METHENY, JACKIE DAWN. M.S. A Genetic Analysis of the Fission-Fusion Roosting Behavior of Tree-Roosting Maternity Colonies of Big Brown Bats *(Eptesicus fuscus)*. (2006)
Directed by Dr. Matina C. Kalcounis-Rüppell and Dr. R. Mark Brigham. 93 pp.

In Cypress Hills Interprovincial Park, Saskatchewan, Canada, tree-roosting big brown bats (*Eptesicus fuscus*) exhibit fission-fusion roosting behavior and are philopatric to one of three non-overlapping roosting areas. Bats switch roost trees and potentially roost-mates about every two days, and bats appear to have preferred roost-mates. To assess whether genetic relationships mediate fission-fusion behavior in tree-roosting bats, I combined genetic analyses (microsatellite loci and mitochondrial DNA) with behavioral studies. First, I determined whether female philopatry produced genetic subdivision among the roosting areas. Second, I examined roosting associations within one roosting area to determine whether roost-mate decisions were based on genetic relationships. I found that female-mediated gene flow was restricted between roosting areas while male-mediated gene flow was not. Roosting associations were not influenced by genetic relationships. Mating and dispersal behavior of *E. fuscus* generate group members that are generally not closely related, and bats do not preferentially roost with closely related or matrilineal females. Thus, kin selection is an unlikely explanation for preferred roost-mates, group stability, and cooperation in tree-roosting *E. fuscus*.

A GENETIC ANALYSIS OF THE FISSION-FUSION ROOSTING BEHAVIOR OF TREE-ROOSTING MATERNITY COLONIES OF BIG BROWN BATS (*EPTESICUS FUSCUS*)

by

Jackie Dawn Metheny

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the requirements for the Degree
Master of Science

Greensboro
2006

Approved by

Matina Kalcounis-Rüppell_____
Committee Co-Chair
R Mark Brigham_____
Committee Co-Chair

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Co-Chair__Matina Kalcounis-Rüppell_____

Committee Co-Chair__R Mark Brigham_____

Committee Members__Malcolm Schug_____

__Olav Rueppell_____

_23 October 2006_____
Date of Acceptance by Committee

_23 October 2006_____
Date of Final Oral Examination

ACKNOWLEDGMENTS

I thank Drs Matina Kalcounis-Rüppell and Mark Brigham for being my co-advisers and arranging the field and lab components of my project. I appreciate the time, advice, encouragement, guidance, and friendship provided by Matina and Mark. Matina was instrumental in coaxing this thesis into reality. I am also grateful to Matina for the experience of working on ultrasonic vocalizations in *Peromyscus* in California.

I am indebted to Matina Kalcounis-Rüppell (and her field assistants), Craig Willis (and his field assistants), and Kristen Kolar (and her field assistants) for creating the trails through the study site and providing the ecological and behavioral foundations for this project. Kristen Kolar and Melissa Ranalli provided field training, enthusiasm, and laughter. Kristen Kolar saved me hours of frustration by designing and building a modified harp trap for use in trapping tree-roosting bats. I am thankful to the tree-climbing skills of Devin Arbuthnott and the hard work of Kristin Bondo in the field.

I am grateful to Maarten Vonhof for providing advice regarding his primers and providing access to unpublished data on allele frequencies. Cori Lausen and Isabelle Delisle kindly responded to my questions about mitochondrial DNA sequencing and provided unpublished sequences for comparison. I appreciate my interactions with Dave Remington which improved my ability to generate genotypes and sequences using the MegaBACE® in his lab and also improved my analyses of the genotypes and sequences. In addition, LaToya Wright provided training and company in the genetics lab. I am grateful to the following individuals for providing tissue samples: Juliet Craig, Craig Willis via Cori Lausen, and Matina Kalcounis-Rüppell (MCKR samples were collected

during the Southeastern Bat Diversity Network 2004 Bat Blitz). Cheryl Logan kindly lent me her office and Macintosh computer for calculating relatedness. I am thankful to Olav Rueppell for writing the Microsoft Excel macro for my randomization tests.

I appreciate the use of the Department of Biology University of Regina Field Station. I am grateful for the cooperation and hospitality of the West Block of Cypress Hills and Fort Walsh administrators and employees. In particular, I thank Kevin Redden for good company and assistance. I also acknowledge the generosity and hospitality of nearby ranchers.

I am grateful for discussions and companionship from both the Kalcounis-Rüppell Bat and Mouse lab (in particular Vicki Payne) at the University of North Carolina at Greensboro and the Brigham Bat and Bird lab from the University of Regina.

My committee members, Olav Rueppell, Malcolm Schug, Mark Brigham, and Matina Kalcounis-Rüppell, provided constructive comments and helpful suggestions that improved my project and thesis.

Funding was provided by the University of North Carolina at Greensboro Biology Department, the University of Regina, the Natural Science and Engineering Research Council of Canada, and the North Carolina Academy of Science (Bryden Award).

As always, my family provided constant emotional and financial support. I especially appreciate the frequent cards from my grandparents, George and Louise Krynicki. Finally, my day to day emotional state was improved by Christy Walter.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

GENERAL INTRODUCTION

The level of genetic subdivision among adjacent social groups is thought to be an important evolutionary force in mammalian populations (Storz, 1999). Interrelated behavioral factors that impact the genetic subdivision among and within social groups include: mating, dispersal, and formation of new social groups (Storz, 1999). These behavioral factors are often difficult to observe directly, but genetic studies can estimate the genetic subdivision among populations. Evidence for genetic subdivision among social groups is used along with behavioral observations to infer mating and dispersal behavior.

The kin composition within social groups is also linked to mating, dispersal, and the formation of new social groups. In particular, the number of breeders in a social group, the genetic relationships among these breeders, and the extent of variation in parentage among same-sex breeders are important characteristics that determine the kin composition of social groups (reviewed by Ross, 2001). Dispersal behavior of juveniles influences the genetic relationships of breeders in the social group. If individuals breed within their natal social group, the relatedness between breeders will increase and the overall relatedness of the social group will increase. The formation of new social groups

is also important. For example, new social groups formed by related individuals will increase the relatedness of breeders and the relatedness of the social group. These behavioral characteristics determine the kin composition of social groups by impacting the number of matrilines and patrilines present and the relatedness of individuals within and between matrilines and patrilines. The kin composition of the social group determines whether kin selection has the potential to be an important selection pressure for group stability and cooperation within the social group.

Many bat species live in social groups and are difficult to study with direct observations due to their use of inaccessible roost sites, nocturnal behavior, and ability to fly long distances. Social groups of tree-roosting big brown bats (*Eptesicus fuscus*) exhibit fission-fusion roosting behavior and are loyal to one of three non-overlapping roosting areas within my study area. Each roosting area contains approximately 30 adult females with their young. In a fission-fusion system of roosting the entire group regularly splits into subgroups that are spatially distinct. Females switch subgroups about every two days. Frequent roost switching among subgroups provides group members with the opportunity to associate more or less frequently with some member relative to others.

This fission-fusion system of roosting provides an excellent opportunity to test whether bats select roost-mates based on relatedness or matrilineal relationships. If bats prefer to roost with related and/or matrilineal females, then kin selection may be an important selection pressure for roost-mate selection. The importance of kin selection is most likely linked to the kin composition of the social group, which is influenced by mating, dispersal, and the formation of social groups (see above). The three adjacent

roosting areas provide an opportunity to infer mating and dispersal behaviors of *E. fuscus* through estimates of genetic subdivision.

To assess whether genetic relationships mediate fission-fusion behavior within social groups of these tree-roosting bats, I combined genetic analyses with behavioral studies. First, I investigate the genetic subdivision among roosting areas to infer the mating and dispersal behavior of *E. fuscus* (CHAPTER II). The mating and dispersal behavior of *E. fuscus* influences the kin composition of social groups and opportunities for interacting preferentially with kin. Second, I examine roosting associations within one roosting area to determine whether roost-mate decisions are based on relatedness and/or matrilineal relationships (CHAPTER III).

My purpose in CHAPTER II is to determine if the apparent female philopatry to roosting areas creates genetic subdivision between adjacent roosting areas. My first objective is to quantify the genetic variability within RA1, RA2, and RA3 using both nuclear microsatellite loci (biparentally inherited) and mitochondrial DNA sequences (maternally inherited). My second objective is to determine if the observed female philopatry will lead to nuclear (F_{st}) and/or maternal (Φ_{st}) genetic subdivision (determined from both biparentally and maternally inherited markers) between RA1, RA2, and RA3. Using the results of these objectives, I infer mating and dispersal behavior and comment on the possible influence of mating and dispersal behaviors on the kin composition of roosting areas.

The purpose of CHAPTER III is to combine knowledge of the roosting associations of individual *E. fuscus* from one roosting areas in Cypress Hills with genetic

analyses to determine whether relatedness and/or matrilineal relationships impact roosting associations. I determine roosting associations from two sources: (1) pairs of bats with a pairwise sharing index (PSI) based on radiotelemetry data (Willis and Brigham, 2004) and (2) roost-tree trapping events (groups of roost-mates). I evaluate the genetic relationships (relatedness and maternal lineages) between bats using both nuclear and mitochondrial DNA markers. My first objective is to determine if roosting associations based on PSI and root-tree trapping events varies with relatedness. I evaluate the relationship between PSIs (Willis and Brigham, 2004) and relatedness using a Mantel test. I calculate the average relatedness of the groups of roost-mates to determine whether roost-mates have a higher relatedness than expected by random chance (evaluated with a randomization test). My second objective is to determine if roosting associations are influenced by matrilineal relationships. I compare the average PSI of pairs of bats within the same matriline to the average PSI of bats that came from different matrilines. I also compare the distribution of matrilines within bats trapped from the same roost-tree to the expected distribution of matrilines to determine if bats prefer to roost with individuals from the same matriline. I use the results from these objectives to determine if roost-mates decisions are based on the genetic relationships of the group members and to infer the importance of kin selection for group stability.

CHAPTER II

GENETIC SUBDIVISION AMONG ROOSTING AREAS

Abstract

The genetic variation between adjacent social groups is thought to be an important force in mammalian populations. When mating and dispersal behaviors are difficult to observe directly, estimates of genetic subdivision between social groups are used to infer mating and dispersal behavior and understand the distribution of genetic variation among social groups. Tree-roosting big brown bats (*Eptesicus fuscus*) exhibit fission-fusion roosting behavior and roost in three non-overlapping roosting areas. Adult females are loyal to the same roosting area within and between seasons. The purpose of my study was to determine if female philopatry creates genetic subdivision between adjacent roosting areas. I used a 274 base pair segment of the mitochondrial DNA control region (maternally inherited) and nine microsatellite loci (biparentally inherited) to determine genetic subdivision. I found that female-mediated gene flow was restricted ($\Phi_{st} = 0.145$) between roosting areas while male-mediated gene flow was not ($F_{st} = 0.015$). Male-mediated gene flow between roosting areas likely occurs during fall swarming and/or hibernation when males and females from multiple natal roosting areas have the opportunity to mate. Although female-mediated gene flow was restricted

between roosting areas (Φ_{st} = 0.145), female-mediated gene flow is greater than observed in the Bechstein's bat (*Myotis bechsteinii*; F_{st} = 0.961 based on mitochondrial DNA), which also exhibits fission-fusion roosting behavior. Unlike *M. bechsteinii*, the maternal genetic subdivision for *E. fuscus* does not suggest a closed female society.

Introduction

The level of genetic subdivision among adjacent social groups is thought to be an important evolutionary force in mammalian populations (Storz, 1999). Interrelated behavioral factors that impact the genetic subdivision among and within social groups include: mating, dispersal, and formation of new social groups (Storz, 1999). These behavioral factors are often difficult to observe directly, but genetic studies can estimate the genetic subdivision among populations. Evidence for genetic subdivision among populations is used along with behavioral observations to infer mating and dispersal behavior. For example, male and female dispersal is understood by comparing the genetic subdivision among groups with uniparentally and biparentally inherited molecular markers (e.g. Petit et al., 2001).

Bat species are difficult to directly observe due to the use of inaccessible roost sites, nocturnal behavior, and the ability to fly and disperse over large distances. To understand the genetic subdivision among groups of both migratory and non-migratory bat species, previous studies have combined genetic analyses with behavioral data to infer mating systems and dispersal. For migratory species there is evidence for both weakly subdivided (*Tadarida brasiliensis*, Russell et al., 2005; *Pteropus* spp, Webb and Tidemann, 1996; *Leptonycteris curasoae*, Wilkinson and Fleming, 1996) and subdivided

populations *(Miniopterus schreibersii natalensis*, Miller-Butterworth et al., 2003). One European migratory species, *Nyctalus noctula*, exhibits weak population subdivision with panmictic units as wide as 3000 km with higher male-mediated gene flow than female-mediated gene flow (Petit and Mayer, 1999). In general, populations of non-migratory species show a pattern of weak subdivision with biparentally inherited markers and moderate to high subdivision with maternally inherited markers due to high male-mediated gene flow and female philopatry to natal roosting areas. Although the extent of male and female gene flow and colony subdivision varies among species (Burland et al., 2001; Castella et al., 2001; Kerth et al., 2000; Kerth et al., 2002a; Rossiter et al., 2000a; Worthington Wilmer et al., 1999; Worthington Wilmer et al., 1994).

The majority of non-migratory species studied thus far (see examples above) are at least partial gleaners (i.e. species which prey to some extent on non-flying arthropods) that generally fly short distances and roost in artificial structures during the summer. Bats that glean are often, although not always, characterized by slow and maneuverable flight, and smaller foraging and dispersal ranges, which is a consequence of low wing aspect ratio and low wing loading (Jones et al., 1995; Norberg and Rayner, 1987). For example, Entwistle et al (2000) demonstrate that among a sample of European bats, species with higher aspect ratio are classified as migratory whereas bat species with lower aspect ratios are classified as non-migratory. The ability of bat species to disperse from their natal roosting area and to make long distance movements to mating sites is expected to directly impact the genetic variation within and between roosting areas. Few studies have examined the genetic subdivision of non-migratory bats roosting in natural conditions or

non-migratory species capable of long distance flights. The historical processes (e.g. isolation in refugia) and current behavioral mechanisms (e.g. mating, dispersal, and new social group formation) in a tree-roosting species capable of long distance flight are likely to be different from partial gleaners roosting in artificial structures. Here, I investigate the genetic subdivision of naturally tree-roosting aggregations of a non-migratory species capable of long distance flight, the big brown bat (*Eptesicus fuscus*).

Within North America, *E. fuscus* is a common, insectivorous, medium-sized (11-23 g) species (Kurta and Baker, 1990). As in other temperate bat species, the mating system of *E. fuscus* is likely promiscuous (McCracken and Wilkinson, 2000), although there are few data. In the fall, *E. fuscus* moves from summer roosting sites to hibernation sites. In the eastern United States, *E. fuscus* are capable of traveling 228 km from summer roosts to hibernation sites (Barbour and Davis, 1969), but commonly travel less than 48 km to hibernate (Mumford, 1958). In Colorado, *E. fuscus* are reported to travel 24.5 to 87.5 km from summer roosts to hibernation sites in rock crevices (Neubaum et al., 2006). Copulation begins in September with a peak during fall swarming and continues in hibernation sites until March. Females store sperm until spring arousal from hibernation when ovulation and fertilization occur (Wimsatt, 1944). In the spring, males and females leave hibernation sites and return to summer roosting sites (Phillips, 1966). Females have one litter per year, and litter size varies from one (in western North America and Caribbean) to two (in eastern North America; Kurta and Baker, 1990). Where litters of two occur, multiple paternity has been observed (Vonhof et al., 2006). In the summer, females form groups (also known as maternity colonies or aggregations) ranging in size

from a few to hundreds of individuals in manmade structures, tree cavities, and rock crevices (Kurta and Baker, 1990), while males often roost alone.

For the past 13 years, the roosting behavior of tree-roosting groups of *E. fuscus* has been studied in the Cypress Hills Interprovincial Park, Saskatchewan, Canada (henceforth Cypress Hills). In the Cypress Hills, *E. fuscus* roost in cavities of trembling aspen trees (*Populus tremuloides*; Kalcounis and Brigham, 1998; Willis and Brigham, 2004; Willis et al., 2003; Willis et al., 2006) in groups consisting of adult breeding females, nonbreeding females, and young of the year (Willis and Brigham, 2004). Of the three non-overlapping roosting areas previously described (RA1, RA2, and RA3 as in Willis and Brigham, 2004), the most sampling has been done in RA1. The resident group using the roosting area is approximately 30 adult females with a variable number of young, and the group conforms to a fission-fusion model of roosting (Willis and Brigham, 2004). Females are loyal to the same roosting area within and between years despite nearby groups approximately 2 km away, and female juveniles return to their natal group (Kristen Kolar and JDM, unpublished; Willis and Brigham, 2004). Adult females are observed to join roosting areas between years, but whether these adult females represent natal juveniles that escaped sampling or immigrants from another roosting area is not known. On rare occasions adult males have been caught at foraging sites, but no males are found in female roosting areas (Kristen Kolar and JDM, unpublished; Willis and Brigham, 2004). Females and young leave the study site in the fall for unknown hibernation site(s).

The purpose of my study is to determine if the apparent female philopatry to roosting areas creates genetic subdivision between adjacent roosting areas. My first objective is to quantify the genetic variability within RA1, RA2, and RA3 using both nuclear microsatellite loci (biparentally inherited) and mitochondrial DNA sequences (maternally inherited). My second objective is to determine if the observed female philopatry will lead to genetic subdivision (determined from both biparentally and maternally inherited markers) between RA1, RA2, and RA3.

Material and Methods

Field Methods

All field work was conducted in Cypress Hills (49°34'N, 109°53'W). The Cypress Hills are a raised upland area not glaciated during the late Wisconsin glaciation with an east-west orientation which is surrounded by the Canadian prairies. The area is made up of 50% grassland, 45% woodland, and 5% wetland (Sauchyn, 1993). Forest vegetation consists of lodgepole pine (*Pinus contorta*) forest in dry, high elevations (>1300 m) and white spruce (*Picea glauca*) forest with understory in wet areas (Sauchyn, 1993). Details of the roosting behavior and social structure of *E. fuscus* in the Cypress Hills are mentioned above (see Introduction).

