al. 1993; Wethington et al. 1996), and exhibit philopatry to specific maternity caves (U.S. Fish and Wildlife Service 1984; Clark 1991; U.S. Fish and Wildlife Service 1995; Clark et al. 1996a; Weyandt et al. 2005) making protection of caves in this region crucial to their survival. As male Ozark big-eared bats remain solitary during the spring and summer, little is known about their movements among caves. Current management practices include annual emergence counts and gating essential caves to prevent human disturbance. Since implementation of these management practices, population sizes have remained relatively stable with an estimated 1,700 individuals in the 1980s and 1,600–2,300 during the 1990s (U.S. Fish and Wildlife Service 1995). Recent cave counts estimate there are 1,600–1,800 individuals (Graening et al. 2011) distributed across 19 essential maternity caves and hibernacula in Oklahoma (12 caves) and Arkansas (7 caves; Stark 2008; Graening et al. 2011).

For species with small fragmented populations, like Ozark big-eared bats, a fundamental concern is reduced genetic variation as these species may not be able to withstand diverse and multilocus selection pressures from future environmental challenges. Small populations are susceptible to inbreeding and may become vulnerable to effects of genetic drift and fixation of alleles, particularly when there are reductions in gene flow among subpopulations (Frankham 1995; Allendorf and Luikart 2007). Effective management strategies should first determine the partitioning of genetic variation within and among populations and aim to preserve or restore the natural patterns of gene flow (Crandall et al. 2000). Using a buffer of 7.3 km, representing average nightly movements of females (Clark et al. 1993), the essential caves for Ozark

big-eared bats form four geographic clusters (Fig. 3.1). It is possible, based on known nightly movements of female Ozark big-eared bats that these four geographic clusters represent ESUs or MUs. Therefore, the first objective of this study was to evaluate levels of genetic connectedness among populations and identify conservation units.

The second objective of this study was to compare current population genetic characteristics of Ozark big-eared bats with those of the same Oklahoma populations examined 10 years earlier (Weyandt et al. 2005). Genetic monitoring of endangered species is important to the success of their management because small populations are threatened with relatively rapid changes in effective population size, levels of genetic variation within populations, and differentiation among populations (Frankham 1995; Allendorf and Luikart 2007; Schwartz et al. 2007). Such changes can be a concern if they lead to inbreeding and associated negative effects on fitness. A temporal genetic analysis of museum specimens from 1912 to 1981 revealed that grizzly bears (*Ursus arctos*) in Yellowstone National Park had a low effective population size and experienced a gradual decline in diversity, but rates of inbreeding appeared less severe than hypothesized (Miller and Waits 2003). Long-term monitoring can also detect periods of genetic stability or periods with a loss of diversity. For example, genetic analysis of temporal samples of Atlantic salmon (*Salmo salar*) from 1963 to 2001 indicated a reduction in effective population size for the 1995 and 2001 samples but little change in genetic diversity from 1963 to 1981 (Lage and Kornfield 2006). Genetic monitoring allows evaluation of the efficacy of management programs and permits early recognition of the need for a change in management action (Schwartz et al. 2007). Results from this

analysis of temporal change in genetic structure of Ozark big-eared bats might aid wildlife officials as they make future decisions regarding conservation of this taxon.

Methods

Current Population Characteristics

Sampling—Ozark big-eared bats were captured with mist nets and released immediately after a 3-mm biopsy punch (Worthington Wilmer and Barratt 1996) was taken from the plagiopatagium of each wing. All protocols for capturing and obtaining wing punches were conducted using procedures approved by the Oklahoma State University Animal Care and Use Committee (ACUC AS-11-1) and under U. S. Fish and Wildlife Permit TE35152A-0 to R. A. Van Den Bussche. Wing punches were collected from 110 Ozark big-eared bats (100 in Oklahoma and 10 in Arkansas) during 2011–2013. Samples from 20 Ozark big-eared bats were collected at each of the following maternity colonies: AD-10, AD-125, AD-18, and AD-13 (Adair Co., Oklahoma; Fig. 3.2). Nineteen individuals were sampled from SQ-1 (Sequoya Co., Oklahoma) and one from CZ-18, (Cherokee Co., Oklahoma; Fig 3.2). Samples were collected from 10 individuals in FR-17BT1 (Franklin Co., Arkansas; Fig. 3.2). All wing punches were stored in lysis buffer until DNA could be isolated in the laboratory following standard protocols (Longmire et al. 1997).

Mitochondrial sequencing—To characterize levels of genetic variation within colonies and connectivity among colonies of Ozark big-eared bats due to female dispersal, approximately 480 base pairs (bp) of the mitochondrial D-loop region were amplified via polymerase chain reaction (PCR) and sequenced using primers from Wilkinson and

Chapman (1991). PCR amplifications were carried out in 30 µL reactions containing 4–8 ng of DNA, 2 mM $MgCl_2$, 0.14 mM of each deoxynucleoside triphosphate, 0.15 µM of each primer, 0.8 mg/mL bovine serum albumin, 2X buffer, 1 unit Go*Taq* polymerase (Promega), and ddH_2O to volume. The thermal profile comprised an initial denaturation step of 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The Wizard SV Gel PCR Prep DNA Purification System (Promega) was used to purify PCR products that were subsequently sequenced using Big Dye chain terminators on an ABI 3130 Genetic Analyzer (Applied Biosystems Inc.). Fragments were aligned with haplotypes presented in Weyandt et al. (2005) and examined for variable nucleotide positions using Geneious v. 5.5.6 (Biomatters Ltd.). Number of mitochondrial haplotypes, haplotype diversity (h), nucleotide diversity (π), and *F*-statistics were calculated in Arlequin v. 3.5.1.3 (Excoffier and Lischer 2010).

