Genetic population structure within and among little brown bat (*Myotis lucifugus*) maternity colonies within mainland Nova Scotia, Canada

by

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Genetic population structure within and among little brown bat *(Myotis lucifugus)* maternity colonies within mainland Nova Scotia, Canada.

By Krista Arseneault, December 2013

Abstract

A common trait among mammals is group living, where individuals come together as a temporary or permanent group. In little brown bats (*Myotis lucifugus*), this is a common behavior of hibernation and at maternity roosts. During the summer, females will gather at maternity roosts to give birth and raise young. It has been suggested from observational studies that maternity colonies are structured based on female philopatry and male dispersal, and the objective of this project was to test this prediction. Population genetic structure was assessed at maternity roosts in mainland Nova Scotia using two molecular markers. Over three years, samples were collected from 505 adult females at 14 roosts (n=13-92 per roost). DNA was extracted and a section of the mitochondrial control region and 10 microsatellite loci were analyzed. Colonies were found to have significant differentiation of mitochondrial haplotypes and microsatellite allele frequencies, with F_{ST} for mitochondrial DNA being tenfold higher than for the nuclear markers. This pattern is consistent with female philopatry and male dispersal, as is often observed in mammals.

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Introduction

Fidelity to a specific location and/or group of other individuals is common among mammals (Clutton-Brock 1989; Matthiopoulos *et al.* 2005). Group living has fitness benefits as, for example, remaining within the natal area with known individuals may result in increased food availability by cooperation within known individuals and predator avoidance which can aid in the survival of an individual and their offspring (Kokko & López-Sepulcre 2006) (i.e., philopatry; (Clutton-Brock & Lukas 2012). Such groups may persist for short periods, as in elephant seals (*Mirounga leonina*) who come together to give birth from September until the pups are weaned in November each year (McCann 1980); to more permanent social groups such as that seen in sperm whales (*Physeter macrocephalus)* where female pods persist for a lifetime, juvenile males disperse at around age 8 while female offspring remain (Christal *et al.* 1998). In mammals, female site fidelity and male dispersal is common and typically attributed to inbreeding avoidance and increased access to mates and resources (Greenwood 1980; Pusey & Wolf 1996). Females who remain within their natal group have the benefit of previous information of resource distribution. As the competition for local resources among kin may reduce an individual's inclusive fitness, the dispersal of one sex—males— results in less competition among the remaining individuals (Perrin & Mazalov 2000). The reproductive fitness of males is limited by female availability, as males have little investment into offspring development, whereas a female's reproductive fitness is

limited by resource availability (Zeveloff & Boyce 1980). Therefore, cooperation among females may increase access to resources, which may increase their fitness.

Although group living is known to be a common trait among mammals, the composition and structure of these groups varies among species as a function of the intensity and extent of gene flow (Rossiter *et al.* 2011). For example, when the incidence of dispersal of individuals from one area to another is restricted by distance or geographic barriers (i.e., reduced gene flow) genetic differentiation may occur and can result in multiple populations (Slatkin 1995). In contrast, species with high vagility, such as bats, may have high rates of gene flow across large spatial scales, preventing genetic differentiation, and resulting in panmixia. Identifying the extent and patterns of gene flow is fundamental to our understanding of population structuring and the evolutionary history of a species. With time and limited gene flow, populations may genetically diverge due to natural selection, genetic drift and other agents of evolution causing allele frequency changes within populations (Hedrick 2001).

Bats show incredible diversity in ecology (Kunz *et al.* 2011). Those species that are year-round residents of temperate areas typically have a three stage annual cycle involving hibernation during the winter, segregation of the sexes in the summer when females form maternal colonies to give birth and raise young while males roost solitarily, followed by a period of swarming (Fenton & Barclay 1980). During swarming and hibernation, bats from a large range of summering areas may congregate, and, since mating is one reason why bats congregate (Thomas *et al.* 1979; Glover & Altringham 2008), the large groups from a wide range of summering

areas promotes a high degree of gene flow (Glover & Altringham 2008). Additionally, during swarming females and males may swarm at multiple sites promoting even more gene flow. As bats are highly vagile, it is expected that bats will show a low degree spatial structuring at nuclear loci due to high intensity gene flow across large spatial scales (Furmankiewicz & Altringham 2006; Norquay *et al.* 2013).

The population structure and relatedness of individuals with a species are difficult to study using traditional observational methods owing to their cryptic nature (Detwiler *et al.* 2010) or their propensity to have long lives and be highly vagile (Podlutsky *et al.* 2005). Such cases make the indirect means, such as molecular techniques, of making inference about movement useful. Molecular techniques can be used to make inferences on the extent of dispersal and characterize population structuring. Many population genetic techniques involve using neutral molecular markers, which are not directly influenced by selection (DeWoody 2005), and may include both nuclear and mitochondrial DNA. As mitochondrial DNA is maternally inherited with effectively no recombination in the majority of mammals, it is most informative about movement and structuring patterns of females (Rokas *et al.* 2003; DeWoody 2005). Nuclear DNA, on the other hand, is bi-parentally inherited with recombination, and therefore provides information on gene flow between the sexes, making it ideal for the study of relatedness and parentage.