Three roosting areas (RA1, RA2, and RA3 from Willis and Brigham, 2004) were sampled (Figure 1). From June to August during 2000-2005, bats were trapped at roost sites using a modified harp trap (Kunz, 1988; modified and built by Kristen Kolar) or mist nets in RA1 about every two weeks, or trapped at foraging sites with mist nets sites (Kristen Kolar and JDM, unpublished; Willis and Brigham, 2004). Given the previous

intensive sampling in RA1 (Kristen Kolar, unpublished; Willis and Brigham, 2004), I expected that all adult females present during 2004-2005 would be captured in RA1. Additionally, bats were trapped in RA2 and RA3 during 2000-2002 and 2005. Captured bats were tagged with numbered split-ring plastic forearm bands (National Band and Tag Company, Newport, KY). During 2003-2005 captured bats were injected subcutaneously with Trovan ID-100 implantable transponders (Eidap Inc., Sherwood Park, AB). Upon capture, the identity and age of each bat were recorded. Juveniles were distinguished from adults based on the fusion of phalangeal epiphyses (Anthony, 1988). For all individuals captured, two wing biopsies (3 mm diameter; one from each wing) were taken and stored in saturated NaCl solution with 20% DMSO (Vonhof et al., 2006) or ethanol (80-95%), refrigerated during the field season, and then frozen at -20^{o}C for storage until DNA extraction.

All field methods and animal handling protocols were approved by the University of Regina President's Committee on Animal Care and in accordance with the Guidelines of the Canadian Council on Animal Care.

Microsatellite Amplification and Genotyping

Total genomic DNA was extracted from tissue biopsies using a DNeasy® Tissue Extraction Kit (QIAGEN). Nine microsatellite loci (Table 1) were amplified in 25 µl polymerase chain reactions (PCRs) using a Mastercycler Gradient Thermocycler (Eppendorf). General PCR conditions were 2-16 ng DNA template, 1 X PCR buffer (Promega; 50 mM Tris-HCl, pH 8.0 at 25^{o}C, 100 mM NaCl, 0.1 mM EDTA, 1mM DTT, 50% glycerol, 1% Triton® X-100), 1.25 units Taq polymerase (Promega), 0.40 µM each

primer, 0.1 mM each dNTP, and 1.5-3.0 mM $MgCl_2$ (Table 1). PCR amplification included a 3 min denaturation cycle at 95°C; 30 cycles of 1 min at 95°C, 1 min at annealing temperature (Table 1), and 2 min at 72°C; and an ending extension of 8 min at 72°C. For a sample of the reactions, amplification of the correct fragment was verified by removing 5 µl of the PCR product to visualize in a gel. The 5 µl PCR product was run out in a 1% agarose gel in 1% TBE buffer with a 100 base pair (bp) DNA step ladder (Promega), stained with SYBR® Gold nucleic acid gel stain (Molecular Probes®), and visualized on an illuminator to confirm amplification of the desired fragment.

To determine the size of the fragments (i.e. alleles), the PCR product (20 or 25 µl) was desalted with MultiScreen™ dialysis plates (Millipore; 0.05 µm pore size) in 0.1 X TE buffer for 15 to 20 minutes before being loaded into a MegaBACE® 500 sequencer with an in-lane standard (ET400-R; GE Healthcare). The size of each fragment was determined by visual inspection of the raw data (generated by the sequencer) in Fragment Profiler®. To reduce scoring errors, at least two identical runs were conducted for each individual at each locus with independent PCR amplifications. Alleles were assigned by visually binning the fragment sizes. To ensure correct assignment, the allele sizes and distribution for EF1, EF6, EF14, EF15, EF20, G9, and TT20 were compared with a larger data set compiled by Maarten Vonhof (unpublished; Vonhof did not use BE22 or G25).

Mitochondrial DNA Amplification, Sequencing, and Haplotypes

A portion of the mitochondrial DNA control region was PCR-amplified using the primers L16517 5'-CATCTGGTTCTTACTTCAGG-3' (Fumagalli et al., 1996) and sH651 5'-AAGGCTAGGACCAAACCT-3' (Castella et al., 2001), which is a shorter

version of the primer H00641 (Kocher et al., 1989). These primers amplify the second hypervariable domain (HVII).

For each adult, 2 µl of extracted genomic DNA was quantified with a ND-1000 Spectrometer (NanoDrop Technologies, Wilmington, DE) and diluted with sterile double distilled water to a concentration of one ng/µl to standardize the template DNA concentrations for all samples. PCR amplifications were in a total volume of 25 µl and contained 12.5 ng of diluted DNA (12.5 µl), 1 X PCR buffer (Promega), 1.0 µM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPS and 1 unit of Taq (Promega). PCR cycling conditions were 94^{o}C for 3 min and then 30 cycles of 94^{o}C for 1 min, 54^{o}C for 1 min, and 72^{o}C for 1.5 min. For each sample, 5 PCR products (total volume = 125 µl) were run out in 1% agarose gels. The bands for HVII were approximately 1000 to 1200 bp in length. The desired bands were cut out of the gel, combined, and purified using an IsoPure^{TM} Gel Extraction Prep Kit (Denville Scientific Inc., Metuchen, NJ). Purified DNA from the gel extraction was quantified and diluted to 10 ng/µl. If the concentration of the purified DNA was less than 10 ng/µl, then 1-3 µl of the purified PCR product was diluted in 99 to 97 µl of sterile double distilled water. The diluted purified DNA was used (instead of diluted template DNA) to PCR-amplify 5 more reactions using the procedure described above to achieve the desired concentration (at least 10 ng/µl).

Sequencing was done using a MegaBACE® 500 sequencer and an ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (GE Healthcare). The sequencing reaction was in total volume of 20 µl with 10 µl of purified DNA (100 ng total), 8 µl of sequencing mix, and 2 µl of primer (2.5 uM) as

recommended by the manufacturer. Cycling conditions were 25 cycles of 95°C for 20 s, 50°C for 15 s, and 60°C for 1 min. Ethanol precipitation was used for post-reaction cleanup. Both forward and reverse sequences were determined for each sample. However, the primers used for PCR-amplification amplify a 1000 to 1200 bp segment of DNA, which is largely a 6-bp repeating region after the first 300 bp (as in Castella et al., 2001; Fumagalli et al., 1996). A reverse primer was designed (5'-ATGCGTATGTCCTGAGACCA -3') to sequence the first 300 bp before the repeat region in both orientations. I used L16517 as the forward primer.

For each sample, forward and reverse sequences were aligned in BioEdit (Hall, 1999) using the ClustalW multiple alignment feature (Thompson et al., 1994). Discrepancies between forward and reverse sequences were resolved by manually comparing chromatograms in BioEdit. If discrepancies were not resolved, another forward and reverse sequence was amplified and sequenced. After every sample was corrected, all sequences were aligned. Any nucleotide differences (insertions, deletions, or substitutions) that occurred in only one sequence were manually checked in the reverse and forward chromatograms to ensure accuracy. Individual bats with the same sequence belong to the same haplotype.

Cytochrome b Amplifications

After the HVII haplotypes were determined, one or two adults from each Cypress Hills HVII haplotype were also sequenced at the cytochrome *b* region of mitochondrial DNA. The primers used to amplify cytochrome *b* were mcb398 5'-TACCATGAGGACAAATATCATTCTG-3' and mcb869 5'-CCTCCTAGTTTGTTAGGGATTGATCG-3'(Verma and Singh, 2003). The primer

numbers (398 and 869) refer to the position of the 5' base of the primers in the complete cytochrome *b* sequence of *Antilope cervicapra* (NCBI Accession no. AF022058) (Verma and Singh, 2003). PCR conditions for cytochrome *b* were 12.5 ng of diluted DNA (12.5 µl), 1 X PCR buffer (Promega), 1.0 µM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPS and 1.5 units of Taq (Promega). PCR cycling conditions were 94°C for 3 min; 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 8 min. For each sample, 2 PCR reactions were run out in 1% agarose gels. Bands were nearly 500 bp in length. The desired bands were cut out of the gel and purified using an IsoPure™ Gel Extraction Prep Kit (Denville Scientific Inc.). Purified PCR products were diluted to a concentration of 5 ng/µl. Sequencing and alignments were done as above for HVII, but with the mcb primers and 50 ng of purified DNA instead of 100 ng.

Statistical Analysis

Roost areas were known to contain about 30 philopatric adult females (Willis and Brigham, 2004). Between years many of the individuals were expected to be the same. Due to this continuity between years, all adult females sampled from the same roosting area were grouped together for analyses. Combining adults from different years could conceal year to year genetic variation and genetic subdivision between or within roosting areas caused by the presence of a few genetically different individuals, therefore I also grouped individuals by year and roosting area. I refer to these different groups of samples as subsets.

Genetic Variation

Genetic variation for the microsatellite loci, described as the number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e), was

calculated using the software program Cervus 2.0 (Marshall et al., 1998). For mitochondrial DNA sequences, gene diversity (h), nucleotide diversity (π), and the number of haplotyes (N_h) were calculated using Arlequin v 2.0 (Schneider et al., 2000). The number of pairwise difference between and within subsets was also calculated using Arlequin 2.0. To visualize the differences between haplotypes a minimum spanning network was generated using TCS (Clement et al., 2000) as described by Templeton et al (1992) and drawn with Adobe®Illustrator®CS v 11.0.0. In addition, *E. fuscus* sampled from outside the Cypress Hills were also sequenced to assess the variability of the HVII region across a wider geographic range. Tissue samples were taken from eight bats caught near Kootenay Lake, British Columbia (hereafter BC; linear distance of approximately 600 km from Cypress Hills; tissue punches provided by Juliet Craig); two from Regina, Saskatchewan (linear distance of approximately 380 km; tissue punches provided by Kristen Kolar); and five from the Uwharrie National Forest, North Carolina (hereafter NC; linear distance of approximately 2800 km from Cypress Hills; tissue punches provided by Matina C. Kalcounis-Rüppell).

Genetic Subdivision

For nuclear genetic subdivision, pairwise F_{st} (Weir and Cockerham, 1984) was calculated in Microsatellite Analyzer (MSA; Dieringer and Schloetter, 2003) and tested for statistical significance by permuting genotypes 10,000 times which does not require loci to be in Hardy-Weinberg equilibrium (Goudet et al., 1996). For mitochondrial DNA, pairwise Φ_{st} was calculated and tested for statistical significance in Arlequin v 2.0.

Results

Samples

Bats genotyped at microsatellite loci

Tissue samples were collected from 116 adult and juvenile bats from Cypress Hills and genotyped at nine microsatellite loci. Of these, there were 70 adult females (Table 2) and one adult male sampled during 2002-2005. One adult female and the adult male were caught foraging and could not be assigned to roosting areas. In total, 48 adult females were sampled from RA1 during 2002-2005, but not all of these females were present in RA1 in the same year (see Table 3). Of these, 3 were female juveniles born in RA1 that returned in subsequent years. Furthermore, 13 adult females joined RA1. The matrilines of the immigrant females were consistent with matrilines from RA1 but were also consistent with matrilines from either RA2 (n = 8) or RA3 (n = 5). Using genotypes from the nine microsatellite loci, 38.5% (5/13) of adult immigrants were assigned to putative mothers in RA1, 46.2% (6/13) were not assigned to putative mothers in RA1 and one was assigned to a putative mother in RA2, and 15.4% (2/13) were not resolved because immigrants mismatched at only one locus with a RA1 putative mother. From 2003-2005, all the adult females from RA1 were sampled during each year. Samples from RA2 included 19 adult females (Table 2). Only one bat was observed to switch roosting areas between RA2 and RA1 (included in both the RA1 and RA2 adult female totals above; see Table 3). This bat was included in both RA1 and RA2 in all analyses. From RA3, only three adults were sampled. RA3 was included in the genetic variation analyses but excluded from the genetic subdivision analyses due to the small sample size.

Bats sequenced at HVII region

HVII sequences were obtained for 70 adult bats (69 females and one male) collected during 2003-2005 from Cypress Hills. The sample from one female adult caught in RA2 failed to amplify after multiple attempts and was not sequenced (individual was successfully genotyped at microsatellite loci). In addition, five bats from NC, eight bats from BC, and two bats from Regina were sequenced (n = 15 bats from outside of Cypress Hills).

Microsatellite Genetic Variability

The nine loci were polymorphic with the number of alleles per locus ranging from 4 to 27 with a mean of 9.8 (Table 4). The loci with the lowest levels of variation were BE22 and TT20 with 4 and 7 alleles respectively. The expected heterozygosity and number of alleles per locus were similar between roosting areas and from year to year within RA1 (Table 5).

Microsatellite Genetic Subdivision

Nuclear genetic subdivision was nearly absent indicating high gene flow between the roosting areas (Table 6). The only statistically significant pairwise F_{st} was between adult females in RA2 during 2002-2005 and female adults in RA1 during 2002 (F_{st} = 0.024, $p < 0.01$). The other pairwise F_{st} comparisons between RA2 and RA1 were not statistically significant. Taken together, these data provided evidence for high gene flow between RA1 and RA2.

Mitochondrial DNA Diversity

The initial 273 or 274 bp of the HVII region was successfully amplified for both forward and reverse sequences. Due to three insertions, the aligned sequences spanned

276 bp. The six bp repeat region 'CATACG' began at approximately 315 bp and continued for at least 200 bp (at least 30 repeat units) although the quality of the sequence became too poor after about 500 bp to determine the entire length of the repeat region.

From 85 sequences (see above), there were 18 haplotypes with 47 variable sites and three gaps (Table 7). Of these 18 haplotypes, nine occurred in the Cypress Hills while the others occurred in BC (n = 3), NC (n = 5), and Regina (n = 1) (Table 8). If the BC haplotypes were excluded, there were 15 haplotypes with 28 variable sites and two gaps. A minimum spanning network was used to visualize the number of mutations among the haplotypes from the Cypress Hills, NC, and Regina (Figure 2). BC haplotypes were not included in the minimum spanning network because they were too divergent from the other haplotypes (>15 mutations).

The transversions among haplotypes also indicated divergence between haplotypes west (H01-03) and east of the Rocky Mountains (H04-18). Within western or eastern haplotypes, no transversions were observed while 12 transversions at the following base pair positions 157, 200, 203, 212, 214, 215, 222, 225, 248, 260, 261, and 270 occurred between western and eastern haplotypes (Table 7). In addition, at four sites (214, 225, 262, and 270 bp) three nucleotides were present (Table 7). In each of these cases, a pyrimidine transition was present in the eastern haplotypes while the western haplotype was monomorphic for a purine (A or G). The divergence between eastern and western haplotypes indicated that the Rocky Mountains were a barrier to maternal gene flow in *E. fuscus*, therefore BC haplotypes were excluded from most analyses and discussion.

Most of the nine haplotypes found in the Cypress Hills were present in more than one roosting area (Table 8). In RA2, seven haplotypes were present, and two of these haplotypes (H13 and H14) were unique to RA2. The adult male captured in the Cypress Hills also had H14. The six haplotypes in RA1 were found in either RA2 or RA3. Although RA1 had a greater sample size (n = 48) than RA2 (n = 18), RA2 had more haplotypes than RA1. RA3, with a sample size of three, had three haplotypes. The most divergent haplotype found in Cypress Hills (H17) was found in RA1, RA3, and the female from unknown roosting area, but not in RA2. Haplotypes (H04-08) from NC were unique, but very similar to the Cypress Hills haplotypes (Figure 2). The Regina samples had one unique haplotype (H18) while the other (H09) was found in Cypress Hills. H18 was similar to the other Cypress Hills haplotypes. The average pairwise differences among roosting areas within Cypress Hills, NC, and Regina haplotypes were 3.2 to 8.7. The average pairwise differences for haplotypes within roosting areas, NC, and Regina ranged from 1.3 to 12.

Overall, gene diversity was high (0.679-1.000), and for sample sizes greater than eight, gene diversity was almost identical (0.788-0.809; Table 9). Nucleotide diversity was lowest in BC and RA2 (0.0048, 0.0072) while RA1 and RA3 had the highest levels of nucleotide diversity (0.0273 to 0.0438). High nucleotide diversity in RA1 and RA3 most likely reflected the presence of the most divergent Cypress Hills haplotype (H17; see Figure 2 and Table 7).

The cytochrome *b* region was sequenced (330 bp) from one bat from NC, BC, and Regina. From the Cypress Hills, two bats from each of the more common HVII

haplotypes (H09, H10, H15, H16, and H17) were sequenced at cytochrome *b* region, and only one bat from each of the four less common HVII haplotypes. From 17 total sequences, there were four haplotypes (HC1-4) with 25 variable sites and no gaps (Table 10). HC1 was the most common haplotype (n = 12) and contained bats from nine different HVII haplotypes (H10-H16, H18, H07) from the following locations: RA1, RA2, RA3, NC, and Regina. HC3 (n = 2) was very similar to HC1 with only one transition and contained bats from H09. HC2 (n = 2) contained bats from the most divergent HVII haplotype (H17). As with HVII, the BC sample (HC4) proved to be quite divergent. Interestingly, HC2 has two transitions and one transversion that separate this haplotype from the others, and all three substitutions were shared with HC4, suggesting that H17 might have originated in the west near the Rocky Mountains.

Mitochondrial DNA Subdivision

Statistically significant maternal genetic subdivision was detected in all comparisons between RA2 and RA1 (Φ_{st} range of 0.145 to 0.229; Table 11). These data provided strong evidence for limited female-mediated gene flow between RA1 and RA2.

Discussion

I found that female-mediated gene flow was restricted between RA1 and RA2, while biparentally-mediated gene flow was not. The genetic subdivision at the maternally inherited marker was likely caused by female philopatry and supports the behavioral observations of female philopatry in tree-roosting *E. fuscus*. My results indicated that there was high male-mediated gene flow between the roosting areas that eliminates genetic subdivision that would otherwise result due to the philopatry of females.

Although I had a larger sample size from RA1 (n = 48 adult females) than RA2 (n = 19), RA1 had fewer haplotypes (n = 6) than RA2 (n = 7). A higher number of haplotypes in RA2 provides evidence that most, if not all, haplotypes were sampled. Within RA2, I found three haplotypes that were not present in RA1 (which was intensively trapped and most, if not all, adult females were sampled). These observations provide evidence that the results were not an artifact of a smaller sample size in RA2.

Female philopatry coupled with high male-mediated gene flow among roosting areas has been observed in other temperate non-migratory bat species (see examples below). The majority of these species are at least partial gleaners (i.e. prey on nonairborne insects) that roost in man-made bat boxes or buildings during the summer. My study is different from previous studies because *E. fuscus* is using natural tree roost sites and an aerial foraging strategy that might produce a different distribution of genetic variation within this species relative to other non-migratory species. In addition, studying the genetic subdivision of tree-roosting *E. fuscus* in a forest environment will facilitate comparisons with future studies in managed forests to clarify the impact of forest management on the genetic structure of tree-roosting bats.

Male-mediated gene flow among roosting areas occurs in two ways. First, males might disperse from their natal roosting area to nearby roosting habitat. Males likely mate with females from their own natal roosting area as well as nearby roosting areas during the fall before bats leave summer roosting sites. This is the case for the well studied greater horseshoe bat (*Rhinolophus ferrumequinum*). Males generally defend a mating territory, mate, and hibernate within about 25 km of their natal roost (Ransome, 1990).