Microsatellite genotyping—Amplification of 15 polymorphic tetranucleotide microsatellite loci (B106, D7, D5, D107, D109, D6, B107, D110, D108, B7, A110, B6, D1, B105, D123; described in Lee et al. 2012) was attempted in all individuals to evaluate levels of genetic variation within colonies and connectivity among colonies due to male and female dispersal. Multiplex PCR reactions included 14–28 ng of DNA, 20.0 µl True Allele PCR mix (Applied Biosystems, Inc.), 0.15 µM of each primer and ddH_2O to a final volume of 30 µl. The following thermal profile was used: 95°C for 12 min, 35 cycles of 94°C for 40 sec, 57°C for 40 sec, 72°C for 30 sec, and 72°C for 4 min. An ABI 3130 Genetic Analyzer was used to visualize PCR products by adding 9.0 µl of formamide (Applied Biosystems, Inc.) and 0.5 µl of ROX size standard (Applied

Biosystems, Inc.) to 1.0 µl PCR product. GeneMapper 4.0 (Applied Biosystems, Inc.) was used to genotype individuals.

Microchecker (Van Oosterhout 2004) was used to evaluate microsatellite loci for the presence of null alleles, heterozygote deficiency, and scoring errors. Tests of Hardy-Weinberg Equilibrium and linkage disequilibrium were performed in GENEPOP v. 4.0 (http://genepop.curtain.edu.au/; Raymond and Rousset 1995; Rousset 2008). Statistical significance was evaluated with 100,000 Markov Chain Monte Carlo (MCMC) simulations and sequential Bonferroni correction to account for inflated type one error rates (Rice 1989). GenAlEx v. 6 (Peakall and Smouse 2006) was used to calculate observed/expected heterozygosity (H_o/H_e) for each cave, analysis of molecular variance (AMOVA), *F*-statistics, and Principal Coordinate Analysis (PCA). HP-RARE (Kalinowski 2005) was used to calculate allelic richness (*A*) corrected for differences in sample size among caves. STRUCTURE v. 2.3.2 (Pritchard et al. 2000; Falush 2003; Falush 2007) was used for a Bayesian analysis of ancestry, allowing for admixture) to assess the most likely population assignment for each individual and to determine the number of populations (*K*) represented by the dataset. For the latter, each of seven potential *K* values (1–7) was run 10 times with a burn-in period and number of iterations set at 30,000 and 100,000, respectively. The average Ln probability of the data for each value of K was calculated and used to determine the number of different genetic groups. The correction method from Evanno et al. (2005) was not used because it cannot determine if $K = 1$ is a better fit to the data than $K = 2$. An additional population assignment test was performed in Arlequin v. 3.5.1.3 (Excoffier and Lischer 2010) with 100,000 MCMC. Data from each cave except CZ-18 (due to small sample size) were

used in BOTTLENECK v. 1.2.02 (Cornuet and Luikart 1996) to test for a recent reduction in effective populations size (N_e), which is evident by an excess of heterozygosity relative to allele frequency. Both the Sign test and one-tailed Wilcoxon test were used with 1,000 replications and the following mutation models: stepwise (SMM), infinite allele (IAM), and two-phase model (TPM; 70% stepwise component). Finally, N_e for each cave was estimated using the linkage disequilibrium method in LD-N_e (Waples and Do 2008), excluding alleles with frequencies less than 0.03 because there were fewer than 25 samples per population (Waples and Do 2010). These values were then compared with census estimates (N_C) of each cave in Oklahoma made by U.S. Fish and Wildlife personnel based on visual counts or video recordings using infrared lighting.

10-year Comparison

To directly compare genetic characteristics of the populations from 2002–2003 to 2011–2013, haplotypes/genotypes needed to be generated using the same loci. Haplotypes from the same mitochondrial marker (D-loop) used in 2002–2003 were generated for 2011–2013 samples for the first objective; however, different microsatellite markers were used for the 2011–2013 samples. Therefore, the 2011–2013 individuals were also genotyped for the loci used in Weyandt et al. (2005). These dinucleotide loci, (EF1, EF21, EF15, EF6, EF13; described by Vonhof et al. 2002) were amplified in all individuals, but PCR reactions were not multiplexed due to different annealing temperatures. PCR reactions included 4–8 ng of DNA, 9.0 µl True Allele PCR mix (Applied Biosystems, Inc.), 0.5 µl of each primer and ddH_2O to a final volume of 15 µl. The same thermal profile described earlier for microsatellite loci was used except the

annealing temperature ranged from 40–55°C. PCR products were visualized and genotyped as described above.

Measures of haplotype diversity (h), nucleotide diversity (π), and number of haplotypes with bats from 2011–2013 were compared using the mitochondrial data. HP-RARE (Kalinowski 2005) was used to calculate microsatellite allelic richness for caves sampled in 2002–2003 and 2011–2013 to look for changes in genetic diversity. I used GenAlEx v. 6 (Peakall and Smouse 2006) for an AMOVA (data partitioned by sampling interval, 2002–2003 and 2011–2013) to compute F-statistics and a PCA. I used STRUCTURE v. 2.3.2 (Pritchard et al. 2000; Falush 2003; Falush 2007) with prior knowledge of the group assignment, therefore the program was only run with $K = 2$ using a burn-in period of 30,000 and 100,000 iterations to evaluate whether the individuals were genetically grouped according to collecting period. An additional population assignment test and exact test of population differentiation were performed in Arlequin v. 3.5.1.3 (Excoffier and Lischer 2010) with 100,000 MCMC replicates.