Molecular studies have recently discovered interesting social patterns among bats. Some bat species have strictly closed maternity colonies, such as the Bechstein's bats (*Myotis bechsteinii*), where individuals form closed groups of 20-40

adult females that regularly split into smaller groups for temporary roosting (Kerth & Van Schaik 2011). While these smaller roosting groups may vary in composition, they will always be subsets of individuals from the larger maternity colony (Kerth & Van Schaik 2011). Unlike most other species, males of the greater sac-winged bats (*Saccopteryx bilineata*) are philopatric while females disperse (Nagy *et al.* 2007).

The little brown bat, *Myotis lucifugus*, is a widely distributed species in North America (Fenton & Barclay 1980). This species is a typical temperate bat with an annual cycle involving communal hibernation during the winter and an apparent segregation of the sexes during the summer. They are capable of long distance movement, a long term mark recapture study found the median movement of bats between summering colonies to be 431 kilometers and the median movement of bats between summering sites and hibernacula to be 463 kilometers (Norquay *et al.* 2013). During the summer, females will gather at maternity colonies to give birth and raise young, these maternity colonies are thought to be structured through maternally directed site fidelity, with male dispersal from their natal roosts (Lewis 1995; Davis & Whitaker 2002). A previous study using cytochrome *b* mitochondrial markers and nuclear DNA markers found significant and slightly higher structure using the mitochondrial marker than nuclear markers (Dixon 2011). Higher structure inferred from mitochondrial DNA than nuclear DNA structure, suggests a pattern of female philopatry and male dispersal. Cytochrome *b* is most often used for species level studies because it has a low substitution rate, therefore the degree of population genetic structuring within the group may be higher than is estimated by this marker.

While it is known that adult females roost together, the degree of relatedness among these individuals is currently unknown (Pearl & Fenton 1996). As related individuals will share a proportion of their genes, helping kin raise their offspring can be beneficial. If females roosting together in maternity colonies are closely related, the formation of these groups may be explained by kin selection, where helping a related individual can increase ones inclusive fitness (Hamilton 1964). Analysis of both nuclear and mitochondrial DNA markers are needed to obtain information on patterns of population structure (i.e., how bats are divided into reproductively segregated groups), and the level of maternal site fidelity to roosts. If maternity colonies are structured through maternally directed site fidelity with male dispersal, than I predict that there will be stronger patterns of structuring at a maternally inherited marker, such as mitochondrial DNA, than in a bi-parentally inherited marker such as nuclear DNA.

Because current populations of bats face risks of declines due to both a fungal pathogen and wind turbines, knowledge of the movement, as well as the structuring and relatedness of colonies is important for species management. *Pseudogymnoascus destructans,* the fungal pathogen responsible for white-nose syndrome (Gargas *et al.* 2009; Blehert *et al.* 2009), grows and spreads within the hibernacula. As of 2010, it was responsible for declines of up to 99% in some little brown bat populations (Frick *et al.* 2010). Because white-nose syndrome is spread by the contact of individuals, the movement of individuals between colonies is an important aspect of species management.

As more environmentally sustainable energy sources are currently highly sought after in North America, the use of wind energy from wind turbines becomes a desirable energy source (Kunz *et al.* 2007). One possible ecological issue associated with wind turbines is bat strikes, where bats are being struck and killed by the rotating blades of the wind turbines; the reason that bats are attracted to the blades is currently unknown. Bat fatalities have been found at wind turbines in the US, Canada and Europe (Kunz *et al.* 2007). Although most of the species affected include long-distance migratory bats travelling through the developed area (Arnett *et al.* 2008), bats of species that are year-round residents of temperate areas have been killed in significant numbers at many wind farms as well. If females and juveniles from a maternity roost are using many distant wintering sites, they may travel through wind turbine areas, increasing the likelihood of being struck and killed by the rotating blades.

To understand bat movement patterns and roost fidelity of females, molecular techniques were used to infer population genetic structure at maternity colonies in mainland Nova Scotia. It is known that females roost together in the summer, yet the composition of these roosts is unknown and the goal of this thesis is to characterize these roost groups. It is predicted that maternity colonies will have a higher degree of mitochondrial DNA structure than nuclear DNA structure as inferred by F_{ST} values. If relatives are roosting together each year, we expect to see a higher degree of relatedness within roost site than between roost sites. In addition to providing information regarding bat biology and roosting behavior, these data

may also aid management initiatives by providing information on the movement between roost sites as well as between summering and wintering sites.