Adjacent maternity colonies of *R. ferrumequinum* generally do not have nuclear genetic subdivision (Rossiter et al., 2000a). Females typically give birth to offspring sired by males from within and outside their own natal roosting area, and females mate with the same male in consecutive years. In addition, matrilineal females often share the same breeding partners without increasing inbreeding, and long-term (10 year) male reproductive skew has been observed (Rossiter et al., 2000b; Rossiter et al., 2006; Rossiter et al., 2005).

Second, males and females from adjacent and/or distant roosting areas likely mate during fall swarming and/or at hibernation sites. In the summer, males could remain within or near their natal roosting area or disperse. Several species are thought to mate with partners from multiple summer roosting areas at swarming or hibernation sites including: Bechstein's bat *(Myotis bechsteinii*, Kerth et al., 2003; Kerth and Morf, 2004), brown long-eared bats *(Plecotus auritus*, Burland et al., 1999; Burland et al., 2001; Veith et al., 2004), and Natterer's bat (*Myotis nattereri*, Rivers et al., 2005). For *E. fuscus* from Cypress Hills, the movements and roosting behaviors of males are simply not known. Males are seldom captured within the study site and never captured at roosting sites with females. The swarming and hibernation sites of both males and females are unknown. Thus, the behavioral mechanism that produces male-mediated gene flow between roosting areas of *E. fuscus* remains unknown.

Of the species studied thus far, *M. bechsteinii* is the most similar to my data for *E. fuscus* from Cypress Hills with respect to roosting behavior. Like *E. fuscus*, *M. bechsteinii* is a naturally tree-roosting bat that exhibits a fission-fusion system of roosting

behavior with nonrandom roosting associations and female philopatry. However, one major difference is that published studies on *M. bechsteinii* are for bats roosting in man-made bat boxes erected throughout forest habitat (e.g. Kerth and Konig, 1999). Unlike *E. fuscus*, in *M. bechsteinii* there is strong genetic subdivision based on mitochondrial DNA markers (F_{st} = 0.961) and weak, but significant, nuclear genetic subdivision (F_{st} = 0.015) among roosting areas (Kerth et al., 2000; Kerth et al., 2002a). Female *M. bechsteinii* forage almost exclusively in areas of closed forest and glean prey within a foraging home range that overlaps little with other individuals (Kerth et al., 2001). Kerth et al (2002a) suggested that extreme male-biased dispersal and complete female philopatry in *M. bechsteinii* results from inbreeding avoidance and competition for limited resources. When confronted with conspecifics from foreign roosting areas in confrontation tests, female *M. bechsteinii* respond aggressively, which suggests that females defend their roosting area from immigrants because resources are limited (Kerth et al., 2002b; Safi and Kerth, 2003).

In contrast, *E. fuscus* is known to be a flexible, generalist aerial feeder that forages successfully in a variety of habitats (reviewed by Agosta, 2002; Fenton and Bogdanowicz, 2002). Female *E. fuscus* in the Cypress Hills do not defend individual foraging home ranges and travel approximately 3-11 km from roost sites to forage in cattle pastures (Arbuthnott and Brigham, submitted). Unlike *M. bechsteinii* which forages only in small nearly exclusive foraging home ranges in closed forests, foraging resources do not appear to be limiting for *E. fuscus*. The need to defend territory from immigrants is likely not as crucial for *E. fuscus* because *E. fuscus* females have less to gain from

excluding immigrants than *M. bechsteinii*. Allowing immigrants to join the roosting area decreases genetic subdivision among roosting areas. The difference in foraging strategy, and consequently resource abundance, may in part explain the much weaker maternal genetic subdivision in female *E. fuscus* (Φ_{st} = 0.145) relative to female *M. bechsteinii* (F_{st} = 0.961 based on mitochondrial DNA).

In addition *M. bechsteinii*, like other gleaners, generally do not fly long distances (maximum male dispersal of 38 km; Schober and Grimmberger, 1998 cited in Kerth et al., 2000) while *E. fuscus* is better suited for longer distance flights (movement to hibernation site of 228 km, Barbour and Davis, 1969). The dispersal distance of individuals, particularly males for species with philopatric females, is critical for determining genetic structure. If there is long distance dispersal in *E. fuscus* or long distance movements from roosting areas to breeding sites, then males and females from both distant and nearby roosting areas are expected to mate. This decreases the genetic subdivision among both distant and nearby roosting areas. For species with shorter dispersal distance, mating is likely to occur between males and females from nearby roosting areas and greater genetic subdivision will occur among distant roosting areas. Multiple mating among males and females from both distant and nearby roosting areas will decrease nuclear genetic subdivision among the roosting areas more quickly in *E. fuscus* than in *M. bechsteinii*. The difference in long-distance flight capabilities may in part explain the weaker nuclear genetic subdivision in *E. fuscus* relative to *M. bechsteinii*.

However, inbreeding avoidance is likely to be as important for *E. fuscus* as it is for *M. bechsteinii* (Kerth et al., 2002a). Inbreeding avoidance increases juvenile success

in wild *R. ferrumequinum* (Rossiter et al., 2001). Resident groups of *E. fuscus* within roosting areas are relatively small, with approximately 30 individuals, and are therefore susceptible to inbreeding depression in the absence of gene flow from other roosting areas. The negative effects of inbreeding depression can sometimes be offset by purging deleterious alleles; however the population must be able to withstand a high mortality rate. Given that *E. fuscus* has at most two pups per year and not all females are reproductive each year, purging deleterious alleles would likely drive groups of *E. fuscus* to extinction (Hedrick, 1994).

Genetic subdivision at the mitochondrial DNA marker, but not at nuclear markers, reflects historical processes and current restricted female gene flow among the adjacent roosting areas. The presence of one divergent haplotype and eight similar haplotypes (Figure 2 and Table 7) within the Cypress Hills suggests that at least two ancestral lineages colonized the two roosting areas. The Cypress Hills were not glaciated during the Pleistocene glaciation (Sauchyn, 1993), and *E. fuscus* is thought to have been the most widespread Pleistocene bat in North America with fossils from numerous sites including Montana (Kurta and Baker, 1990). Further sampling of *E. fuscus* within the Cypress Hills and surrounding areas could clarify the origin of ancestral lineages and past colonization events by female *E. fuscus*.

In summary, my results indicate that females from the two roosting areas in Cypress Hills were founded by at least two ancestral lineages. Male-mediated gene flow appears to prevent nuclear genetic subdivision between roosting areas of philopatric females. Comparison with a similar bat species, *M. bechsteinii*, suggests that genetic

subdivision in *E. fuscus* might be prevented due to a generalist, aerial foraging strategy and/or the capability of long distance flight. Expanding this study to include additional roosting areas within and around Cypress Hills is of interest for five reasons. First, extensive sampling and radiotracking might locate swarming and hibernations sites which will clarify the mating behavior of *E. fuscus* in the Cypress Hills. Second, extensive sampling will determine whether high male-mediated gene flow prevents genetic subdivision from occurring between distant roosting areas. Third, the distribution of mitochondrial DNA haplotypes will provide insight into the past colonization events of this widespread Pleistocene bat. Fourth, studies of tree-roosting bats in a natural setting can be used to interpret the impact of forest management on tree-roosting bats. Fifth, the Cypress Hills will likely experience a fast burning forest fire in the near future (Kevin Redden, personal communication), and these data will provide the before fire comparison. These additional studies of mating behavior, genetic subdivision, and genetic variation over a larger geographic region are necessary to understand the historical (e.g. colonization and isolation events) and current processes (dispersal, social group formation, and mating behaviors) that generate the genetic variability and subdivision observed in tree-roosting *E. fuscus*.

CHAPTER III

ROOSTING ASSOCIATIONS AND GENETIC RELATIONSHIPS

Abstract

In group living mammals, kin selection is often invoked to explain associations and cooperation between group members, and consequently group stability. Tree-roosting big brown bats (*Eptesicus fuscus*) exhibit fission-fusion roosting behavior and female philopatry. Within one roosting area, adult females switch roost trees and potentially roost-mates approximately every two days. Group members appear to associate frequently with some members and infrequently with others. Kin selection might be a strong selection pressure mediating roost-mate decisions. To assess whether roosting associations were based on genetic relationships within a group of tree-roosting bats with fission-fusion roosting behavior, I combined genetic analyses with behavioral studies. Roosting associations were determined from (1) a pairwise sharing index (PSI) based on pairs of radiotracked bats and (2) roost-tree trapping events. Genetic relationships were inferred from nine microsatellite loci and from 274 base pair segment of the mitochondrial DNA control region. I found that roosting associations were not influenced by relatedness or matrilineal relationships, thus kin selection does not explain roost-mate decisions. For groups of roost-mates there is a trade-off between subgroup size and kin composition, because as subgroup size increased, subgroup relatedness decreased.

Preferred roost-mates, identified by the PSI, might be explained by reciprocity, where bats with high past roosting associations might preferentially cooperate.

Introduction

Kin selection, mutualism or reciprocity, parasitism, coercion, by-product mutualism, and group augmentation might explain the evolutionary processes that produce stable cooperative groups (reviewed by Clutton-Brock, 2002). Kin selection has been used to explain cooperative behavior among group members (e.g. Eberle and Kappeler, 2006; Krakauer, 2005). Kin selection is the process by which traits are favored because of their beneficial effects on the survival of relatives, including offspring (direct fitness) and non-descendant offspring (indirect fitness) (Griffin and West, 2002). Mutualism or reciprocity can take a variety of forms (Clutton-Brock, 2002). For example, reciprocity can occur when two or more individuals exchange beneficial acts in turn ("reciprocal altruism" or "cost-counting" reciprocity). Group augmentation can explain group living when members are related, distantly related or not related at all because it occurs when participating in large groups increases the fitness of individual group members and it benefits individuals further to recruit new members to the group (Kokko et al., 2001). Group augmentation differs slightly from reciprocity because the best strategy for group augmentation is to always help group members while in reciprocity generally group members must reciprocate cooperative behaviors in order for reciprocity to be a stable strategy.

Examining the relatedness of group members in natural populations that exhibit cooperative behaviors can clarify the relative importance of kin selection from other

evolutionary processes like reciprocity and group augmentation. Direct observations are not sufficient to identify closely related individuals. In particular, when parent-offspring pairs are not obvious from observation, genetic analyses are necessary to assign parentage and estimate relatedness among individuals. In addition, for long-lived species with over-lapping generations, observations are impracticable to infer relatedness among adults. While long-term behavioral data are necessary to understand the associations among individuals, genetic analyses are required to determine relatedness among individuals.

Many species of bats spend the summer months in social groups. Social groups, often referred to as colonies, aggregations or simply groups, contain a few to thousands of individuals (Kunz and Lumsden, 2003). The nature of group living in bats appears to be influenced by a variety of factors. Bats may roost together because an optimal group size is desirable. Groups may be important for thermoregulation (Racey and Swift, 1981; Wilde et al., 1995) and/or predator avoidance (Kalcounis and Brigham, 1994; Speakman et al., 1999). In addition to group size, individual composition might be important for information transfer about roost and foraging sites (Kerth and Reckardt, 2003; Kerth et al., 2001; Wilkinson, 1992b) as well as cooperative breeding (Kerth et al., 2001). Reproductive differences can also influence the roosting decisions made by group members (Kerth and Konig, 1999). For example, to accelerate fetal growth, reproductive individuals may benefit from a different roost microclimate relative to non-reproductive individuals (Racey and Swift, 1981; Wilde et al., 1995).

In a fission-fusion system of roosting the entire group regularly splits into subgroups that are spatially distinct. These subgroups continually form, break up, and reform over the course of hours, days, months, and/or years. In a fission-fusion system of roosting, members appear to have the opportunity to associate more or less frequently with some members relative to others. Individuals make decisions about whom to associate with each time the group fissions (splits up into subgroups). If subgroup size is the only important factor for decisions that individuals make about roosting associations, then random associations among individual bats are expected. In other words, the individuals a bat roosts with are predicted to be irrelevant as long as the appropriate numbers of bats are present. However, if the individual composition of the subgroup is important, nonrandom associations among individual members are expected. In this case, the size of the roosting subgroup is irrelevant whereas the particular individuals within the subgroup are more important.

If group members are genetically related, kin selection benefits could influence roosting decisions (Kerth et al., 2001; O'Donnell and Sedgeley, 1999). In bat species that form highly stable and cohesive groups, group relatedness is low (Burland et al., 2001; Kerth et al., 2002b; Rossiter et al., 2002; Storz et al., 2001; Wilkinson, 1985b; Wilkinson, 1992a). It has been suggested that the mating behavior of many bat species prevents high levels of relatedness within a group despite female philopatry because many males from outside the group sire offspring within the group (Burland and Worthington Wilmer, 2001). In addition, low fecundity, small litter size, and high juvenile mortality also decrease relatedness within a group (Wilkinson, 1985b).

A low mean relatedness does not preclude the presence of related females living in groups. In Bechstein's bat *(Myotis bechsteinii)*, 75% of group members live together with a close relative ($r \geq 0.25$) despite low mean relatedness ($r = 0.02$) (Kerth et al., 2002b). Some pairs of brown long-eared bats (*Plecotus auritus*) are close relatives despite group relatedness of almost zero (Burland et al., 2001). Within a group of greater horseshoe bats (*Rhinolophus ferrumequinum*), average relatedness within matrilines (n = 15 matrilines) range from 0.17 to 0.64, whereas the background relatedness is 0.03; suggesting that within matrilines the potential for kin-based cooperation exists (Rossiter et al., 2002). Thus, despite low overall group relatedness, there is potential for related individuals to participate in long-term associations.

With the exception of mother-offspring pairs, bats that live in social groups have never been found to preferentially associate or cooperate with kin (including the studies mentioned above). For example, related individuals do not preferentially roost together or transfer information about novel roosts in *M. bechsteinii* (Kerth and Konig, 1999; Kerth and Reckardt, 2003). In communally nursing *Nycticeius humeralis,* the relatedness of female pairs does not appear to influence their decision to nurse non-descendant young (Wilkinson, 1992a). Roosting and foraging subgroups are not composed of closely related individuals in the greater-spear-nosed bat (*Phylllostomus hastatus*) (McCracken and Bradbury, 1977; Wilkinson and Boughman, 1998).

Matrilineal and/or mother-offspring relationships do explain some types of long-term associations. Overlap in foraging areas between mother-offspring pairs occur for both *M. bechsteinii* and *R. ferrumequinum* (Kerth et al., 2001; Rossiter et al., 2002).

Mother-offspring pairs of *R. ferrumequinum* are the only group members observed to share the same night roost site (Rossiter et al., 2002). Remarkably, matrilineal female *R. ferrumequinum* often share the same breeding partners (intra-lineage polygny) (Rossiter et al., 2005), although the benefit of sharing mates is not clear (Pen and Kerth, 2005).

Common vampire bats (*Desmodus rotundus*) conform to a fission-fusion system of roosting (Wilkinson, 1985a). In *D. rotundus*, food sharing occurs preferentially among matrilineal females and unrelated females with high past associations, indicating that the pair frequently roosted together (Wilkinson, 1985b). For *D. rotundus* reciprocal altruism, not kin selection, best explains food sharing between both females with high past associations and matrilineal females (Wilkinson, 1988). As observed in *D. rotundus*, frequent associations increase the probability of future interactions and consequently the stability of cooperative behaviors between group members. Future interactions provide opportunities for individuals to cooperate and opportunities to recognize and punish "cheaters" by not cooperating in future interactions (cheaters receive a benefit from a cooperative behavior, but do not return the benefit to the individual that cooperated; e.g. Axelrod and Hamilton, 1981).

Bats with a fission-fusion system of roosting, like *D. rotundus*, appear to have the choice of roosting with unrelated or related individuals. A fission-fusion system of roosting provides an excellent opportunity to test whether bats select roost-mates based on relatedness or matrilineal relationships. If bats prefer to roost with related and/or matrilineal females, then kin selection may be an important selection pressure for roost-mate selection. Selection pressures other than kin selection may be more important for

roost-mate decisions. For example, if subgroup size is more important than kin composition, then bats should roost with both unrelated and related individuals at random.

Tree-roosting big brown bats (*Eptesicus fuscus*) from Cypress Hills Interprovincial Park, Saskatchewan, Canada (henceforth Cypress Hills) conform to a fission-fusion system of roosting and are the focus of a long-term behavioral study (e.g. Kalcounis and Brigham, 1998; Willis and Brigham, 2004; Willis et al., 2003). During the summer reproductive season, bats roost exclusively in the cavities of aspen trees (*Populus tremuloides*) (Kalcounis and Brigham, 1998; Willis et al., 2003; Willis et al., 2006). A resident group of approximately 30 individuals, fission into several subgroups which roost in different aspen trees during the day. At night, bats have the opportunity to fission and reform (fusion) subgroups. The resident group is loyal to the same roosting area and trees within the roosting area (Willis and Brigham, 2004) and consists of non-reproductive adult females and reproductive females with young. Solitary bats and males are rarely captured (Kristen Kolar and JDM, personal observation; Willis and Brigham, 2004). Bats switch roost trees and potentially roost-mates about every two days (Willis and Brigham, 2004). Within roosting area one (henceforth "RA1" as in Willis and Brigham, 2004), roosting associations between pairs of *E. fuscus* are nonrandom. In other words, bats have preferred roost-mates even though they roost with all RA1 members throughout the summer (Willis and Brigham, 2004).

The purpose of my project is to combine knowledge of the roosting associations of individuals within this group of *E. fuscus* from Cypress Hills with genetic analyses of

their relationship with each other to determine whether relatedness and/or matrilineal relationships impact roosting associations. I predict that if individual *E. fuscus* select roost-mates based on relatedness, then bats that are regular roost-mates will be more related than bats that rarely roost together. Roosting associations are determined from two sources: (1) a pairwise sharing index (PSI) based on radiotelemetry data (Willis and Brigham, 2004) and (2) roost-tree trapping events (see Methods for details). The genetic relationships (relatedness and maternal lineages) between bats are determined based on both nuclear and mitochondrial DNA markers.

My first objective is to determine if roosting associations based on PSI and root-tree trapping events varies with relatedness. The relationship between PSIs (Willis and Brigham, 2004) and relatedness is evaluated using a Mantel test. I predict that there will be a positive correlation with pairs of bats with high degrees of association (high PSI) having a higher relatedness. The average relatedness of bats captured from the same roost tree during roost-tree trapping events is calculated to determine whether roost-mates have a higher relatedness than expected by random chance. I predict that roost-mates will have higher relatedness than expected at random.