Results

Current Population Characteristics

Mitochondrial sequencing—Analysis of 426 bp of mitochondrial D-loop region from 105 Ozark big-eared bats revealed four haplotypes, three (A, B, C) previously described in Weyandt et al. (2005) and a new haplotype (E) found in AD-18 (Fig. 3.3). Haplotype C and E differed from the most common haplotype (A) by one substitution; however, haplotype B differed from A at 11 nucleotide positions. Haplotype A was found in all caves, and haplotype E was restricted to AD-18 (Fig. 3.3). Haplotype diversity (h) was

low for all caves except AD-18 which had four unique haplotypes (Table 3.1). Nucleotide diversity (π) was low for all caves (Table 3.1), and there were no significant pairwise F_{ST} comparisons among caves (Table 3.2).

Microsatellite genotyping—Final samples sizes using 110 Ozark big-eared bats for each microsatellite locus were as follows: B106 *N* = 106, B105 *N* = 105, B107 *N* = 107, A110 *N* = 86, D5 *N* = 94, D109 *N* = 103, D1 *N* = 107, D110 *N* = 106, D123 *N* = 96, B7 *N* = 107, D7 *N* = 98, D108 *N* = 94, D6 *N* = 102, D107 *N* = 95, B6 *N* = 102. After sequential bonferonni, no loci deviated from HWE, had evidence of null alleles or were in linkage disequilibrium in more than one population. Number of alleles detected at each locus averaged 5.0 and ranged from three (B107, D109, D110) to eight (D1). Allelic richness (*A*) ranged from 2.65–3.10 (Table 3.1). Six of the seven colonies had private alleles with a striking two private alleles for CZ-18, which was only represented by one bat (Table 3.1). AMOVA revealed 86% of the genetic variation was attributable to differences within individuals, 3% among populations, and 11% among individuals. Twelve of 15 pairwise comparisons had significant F_{ST} values, including all comparisons made with Arkansas and all made with SQ-1 samples (Table 3.3). Despite F_{ST} evidence of genetic divergence, there was no notable geographic clustering of samples in the PCA, and the STRUCTURE analysis failed to group the sampled populations in a significant way (results not shown). Ninety-two of 107 individuals (86%) that were genotyped were correctly assigned to the cave of their collection. The individuals not correctly assigned were collected from AD-125, AD-18, AD-13, and SQ-1 and were incorrectly assigned to one or another of these populations or to AD-10. Effective population sizes for each cave

were variable and ranged from 23–101. The N_e to N_C ratios were also variable with a N_e greater than N_C for AD-13 (Table 3.4). With the sign test, all caves that could be evaluated (sample size greater than one) showed evidence of a recent bottleneck under the IAM (P = 0.011 0.048), whereas under the other two mutation models (TPM and SMM) there was only one indication of a bottleneck (TPM; population AD-125; P = 0.003). With the Wilcoxin test statistic, none of the tests were statistically significant; this test is more powerful than the sign test when using less than 20 loci (Piry et al. 1999).

10-year Comparison

It was possible to compare the genetic characteristics of three caves sampled 10 years apart (Table 3.5). Caves AD-10 and AD-125 were sampled in 2002–2003 and 2011–2013. The third comparison involved two separate caves, AD-17 and AD-18, which were sampled in 2002–2003 and 2011–2013, respectively. These caves are located adjacent to each other and are separated by only 100–200 yards. U.S. Fish and Wildlife personnel have observed the same colony of bats switching between these two caves in a single maternity season (Stark 2008).

Four haplotypes were found in 2002–2003 (A, B, C, D) and four haplotypes in 2011–2013 (A, B, C, E; Fig. 3.3). Haplotype A was the most frequently detected haplotype in all caves for each collecting period, but there were observable differences in haplotype frequencies between collecting intervals (Fig. 3.3). Haplotype B was detected in two caves in 2002–2003 but three caves in 2011–2013 (Fig. 3.3). Haplotype C was collected in only one cave in 2002–2003 but in three caves in 2011–2013 (Fig. 3.3). Haplotype D was collected in one cave in 2002–2003 and not detected in 2011–2013,

while haplotype E was only found in AD-18 in 2011–2013 (Fig. 3.3). Haplotype diversity (h) and nucleotide diversity (π) increased over the 10-year interval for some caves but decreased for others (Table 3.5).

Attempts were made to amplify the same microsatellite loci as in Weyandt et al. (2005) on samples from all 110 bats captured from 2011–2013; however, 27 bats could not be amplified for three of the five loci so those individuals were removed from all analyses. Comparisons between three caves sampled in 2002–2003 and again in 2011–2013 showed a decrease in allelic richness for all caves (Table 3.5).

Finally, data were partitioned into collecting periods 2002–2003 and 2011–2013 and treated as two populations. Although results of an exact test of population differentiation were not significant, AMOVA identified 51% of the genetic variation was within individuals, 35% among populations, and 14% among individuals with an F_{ST} of 0.346 (P = 0.001). Individuals from each collecting period grouped together in the PCA (Fig. 3.4), formed two distinct groups in the STRUCTURE analysis (not shown), and were assigned correctly in Arlequin.

Discussion

Current Population Characteristics

Species characterized by fragmented populations with reduced gene flow are susceptible to losses of genetic diversity due to inbreeding and genetic drift (Frankham 1995; Allendorf and Luikart 2007). Despite Ozark big-eared bats having a highly restricted and fragmented distribution, the seven colonies of Ozark big-eared bats examined in this study appear to be maintaining adequate levels of genetic variation. For

example, 1–4 mitochondrial haplotypes were detected in Oklahoma colonies with more than one Ozark big-eared bat sampled (Table 3.1 and Fig. 3.3), and observed heterozygosity was equal to or greater than mean expected heterozygosity in five of the seven colonies examined. Moreover, six of the seven colonies had private alleles and five of the seven colonies had a negative inbreeding coefficient (Table 3.1).