Methods

Sample Collection

Bats were captured at 14 summer maternity roosts on mainland Nova Scotia from 2010-2012, with pairwise distances between roosts of 6 to 295 km. Individuals were caught by hand inside the roost, or in mist nets (Avinet, Dryden, New York), and/or in harp traps (Austbat Research Equipment, Lower Plenty, Victoria, Australia) as they emerged. From each roost, between 13 individuals at small sites and 91 adult females at larger sites were sampled as it has been suggested that sampling 20 individuals from a site is sufficient when nuclear F_{ST} values are greater than 0.05, yet the sample size needs to be increased to infer lower nuclear F_{ST} values (Kalinowski 2004). From each individual a 3-5 mm diameter sample of the plagiopatagium was collected using sterilized instruments (Worthington Wilmer & Barratt 1996), each sample was stored in a 20% DMSO (20% dimethyl sulfoxide, 0.25M disodium EDTA pH 8.0, NaCl saturated (Seutin *et al.* 1991)), and stored at -20°C.

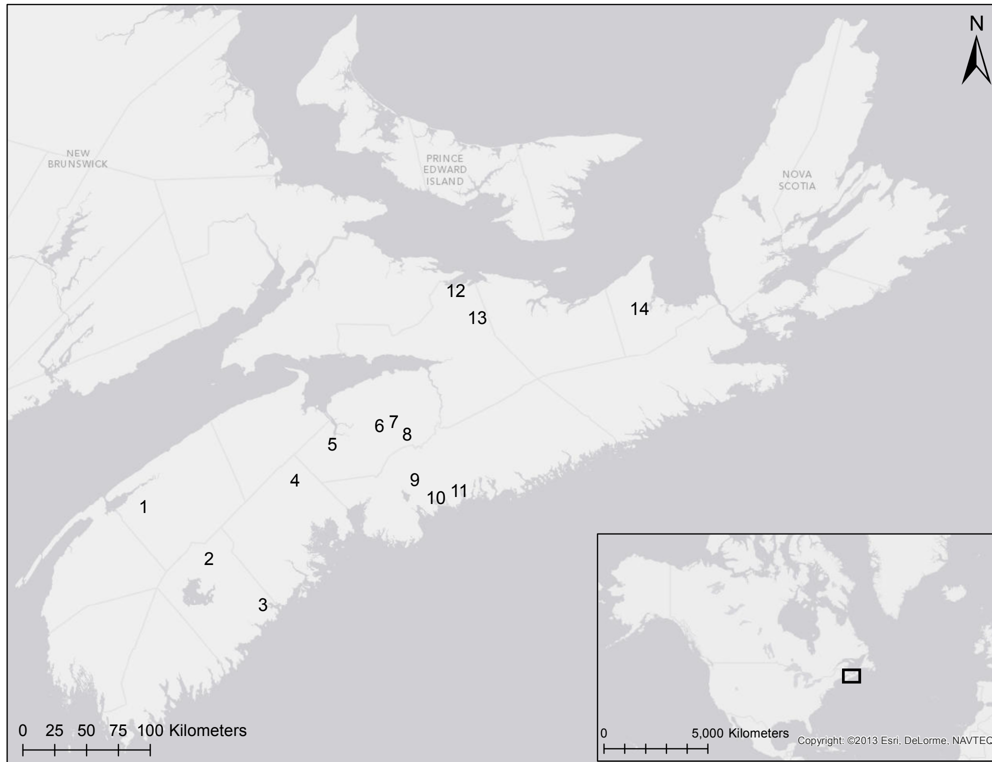

Figure 1: Location of 14 maternity roosts of *Myotis lucifugus* in Nova Scotia that were sampled to collect tissue samples for genetic analysis, 2010 to 2012.

1: Annapolis Royal (AR), 2: Caledonia (CA), 3: Mill Village (MV), 4: New Russell (NR), 5: Martock (MA), 6: Center Rawdon (CR) 7: Upper Rawdon (UR) 8: Renfrew (RE), 9: Waverley (WA), 10: Mineville (MI), 11: West Chezzetcook (WC), 12: Tatamagouche (TT), 13: Earltown (EA), 14: Antigonish (AN).

DNA Extraction

DNA was extracted using standard phenol:chloroform protocols (Sambrook & Russell 2000). The quantity and quality of extracted DNA was evaluated using spectrophotometry and by electrophoresis of DNA through 2% agarose gels stained with SYBR Green I. These procedures resulted in estimates of DNA concentration, and an assessment of the degree of degradation of the DNA in each sample. DNA was stored in $TE_{(0.1)}$ at -20°C until use.