The second objective is to determine if roosting associations are influenced by matrilineal relationships. The average PSI of pairs of bats within the same matriline will be compared to the average PSI of bats that came from different matrilines. I predict that pairs of bats within the same matriline will have a higher mean PSI than pairs of bats that came from different matrilines. I compare the distribution of matrilines within bats trapped from the same roost-tree to the expected distribution of matrilines to determine if

bats prefer to roost with individuals from the same matriline. I predict that matrilines will not be randomly distributed. For bats captured exiting from the same roost tree, I predict that there will be more bats with the same matriline than expected based on a random distribution.

Material and Methods

All field methods and animal handling protocols were approved by the University of Regina President's Committee on Animal Care and in accordance with the Guidelines of the Canadian Council on Animal Care.

All field work was conducted in the Cypress Hills (49°34'N, 109°53'W). The Cypress Hills are a raised upland area not glaciated during the late Wisconsin glaciation with an east-west orientation which is surrounded by the Canadian prairies. The area is made up of 50% grassland, 45% woodland, and 5% wetland (Sauchyn, 1993). Forest vegetation consists of lodgepole pine (*Pinus contorta*) forest in dry, high elevations (>1300 m) and white spruce (*Picea glauca*) forest with understory in wet areas (Sauchyn, 1993). Details of the roosting behavior and social structure of *E. fuscus* in the Cypress Hills are described above (see Introduction).

Roosting Associations

Pairwise Sharing Index (PSI)

Pairwise sharing index values (PSIs) were calculated based on data from simultaneously radiotracked pairs of bats within RA1 in the summers of 2000-2002 (Willis and Brigham, 2004). The PSI index quantifies the degree of non-randomness of roosting associations of two bats radiotracked simultaneously by considering the number of trees each bat roosted in, the number of times the pair was found together and apart,

and the number of times each bat switched roosts (see Willis and Brigham, 2004). Theoretically, PSI values can range from -1 to +1. A positive PSI indicates that the pair spent more time roosting together than expected with random roost-mate and roost site selection (Willis and Brigham, 2004).

Tree-Roost Trapping Events

Roost-tree trapping events occurred as part of the long-term study (e.g. Kalcounis and Brigham, 1998; Willis and Brigham, 2004; Willis et al., 2003) where bats from RA1 were captured as they exited roost trees at dusk. Many roost trees had a single exit and therefore it was possible to capture emerging individuals. Thus, many trapping events provided a sample of most if not all of the bats that were roosting in the same tree (i.e. roost-mates). Occasionally bats evaded capture by remaining in the tree or escaping from the trap during the trapping event. During the summer of 2002-2005, bats were captured leaving roost-trees at dusk using a modified harp trap (Kunz, 1988; modified and built by Kristen Kolar) and/or mist nets set roughly every two weeks (Kristen Kolar and JDM, unpublished; Willis and Brigham, 2004). Captured bats were uniquely tagged for later identification as follows: (1) with numbered split-ring plastic forearm bands (National Band and Tag Company, Newport, KY: 2002-2005) and/or (2) with Trovan ID-100 transponders (during 2003-2005 only) injected subcutaneously into the posterior dorsal surface of the bat (Eidap Inc., Sherwood Park, AB). Upon capture of each bat, the identity (forearm band and/or transponder), mass, sex, age, and reproductive status were recorded. Juveniles were distinguished from adults based on the fusion of phalangeal epiphyses (Anthony, 1988). Reproductive status was assessed by palpation of the

abdomen to detect pregnancy and lactation was detected by the presence of bare patches around the nipples and/or expression of milk.

In order to find the roost sites used by bats, a sample of the individuals captured were fitted with radiotransmitters (≤ 1.0 g BD-2 or BD-2T, Holohil Systems Ltd, Carp, ON) by trimming a small portion of the hair on the interscapular dorsal surface of the bat and then affixing the transmitter to the trimmed area with Skin-Bond® surgical glue (Smith & Nephew United, Inc., Largo, FL). Transmitters weighed less than 5% of the bat's body mass (Aldridge and Brigham, 1988). Bats were tracked to roost sites with an R-1000 receiver (Communications Specialists, Inc., Orange, CA) coupled to a five-element yagi antenna (AF Antronics, Inc., Urbana, IL).

Genetic Relationships

From all individuals captured in 2003-2005 and most of the individuals captured in 2002, two wing biopsies (3 mm diameter; one from each wing) were taken and stored in saturated NaCl solution with 20% DMSO (Vonhof et al., 2006) or ethanol (80-95%). They were refrigerated during the field season and then frozen at -20^{o}C until DNA extraction.

Microsatellite Amplification, Genotyping, and Relatedness

Total genomic DNA was extracted from tissue biopsies using a DNeasy® Tissue Extraction Kit (QIAGEN). Nine microsatellite loci (Table 12) were amplified in 25 µl polymerase chain reactions (PCRs) using a Mastercycler Gradient Thermocycler (Eppendorf). General PCR conditions were 2-16 ng DNA template, 1 X PCR buffer (Promega; 50 mM Tris-HCl, pH 8.0 at 25^{o}C, 100 mM NaCl, 0.1 mM EDTA, 1mM DTT, 50% glycerol, 1% Triton® X-100), 1.25 units Taq polymerase (Promega), 0.40 µM each

primer, 0.1 mM each dNTP, and 1.5-3.0 mM $MgCl_2$ (Table 12). PCR amplification included a 3 min denaturation cycle at 95°C; 30 cycles of 1 min at 95°C, 1 min at annealing temperature (Table 12), and 2 min at 72°C; and an ending extension of 8 min at 72°C. For a sample of the reactions, amplification of the correct fragment was verified by removing 5 µl of the PCR product to visualize in a gel. The 5 µl of PCR product was run out in a 1% agarose gel in 1% TBE buffer with a 100 base pair (bp) DNA step ladder (Promega), stained with SYBR® Gold nucleic acid gel stain (Molecular Probes®), and visualized on an illuminator to confirm amplification of the desired fragment.

To determine the size of the fragments (i.e. alleles), the PCR product (20 or 25 µl) was desalted with MultiScreen™ dialysis plates (Millipore; 0.05 µm pore size) in 0.1 X TE buffer for 15 to 20 minutes before being loaded into a MegaBACE® 500 sequencer with an in-lane standard (ET400-R; GE Healthcare). The size of each fragment was determined by visual inspection of the raw data (generated by the sequencer) in Fragment Profiler®. To reduce scoring errors, at least two identical runs were conducted for each individual at each locus with independent PCR amplifications. Alleles were assigned by visually binning the fragment sizes. To ensure correct assignment, the allele sizes and distribution for EF1, EF6, EF14, EF15, EF20, G9, and TT20 were compared with a larger data set compiled by Maarten Vonhof (unpublished; Vonhof did not use BE22 or G25). Genetic diversity indices (the number of alleles, allele size range, expected heterozygosity, observed heterozygosity, and null allele frequency) were calculated using Cervus 2.0 (Marshall et al., 1998) and were based on the genotypes of female adults in RA1 from 2002-2005.

Pairwise relatedness estimates were calculated using the nine microsatellite loci and Relatedness 5.0.8 (Queller and Goodnight, 1989). When relatives are included in background allele frequencies, relatedness is underestimated (e.g. West et al., 2002). I dealt with this first by excluding all juveniles from contributing to the background allele frequencies. Within the remaining adults, the number of relatives was unknown. To evaluate the reliability of the relatedness estimator, juveniles were assigned to putative mothers using Cervus 2.0 (Marshall et al., 1998) with strict criteria (95% confidence and no loci mismatches between juvenile-mother), and the average pairwise relatedness of the assigned juvenile-mother pairs was determined and compared to the expected result of 0.5. If the assigned juvenile-mother pairs had an average relatedness near the theoretical value of 0.5, then the relatedness estimator was assumed to not underestimate relatedness.

Pairwise relatedness estimates were calculated between all genotyped bats. In each year, the average pairwise relatedness of all adult females and the number of close relatives ($r \geq 0.25$) for each adult were determined. The average pairwise relatedness of all adult females and the number of close relatives for each adult were also averaged over all years. In addition, the average pairwise relatedness of adults within each matriline (see below) was calculated.

Mitochondrial DNA Amplification, Sequencing, and Matrilines

A portion of the mitochondrial DNA control region was PCR-amplified using the primers L16517 5'-CATCTGGTTCTTACTTCAGG-3' (Fumagalli et al., 1996) and sH651 5'-AAGGCTAGGACCAAACCT-3' (Castella et al., 2001), which is a shorter version of the primer H00641 (Kocher et al., 1989). These primers amplify the second hypervariable domain (HVII).

For each adult, 2 µl of extracted genomic DNA was quantified with a ND-1000 Spectrometer (NanoDrop Technologies, Wilmington, DE) and diluted with sterile double distilled water to a concentration of one ng/µl to standardize the template DNA concentrations for all samples. PCR amplifications were in a total volume of 25 µl and contained 12.5 ng of diluted DNA (12.5 µl), 1 X PCR buffer (Promega), 1.0 µM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPS and 1 unit of Taq (Promega). PCR cycling conditions were 94^{o}C for 3 min and then 30 cycles of 94^{o}C for 1 min, 54^{o}C for 1 min, and 72^{o}C for 1.5 min. For each sample, 5 PCR products (total volume = 125 µl) were run out in a 1% agarose gel. The bands for HVII were approximately 1000 to 1200 bp in length. The desired bands were cut out of the gel, combined, and purified using an IsoPure™ Gel Extraction Prep Kit (Denville Scientific Inc., Metuchen, NJ). Purified DNA from the gel extraction was quantified and diluted to 10 ng/µl. If the concentration of the purified DNA was less than 10 ng/µl, then 1-3 µl of the purified PCR product was diluted in 99 to 97 µl of sterile double distilled water. The diluted purified DNA was used (instead of diluted template DNA) to PCR-amplify 5 more reactions using the procedure described above to achieve the desired concentration (at least 10 ng/µl).

Sequencing was done using a MegaBACE® 500 sequencer and an ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (GE Healthcare). The sequencing reaction was in total volume of 20 µl with 10 µl of purified DNA (100 ng total), 8 µl of sequencing mix, and 2 µl of primer (2.5 uM) as recommended by the manufacturer. Cycling conditions were 25 cycles of 95^{o}C for 20 s, 50^{o}C for 15 s, and 60^{o}C for 1 min. Ethanol precipitation was used for post-reaction

cleanup. Both forward and reverse sequences were determined for each sample. However, the primers used for PCR-amplification amplify a 1000 to 1200 bp segment of DNA, which is largely a 6-bp repeating region after the first 300 bp (as in Castella et al., 2001; Fumagalli et al., 1996). A reverse primer was designed (5'-ATGCGTATGTCCTGAGACCA -3') to sequence the first 300 bp before the repeat region in both orientations. I used L16517 as the forward primer.

For each sample, forward and reverse sequences were aligned in BioEdit (Hall, 1999) using the ClustalW multiple alignment feature (Thompson et al., 1994). Discrepancies between forward and reverse sequences were resolved by manually comparing chromatograms in BioEdit. If discrepancies were not resolved, another forward and reverse sequence was amplified and sequenced. After every sample was corrected, all sequences were aligned. Any nucleotide differences (insertions, deletions, or substitutions) that occurred in only one sequence were manually checked in the reverse and forward chromatograms to ensure accuracy. Individual bats were considered to be from the same matriline if they shared the same sequence (haplotype) whereas bats with different sequences (haplotypes) were considered to be from different matrilines.

Statistical Analyses

Objective 1: Roosting Associations and Relatedness

I used a Mantel test to evaluate the correlation between a matrix of PSI values and a matrix of pairwise relatedness in Excel with the aid of XLSTAT v 2006.3 (Addinsoft™; available at www.xlstat.com/en/home). The strength of the correlation was measured with Pearson's correlation coefficient (r) and the statistical significance of r was assessed by a permutation test with 10,000 randomizations. I used an upper-tailed

test to determine whether there was a positive correlation between PSI and pairwise relatedness.

Roost-tree trapping events resulted in the capture of a sample of bats that were roosting in the same tree. A sample of bats caught while exiting the same roost is henceforth called a "trapping group". Young of the year captured in a trapping group were excluded from analyses because relatedness between young and mothers would inflate the average pairwise relatedness of adults in the trapping group. Average pairwise relatedness was obtained by averaging all the pairwise relatedness estimates between individuals within the trapping group.

Randomizations were used to calculate the statistical significance of the average pairwise relatedness of each trapping group. A sample of bats containing the same number of bats observed in the trapping group was randomly selected from all the adult females from RA1. The average pairwise relatedness between the randomly selected individuals was calculated. Randomizations were performed 1000 times for each trapping group in Excel with the aid of a macro programmed in Microsoft Visual Basic (written by Olav Rüppell, see Appendix B). If the observed average pairwise relatedness of the trapping group was distributed in the highest 5% of randomly generated values, then the observed value was considered statistically significant at the 5% level.

Objective 2: Roosting Associations and Matrilines

I used randomizations to determine if the average pairwise relatedness of bats within the same matriline was higher than expected based on random chance to evaluate the background relatedness of adult females belonging to the same matriline. Randomizations (n = 1000) were done in Excel with the aid of macro programmed in

Microsoft Visual Basic (written by Olav Rüppell, see Appendix B). For each randomization, a random sample of individuals, of the same size observed within the matriline, was selected, and the average pairwise relatedness was calculated. The observed value was compared to the randomly generated distribution, and observed values within the highest 5% of randomly generated values were considered statistically significant.

Each pair of bats with a PSI was assigned to one of two groups based on whether the pair shared the same matriline or came from different matrilines. The mean PSI for each group and the difference in mean PSI between groups was calculated with Statistica 7. Randomizations were used to evaluate the statistical significance of the difference in mean PSI between pairs within the same matriline and pairs that came from different matrilines. In each randomization, all pairs of bats with a PSI were pooled together regardless of matriline and randomly assigned without replacement into two groups. Randomly generated groups were limited to the same number of pairs observed for the actual groups (pairs from the same matriline and pairs with different matrilines). For every randomization, the difference in mean PSI between the two randomly generated groups was calculated. Randomizations were repeated 1000 times using the Resampling Software Excel add-in v 3.2 (available at www.resample.com), and a distribution of the difference in means between the two groups was generated. A difference in mean PSI calculated for the observed groups in the highest 5% of the randomly generated differences in mean PSIs was considered statistically significant at the 5% level.

The distribution of matrilines within each trapping group was determined and compared to the expected distribution of matrilines using a chi-squared test. Expected values were calculated by multiplying the frequency of each matriline within RA1 by the trapping group sample size.

Results

Samples

I collected and genotyped tissue samples from 48 adult females in RA1. In 2002, not all tissue samples were available, but during 2003-2005, the genotyped sample represents all the adult females from RA1. In addition, 41 juveniles were collected and genotyped. The loci were polymorphic with 4 to 21 alleles per locus, with high expected and observed heterozygosity based on calculations from female adults (n = 48) in RA1 during 2002-2005 (Table 12). In order to calculate background allele frequencies for the relatedness estimator, additional adults were also sampled from nearby roosting areas (n = 22) that were within four km of RA1. The number of adults in RA1 fluctuated between years, ranging from 26 to 32 adults (n = 32 in 2002 (includes only adults with tissue samples); n = 26 in 2003; n = 30 in 2004; n = 29 in 2005). Three juveniles returned as adults in subsequent years. During 2003-2005, 15% (3 of 20) of the captured juvenile females returned to RA1 after their first winter. Furthermore, 13 adult females joined RA1. The matrilines of the immigrant females were consistent with matrilines from RA1 but were also consist with matrilines from either RA2 (n = 8) or RA3 (n = 5). Using genotypes from the nine microsatellite loci, 38.5% (5/13) of adult immigrants were assigned to putative mothers in RA1, 46.2% (6/13) were not assigned to putative mothers

in RA1 and one was assigned to a putative mother in RA2, and 15.4% (2/13) were not resolved because immigrants mismatched at only one locus with a RA1 putative mother.

At the end of 2004 and in 2005, individuals from RA1 expanded their roosting area to another spatially distinct site that was not used in previous years. I refer to these individuals as belonging to RA1, even though they expanded their roosting area beyond the boundaries given in Willis and Brigham (2004).

Roosting Associations

Pairwise Sharing Index

A PSI value was available for 36 pairs of bats based on data from the summers of 2000-2002 (Willis and Brigham, 2004). The 36 pairs consisted of combinations of 17 different individuals. The PSI values ranged from -0.01 to 0.75 with an average of 0.38.

Trapping Group

For my analyses, 20 trapping groups were used (n = 3 in 2002; n = 6 in 2003; n = 7 in 2004; n = 4 in 2005). Trapping groups ranged in size from 4 to 21 adults (mean = 10.75). Bats were trapped in the study area about every two weeks during June, July and August. Due to logistic constraints, only one tree can be trapped during each trapping event, which means that only six to seven trapping events occur during a year. In 2002 and 2005, RA1 was not as intensively trapped because bats from other roosting areas were also being trapped. In addition, during some trapping events several bats escaped and those trapping events were not included in the analyses.

Genetic Relationships

Relatedness

Background allele frequencies were calculated using all adults from the study area (n = 68, this does not include the three juveniles that returned as adults). The background

allele frequencies calculated from only adults were not the same as the allele frequencies for adults and juveniles combined (data not shown). Juveniles had 15 unique alleles that adults did not share. These alleles occurred at the seven most polymorphic loci (not BE22 and TT20). To calculate relatedness, the unique juvenile alleles were assigned a frequency of 0.00. Pairwise relatedness was calculated for all 116 genotyped bats and a pairwise relatedness matrix for all individuals was generated using the program Relatedness 5.0.8. The average pairwise relatedness of adults within RA1 from 2002-2005 was -0.01. Adults in RA1 had an average of 1.84 (range 0-9) closely related ($r \geq 0.25$) adults within the roosting area.

Mother-offspring pairs were identified using Cervus. Of the 41 juveniles from RA1, 18 were assigned to a candidate mother at the 95% confidence level with no mismatching loci. The putative mother-juvenile pairs had an average pairwise relatedness of 0.49, which was nearly identical to the expected value of 0.5. This provides evidence that the relatedness estimator was not underestimating relatedness.

Matrilines

All samples from RA1 female adults (n = 48; including the three juveniles that returned as adults) were sequenced. The sequences were 273 or 274 base pairs in length with 20 variable sites that resulted in six unique haplotypes or matrilines (Table 13). As part of another study (see CHAPTER II) matrilines from the entire study area have been determined and named. To avoid confusion, the numbers already assigned for the matrilines within RA1 were used (i.e. H17 in CHAPTER II is identical to M17). Variable sites were the result of 19 transitions and one insertion. All six matrilines were present in RA1 each year, but the number of individuals within each matriline differed among years

(Table 14 and Table 15). The relatedness of bats with the same matriline ranged from 0.08 to 0.49 with an average of 0.14 (Table 16).