To protect these levels of variation and adaptive differences of population units, identifying CUs is an important first conservation step. Current distribution and known maximum nightly movement of Ozark big-eared bats (Clark et al. 1993) indicate four clusters that might be either ESUs or MUs. Within CUs, ESU are generally the largest unit, and a commonly used working definition for the establishment of ESUs are populations that are reciprocally monophyletic for mitochondrial DNA and show statistically significant divergence of allele frequencies at nuclear loci (Moritz 1994). Thus, ESUs represent discrete segments of the population that have their own evolutionary trajectory. Unfortunately, it is not possible to determine ESUs based on this study. The initial design of the study was to sample Ozark big-eared bats from several colonies in Arkansas, including those in Marion Co., but with the movement of WNS south and west, land owners determined the benefits of sampling Ozark big-eared bats in these areas for this study did not outweigh the potential risk associated with WNS. I was able to include samples from Franklin Co., Arkansas, which represent a cluster of Ozark big-eared bats demographically isolated from the Oklahoma populations. All individuals possessed mitochondrial haplotype A, which is the most common haplotype throughout Oklahoma. Thus, the first criterion for designation of an ESU, reciprocal monophyly at mitochondrial loci, could not be established.

MUs are typically smaller units within an ESU and represent populations that are demographically isolated. MUs are important for short-term management goals such as monitoring habitat and population status, and protection of MUs is important for long-term persistence of a taxon (Funk et al. 2012). Weyandt et al. (2005) examined fine-scale spatial structure among five colonies of Ozark big-eared bats from a single county in eastern Oklahoma and detected significant genetic differentiation at the mitochondrial loci but no significant genetic differentiation at the five microsatellite loci examined. Weyandt et al. (2005) recommended protecting each of the caves and suggested extirpation of any cave could result in loss of the colony because the females would likely not recolonize. Their results contrast those of the current study in which no significant genetic differentiation at the mitochondrial locus was detected. Differences between this study and the study of Weyandt et al. (2005) are likely related to increased sample sizes of bats in the current study.

Based on the 15 nuclear microsatellite loci, neither STRUCTURE nor the PCA genetic structure among the populations examined, although the AMOVA indicated low but significant level of genetic differentiation ($F_{ST} = 0.03$). In pairwise population comparisons, statistically significant differentiation was detected in all comparisons involving the colony of Ozark big-eared bats from Franklin Co., Arkansas, between colony AD-10 and both AD-125 and AD-13, as well as between AD-13 and AD-125 and all comparisons with SQ-1 (Table 3.3). SQ-1 was not routinely monitored for Ozark big-eared bats until U.S. Fish and Wildlife personnel became aware of its use in 2010 (R. Stark, pers. comm.), and results from this study suggest a need for continued monitoring and designation of SQ-1 as an essential maternity site. Results of the assignment test also

support the presence of genetic substructure with some movement of individuals among colonies in Oklahoma. More specifically, 92 of the 107 individuals genotyped (86%) were correctly assigned to the cave they were collected from, and importantly, all individuals (N = 10) collected from Franklin Co., Arkansas, were correctly assigned to that colony. The 15 incorrectly assigned bats were collected from caves in eastern Oklahoma (AD-13, AD-18, AD-125, and SQ-1) and were assigned to another cave in this area. Interestingly, the maternity colony at AD-13 is the most northern colony sampled whereas the colonies at AD-10 and AD-125 not only serve as maternity colonies, but are also two of the known winter hibernacula (Stark 2008). Cave AD-10 and AD-125 may serve as sites for additional mating opportunities into winter. Taken together, these results suggest the presence of at least two MUs. One MU comprises the colonies in eastern Oklahoma, representing the most western populations of Ozark big-eared bats. The second MU would include the colonies in western Arkansas that are represented in this study by bats from Franklin Co. (Figs. 3.1 and 3.2).

The two populations of Ozark big-eared bats in Marion Co., Arkansas likely represent two additional MUs and may possibly even represent a distinct ESU. The caves in Marion Co. are approximately 136 km from the other colonies in western Arkansas and 160 km from the colonies in Oklahoma. Such distances likely are barriers to gene flow, based on known movements of Ozark big-eared bats (Clark et al. 1993) and the low levels of gene flow indicated in this study between caves in eastern Oklahoma and those in western Arkansas. The bats in Marion Co. probably are genetically isolated from all other known populations of Ozark big-eared bats, but this needs further study.

Managing Ozark big-eared bat populations as at least three MU's (Oklahoma, western Arkansas, and Marion Co., Arkansas) will allow populations to retain locally adapted alleles. Protection of swarming sites and habitat surrounding caves will facilitate gene flow and allow for the introduction of new alleles and maintenance of heterozygosity without subpopulations drifting to allele fixation. Additionally, it is crucial that management plans include protection of individual caves because Ozark big-eared bats are sensitive to human disturbance (Graening et al. 2011). Gates have been placed at the entrance of some caves in eastern Oklahoma to restrict recreational access (Graening et al. 2011) and have not been shown to affect Ozark big-eared bats nor populations of endangered gray bats (*Myotis grisescens*; Martin et al. 2000). I recommend additional gating of caves with priority on locations with high genetic diversity or rare haplotypes or alleles. A combination of these practices promotes opportunities for gene flow, which could slow the rate of loss of genetic variation.

10-year Comparison

Temporal genetic comparisons of other species have been useful for documenting changes in a gene pool over time (Miller and Waits 2003; Lage and Kornfield 2006). In this study it was possible to assess genetic change in Oklahoma Ozark big-eared bat populations sampled in 2002–2003 and 2011–2013 using the same mitochondrial and nuclear markers. The mitochondrial results indicate no significant change over this time period. Haplotype D was detected in a single individual by Weyandt et al. (2005) at AD-14 but was not detected in the current study. Conversely, the current study detected a new haplotype, E, in three individuals from AD-18. Other than these differences, which

likely reflect sampling error, there was no evidence of notable mitochondrial DNA change between the two sampling periods (Fig. 3.3).