Mitochondrial DNA Sequencing

A region of approximately 300 bp of the hypervariable region II of the mitochondrial DNA was amplified with the L16517-forward primer 5' CATCTGGTTCTTACTTCAGG 3') (Fumagalli *et al.* 1996; Castella *et al.* 2001) and KAHVII-reverse primer (designed in lab, 5' GTAGCGTGAATATGTCCTG 3'). The PCR cocktail included 1X PCR buffer (20 mM Tris-HCl, 50mM KCl) (New England Biolabs), 0.2 mM each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 0.3μM of each primer (L16517 and KAHVII), 10 ng DNA, 0.16 mg/mL bovine serum albumin (BSA, Ambion), and 0.05U/μL *Taq* polymerase (New England Biolabs). The cycling conditions were: 1 cycle of 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72 °C for 1 minute; followed by 1 cycle of 64°C for 45 minutes. PCR products were visualized on 2% agarose gels stained with 0.05 μg/mL ethidium bromide. PCR products were cleaned using an enzymatic purification step to remove any unincorporated primers and dNTPs. To do this, for each 1μL of sample to be cleaned, 0.129 μL of Antarctic Phosphatase Buffer, 0.02 μL Antarctic Phosphatase and 0.00614 μL Exonuclease I were added to the sample followed by 2 15-minute PCR cycles (37° C and 80°C), the samples were then held at 10° C for a minimum of 2 minutes. The subsequent sequencing PCR was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and the following conditions; 0.25X Reaction Mix, 1X Sequencing Buffer, 1μL per reaction of 10μM KAHVII primer and 0.05ng DNA/100 bp of desired sequence. After optimization of sequencing procedure, amplified samples were sent to Macrogen Inc for sequencing (Macrogen Inc., Seoul, Korea).

Microsatellite Amplification

Eleven nuclear microsatellites were amplified using 4 multiplex reactions designed for *M. lucifugus* using published conditions (Burns *et al.* 2012). PCR products were then size-separated using the 600 LIZ size standard for comparison (Applied Biosystems) and visualized on an ABI 3500xl genetic analyzer (Life Technologies), and scored manually using GeneMarker software (SoftGenetics, State College, PA). All electropherograms were visually checked to ensure that no alleles were missed. As multiple 96-well plates were necessary to complete all reactions, each plate contained a previously genotyped individual for comparison and a negative standard to ensure that no reagents were contaminated.

Analysis

Sequencing Analysis

Sequences were edited using the program 4-Peaks (Nucleobytes, Netherlands). Sequences were trimmed to remove priming sites from both ends, and base calls were confirmed by eye. Edited sequences were aligned using ClustalX (Larkin *et al.* 2007). Aligned sequences were uploaded to FaBox (Villesen 2007), this permitted identification of variable sites and assignment of unique haplotypes for sequence analysis.

Modelgenerator was used to estimate which model of molecular evolution was most appropriate for the data set, as well as to obtain estimates of the transition:transversion ratio and alpha value for the gamma distribution for accounting for rate heterogeneity in substitution patterns within the sequences (Keane *et al.* 2006).

Analyses of Molecular Variance (AMOVA) was conducted on the mitochondrial sequences to estimate global F_{ST} and Φ_{ST}, as well as pairwise F_{ST} values using Arlequin (Excoffier *et al.* 2005). Pairwise F_{ST} and the Euclidean distance (Google Earth) between each of the colonies was estimated to assess if differentiation correlated with geographic distance.

The ade4 package (Chessel *et al.* 2004) in the statistical program R (R Core Team 2012) was used to test for a pattern of isolation-by-distance. For this analysis a Mantel test was used to assess the correlation of two pairwise matrices: pairwise genetic F_{ST} and geographic distance (km). Significance was tested using Monte-Carlo simulation based on 999 permutations.

SAMOVA (spatial analysis of molecular variance) (Dupanloup *et al.* 2002) analysis was used to define the number of groups of genetically different populations represented by the data. It is used to determine if there is geographical clustering in a molecular dataset, and if so, how many groups can be differentiated. A simulated annealing approach to calculate the number of K (populations) is used, which will result in the highest F_{CT} index (which is the proportion of total genetic variance due to differences between groups of populations). One issue with this program is that it is unable to calculate for the data most likely representing one population.

Microsatellite Analysis

Because samples were often taken on multiple nights from the same site, it was possible that individuals were been sampled multiple times. I used CERVUS (Kalinowski *et al.* 2007) to identify any samples that had the same genotype, and

therefore likely represent the same individual, only one was included in further analysis

CERVUS (Kalinowski *et al.* 2007) was used to calculate observed and expected heterozygosity and test for Hardy-Weinberg equilibrium at each roost. To calculate this, CERVUS uses a likelihood approach with 1000 permutations.

SPaGeDi (Hardy & Vekemans 2002) was used to calculate global F statistic values from the 10 nuclear microsatellite loci. Global F_{ST}, F_{IS} and F_{IT} were calculated using the Weir & Cockerham methods (Weir & Cockerham 1984) with 1000 permutations for significant testing.