Objective 1: Roosting Associations and Relatedness

There was no relationship between PSI and relatedness (Mantel test, $r = 0.012$, p-value = 0.437; Figure 3). The average pairwise relatedness of roost-mates within each trapping event was low (r = -0.01) with a range from -0.11 to 0.07 (Table 17). Of the 20 trapping groups, two had a significantly higher average pairwise relatedness than expected with random selection (Table 17). The two trapping groups (TG18 and TG19) with significantly higher average pairwise relatedness were both from 2005 in the expanded roosting area.

Objective 2: Roosting Associations and Matrilines

The pairs of bats with a PSI were separated into groups based on whether the pair had the same (n = 10 pairs) or a different (n = 26 pairs) matriline. The mean PSI for the group with the same mitochondrial DNA matriline was 0.428 (standard error = 0.208) which was not significantly higher (randomization, p-value = 0.210) than the mean PSI for the group with different mitochondrial DNA haplotypes (mean = 0.360, standard error = 0.191; Figure 4). Matrilines were distributed randomly within each trapping group (average χ^2=0.98, d.f. = 5, average p-value = 0.98; Table 18).

Discussion

Although related individuals would potentially gain indirect fitness benefits from roosting together, I found no evidence that relatedness influenced roosting associations in tree-roosting *E. fuscus*. Relatedness was not correlated with nonrandom roosting associations between pairs. Likewise, the average pairwise relatedness of most trapping

groups was not greater than expected with random roost-mate selection, which suggested that roost-mates were not preferentially roosting with kin. These results provide evidence that individual *E. fuscus* in the Cypress Hills are not using relatedness to select roost-mates.

Roosting associations were not influenced by matrilineal relationships. Relatedness differed from matrilineal relationships because bats within the same matriline had a range of relatedness values and may or may not have been closely related. There was no difference between the mean PSI for pairs of *E. fuscus* from the same matriline and pairs from different matrilines. This indicates that *E. fuscus* have nonrandom roosting associations with both matrilineal females and non-matrilineal females. Roost-mates from trapping events were composed of several matrilines. Individuals had a tendency to roost with bats of the same matriline, although this was not more often than expected when compared to a random distribution of matrilines. These roosting patterns suggest that *E. fuscus* do not preferentially roost with maternal kin. Overall, my results indicate that *E. fuscus* are not preferentially roosting with closely related kin or with individuals from the same matriline.

Previous studies have also demonstrated that relatedness and matrilineal relationships are not correlated with most associations and cooperative behaviors in bats. Like *E. fuscus*, *M. bechsteinii* are also female philopatric, naturally tree-roosting bats with a fission-fusion system of roosting that have nonrandom roosting associations between pairs of bats. These nonrandom roosting associations are not explained by relatedness or matrilineal relationships (Kerth and Konig, 1999). Within a group of *P.*

auritus, females and males are both philopatric, and bats are distributed in clusters throughout building roost sites. Bats within a cluster are in physical contact with one another, but the average relatedness within each cluster is low ($r < 0.25$; Burland et al., 2001). In addition, *M. bechsteinii* are known to transfer information about novel roosts to other roost-mates, but this transfer is also not influenced by the relatedness of the participants (Kerth and Reckardt, 2003). This is also the case for information transfer about foraging and roost locations in the evening bat (*N. humeralis*) (Wilkinson, 1992b). Within *N. humeralis* roosts, females occasionally nurse pups that are not their own, although females do not prefer to nurse pups of related females or pups of females from the same matriline (Wilkinson, 1992a). Both roosting and foraging subgroups are not correlated with relatedness in a more distantly related species, *P. hastatus* (McCracken and Bradbury, 1977; Wilkinson and Boughman, 1998).

In other group living mammals, relatedness may sometimes explain associations and cooperative behaviors. In chimpanzees (*Pan troglodytes*), males affiliate with and cooperate with each other (on hunts and boundary patrols), but these behaviors are not correlated with relatedness (Mitani et al., 2000). Likewise, the composition of humpback whales that travel together (*Megaptera novaeangliae*) are not correlated with relatedness (Valsecchi et al., 2002). Red howler monkeys (*Alouatta seniculus*) have a unique system because unrelated extra-group females form long-term associations (approximately two years) while trying to defend a territory and begin breeding (Pope, 2000). Like *E. fuscus*, unrelated female monkeys form long-term associations, but unlike *E. fuscus*, when

individuals in a group began to breed successfully, one matriline becomes dominant within the group until all breeders have the same matriline.

In contrast to *E. fuscus*, relatedness and matrilineal relationships are important for other group living mammals who employ fission-fusion behavior. For example, in groups of spotted hyenas (*Crocuta crocuta*) with fission-fusion behavior, both males and females are able to distinguish kin from non-kin, and preferentially affiliate and cooperate with kin (e.g. during hunts) (Van Horn et al., 2004; Wahaj et al., 2004). Kin selection explains individual choices in associations and cooperative behaviors, but the evolution and stability of the entire clan, which consists of about 60 philopatric females (1-7 matrilines), males, and offspring, is more likely explained by strong direct fitness benefits accrued by individuals through group living (Van Horn et al., 2004). Likewise, female African elephants (*Loxodonta africana*) conform to a fission-fusion model of interacting where close associations are almost always between mother-daughter or maternal sibling pairs (Archie et al., 2006). Unlike for *C. crocuta*, core social groups of *L. africana* consist of only one matriline (Archie et al., 2006). Matrilineal relationships are important for the permanent fission of core social groups and also in long-term fidelity to bond groups (Archie et al., 2006). Bond groups are large aggregations of several core social groups and often form when newborn calves are vulnerable to predators (Archie et al., 2006). In another mammal which uses fission-fusion behavior, the African lion (*Panthera leo*), group size is smaller, typically with three to seven breeding females (Packer et al., 2001). Within a pride, females are always close genetic relatives and benefit from group territory defense and communally breeding with relatives. Within all three African mammals, *C.*

crocuta, L. africana, and *P. leo*, group members benefit from both close individual associations and membership in a larger group which facilitate group stability and cooperation.

These examples make clear the importance of associating with close kin in social groups varies among different species. The particular life history characteristics of a given species likely determine the processes and thus selection pressures that lead to group stability and cooperation. Bats have a unique life history among mammals (Barclay and Harder, 2003). Unlike other similarly sized mammals, bats, as a whole, reproduce slowly and live long lives. This unique life history is probably due to the morphological, anatomical, and physiological adaptations associated with flight (Barclay and Harder, 2003; Jones and MacLarnon, 2001; Maurer et al., 2004).

Physiological constraints may be particularly influential in shaping the roosting behavior of bats. Both the reproductive condition (reproductive or non-reproductive) and the reproductive period (pregnancy, lactation, post-lactation) have been postulated to influence the roosting behavior of *E. fuscus*. During late pregnancy, big brown bats use fewer multiple hole cavities relative to early pregnancy and lactation, and *E. fuscus* roost with a greater number of different individuals during late pregnancy (Willis and Brigham, 2004; Willis et al., 2006). There is also evidence that reproductive condition accounted for some of the nonrandom roosting associations within RA1 (Willis and Brigham, 2004). Kerth and König (1999) found a similar effect of reproductive condition on the roosting associations of *M. bechsteinii*. Since roosting behavior changes with reproductive condition and during different reproductive periods, the physiological condition of *E.*

fuscus and/or the ambient temperature might constrain roosting behavior and shape the context in which individual associations, cooperation, and group stability evolve.

In addition to physiological constraints, other life history characteristics might shape group living and cooperation in *E. fuscus*. Like other species of bats, *E. fuscus* has a long lifespan (19 years, maximum observed in the wild) and reproduces slowly (Kurta and Baker, 1990). Litter size in *E. fuscus* is only one or two pups per year (Kurta and Baker, 1990). Within the study area, females do not reproduce every year, juvenile mortality is high, and only females are philopatric. In RA1, the group contains both unrelated and related members. The average relatedness of all adults is low, and within RA1, *E. fuscus* associate with and have the opportunity to roost with only a few closely related bats (average ≈ 2, range 0 to 9). I found that relatedness does not explain roosting associations, and consequently, kin selection is unlikely to occur within roost sites. Kin selection is probably not an important selection pressure because associating preferentially with kin might not provide large indirect benefits given the low reproductive rate of individuals, low juvenile return to the roosting area (15%), small litter size, and the low number of closely related females (average ≈ 2).

In addition, if the size of the subgroups formed during fission events are important then *E. fuscus* has a trade-off between the size of the subgroup and the kin composition of the subgroup because as subgroup size increases, subgroup relatedness decreases (this study; modeled and reviewed in: Aviles et al., 2004; Lukas et al., 2005). In the trapping group randomizations, the highest average pairwise relatedness value decreased as the group size increased. For example, with a group size of four, the highest randomly

generated average pairwise relatedness was 0.32 compared to 0.05 for a group size of 21. Group size is likely an important influence on *E. fuscus* roosting behavior because as group size increases, energy expenditure likely decreases (Willis and Brigham, submitted).

Given the life history characteristics of *E. fuscus* and that roosting associations between individuals are not explained by relatedness or matrilineal relationships, group living and cooperation is more likely to have evolved due to reciprocity and/or group augmentation. If close associations with other members increase fitness, then it will be advantageous to form long lasting associations with available females regardless of relatedness (similar to female extra-groups of *A. seniculus*). In particular, non-reproductive *E. fuscus* roost with reproductive bats throughout the summer and have the opportunity to participate in cooperative behaviors that may be energetically expensive for reproductive roost-mates. Alternatively, non-reproductive females might learn skills (e.g. parenting) from associating with reproductive ones. Reciprocity might also be an important factor because bats have preferred roost-mates, and bats with higher associations have more opportunities to reciprocate cooperative behaviors and punish cheaters. In group augmentation, helping other group members may maintain or increase the size of the group which is beneficial to the helper as well as the recipient (Kokko et al., 2001). For bats, group size is important for thermoregulation and potentially for behavioral associations and interactions (e.g. allo-nursing, allogrooming, and information transfer). Reciprocity and group augmentation are not necessarily exclusive of one another, and both might be important for *E. fuscus* given that females do have preferred

roost-mates (Willis and Brigham, 2004) and increasing group size likely benefits all group members (Willis and Brigham, submitted).

CHAPTER IV

GENERAL CONCLUSION

I used a fission-fusion system of tree-roosting *E. fuscus* from Cypress Hills to investigate mating and dispersal behavior and to investigate the influence of genetic relationships on roost-mate decisions. First, I investigated the genetic subdivision among roosting areas to infer mating and dispersal behavior of *E. fuscus* (CHAPTER II). I evaluated genetic subdivision between RA1 and RA2 using both biparentally inherited microsatellite loci and maternally inherited mitochondrial DNA sequences. Second, I examined roosting associations within one roosting area to determine whether roost-mate decisions were based on relatedness (microsatellite loci) and/or matrilineal relationships (matrilines; CHAPTER III). I used roosting associations from pairs of bats (PSI; Willis and Brigham, 2004) and groups of roost-mates (roost-tree trapping data). I determined if roosting associations based on pairs of bats and groups of roost-mates varied with relatedness or matrilineal relationships.

In CHAPTER II, I found that female-mediated gene flow was restricted between RA1 and RA2, while biparentally-mediated gene flow was not. The maternal genetic subdivision was likely caused by female philopatry, while male-mediated gene flow, which likely occurs at fall swarming or hibernation sites, prevented nuclear genetic

subdivision. I also found that these two roosting areas were likely founded by two ancestral lineages. In CHAPTER III, I found that relatedness and matrilineal relationships did not influence roosting associations between pairs of bats or groups of roost-mates. Female *E. fuscus* were not preferentially roosting with closely related kin or with individuals from the same matriline. Together, my results suggest that mating and dispersal behavior decrease overall relatedness among females within roosting areas despite female philopatry and reduce the importance of kin selection as an evolutionary force in group stability and cooperation in tree-roosting *E. fuscus*.

Mating and dispersal behavior impact the kin composition of social groups (reviewed by Ross, 2001; Storz, 1999). In my study area, male-mediated gene flow suggests males mate with females that are not from their natal roosting areas. This male mating behavior prevents nuclear genetic subdivision between adjacent roosting areas and decreases the overall relatedness of adult females within each roosting area. Male-mediated gene flow might also prevent high levels of relatedness within a matriline because matrilineal females mate with multiple unrelated males. Furthermore, female-mediated gene flow is not completely restricted between the two roosting areas as indicated by Φ_{st} value, immigrant genotypes, and behavioral observations of one bat switching roosting areas. Female immigrants reduce both maternal and nuclear genetic subdivision between roosting areas and decrease the overall relatedness of adult females within roosting areas. In addition to these mating and dispersal behaviors, the slow reproductive rate of females (one to two pups per year) and high juvenile mortality could decrease the relatedness of females within a roosting area (Wilkinson, 1985b).

My results from CHAPTER II indicate that females from the two roosting areas in Cypress Hills were founded by at least two ancestral lineages. Male-mediated gene flow appears to prevent nuclear genetic subdivision between roosting areas of philopatric females. Comparison with a similar bat species, *M. bechsteinii*, suggests that genetic subdivision in *E. fuscus* might be prevented due to a generalist, aerial foraging strategy and/or the capability of long distance flight. Additional studies of mating behavior, genetic subdivision, and genetic variation over a larger geographic region are necessary to understand the historical (e.g. colonization and isolation events) and current processes (dispersal, social group formation, and mating behaviors) that generate the genetic variability and subdivision observed in tree-roosting *E. fuscus*. Additionally, studying the genetic subdivision of tree-roosting *E. fuscus* in a natural forest environment will facilitate comparisons with future studies in managed forests to clarify the impact of forest management on the genetic structure of tree-roosting bats.

The inferred mating and dispersal behavior in combination with a slow reproductive rate and high juvenile mortality suggests that high levels of relatedness are not likely to accumulate within roosting areas (Burland and Worthington Wilmer, 2001; Ross, 2001; Storz, 1999; Wilkinson, 1985b). Within RA1, the average relatedness is low and each adult female has approximately two (range 0-9) closely related group members ($r \geq 0.25$). Females did not prefer to roost with related or matrilineal group members. Given that most females have on average less then two closely related group members, there is a trade-off between subgroup size and relatedness because as subgroup size increases, relatedness decreases.

The lack of kin within the roosting area likely decreases the importance of kin selection as a selection pressure for the stability of group living. Females are not likely to accrue large indirect fitness benefits by preferentially associating and cooperating with related individuals because of high juvenile mortality, the low reproductive rate, and less than two closely related group members within the roosting area. Instead, individuals might be more successful by associating and cooperating with any available female regardless of genetic relationships. An individual might be motivated to associate and/or cooperate with group members because a larger group size is beneficial to the individual (group augmentation; Kokko et al., 2001). A larger group size might be important for *E. fuscus* due to the costs of thermoregulation (Willis and Brigham, submitted) or cumulative knowledge about roost and foraging sites (Kerth et al., 2000; Kerth et al., 2001; Willis et al., 2006). However, group augmentation does not explain preferred roost-mates. Reciprocity might lead to preferred roost-mates because individuals that frequently roost together have more opportunities to reciprocate cooperative behaviors (Axelrod and Hamilton, 1981; Clutton-Brock, 2002; Wilkinson, 1988). Group living in tree-roosting *E. fuscus* is likely due to group augmentation and/or reciprocity rather than kin selection.

LITERATURE CITED

Agosta SJ, 2002. Habitat use, diet and roost selection by the big brown bat (*Eptesicus fuscus*) in North America: a case for conserving an abundant species. Mammal Review 32:179-198.

Aldridge HDJN, Brigham R, 1988. Load carrying and maneuverability in an insectivorous bat-a test of the 5-percent rule of radio-telemetry. Journal of Mammalogy 69:379-382.

Anthony EL, 1988. Age determination in bats. In: Ecological and behavioral methods for the study of bats (Kunz TH, ed). Washington, D.C.: Smithsonian Institution Press.

Arbuthnott D, Brigham RM, submitted. The influence of a local temperature inversion on the foraging behaviour of big brown bats, *Eptesicus fuscus*. Acta Chiropterologica.

Archie EA, Moss CJ, Alberts SC, 2006. The ties that bind: genetic relatedness predicts the fission and fusion of social groups in wild African elephants. Proceedings of the Royal Society Biological Sciences Series B 273:513-522.

Aviles L, Fletcher JA, Cutter AD, 2004. The kin composition of social groups: Trading group size for degree of altruism. American Naturalist 164:132-144.

Axelrod R, Hamilton W, 1981. The evolution of cooperation. Science (Washington D C) 211:1390-1396.

Barbour RW, Davis WH, 1969. Bats of America. Lexington: University Press Kentucky.

Barclay RMR, Harder LD, 2003. Life histories of bats: life in the slow lane. In: Bat Ecology (Kunz TH, Fenton BM, eds). Chicago: The University of Chicago Press.

Burland TM, Barratt EM, Beaumont MA, Racey PA, 1999. Population genetic structure and gene flow in a gleaning bat, *Plecotus auritus*. Proceedings of the Royal Society Biological Sciences Series B 266:975-980.

Burland TM, Barratt EM, Nichols RA, Racey PA, 2001. Mating patterns, relatedness and the basis of natal philopatry in the brown long-eared bat, *Plecotus auritus*. Molecular Ecology 10:1309-1321.

Burland TM, Worthington Wilmer J, 2001. Seeing in the dark: molecular approaches to the study of bat populations. Biological Reviews 76:389-409.

Castella V, Ruedi M, 2000. Characterization of highly variable microsatellite loci in the bat *Myotis myotis* (Chiroptera: Vespertilionidae). Molecular Ecology 9:1000-1002.

Castella V, Ruedi M, Excoffier L, 2001. Contrasted patterns of mitochondrial and nuclear structure among nursery colonies of the bat *Myotis myotis*. Journal of Evolutionary Biology 14:708-720.

Clement M, Posada D, Crandall K, 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657-1660.

Clutton-Brock T, 2002. Breeding together: Kin selection and mutualism in cooperative vertebrates. Science (Washington D C) 296:69-72.

Dieringer D, Schloetter C, 2003. Microsatellite analyzer (MSA): A platform independent analysis tool for large microsatellite data sets. Molecular Ecology Notes 3:167-169.

Eberle M, Kappeler PM, 2006. Family insurance: kin selection and cooperative breeding in a soliatry primate *(Microcebus murinus)*. Behavioral Ecology and Sociobiology 60:582-588.

Entwistle AC, Racey PA, Speakman JR, 2000. Social and population structure of a gleaning bat, *Plecotus auritus*. Journal of the Zoological Society of London 252:11-17.

Fenton BM, Bogdanowicz W, 2002. Relationships between external morphology and foraging behavior: bats in the genus *Myotis*. Canadian Journal of Zoology 80:1004-1013.

Fumagalli L, Taberlet P, Favre L, Hausser J, 1996. Origin and evolution of homologous repeated sequences in the mitochondrial DNA control region of shrews. Molecular Biology and Evolution 13:31-46.