With regard to the nuclear microsatellite loci, it appears that these small and somewhat isolated colonies have been subjected to erosion of genetic diversity through a loss of alleles, via genetic drift. The loss of diversity was pronounced such that the combined populations in 2002–2003 were well differentiated from those collected in 2011–2013 (F_{ST} = 0.346; P = 0.001). Individuals from the two collecting periods were assigned to separate genetic clusters in STRUCTURE analysis, and they formed distinct groups in the plot of PCA1 and 2 (Fig. 3.4).

Despite these results, colonies appeared to have stable effective populations sizes (Table 3.4) and no evidence of inbreeding (Table 3.1) or a population bottleneck. Effective population size estimates were low compared to the 50/500 rule which states N_e values should be no less than 50 to balance the short-term effects of inbreeding depression and no less than 500 to maintain evolutionary potential (Franklin 1980; Franklin and Frankham 1998). Although N_e values were greater than 50 in only two colonies (AD-125 and AD-13; Table 3.4), there are only 1,600–1,800 individuals spread throughout 19 maternity or winter hibernating sites. Encouraging results were found in the N_e: N_C ratios with values typical of other wild populations (AD-10 and AD-18) or higher (AD-125, AD-13, and SQ-1; Table 3.4). Frankham (1995) compared estimates of effective population size in over 100 animal and plant species and found N_e averages 10% of N_C. Waples (2002) used a temporal method for N_e estimates and suggested an average of 20% was more accurate. More recently, Palstra and Ruzzante (2008) reviewed 83 studies and reported a median N_e:N_C of 0.14. Interestingly, N_e of AD-13 was even greater

than census counts. This can occur in situations where all individuals contribute equal numbers of progeny because variation in reproductive success decreases N_e (Allendorf and Luikart 2007).

Continued temporal genetic monitoring along with annual emergence counts of Ozark big-eared bats is crucial because of the small size and fragmented nature of the populations. Annual emergence counts allow an estimation of population size; however, these counts provide no information regarding genetic characteristics of the gene pool. The small and somewhat isolated nature of colonies subject Ozark big-eared bats to decreases in genetic variation. If gene flow were limited for some reason, inbreeding could become a management concern and potentially affect the fitness in Ozark big-eared bats. For example, the degree of outbreeding has been associated with survival in wild populations of greater horseshoe bats (*Rhinolophus ferrumequinum*; Rossiter et al. 2001). Even though census estimates of Ozark big-eared bats have been relatively stable (Graening et al. 2011), continued genetic monitoring with the same molecular markers, specifically the microsatellite markers developed for Ozark big-eared bats (Lee et al. 2012), should be used to monitor changes in H_o, allelic richness, and N_e as well as levels of gene flow among populations. Effective population size of Ozark big-eared bats is unlikely to ever approach 500, but providing continuous suitable habitat connecting populations can facilitate gene flow and maintain genetic variability. The development of a genetic monitoring program will facilitate adaptive management of Ozark big-eared bats by altering management strategies in a way that reflects the changes in the genetics of the populations. Moreover, additional colonies of Ozark big-eared bats throughout Arkansas, especially those in Marion Co., should be included in future studies.

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Figure Captions

Figure 3.1 Distribution of Ozark big-eared bats (*Corynorhinus townsendii ingens*), taken from (Graening et al. 2011). Black circles indicate currently used caves (essential and limited use), gray circles indicate historically used caves, and white circles indicate caves with potentially suitable habitat. Radial buffers of 7.3 km represent the maximum nightly foraging distance.

Figure 3.2 Map of eastern Oklahoma and western Arkansas with caves sampled in this study indicated by triangles.

Figure 3.3 Frequencies of mitochondrial D-loop region haplotypes found in colonies of Ozark big-eared bats (*Corynorhinus townsendii ingens*). A) Colonies sampled in 2011–2013. B) Colonies sampled in 2002–2003 by Weyandt et al. (2005). Caves AD-17 and AD-18 can be directly compared because they are adjoining and used by the same population of Ozark big-eared bats.

Figure 3.4 Principal coordinate analysis of Ozark big-eared bat (*Corynorhinus townsendii ingens*) based on five microsatellite loci used in Weyandt et al. (2005). Data partitioned by collecting period (2002–2003 and 2011–2013).

Table 3.1 Population genetic characteristics from colonies of Ozark big-eared bats (*Corynorhinus townsendii ingens*) from seven caves: Number of individuals sampled (*N*), number of mitochondrial haplotypes (*a*), haplotype diversity (*h*), nucleotide diversity (π), allelic richness corrected for sample size differences and averaged over all loci (*A*), number of private microsatellite alleles (*P*), mean observed heterozygosity (H_o), mean expected heterozygosity (H_e), and fixation index (*F*) for each cave sampled.

Pop	N^{mtDNA}	N^{msats}	*a*	*h*	π	*A*	*P*	H_o	H_e	*F*
AD-10	20	20	2	0.100	0.003	3.10	2	0.646	0.613	-0.058
AD-125	20	20	3	0.195	0.003	3.04	3	0.667	0.617	-0.093
AD-18	20	18	4	0.500	0.004	2.98	1	0.577	0.602	0.034
AD-13	20	20	2	0.268	0.001	2.68	3	0.564	0.533	-0.060
SQ-1	16	18	1	0.000	0.000	2.65	1	0.521	0.589	0.124
CZ-18	1	1	1	0.000	0.000	–	2	0.733	0.367	-1.000
FR-17BT1	8	10	1	0.000	0.000	2.65	0	0.542	0.507	-0.075

Table 3.2 Pairwise population F_{ST} values between colonies of Ozark big-eared bats (*Corynorhinus townsendii ingens*) based on haplotypes (below diagonal). Probability values based on 10,000 permutations (above diagonal). CZ-18 was removed from analysis because of small sample size.