GenePop (Raymond & Rousset 1995; Rousset 2008) was used to estimate pairwise F_{ST} for nuclear DNA genotypes. Pairwise F_{ST} values were calculated using the Weir & Cockerham methods (Weir & Cockerham 1984) with 1000 permutations for significant testing. Isolation-by-distance was tested for the microsatellite data using the same approach as for the mitochondrial sequences.

STRUCTURE 2.3.1 (Pritchard *et al.* 2000; Falush *et al.* 2007) was used to estimate the number of genetically distinct clusters (K) with the highest likelihood. It was run for 10,000,000 steps after an initial burn-in period of 1,000,000 steps for K=1-14. Each possible K was replicated 3 times and results of LnP(K) versus each K was graphed to visualize results. A model of correlation of allele frequencies across populations was selected as it was expected that populations would have a similar evolutionary history, as well as the admixture model to allow for mixed ancestry of individuals within each population

Structurama (Huelsenbeck *et al.* 2011) calculates the posterior probability of individuals being assigned to a population, and from this it estimates the most likely number of populations. It does not require prior information on the number of populations as this is treated as a random variable. Analysis was run three times for a Gamma distribution value of (1,1) and (1.001,0.0001) for 1000 permutations each. The Gamma values refer to the shape and parameter of the probability distribution, changing only how tightly groups are clustered.

Relatedness

The patterns suggested from F_{ST} values are difficult to compare to other studies, therefore patterns of relatedness within colonies will also be estimated to infer if the observed patterns are biologically relevant. STORM (software for testing hypothesis of relatedness and mating patterns) was used to test within group relatedness to see if individuals sampled at a roost site were more closely related than expected by chance (Frasier 2008). STORM uses sampled nuclear genotypes, allele frequencies of each loci and known grouping of individuals to calculate the average pairwise relatedness values within the grouping to give an overall average for each observed group. The observed within group relatedness can then be compared to 1000 iterations of expected relatedness calculated by STORM to estimate p-values for each group. Expected relatedness values are estimated by the program by randomizing genotypes between groups while keeping group size constant with sampled groups.

Results

Mitochondrial DNA

I sequenced a 294 base pair section of the hypervariable region II within control region of 423 adult female *M. lucifugus* from 14 maternity colonies in mainland Nova Scotia (Table 1). There were 37 variable sites resulting in 47 unique haplotypes with a nucleotide polymorphism estimate of 0.125. Of the 47 haplotypes, 27 were private to a single group of individuals (i.e., colony).

Table 1: Summary statistics of mitochondrial DNA hypervariable II region sequences obtained from mainland Nova Scotia. Sequences obtained (s), haplotypes found (h), private haplotypes (p) haplotype diversity, nucleotide diversity and gene diversity for the adult females sampled at 14 maternity colonies. Map number refers to locations on map in Figure 1.

Map Number	Colony	s	h	p	Haplotype Diversity	Nucleotide Diversity	Gene Diversity
1	Annapolis Royal	27	12	4	0.239	0.0156	0.897
2	Caledonia	20	8	1	0.305	0.0147	0.837
3	Mill Village	69	14	6	0.233	0.0128	0.824
4	New Russell	13	7	1	0.392	0.0135	0.885
5	Martock	29	6	0	0.317	0.012	0.741
6	Center Rawdon	23	8	0	0.327	0.0168	0.854
7	Upper Rawdon	24	9	1	0.336	0.0162	0.851
8	Renfrew	28	8	1	0.293	0.0141	0.77
9	Waverley	19	10	1	0.338	0.0162	0.93
10	Mineville	24	8	0	0.358	0.0148	0.877
11	West Chezzetcook	18	8	2	0.246	0.011	0.64
12	Tatamagouche	86	12	3	0.208	0.0136	0.775
13	Earltown	13	4	0	0.347	0.0131	0.603
14	Antigonish	30	12	7	0.288	0.0198	0.917
	Total	**423**	**47**	**27**			

The global mitochondrial F_{ST} estimate was 0.114 ($p < 0.001$), with pairwise F_{ST} values between colonies ranging from 0 to 0.40 (Table 2). Among colony

variation accounted for 11.41 % of the variation, and within colony variation accounted for the remaining 88.59 %. The global Φ_{ST} estimate was 0.12924 ($p < 0.0001$) with 12.90 % of the variation occurring among populations and 87.10% of the variation occurring within populations. In total 28% (26/91) of the pairwise F_{ST} values are statistically different from zero after Bonferroni correction. Of these, pairwise F_{ST} values for Tatamagouche with 10 of the other roosts were statically significant. Tatamagouche was the colony with largest sample size, 86 sampled adult females. The colony sampled at West Chezzetcook, with 18 samples, showed a similar pattern and was significantly different from 7 other roosts (Table 2).