Goudet J, Raymond M, De Meeues T, Rousset F, 1996. Testing differentiation in diploid populations. Genetics 144:1933-1940.

Griffin AS, West SA, 2002. Kin selection: Fact and fiction. Trends in Ecology and Evolution 17:15-21.

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95-98.

Hedrick P, 1994. Purging inbreeding depression and the probability of extinction: full-sib mating. Heredity 73:363-372.

Jones G, Duverge PL, Ransome RD, 1995. Conservation biology of an endangered species: field studies of greater horseshoe bats. Symposia of the Zoological Society of London 67:309-324.

Jones KE, MacLarnon A, 2001. Bat life histories: testing models of mammalian life-history evolution. Evolutionary Ecology Research 3:465-476.

Kalcounis MC, Brigham RM, 1994. Impact of predation risk on emergence by little brown bats, *Myotis lucifugus* (Chiroptera, Vespertilionidae), from a maternity colony. Ethology 98:201-209.

Kalcounis MC, Brigham RM, 1998. Secondary use of aspen cavities by tree-roosting big brown bats. Journal of Wildlife Management 62:603-611.

Kerth G, Kiefer A, Trappmann C, Weishaar M, 2003. High gene diversity at swarming sites suggest hot spots for gene flow in the endangered Bechstein's bat. Conservation Genetics 4:491-499.

Kerth G, Konig B, 1999. Fission, fusion and nonrandom associations in female Bechstein's bats *(Myotis bechsteinii)*. Behaviour 136:1187-1202.

Kerth G, Mayer F, Konig B, 2000. Mitochondrial DNA (mtDNA) reveals that female Bechstein's bats live in closed societies. Molecular Ecology 9:793-800.

Kerth G, Mayer F, Petit E, 2002a. Extreme sex-biased dispersal in the communally breeding, nonmigratory Bechstein's bat *(Myotis bechsteinii)*. Molecular Ecology 11:1491-1498.

Kerth G, Morf L, 2004. Behavioural and genetic data suggest that Bechstein's bats predominantly mate outside the breeding habitat. Ethology 110:987-999.

Kerth G, Reckardt K, 2003. Information transfer about roosts in female Bechstein's bats: An experimental field study. Proceedings of the Royal Society Biological Sciences Series B 270:511-515.

Kerth G, Safi K, Konig B, 2002b. Mean colony relatedness is a poor predictor of colony structure and female philopatry in the communally breeding Bechstein's bat *(Myotis bechsteinii)*. Behavioral Ecology and Sociobiology 52:203-210.

Kerth G, Wagner M, Konig B, 2001. Roosting together, foraging apart: Information transfer about food is unlikely to explain sociality in female Bechstein's bats *(Myotis bechsteinii)*. Behavioral Ecology and Sociobiology 50:283-291.

Kocher TD, Thomas WK, Meyer SA, Edwards SV, Paabo S, Villablanca FX, Wilson AC, 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and

sequencing with conserved primers. Proceedings of the National Academy of Sciences of the United States of America 86:6196-6200.

Kokko H, Johnstone RA, Clutton-Brock TH, 2001. The evolution of cooperative breeding through group augmentation. Proceedings of the Royal Society Biological Sciences Series B 268:187-196.

Krakauer AH, 2005. Kin selection and cooperative courtship in wild turkeys. Nature (London) 434:69-72.

Kunz TH, 1988. Ecological and behavioral methods for the study of bats. Washington, D.C.: Smithsonian Institution Press.

Kunz TH, Lumsden LF, 2003. Ecology of cavity and foliage roosting bats. In: Bat Ecology (Kunz TH, Fenton BM, eds). Chicago: The University of Chicago Press.

Kurta A, Baker R, 1990. Mammalian Species: *Eptesicus fuscus*. The American Society of Mammalogists 356:1-10.

Lukas D, Reynolds V, Boesch C, Vigilant L, 2005. To what extent does living in a group mean living with kin? Molecular Ecology 14:2181-2196.

Marshall T, Slate J, Kruuk L, Pemberton JM, 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7:639-655.

Maurer BA, Brown JH, Dayan T, Enquist BJ, Ernest SKM, Hadly EA, Haskell JP, Jablonski D, Jones KE, Kaufman DM, Lyons SK, Niklas KJ, Porter WP, Roy K, Smith FA, Tiffney B, Willig MR, 2004. Similarities in body size distributions of small-bodied flying vertebrates. Evolutionary Ecology Research 6:783-797.

McCracken G, Wilkinson G, 2000. Bat mating systems. In: Reproductive Biology of Bats (Krutzsch P, Creighton E, eds). New York: Academic Press; 321-362.

McCracken GF, Bradbury JW, 1977. Paternity and genetic heterogeneity in the polygynous bat *Phyllostomus hastatus*. Science (Washington D C) 198:303-306.

Miller-Butterworth CM, Jacobs DS, Harley EH, 2003. Strong population substructure is correlated with morphology and ecology in a migratory bat. Nature (London) 424:187-191.

Mitani JC, Merriwether DA, Zhang C, 2000. Male affiliation, cooperation and kinship in wild chimpanzees. Animal Behaviour 59:885-893.

Mumford R, 1958. Population turnover in wintering bats in Indiana. Journal of Mammalogy 39:153-161.

Neubaum DJ, O'Shea TJ, Wilson KR, 2006. Autumn migration and selection of rock crevices as hibernacula by big brown bats in Colorado. Journal of Mammalogy 87:270-479.

Norberg UM, Rayner JM, 1987. Ecological morphology and flight in bats (Mammalia; Chiroptera): wing adaptations, flight performance, foraging strategy and echolocation. Philosophical Transactions of the Royal Society of London B Biological Sciences 316:335-427.

O'Donnell CFJ, Sedgeley JA, 1999. Use of roosts by the long-tailed bat, *Chalinolobus tuberculatus*, in temperate rainforest in New Zealand. Journal of Mammalogy 80:913-923.

Packer C, Pusey AE, Eberly LE, 2001. Egalitarianism in female African lions. Science (Washington D C) 293:690-693.

Pen I, Kerth G, 2005. Mate choice: female relatives share sexual partners in bats. Current Biology 15:927-929.

Petit E, Balloux F, Goudet J, 2001. Sex-biased dispersal in a migratory bat: A characterization using sex-specific demographic parameters. In: Evolution; 635-640.

Petit E, Mayer F, 1999. Male dispersal in the noctule bat (*Nyctalus noctula*): Where are the limits? Proceedings of the Royal Society Biological Sciences Series B 266:1717-1722.

Phillips GL, 1966. Ecology of the big brown bat (Chiroptera: Vespertilionidae) in northeastern Kansas. The American Midland Naturalist 78:251-252.

Pope TR, 2000. Reproductive success increases with degree of kinship in cooperative coalitions of female red howler monkeys (*Alouatta seniculus*). Behavioral Ecology and Sociobiology 48:253-267.

Queller DC, Goodnight KF, 1989. Estimating relatedness using genetic markers. Evolution 43:258-275.

Racey P, Swift S, 1981. Variation in gestation length in a colony of pipistrelle bats (*Pipistrellus pipistrellus*) from year to year. Journal of Reproduction and Fertility 61:123-129.

Ransome R, 1990. The Natural History of Hibernating Bats. London: Christopher Helm.

Rivers NM, Butlin RK, Altringham JD, 2005. Genetic population structure of Natterer's bats explained by mating at swarming sites and philopatry. Molecular Ecology 14:4299-4312.

Ross KG, 2001. Molecular ecology of social behaviour: analyses of breeding systems and genetic structure. Molecular Ecology 10:265-284.

Rossiter SJ, Jones G, Ransome RD, Barratt EM, 2000a. Genetic variation and population structure in the endangered greater horseshoe bat *Rhinolophus ferrumequinum*. Molecular Ecology 9:1131-1135.

Rossiter SJ, Jones G, Ransome RD, Barratt EM, 2000b. Parentage, reproductive success and breeding behaviour in the greater horseshoe bat (*Rhinolophus ferrumequinum*). Proceedings of the Royal Society Biological Sciences Series B 267:545-551.

Rossiter SJ, Jones G, Ransome RD, Barratt EM, 2001. Outbreeding increases offspring survival in wild greater horseshoe bats (*Rhinolophus ferrumequinum*). Proceedings of the Royal Society Biological Sciences Series B 268:1055-1061.

Rossiter SJ, Jones G, Ransome RD, Barratt EM, 2002. Relatedness structure and kin-biased foraging in the greater horseshoe bat (*Rhinolophus ferrumequinum*). Behavioral Ecology and Sociobiology 51:510-518.

Rossiter SJ, Ransome R, Faulkes CG, Dawson DA, Jones G, 2006. Long-term paternity skew and the oppurtunity for selection in a mammal with reversed sexual size dimorphism. Molecular Ecology 15:3035-3043.

Rossiter SJ, Ransome RD, Faulkes CG, Le Comber SC, Jones G, 2005. Mate fidelity and intra-lineage polygyny in greater horseshoe bats. Nature (London) 437:408-411.

Russell AL, Medellin RA, McCracken GF, 2005. Genetic variation and migration in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*). Molecular Ecology 14:2207-2222.

Safi K, Kerth G, 2003. Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. Animal Behaviour 65:363-369.

Sauchyn D, 1993. Quaternary and late tertiary landscape evolution in the western Cypress Hills. In: Quaternary and late tertiary landscapes of southwestern Saskatchewan and adjacent areas (Sauchyn D, ed). Regina: Canadian Plains Research Centre; 46-58.

Schneider S, Roessli D, Excoffier L, 2000. Arlequin ver. 2.000: A software for poulation genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Swizerland.

Schober W, Grimmberger E, 1998. Die Fledermäuse Europas: kennen — bestimmen — schützen. 2nd edn, Franckh-Kosmos-Verlag, Stuttgart.

Speakman JR, Irwin N, Tallach N, Stone R, 1999. Effect of roost size on the emergence behaviour of pipistrelle bats. Animal Behaviour 58:787-795.

Storz JF, 1999. Genetic consequences of mammalian social structure. Journal of Mammalogy 80:553-569.

Storz JF, Bhat HR, Kunz TH, 2001. Genetic consequences of polygyny and social structure in an Indian fruit bat, *Cynopterus sphinx*. I. Inbreeding, outbreeding, and population subdivision. Evolution 55:1215-1223.

Templeton A, Crandall K, Sing C, 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619-633.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673-4680.

Valsecchi E, Hale P, Corkeron P, Amos W, 2002. Social structure in migrating humpback whales (*Megaptera novaeangliae*). Molecular Ecology 11:507-518.

Van Horn RC, Engh AL, Scribner KT, Funk SM, Holekamp KE, 2004. Behavioural structuring of relatedness in the spotted hyena (*Crocuta crocuta*) suggests direct fitness benefits of clan-level cooperation. Molecular Ecology 13:449-458.

Veith M, Beer N, Kiefer A, Johannesen J, Seitz A, 2004. The role of swarming sites for maintaining gene flow in the brown long-eared bat (*Plecotus auritus*). Heredity 93:342-349.

Verma S, Singh L, 2003. Novel universal primers establish identity of an enormous number of animal species for forensic application. Molecular Ecology Notes 3:28-31.

Vonhof MJ, Barber D, Fenton MB, Strobeck C, 2006. A tale of two siblings: multiple paternity in big brown bats (*Eptesicus fuscus*) demonstrated using microsatellite markers. Molecular Ecology 15:241-247.

Vonhof MJ, Davis CS, Fenton MB, Strobeck C, 2002. Characterization of dinucleotide microsatellite loci in big brown bats (*Eptesicus fuscus*), and their use in other North American vespertilionid bats. Molecular Ecology Notes 2:167-169.

Vonhof MJ, Davis CS, Strobeck C, Fenton MB, 2001. Characterization of microsatellite loci in Spix's disk-winged bats (*Thyroptera tricolor*). Molecular Ecology Notes 1:73-75.

Wahaj SA, Van Horn RC, Van Horn TL, Dreyer R, Hilgris R, Schwarz J, Holekamp KE, 2004. Kin discrimination in the spotted hyena (*Crocuta crocuta*): nepotism among siblings. Behavioral Ecology and Sociobiology 56:237-247.

Webb NJ, Tidemann CR, 1996. Mobility of Australian flying-foxes, *Pteropus* spp. (Megachiroptera): Evidence from genetic variation. In: Proceedings of the Royal Society of London Series B Biological Sciences; 497-502.

Weir B, Cockerham C, 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370.

West SA, Pen I, Griffin AS, 2002. Cooperation and competition between relatives. Science (Washington D C) 296:72-75.

Wilde CJ, Kerr MA, Knight CH, Racey PA, 1995. Lactation in Vespertilionid bats. Symposium of the Zoological Society of London 67:139-149.

Wilkinson GS, 1985a. The social organization of the common vampire bat *Desmodus rotundus* I. Pattern and cause of association. Behavioral Ecology and Sociobiology 17:111-122.

Wilkinson GS, 1985b. The social organization of the common vampire bat *Desmodus rotundus* II. Mating system genetic structure and relatedness. Behavioral Ecology and Sociobiology 17:123-134.

Wilkinson GS, 1988. Reciprocal altruism in bats and other mammals. Ethology and Sociobiology 9:85-100.

Wilkinson GS, 1992a. Communal nursing in the evening bat, *Nycticeius humeralis*. Behavioral Ecology and Sociobiology 31:225-235.

Wilkinson GS, 1992b. Information transfer at evening bat colonies. Animal Behaviour 44:501-518.

Wilkinson GS, Boughman JW, 1998. Social calls coordinate foraging in greater spear-nosed bats. Animal Behaviour 55:337-350.

Wilkinson GS, Fleming TH, 1996. Migration and evolution of lesser long-nosed bats *Leptonycteris curasoae*, inferred from mitochondrial DNA. Molecular Ecology 5:329-339.

Willis CKR, Brigham RM, 2004. Roost switching, roost sharing and social cohesion: forest-dwelling big brown bats, *Eptesicus fuscus*, conform to the fission-fusion model. Animal Behaviour 68:495-505.

Willis CKR, Brigham RM, submitted. Social thermoregulation exerts more influence than microclimate on forest roost preferences by a cavity-dwelling bat. Behavioral Ecology and Sociobiology.

Willis CKR, Kolar KA, Karst AL, Kalcounis-Rueppell MC, Brigham RM, 2003. Medium- and long-term reuse of trembling aspen cavities as roosts by big brown bats (*Eptesicus fuscus*). Acta Chiropterologica 5:85-90.

Willis CKR, Voss CM, Brigham RM, 2006. Roost selection by forest-living female big brown bats (*Eptesicus fuscus*). Journal of Mammalogy 87:345-350.

Wimsatt WA, 1944. Further studies on the survival of spermatozoa in the female reproductive tract of the bat. Anatomical Record 88:193-204.

Worthington Wilmer J, Hall L, Barratt E, Moritz C, 1999. Genetic structure and male-mediated gene flow in the ghost bat (*Macroderma gigas*). Evolution 53:1582-1591.

Worthington Wilmer J, Moritz C, Hall L, Toop J, 1994. Extreme population structuring in the threatened ghost bat, *Macroderma gigas*: Evidence for mitochondrial DNA. Proceedings of the Royal Society of London Series B Biological Sciences 257:193-198.

APPENDIX A. TABLES AND FIGURES

Table 1. Description of the nine microsatellite loci used to estimate nuclear genetic variability and subdivision. Variable PCR conditions are listed and differed with respect to the concentration of $MgCl_2$ (mM) and annealing temperature ^{o}C (T_a). The source of the primers used to amplify each locus and the original species that each locus was designed for are given.

Locus	Reverse and Forward Primer	A	$MgCl_2$	T_a	Original Species	Citation
EF1	ATC TGG GCA ATG ATA CCT TT GCA GGC TGG GCT GAG	27	1.5	48.4	*Eptesicus fuscus*	Vonhof et al (2002)
EF6	ATC ACA TTT TTG AAG CAT ATC TGT TTT TCT CTC CTT AT	22	3	46.1	*Eptesicus fuscus*	Vonhof et al (2002)
EF14	ATC ATA TAT TTG TGT TCT GG AAA ATC AGC TAT GTA GCA C	16	3	46.1	*Eptesicus fuscus*	Vonhof et al (2002)
EF15	AGC AGC AAA GGG GAC TAC GA GAG AAG CAG GGA GGG CAT TT	26	2	43.0	*Eptesicus fuscus*	Vonhof et al (2002)
EF20	TTA TCT TTG CCG TGG TT CCC CAC AAT GCC ATT A	20	3	46.1	*Eptesicus fuscus*	Vonhof et al (2002)
G9	AGG GGA CAT ACA AGA ATC AAC C TAA TTT CTC CAC TGA ACT CCC C	26	3	51.0	*Myotis myotis*	Castella & Ruedi (2000)
G25	TCC TTC CCA TTT CTG TGA GG CCA TTT CAT CCA TCC AGT CC	10	2	51.0	*Myotis myotis*	Castella & Ruedi (2000)
BE 22	CTG ATG CAA GAC CCC TTA CAA C ACG GCA GCA GTG AAA TCA GA	4	3	53.8	*Myotis bechsteinii*	Kerth et al (2002a)
TT20	TCT TAC CTC TTT TCC TGC TTT TTT TCT TCT GTG TTA CC	7	2	46.1	*Thyroptera tricolor*	Vonhof et al (2001)

Table 2. Number of tissue samples collected from adult females during each year from roosting area one, two, and three (RA1, RA2, RA3). The number of unique adult females captured from each roosting area during all years combined is listed. Note that due to female philopatry the total number of unique individuals from RA1 and RA2 was not equal to the sum of individuals sampled each year.

Year	RA	n
2002	RA1	32
2003	RA1	26
2004	RA1	30
2005	RA1	29
2002-2005	RA1	48
2002	RA2	7
2005	RA2	13
2002, 2005	RA2	19
2002	RA3	3

Table 3.
The distribution of adult females from roosting area one (RA1, n = 48) and roosting area two (RA2, n = 19) during 2002-2005. Years are listed by the last two digits. All adult females present in the specified roosting area in each year are represented by their forearm band number (ID). In RA1, female adults are listed according to the number of years they were present in the roosting area. A blank space indicates that a bat was not caught during that year or the individual was a juvenile. Note that individuals 168, 182, and 202 were juveniles that returned in subsequent years and were not listed in the year of their birth. Also note that bat 115 switched from RA2 to RA1.