	AD-10	AD-125	AD-18	AD-13	SQ-1	FR-17BT1
AD-10	–	0.991	0.505	0.631	0.991	0.991
AD-125	0	–	0.685	0.991	0.514	0.784
AD-18	0	0	–	0.369	0.081	0.514
AD-13	0.001	0	0.002	–	0.270	0.559
SQ-1	0	0.006	0.035	0.101	–	0.991
FR-17BT1	0	0	0	0.026	0	–

Table 3.3 Pairwise population F_{ST} values between colonies of Ozark big-eared bats (*Corynorhinus townsendii ingens*) based on microsatellites (below diagonal). Probability values are above diagonal with bolding indicating significance. CZ-18 was removed from analysis because of small sample size.

	AD-10	AD-125	AD-18	AD-13	SQ-1	FR-17BT1
AD-10	–	**0.011**	0.098	**0.002**	**0.001**	**0.001**
AD-125	0.021	–	0.273	**0.048**	**0.005**	**0.001**
AD-18	0.011	0.003	–	0.161	**0.003**	**0.001**
AD-13	0.037	0.015	0.007	–	**0.002**	**0.001**
SQ-1	0.033	0.029	0.026	0.037	–	**0.001**
FR-17BT1	0.059	0.074	0.060	0.095	0.065	–

Table 3.4 Comparison of effective population size (N_e) as calculated by LD-N_e (Waples and Do 2008) and census size (N_C) estimates based on visual counts using infrared night vision (R. Stark, pers. comm.) of Ozark big-eared bats (*Corynorhinus townsendii ingens*) maternity colonies. 95% confidence intervals for N_e estimates are shown in brackets. CZ-18 was removed from analysis because of small sample size.

Cave	N_e	N_C	N_e/ N_C
AD-10	42 [20–253]	234	0.177
AD-125	65 [25–∞]	114	0.567
AD-18	29 [15–86]	245	0.116
AD-13	101 [24–∞]	57	1.765
SQ-1	27 [11–10,188]	61	0.443
FR-17BT1	23 [6–∞]	–	–

Table 3.5 Population genetic characteristics from colonies of Ozark big-eared bats (*Corynorhinus townsendii ingens*) from caves sampled in 2002–2003 and 2011–2013: Number of individuals sampled (*N*), number of mitochondrial haplotypes (*a*), haplotype diversity (*h*), nucleotide diversity (π), allelic richness corrected for sample size differences and averaged over all loci (*A*), and mean observed heterozygosity (H_o). Results for 2002–2003 taken from Weyandt et al. (2005) except for allelic richness which was calculated in this study.

Pop	Date	*N*	*a*	*h*	π	*A*	H_o
AD-10	2011–2013	20	2	0.100	0.003	2.62	0.562
AD-10	2002–2003	21	1	0.000	0.000	4.67	0.524
AD-125	2011–2013	20	3	0.195	0.003	2.79	0.507
AD-125	2002–2003	20	2	0.268	0.006	4.31	0.540
AD-18	2011–2013	20	4	0.500	0.004	2.67	0.437
AD-17	2002–2003	10	2	0.356	0.001	4.6	0.480