Table 2: Pairwise F_{ST} values for 14 maternity colonies of *Myotis lucifugus* in Mainland Nova Scotia (2010-2012). Mitochondrial F_{ST} values above the diagonal as calculated by Arlequin, and nuclear F_{ST} values below the diagonal as calculated by GenePop. Bold indicates statistical significance with Bonferroni correction.

	AR (1)	AN (14)	CA (2)	EA (13)	MA (5)	MI (10)	MV (3)	NR (4)	CR (6)	UR (7)	RE (8)	TT (12)	WA (9)	WC (11)
AR		**0.097**	0.050	0.160	0.046	0.046	0.074	0.089	0.034	0.069	**0.147**	0.060	-0.005	**0.303**
AN	0.006		0.062	0.061	**0.112**	**0.104**	0.022	0.062	0.025	0.023	0.021	**0.201**	0.040	0.133
CA	0.000	0.014		0.101	0.059	0.080	0.031	0.115	0.053	0.069	0.062	**0.195**	0.052	**0.268**
EA	0.008	0.009	0.014		0.140	**0.167**	**0.122**	**0.218**	0.115	0.150	0.117	**0.292**	0.148	**0.347**
MA	0.003	-0.001	0.003	0.006		0.014	**0.124**	0.139	0.106	0.110	**0.170**	**0.124**	0.089	**0.394**
MI	0.012	-0.006	0.016	0.008	0.001		0.080	0.038	0.054	0.053	**0.139**	**0.073**	0.044	**0.314**
MV	0.000	0.003	0.000	0.000	0.002	0.006		0.009	-0.022	-0.017	-0.015	**0.187**	0.009	0.099
NR	0.008	0.009	0.009	0.009	0.003	0.014	0.009		-0.014	-0.033	0.071	0.101	0.011	0.157
CR	0.000	0.000	0.009	0.009	0.004	-0.002	0.004	0.014		-0.023	0.023	**0.117**	-0.030	0.122
UR	0.011	0.005	0.012	0.005	0.005	0.006	0.004	0.014	0.004		0.018	**0.138**	-0.003	0.096
RE	0.002	0.007	0.001	0.001	0.003	0.004	0.003	-0.002	0.001	0.005		**0.271**	0.074	0.086
TT	0.005	0.004	0.005	0.007	0.004	0.001	0.001	0.009	-0.005	0.007	0.003		0.078	**0.380**
WA	0.003	-0.002	0.013	0.000	0.002	-0.006	0.006	0.000	0.002	0.008	0.001	0.003		**0.195**
WC	0.009	0.006	0.011	0.012	0.007	-0.008	0.001	0.013	0.002	0.003	0.006	-0.008	-0.001	

Map reference

1: Annapolis Royal (AR), 2: Caledonia (CA), 3: Mill Village (MV), 4: New Russell (NR), 5: Martock (MA), 6: Center Rawdon (CR) 7: Upper Rawdon (UR) 8: Renfrew (RE), 9: Waverley (WA), 10: Mineville (MI), 11: West Chezzetcook (WC), 12: Tatamagouche (TT), 13: Earltown (EA), 14: Antigonish (AN).

Table 3: Euclidian pairwise distance (km) between the 14 maternity colonies of *Myotis lucifugus* in mainland Nova Scotia used for isolation by distance calculation.

	AR	AN	CA	EA	MA	MI	MV	NR	RC	RH	RE	TT	WA	WC
AR		295	48	86	112	86	169	88	139	150	153	211	154	178
AN			271	86	181	264	140	210	157	150	145	100	155	140
CA				200	93.5	43	136	66	122	126	130	203	123	145
EA					211	200	101	136	77	73	74	72	94	95
MA						97	68	28	27	33	42	108	50	76
MI							118	71	118	123	123	204	110	127
MV								83	53	49	38	113	16	10
NR									57	61	69	137	68	95
RC										6	17	86	36	59
RH											12	83	34	55
RE												83	25	42
TT													166	110
WA														26