RA1 02 ID	RA1 03 ID	RA1 04 ID	RA1 05 ID	RA2 02 ID	RA2 05 ID
7	7	7	7	114	
18	18	18	18	122	
35	35	35	35	125	
41	41	41	41	126	
82	82	82	82	127	127
84	84	84	84	128	
85	85	85	85		216
86	86	86	86		217
90	90	90	90		218
91	91	91	91		219
98	98	98	98		220
100	100	100	100		222
131	131	131	131		223
138	138	138	138		224
22	22	22			225
	115	115	115	115	
135	135	135			226
	153	153	153		227
	154	154	154		263
	155	155	155		
	181	181	181		
	157	157	157		
	158	158	158		
92	92				
109	109				
	156	156			
		168	168		
		188	188		
		192	192		
		193	193		
		205	205		
		206	206		
8					
27					
28					
34					
45					
83					
87					
88					
89					
93					
94					
136					
137					
142					
			182		
			202		

Table 4. Genetic variation calculated from 116 bats from the study area genotyped at nine microsatellite loci. Genetic variation is described as the allele size range in base pairs (bp), number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e). The null allele frequency is also given.

Locus	Allele size range (bp)	A	H_o	H_e	Null allele frequency
EF1	157-217	27	0.914	0.918	+0.001
EF6	165-203	22	0.791	0.891	+0.058
EF14	96-142	16	0.835	0.860	+0.013
EF15	103-160	26	0.876	0.891	+0.006
EF20	86-115	15	0.914	0.877	-0.024
G9	115-173	27	0.886	0.900	+0.004
G25	112-146	10	0.609	0.681	+0.067
BE22	131-135	4	0.509	0.545	+0.047
TT20	180-190	7	0.621	0.602	-0.012

Table 5. Genetic variation within each subset. Adult females were categorized into subsets based on roosting area (RA) and year. For each subset, the sample size (n), number of alleles per locus, and expected heterozygosity are listed. The total number of alleles from the 116 bats genotyped from the study area (Total) is also provided.

			Number of Alleles Observed								
n	RA	Year	EF1	EF6	EF14	EF15	EF20	G9	G25	BE22	TT20
		Total	27	22	16	26	15	27	10	4	7
32	RA1	2002	16	13	11	17	12	15	6	4	7
7	RA2	2002	10	11	6	9	8	10	5	4	3
3	RA3	2002	4	6	5	3	6	2	2	3	2
26	RA1	2003	15	14	12	16	12	15	7	4	6
30	RA1	2004	16	14	14	16	13	19	8	4	6
29	RA1	2005	16	13	14	17	13	19	8	4	6
12	RA2	2005	13	11	10	12	10	12	6	4	4
48	RA1	02 to 05	19	16	15	20	14	21	9	4	7
19	RA2	02 to 05	17	15	11	14	12	18	7	4	4
			Expected Heterozygosity								
32	RA1	2002	0.898	0.873	0.853	0.852	0.871	0.912	0.680	0.502	0.683
7	RA2	2002	0.956	0.967	0.813	0.934	0.912	0.923	0.758	0.670	0.484
3	RA3	2002	0.800	1.000	0.933	0.733	1.000	0.333	0.533	0.600	0.333
26	RA1	2003	0.905	0.906	0.863	0.865	0.870	0.917	0.748	0.542	0.632
30	RA1	2004	0.901	0.899	0.873	0.875	0.873	0.915	0.712	0.544	0.611
29	RA1	2005	0.904	0.887	0.877	0.893	0.881	0.917	0.702	0.535	0.593
12	RA2	2005	0.902	0.931	0.828	0.906	0.877	0.898	0.551	0.612	0.455
48	RA1	02 to 05	0.906	0.874	0.866	0.890	0.875	0.925	0.708	0.533	0.640
19	RA2	02 to 05	0.923	0.935	0.801	0.911	0.879	0.915	0.644	0.622	0.440

Table 6. Pairwise Fst values for each comparison between roosting area one (RA1) and roosting area two (RA2). Years are listed by the last two digits (e.g. 01 is 2001). Each subset contained only adult females from the specified year. When year is not specified, the subset combines adult females from all years. Other subsets are not listed due to small sample sizes (refer to Table 2 for sample sizes). F_{st} values are below the diagonal while p-values are above the diagonal. The statistical significance of the p-values are indicated by either ns (not significant) or ** (p-value < 0.01).

	02 RA1	02 RA2	03 RA1	04 RA1	05 RA1	05 RA2	RA1	RA2
02 RA1		ns	ns	ns	ns	ns	ns	**
02 RA2	0.018		ns	ns	ns	ns	ns	ns
03 RA1	-0.013	0.004		ns	ns	ns	ns	ns
04 RA1	-0.009	0.003	-0.016		ns	ns	ns	ns
05 RA1	-0.009	0.002	-0.015	-0.016		ns	ns	ns
05 RA2	0.019	-0.022	0.011	0.007	0.006		ns	ns
RA1	-0.010	0.008	-0.011	-0.011	-0.011	0.011		ns
RA2	0.024	-0.038	0.013	0.011	0.010	-0.030	0.015	

Table 7 (continued on next page). The variable sites within 273-274 base pair region of HVII. Due to three insertions, the aligned sequences span 276 bp. From the 85 sequences, 18 haplotypes were described based on 47 variable sites and three gaps. Position refers to the nucleotide position (gaps are counted).

Position	52	103	149	150	155	157	180	183	190	191	192	193	200	203	204	206	212	214	215	216	217	219	222	224
H01	G	C	C	G	T	C	A	G	C	T	G	A	G	C	C	G	A	A	T	A	T	C	T	C
H02	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H03	.	T	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H04	.	T	T	A	C	G	.	.	T	.	.	G	C	G	T	.	T	C	A	G	.	T	G	T
H05	.	T	T	A	C	G	.	.	T	.	.	G	C	G	T	.	T	C	A	G	.	T	G	T
H06	.	T	T	A	C	G	.	.	T	.	.	.	C	G	.	.	T	C	A	G	.	T	G	T
H07	.	T	T	A	C	G	.	.	T	.	.	G	C	G	T	.	T	C	A	G	.	T	G	T
H08	.	T	T	A	C	G	.	.	T	.	A	.	C	G	T	.	T	C	A	G	.	T	G	T
H09	.	T	T	A	C	G	.	.	T	C	.	G	C	G	T	.	T	C	A	G	.	T	G	T
H10	.	T	T	A	C	G	.	.	T	.	.	G	C	G	T	.	T	C	A	.	.	T	G	T
H11	.	T	T	A	C	G	.	.	T	.	.	G	C	G	T	.	T	C	A	G	.	T	G	T
H12	.	T	T	A	C	G	.	.	T	.	.	G	C	G	T	.	T	C	A	G	.	T	G	T
H13	.	T	T	A	C	G	.	.	T	.	.	G	C	G	T	.	T	C	A	G	.	T	G	T
H14	.	T	T	A	C	G	.	.	T	.	.	.	C	G	T	.	T	C	A	G	.	T	G	T
H15	.	T	T	A	C	G	.	.	T	.	.	.	C	G	T	A	T	C	A	G	.	T	G	T
H16	.	T	T	A	C	G	.	.	T	.	A	G	C	G	T	.	T	C	A	G	.	T	G	T
H17	A	T	T	A	C	G	.	A	T	.	A	G	C	G	T	A	T	T	A	.	C	T	G	T
H18	.	T	T	A	C	G	.	A	T	C	.	G	C	G	T	.	T	C	A	G	.	T	G	T

Table 7 continued.

	225	228	230	231	236	238	240	242	245	246	248	253	255	259	260	261	262	263	267	268	270	271	273	274	275	276
H01	A	-	T	A	C	G	G	T	T	A	G	C	-	A	C	G	G	A	T	A	G	T	C	A	C	A
H02	.	-	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	T	.	.	.
H03	.	-	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	T	.	.	.
H04	T	-	.	.	T	A	.	C	-	.	C	T	A	.	G	T	T	.	.	.	T	.	.	G	T	.
H05	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	C	.	.	.	T	.	.	G	T	.
H06	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	T	.	.	.	C	C	.	G	T	.
H07	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	T	.	.	G	T	.	.	G	T	.
H08	T	-	.	G	T	.	.	C	-	.	C	T	-	.	G	T	T	G	.	.	T	.	.	G	T	.
H09	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	T	.	.	.	T	.	.	G	T	.
H10	T	-	.	.	T	A	.	C	-	.	C	T	-	G	G	T	T	.	.	.	T	.	.	G	T	.
H11	T	-	.	G	T	A	.	C	-	.	C	T	-	G	G	T	T	.	.	.	T	.	.	G	T	.
H12	T	-	.	.	T	.	.	C	-	.	C	T	-	.	G	T	T	.	.	.	T	.	.	G	T	.
H13	T	-	.	.	T	A	.	.	-	.	C	T	-	.	G	T	T	.	.	.	T	.	.	G	T	.
H14	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	T	.	.	.	T	.	.	G	T	.
H15	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	T	.	.	.	T	.	.	G	T	.
H16	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	T	.	.	.	T	.	.	G	T	.
H17	C	A	C	.	.	A	A	C	-	G	C	T	-	.	G	T	T	.	C	G	T	.	.	G	T	G
H18	T	-	.	.	T	A	.	C	-	.	C	T	-	.	G	T	T	.	.	.	C	.	T	G	T	.

Table 8. The distribution of haplotypes within roosting areas, geographic regions, and unknown roosting areas. The haplotype is listed across the first row, and the location of the haplotype is given in the first column (BC = British Columbia; NC = North Carolina; Regina refers to Regina, Saskatchewan; RA = roosting area). Bats from unknown roosting areas are noted as "Unk" and the sex as "F" for female or "M" for male. The number of bats sampled from each location (n) is given. Below the dotted lines the haplotypes present during each year in roosting area one (RA1) are listed. The total number of bats sequenced was 85, but one bat switched roost sites and is included in totals for both RA1 and RA2 making the total equal to 86.

	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12	H13	H14	H15	H16	H17	H18	n
BC	3	4	1																8
NC				1	1	1	1	1											5
Regina									1									1	2
RA1									7	8		2			14	4	13		48
RA2									2		2	1	2	1	2	8			18
RA3										1	1						1		3
Unk F																	1		1
Unk M														1					1
TOTAL	3	4	1	1	1	1	1	1	10	9	3	3	2	2	16	12	15	1	
2002 RA1									4	4		2			11	2	9		32
2003 RA1									6	3		1			6	2	8		26
2004 RA1									6	5		1			6	2	10		30
2005 RA1									4	6		1			6	2	10		29

Table 9. The maternal genetic diversity within each subset. The genetic diversity, based on HVII sequences, is described by the number of haplotypes (H_n), the haplotype diversity (h), and the nucleotide diversity (π). The sample size (n) of each subset is also listed. Subsets are based on the roosting area and year, and they contain only adult females. When the year is not specified, the subset contains adult females from all years combined.

Subset	n	H_n	h	π
2002 RA1	32	6	0.788	0.0289
2003 RA1	26	6	0.809	0.0303
2004 RA1	30	6	0.802	0.0311
2005 RA1	29	6	0.798	0.0314
RA1	48	6	0.799	0.0273
RA2	18	7	0.791	0.0072
RA3	3	3	1.000	0.0438
BC	8	3	0.679	0.0048
NC	5	5	1.000	0.0168
Regina	2	2	1.000	0.0110
Average	20.1	5	0.847	0.0233

Table 10. Variable sites within 330 base pairs of the cytochrome *b* region. Four haplotypes (HC1-4) were described by 25 variable sites from 17 individuals. The sample size (n) for each cytochrome *b* haplotype is given. The "HVII" column contains the HVII haplotypes of bats with the same cytochrome *b* haplotype. The "Location" column contains the roosting area or geographic location of the bats sampled.

n	HVII	Location	cyt b	7	13	19	46	64	100	109	115	121	127	130	151	175	183	211	229	235	238	250	274	283	304	307	310	319
12	H10-16, H07, H18	RA1-3, NC, Regina	HC1	T	A	C	T	A	T	C	T	C	C	C	G	T	G	C	T	A	A	T	A	T	C	C	T	T
2	H17	RA1, RA3	HC2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	.	.	.	T	.	.	.
2	H09	RA1, RA2, Regina	HC3	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
1	H01	BC	HC4	C	G	T	C	C	.	T	C	T	T	T	T	C	A	T	C	G	T	C	G	C	T	T	C	C

Table 11. Pairwise Φ_{st} values for each comparison between roosting area one (RA1) and roosting area two (RA2). Years are listed based on the last two digits. Each subset contains only adult females from the specified year. When the year is not specified, the subset combines adult females from all years. Other subsets are not included due to small sample sizes. Sample size (n) is given. Φ_{st} values are below the diagonal while p-values are above the diagonal. The statistical significance of the p-values are indicated by the following symbols: ns (not significant), * (p-value < 0.05), and ** (p-value <0.01).

N		RA2	RA1	02 RA1	03 RA1	04 RA1	05 RA1
18	RA2		*	**	**	**	**
48	RA1	0.145		ns	ns	ns	ns
32	02 RA1	0.180	-0.024		ns	ns	ns
26	03 RA1	0.193	-0.022	-0.030		ns	ns
30	04 RA1	0.212	-0.012	-0.022	-0.035		ns
29	05 RA1	0.229	-0.008	-0.020	-0.031	-0.034	

Table 12. PCR conditions for microsatellite loci amplifications and genetic diversity measures. PCR conditions differed with respect to the concentration of $MgCl_2$ (mM) and annealing temperature (T_a). Allele size range in base pairs (bp), number of alleles per locus (A), expected heterozygosity (H_e), observed heterozygosity (H_o), and null allele frequency for each locus were calculated from adult females (n = 48) in roosting area one. The source species and source of the primers for each locus is also listed.

Locus	$MgCl_2$ (mM)	T_a (^oC)	Allele size range (bp)	A	H_o	H_e	Null allele frequency	Source Species	Source of the Primers
EF1	1.5	48.4	157-217	19	0.917	0.904	-0.014	*Eptesicus fuscus*	Vonhof et al (2002)
EF6	3.0	46.1	165-195	16	0.813	0.875	+0.031	*Eptesicus fuscus*	Vonhof et al (2002)
EF14	3.0	46.1	96-138	15	0.938	0.870	-0.047	*Eptesicus fuscus*	Vonhof et al (2002)
EF15	2.0	43.0	103-146	18	0.872	0.881	+0.003	*Eptesicus fuscus*	Vonhof et al (2002)
EF20	3.0	46.1	86-115	14	0.938	0.875	-0.041	*Eptesicus fuscus*	Vonhof et al (2002)
G9	3.0	51.0	115-173	20	0.913	0.918	-0.004	*Myotis myotis*	Castella & Ruedi (2000)
G25	2.0	51.0	112-140	8	0.660	0.706	+0.044	*Myotis myotis*	Castella & Ruedi (2000)
BE22	3.0	53.8	131-135	4	0.479	0.531	+0.071	*Myotis bechsteinii*	Kerth et al (2002a)
TT20	2.0	46.1	180-190	7	0.688	0.639	-0.044	*Thyroptera tricolor*	Vonhof et al (2001)

Table 13. HVII mitochondrial DNA matrilines (haplotypes). The 20 variable sites found in a 273-274 base pair HVII region of mitochondrial DNA sequenced for every adult female (n = 48) in roosting area one (RA1). The variable sites consisted of 19 transitions and one insertion, which produced six unique matrilines (or haplotypes).

	Nucleotide Position (base pairs)																			
Matrilines	52	183	191	192	193	206	214	216	217	225	228	230	236	238	240	245	257	265	266	274
M09	G	G	C	G	G	G	C	G	T	T	-	T	T	A	G	A	A	T	A	A
M10	.	.	T	.	.	.	.	A	.	.	-	.	.	.	.	.	G	.	.	.
M12	.	.	T	.	.	.	.	.	.	.	-	.	.	G	.	.	.	.	.	.
M15	.	.	T	.	A	A	.	.	.	.	-	.	.	.	.	.	.	.	.	.
M16	.	.	T	A	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.
M17	A	A	T	A	.	A	T	A	C	C	A	C	C	.	A	G	.	C	G	G

Table 14. Roosting area one (RA1) colony composition from 2002-2005. The forearm band number (ID) and matriline (M) of each female adult (n = 48) is given. A blank space indicates that the individual was not present in RA1 or the individual was a juvenile that year. Note that individuals 168, 182, and 202 were juveniles that returned in subsequent years and were not listed in the year of their birth.

2002		2003		2004		2005	
ID	M	ID	M	ID	M	ID	M
7	M12	7	M12	7	M12	7	M12
8	M10						
18	M15	18	M15	18	M15	18	M15
22	M09	22	M09	22	M09		
27	M17						
28	M15						
34	M12						
35	M17	35	M17	35	M17	35	M17
41	M17	41	M17	41	M17	41	M17
45	M17						
82	M17	82	M17	82	M17	82	M17
83	M10						
84	M09	84	M09	84	M09	84	M09
85	M15	85	M15	85	M15	85	M15
86	M15	86	M15	86	M15	86	M15
87	M16						
88	M16						
89	M15						
90	M10	90	M10	90	M10	90	M10
91	M09	91	M09	91	M09	91	M09
92	M15	92	M15				
93	M15						
94	M15						
98	M17	98	M17	98	M17	98	M17
100	M17	100	M17	100	M17	100	M17
109	M15	109	M15				
		115	M09	115	M09	115	M09
131	M10	131	M10	131	M10	131	M10
135	M17	135	M17	135	M17		
136	M15						
137	M15						
138	M17	138	M17	138	M17	138	M17
142	M09						
		153	M16	153	M16	153	M16
		154	M17	154	M17	154	M17
		155	M15	155	M15	155	M15
		156	M09	156	M09		
		157	M16	157	M16	157	M16
		158	M09	158	M09	158	M09
				168	M17	168	M17
		181	M10	181	M10	181	M10
						182	M10
				188	M17	188	M17
				192	M10	192	M10
				193	M15	193	M15
						202	M17
				205	M15	205	M15
				206	M10	206	M10

Table 15. Individuals belonging to the six matrilines from roosting area one (RA1) during 2002-2005. The number of individuals in each matriline in each year is shown. RA1* indicates the distribution of matrilines among all the adult females present from 2002-2005.

	Matrilines						
	M09	M10	M12	M15	M16	M17	Total
RA1*	7	8	2	14	4	13	48
2002 RA1	4	4	2	11	2	9	32
2003 RA1	6	3	1	6	2	8	26
2004 RA1	6	5	1	6	2	10	30
2005 RA1	4	6	1	6	2	10	29

Table 16. Average pairwise relatedness of adult females within each matriline in roosting area one (RA1) during 2002-2005. The number of individuals within each matriline (n) and the p-value (determined from 1000 randomizations, see text) are also given.

Matriline	Average pairwise relatedness	n	p-value
M09	0.08	7	0.043
M10	0.17	8	<0.001
M12	0.09	2	0.216
M15	0.02	14	0.115
M16	0.49	4	<0.001
M17	0.01	13	0.156
Average	0.14	8	

Table 17. Average pairwise relatedness of adult females within each of the 20 trapping groups. The sample size (n) for each trapping group refers only to adult females. The p-value was based on 1000 randomizations (see text). Only TG18 and TG19 had statistically significant p-values.