Figure 3.1

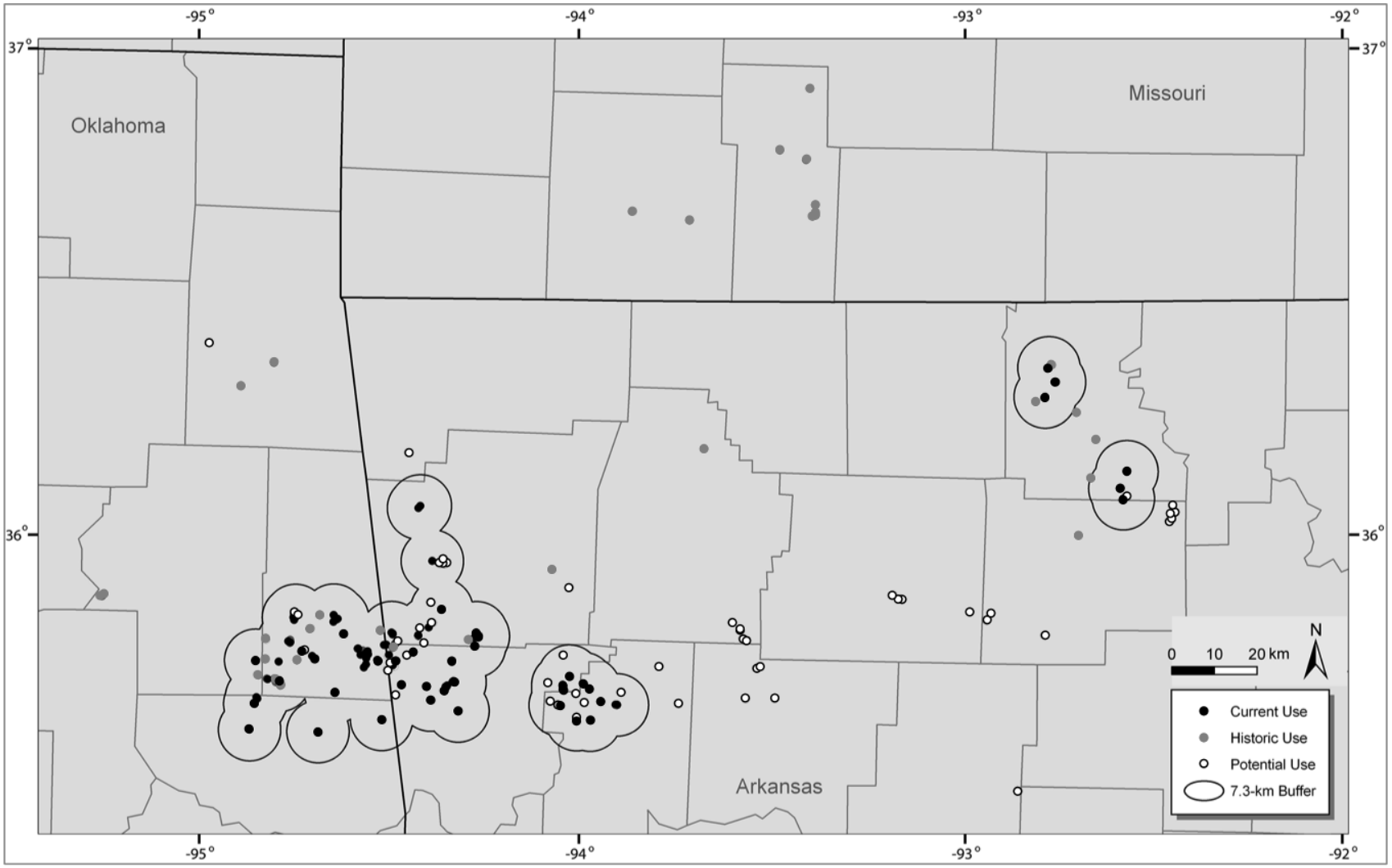

-95°
-94°
-93°
-92°
37°
36°
Oklahoma
Missouri
Arkansas
0 10 20 km
N
Current Use
Historic Use
Potential Use
7.3-km Buffer

Figure 3.2

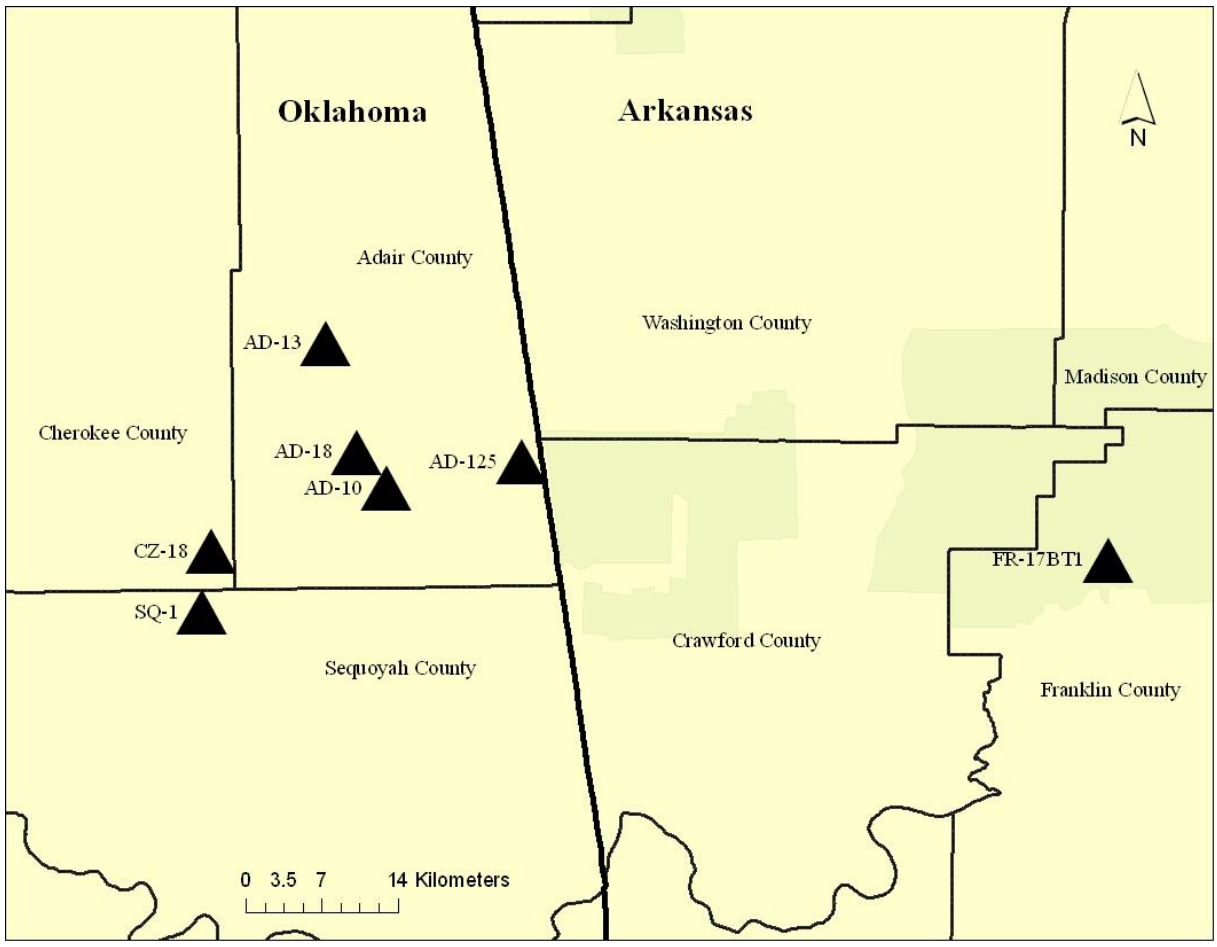

Oklahoma
Arkansas
N
Adair County
Washington County
AD-13
Madison County
Cherokee County
AD-18
AD-125
AD-10
CZ-18
FR-17BT1
SQ-1
Crawford County
Sequoyah County
Franklin County
0 3.5 7 14 Kilometers