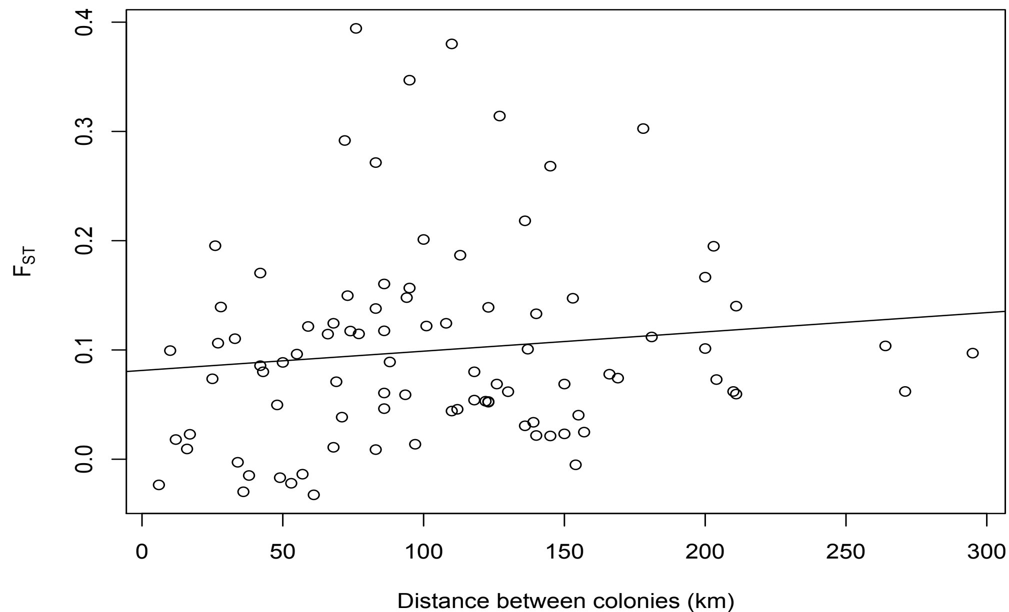

Figure 2: Isolation by Distance for mitochondrial DNA hypervariable region II sequences for little brown bat maternity colonies in mainland Nova Scotia. Pairwise F_{ST} versus distance between colonies (km) (df=89, R^2=0.0033, p=0.2577)

Maternity colonies in mainland Nova Scotia did not show a significant pattern of mitochondrial DNA isolation by distance (p=0.2577; Figure 2). The distances separating colonies ranges from 6-295 km (Table 3), yet the colonies with the highest F_{ST} value were 75-125 kilometers apart (Table 3).

Table 4: F_{SC}, F_{ST}, and F_{CT} calculated by SAMOVA for mitochondrial DNA haplotypes at 14 maternity colonies in mainland Nova Scotia to estimate the most genetically differentiated clusters of populations.

K	2	3	4	5	6	7	8	9	10	11	12	13
F_{SC}	**0.10**	0.09	0.06	0.03	0.02	0.02	0.01	-0.01	-0.01	-0.02	-0.02	-0.03
F_{ST}	**0.25**	0.22	0.16	0.14	0.13	0.13	0.13	0.12	0.12	0.12	0.12	0.12
F_{CT}	**0.17**	0.14	0.11	0.11	0.11	0.12	0.12	0.13	0.13	0.13	0.13	0.14

SAMOVA analysis resulted in the highest F_{CT} value at K=2 indicating that the sampled individuals represent, at most, 2 genetically distinct populations. The first was composed of the individuals sampled at West Chezzetcook, and the second included all remaining individuals.

Analysis of Nuclear Loci

I genotyped 13 - 87 adult females at each of the 14 maternity colonies at 11 microsatellite loci. One primer set, Mluc29 (Burns *et al.* 2012), did not amplify well in the majority of samples and therefore was excluded, leaving 10 loci for analysis. Overall, within the 10 loci, the average number of alleles per locus was 10.3 (range: 6.2 -13.9). Observed heterozygosity per colony was 3-17% lower than expected.

Table 5: Summary statistics of 10 polymorphic nuclear DNA loci obtained from *M. lucifugus* from mainland Nova Scotia. Sample size (n), average alleles per loci (A), observed heterozygosity (H_o) and expected heterozygosity (H_E) for the adult females sampled at 14 maternity colonies. Map number refers to locations on map in Figure 1.

Map Number	Colony	*n*	A	H_o	H_e
1	Annapolis Royal	28	10.6	0.683	0.814
2	Caledonia	21	9.6	0.697	0.811
3	Mill Village	68	14.0	0.701	0.815
4	New Russell	14	7.9	0.646	0.821
5	Martock	56	12.9	0.723	0.821
6	Center Rawdon	44	12.8	0.699	0.812
7	Upper Rawdon	37	12.5	0.733	0.803
8	Renfrew	25	9.4	0.727	0.814
9	Waverley	19	6.2	0.670	0.794
10	Mineville	26	7.3	0.619	0.707
11	West Chezzetcook	18	7.3	0.715	0.808
12	Tatamagouche	87	13.9	0.741	0.823
13	Earltown	13	7.6	0.770	0.800
14	Antigonish	30	11.3	0.736	0.829

Nuclear DNA suggested there was population structure (global $F_{ST} = 0.0034$, $p=0.0014$), albeit weak. F_{IS} (0.1197 ($p<0.001$)) was higher than F_{ST} (Table 6), suggesting that sampled individuals may be close relatives.

Table 6: Nuclear global F-statistics (F_{ST}, F_{IS} and F_{IT}) estimated using GenePop and SPaGeDi from adult female little brown bats sampled at 14 maternity colonies.