Trapping Group	Average pairwise relatedness	n	p-value
TG1	0.00	19	0.216
TG2	-0.04	4	0.590
TG3	-0.03	4	0.588
TG4	0.01	8	0.213
TG5	-0.03	17	0.832
TG6	-0.02	10	0.549
TG7	-0.11	9	1.000
TG8	-0.01	7	0.401
TG9	0.00	9	0.343
TG10	-0.04	5	0.604
TG11	-0.02	21	0.606
TG12	0.02	14	0.136
TG13	-0.01	14	0.469
TG14	-0.03	19	0.819
TG15	0.04	9	0.095
TG16	-0.02	6	0.570
TG17	-0.01	16	0.366
TG18	0.07	9	0.029
TG19	0.07	10	0.010
TG20	0.06	5	0.151
Average	-0.01		

Table 18. The observed and expected distribution of matrilines in trapping groups from roosting area one (RA1). For each trapping group (n = 20 from 2002-2005), the number of adult females observed and expected from each matriline (M) is given.

	Observed distribution of matrilines in each trapping group																			
M	TG 1	TG 2	TG 3	TG 4	TG 5	TG 6	TG 7	TG 8	TG 9	TG 10	TG 11	TG 12	TG 13	TG 14	TG 15	TG 16	TG 17	TG 18	TG 19	TG 20
M09	3	0	0	3	4	3	0	2	4	1	6	3	1	1	1	0	2	2	3	1
M10	3	0	0	0	1	2	1	0	1	2	3	1	2	2	4	2	3	2	3	1
M12	2	0	0	0	1	1	1	0	0	0	1	1	1	1	0	0	1	0	0	0
M15	7	1	2	1	5	2	2	0	2	0	2	3	1	3	4	2	3	2	1	1
M16	2	0	0	1	1	1	1	1	0	0	2	2	2	2	0	1	2	0	1	0
M17	2	3	2	3	5	1	4	4	2	2	7	4	7	10	0	1	5	3	2	2
Total	19	4	4	8	17	10	9	7	9	5	21	14	14	19	9	6	16	9	10	5
	Expected distribution of matrilines in each trapping group																			
M09	2.4	0.5	0.5	1.8	3.9	2.3	2.1	1.6	2.1	1.0	4.2	2.8	2.8	3.8	1.8	1.2	2.2	1.2	1.4	0.7
M10	2.4	0.5	0.5	0.9	2.0	1.2	1.0	0.8	1.0	0.8	3.5	2.3	2.3	3.2	1.5	1.0	3.3	1.9	2.1	1.0
M12	1.2	0.3	0.3	0.3	0.7	0.4	0.3	0.3	0.3	0.2	0.7	0.5	0.5	0.6	0.3	0.2	0.6	0.3	0.3	0.2
M15	6.5	1.4	1.4	1.8	3.9	2.3	2.1	1.6	2.1	1.0	4.2	2.8	2.8	3.8	1.8	1.2	3.3	1.9	2.1	1.0
M16	1.2	0.3	0.3	0.6	1.3	0.8	0.7	0.5	0.7	0.3	1.4	0.9	0.9	1.3	0.6	0.4	1.1	0.6	0.7	0.3
M17	5.3	1.1	1.1	2.5	5.2	3.1	2.8	2.2	2.8	1.7	7.0	4.7	4.7	6.3	3.0	2.0	5.5	3.1	3.4	1.7
Total	19	4	4	8	17	10	9	7	9	5	21	14	14	19	9	6	16	9	10	5
	χ^2 Statistical test results																			
χ^2	0.61	0.45	0.78	0.75	0.96	0.65	0.55	0.45	0.69	0.67	0.79	0.74	0.37	0.37	0.05	0.50	0.94	0.92	0.55	0.98
p	0.99	0.99	0.98	0.98	0.97	0.99	0.99	0.99	0.98	0.98	0.98	0.98	1.00	1.00	1.00	0.99	0.97	0.97	0.99	0.96
d.f.	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

Figure 1. Three non-overlapping roosting areas (RA1, RA2, and RA3) located within Cypress Hills Interprovincial Park, Saskatchewan, Canada. Forested areas are shaded gray. Thin lines represent roads, while the thick line represents Battle Creek. Roosting areas (RA) are indicated by ovals. Map reprinted from Willis and Brigham (2004) with permission from Elsevier.

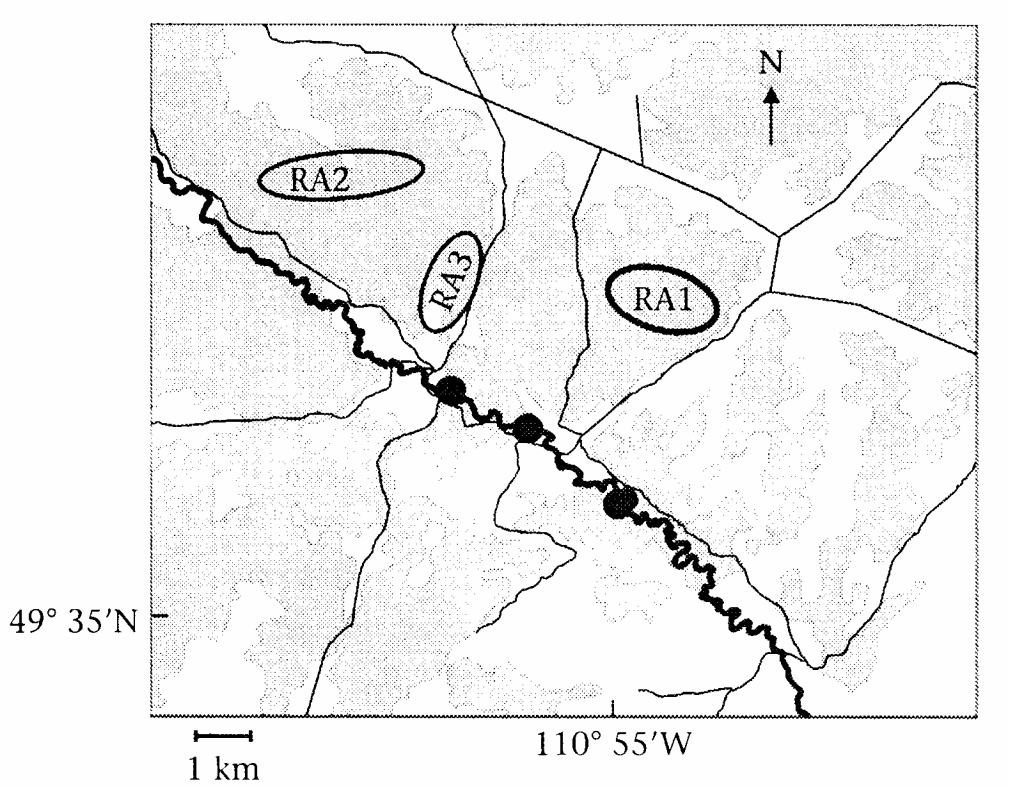


Figure 2. Representation of a minimum spanning network to provide a visual image of the number of mutations between the HVII haplotypes (H04-H18). The British Columbia (BC) haplotypes (H01-03) are not included, because of the large number of mutations (>15) when compared to any of the other haplotypes. Open circles with the haplotype name inside represent observed haplotypes. The relative frequency of the haplotype is represented by the size of the circle and also noted with the sample size (n). Smaller closed circles denote haplotypes not observed but expected intermediates between haplotypes. Each line (regardless of length) between closed or open circles indicates one mutation. For example, there are two mutations between H16 and H07.

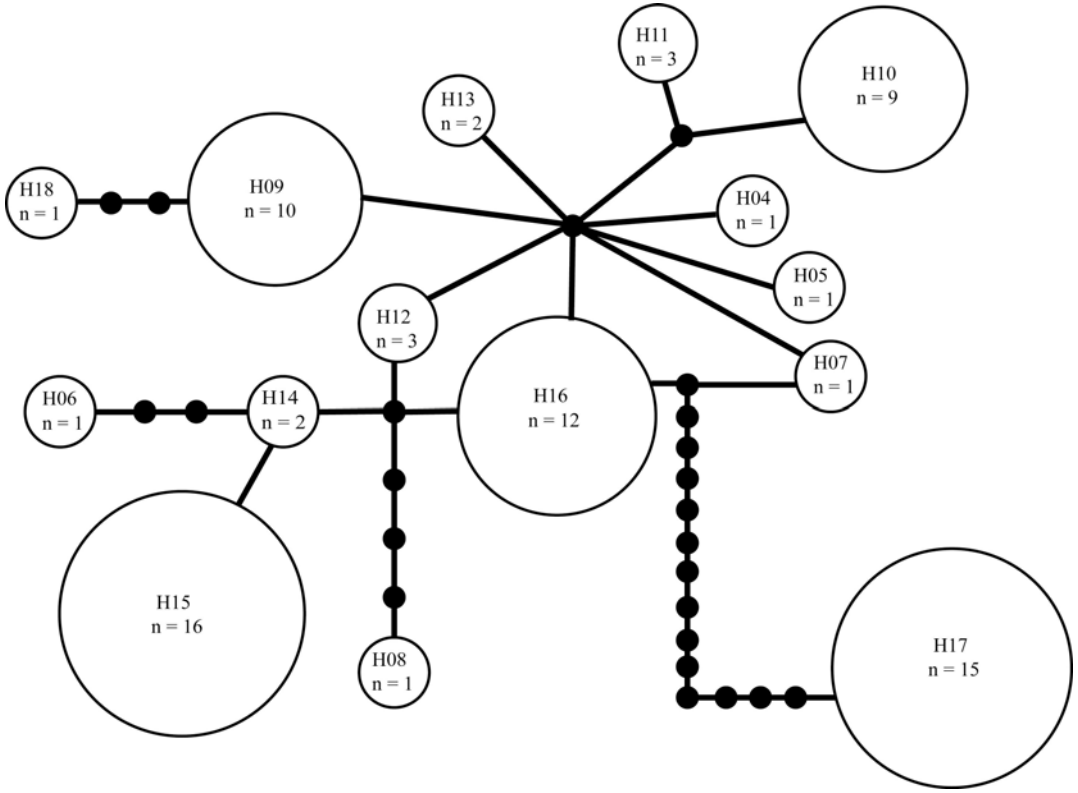


Figure 3. A scatter plot demonstrating the relationship between PSI value and pairwise relatedness. PSI values are taken from Willis and Brigham (2004) based on data from 36 pairwise combinations of 17 different adult females from roosting area one (RA1) during 2000-2002.

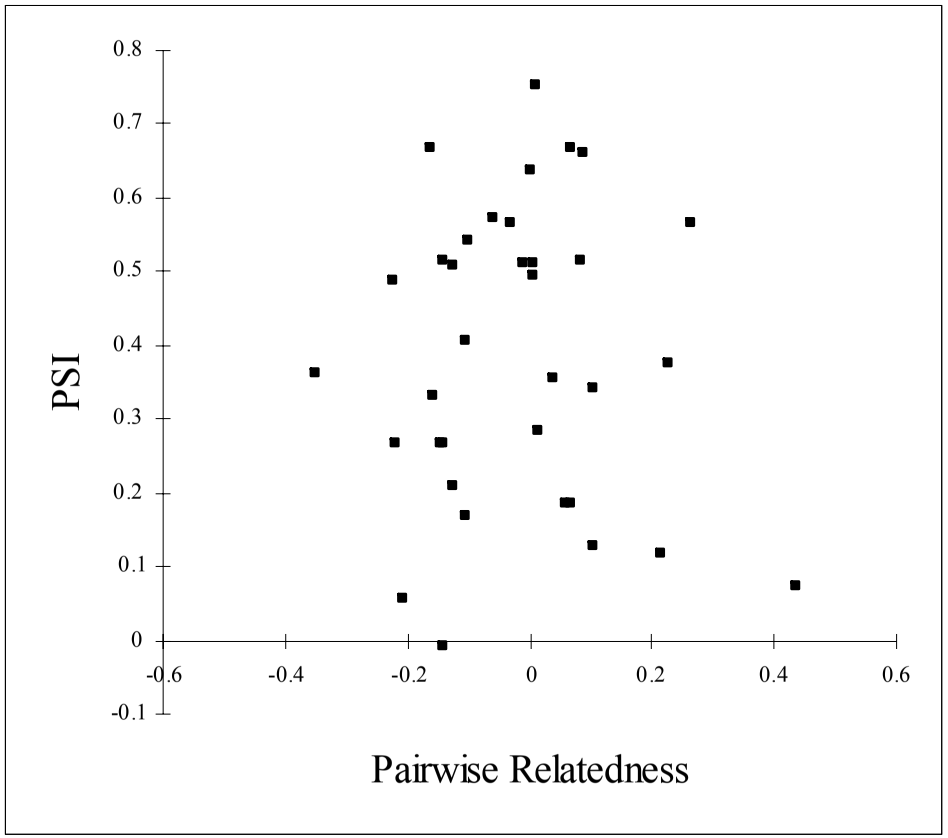
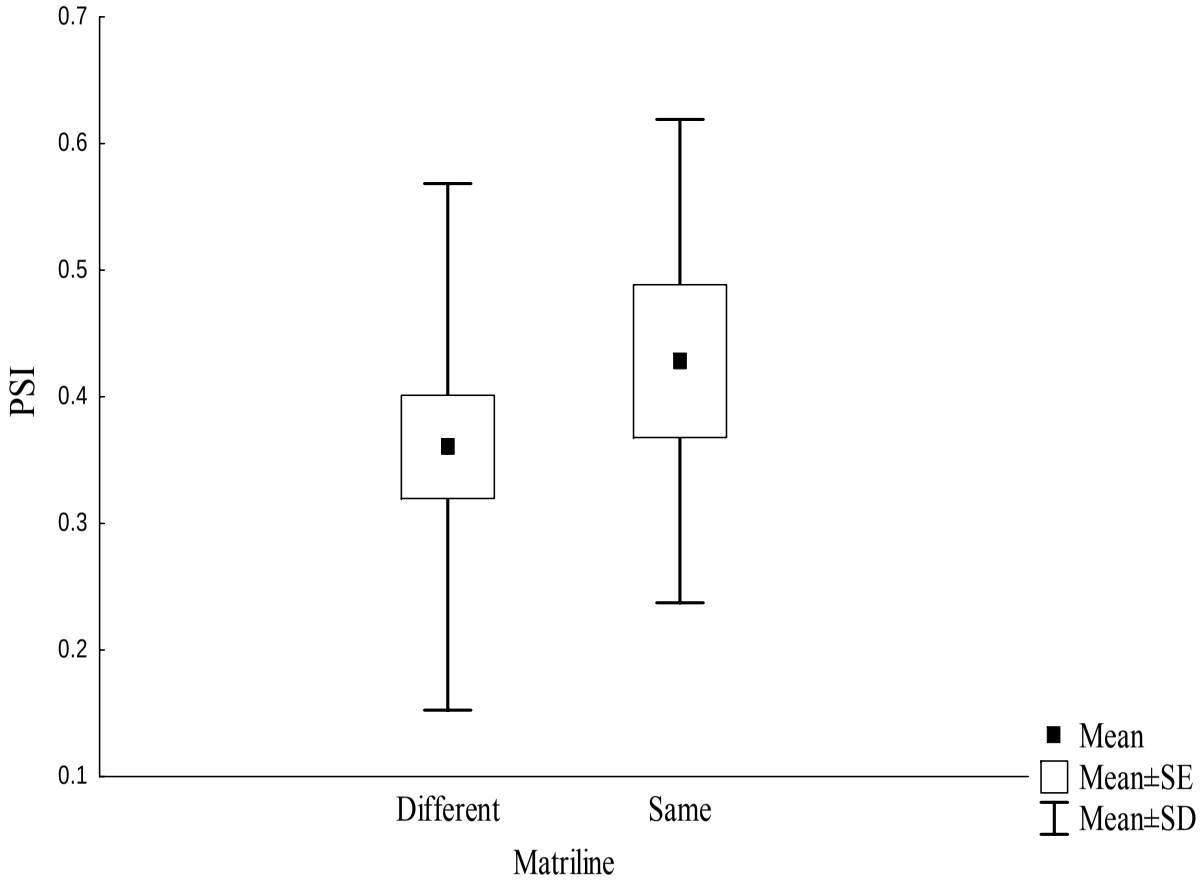


Figure 4. The mean pairwise sharing index (PSI) for 10 pairs of bats with the same matriline and 26 pairs of bats from different matrilines. See legend for an explanation of symbols. PSI values are the same as those in Figure 3.



APPENDIX B. MICROSOFT VISUAL BASIC COMMANDS

The Microsoft Visual Basic commands for the Excel macro (written by Olav Rueppell) used to perform randomizations. For each randomization, a random number of individuals were selected, and their pairwise relatedness values were obtained from a full matrix. The average pairwise relatedness value was calculated. Selected individuals, pairwise relatedness, and average pairwise relatedness were generated as output in Excel.

```
   Dim ind(22) As Integer, r(400) As Single

   bouts = 1000 'number of resamples
   matrixstart = 3 'row or column #
   matrixend = 50   ' row or column #
   grps = InputBox("Groupsize (2-21)", "Hello Jackie", 5)
   grpsize = Val(grps)

   For i = 1 To bouts

      For ii = 1 To 22
         ind(ii) = -1
      Next ii

      For ii = 1 To 400
         r(ii) = -9
      Next ii

Randomize
      ii = 0
      Do
         ii = ii + 1
         Do
            testok = True
            ind(ii) = Int(((matrixend - matrixstart + 1) * Rnd) + matrixstart)

            iii = 1
            Do While testok And iii < 22
               If (ind(ii) = ind(iii)) And (ii <> iii) Then testok = False
            iii = iii + 1
            Loop
         Loop Until testok
      Loop Until ii = grpsize

      Sheets("RA1 adults full matrix").Select
      i4 = 1
```

```
        For ii = 1 To 22
           If ind(ii) > 0 Then
              For iii = ii + 1 To 22
                 If ind(iii) > 0 Then
                    r(i4) = Cells(ind(ii), ind(iii))
                    i4 = i4 + 1
                 End If
              Next iii
           End If
        Next ii

        Sheets("results").Select

        colum = 1
        ii = 1

        Do While ind(ii) > 0
           Cells(i, ii) = ind(ii)
           ii = ii + 1
           colum = colum + 1
        Loop

        ii = 1
        averag = 0
        Do While r(ii) > -2
           Cells(i, ii + colum) = r(ii)
           averag = averag + r(ii)
           ii = ii + 1
        Loop

        Cells(i, ii + colum + 2) = averag / (ii - 1)

     Next i

End Sub
```