Figure 3.3

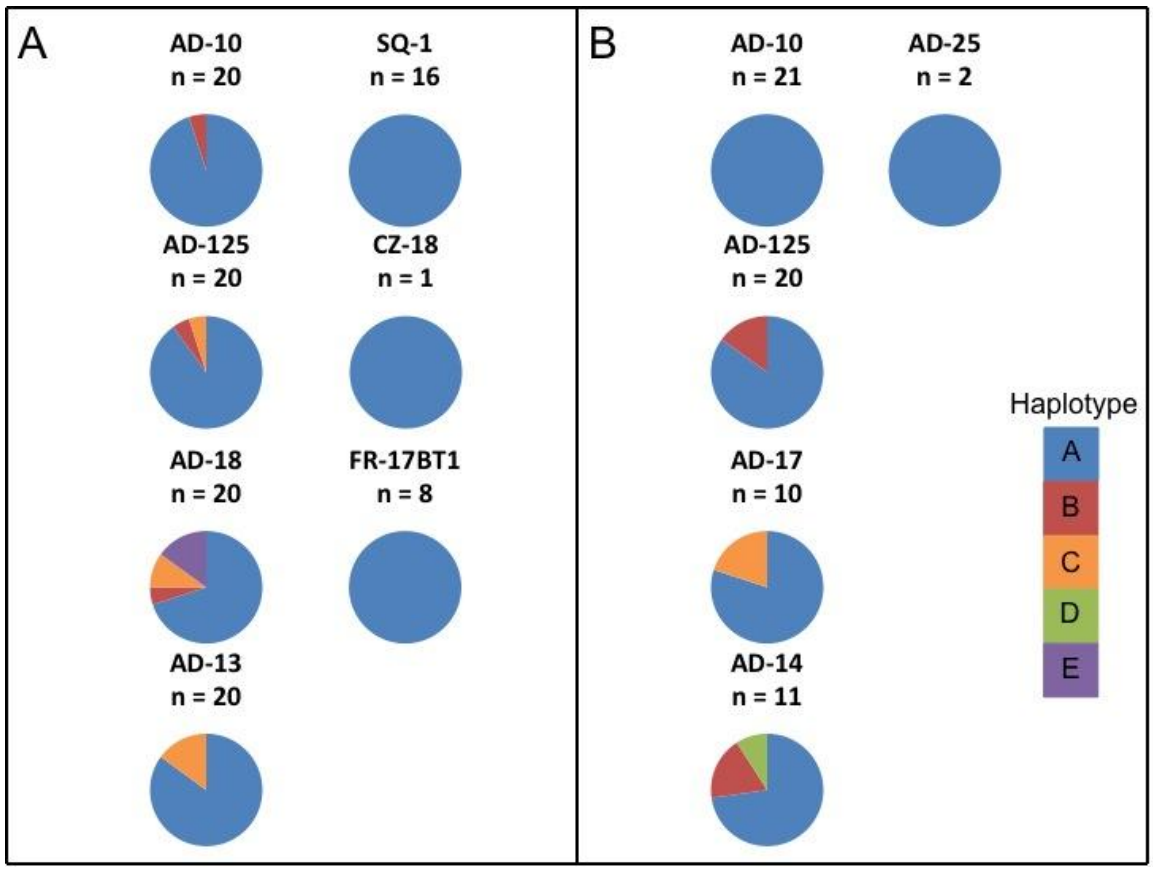
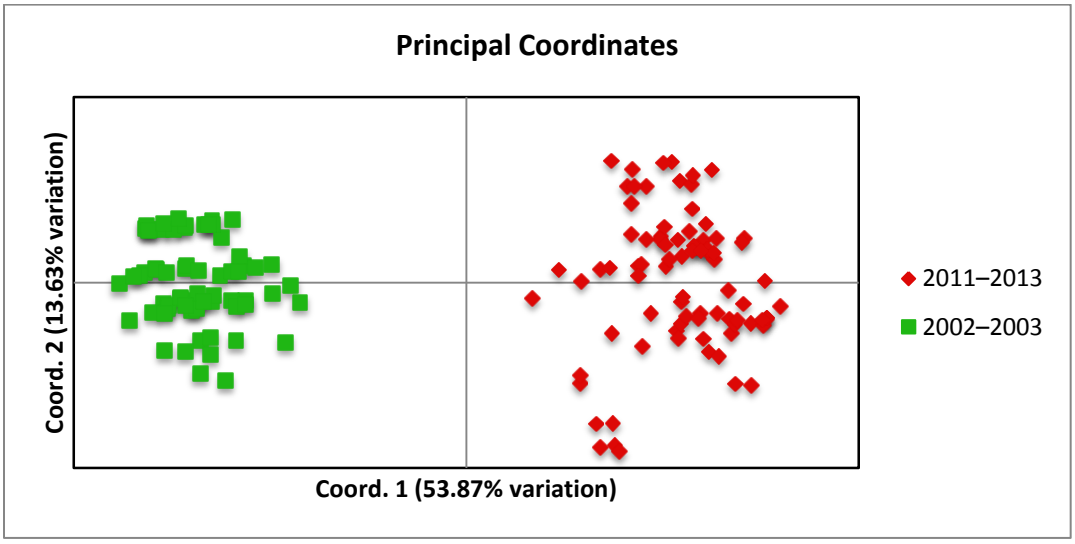

A
AD-10
n = 20
SQ-1
n = 16
AD-125
n = 20
CZ-18
n = 1
AD-18
n = 20
FR-17BT1
n = 8
AD-13
n = 20
B
AD-10
n = 21
AD-25
n = 2
AD-125
n = 20
AD-17
n = 10
AD-14
n = 11
Haplotype
A
B
C
D
E

Figure 3.4


Principal Coordinates
Coord. 2 (13.63% variation)
Coord. 1 (53.87% variation)
2011–2013
2002–2003

VITA

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