	F_{it}	F_{is}	F_{st}
Global	0.1227	0.1197	0.0034
p-Value	<0.001	<0.001	0.0014

No pattern of isolation by distance was evident (df=89, R^2= -0.0099, p= 0.735) (Figure 3). Pairwise nuclear F_{ST} values were highest between colonies between 50 and 150 km apart, with lower F_{ST} values between the more distant roosts (Figure 3).

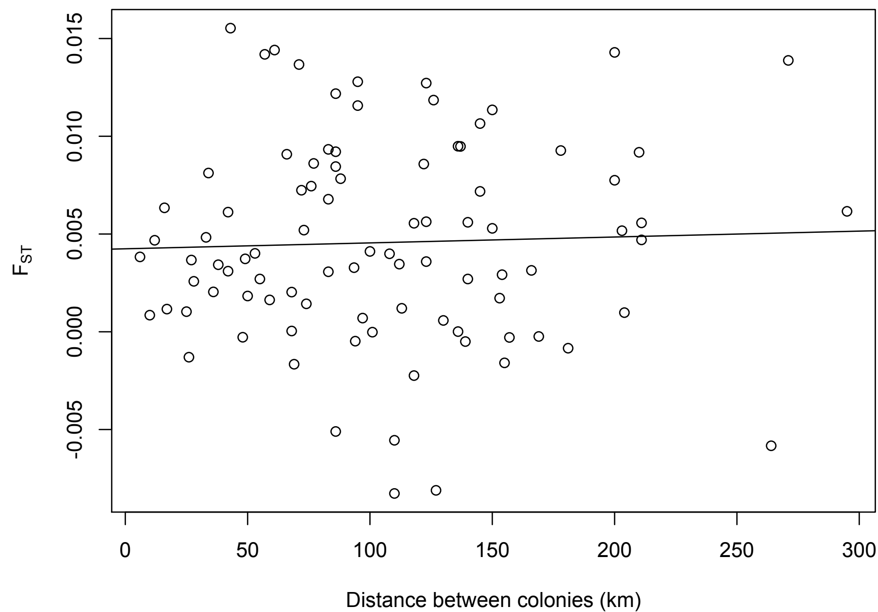

Figure 3: Isolation by Distance for 10 polymorphic nuclear DNA microsatellite loci. Pairwise genetic Fst versus Distance between colonies (km) of female little brown bats sampled at 14 maternity roosts on mainland Nova Scotia, Canada, 2010-2012 (df=89, R^2= -0.0099, p= 0.735)

Using two cluster analysis methods, mainland Nova Scotia was found to most likely represent one large population formed of all the small roost sites (Figures 4 & 5). STRUCTURE found the highest probability of k=1 with the lowest LnP(K) of the 14 options, while Structurama found k=2 with a 95% probability.

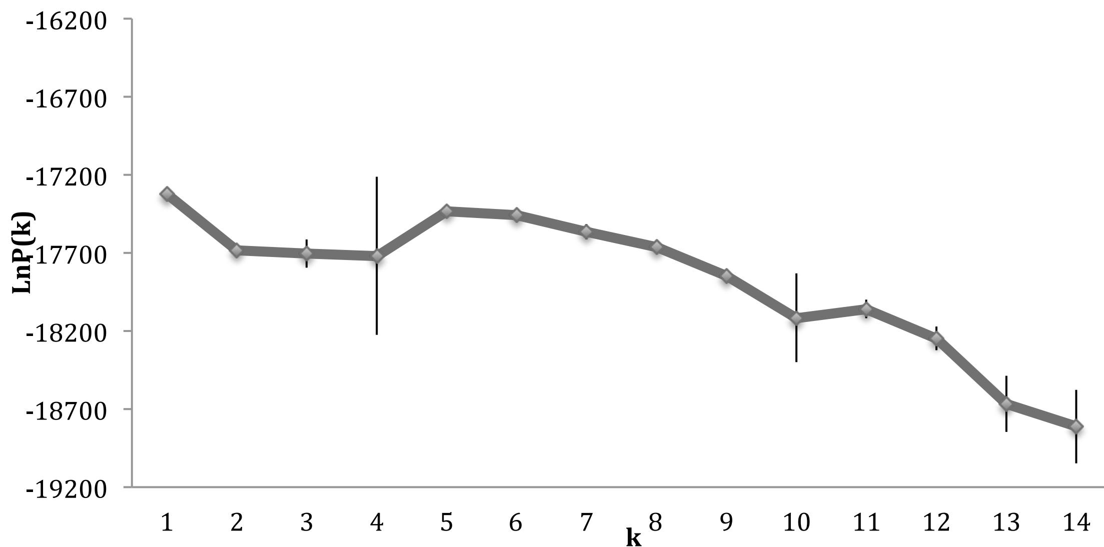

Figure 4: Cluster analysis. Log probability of each value of k from k=1 to k=14 as calculated by the program STRUCTURE using 10 polymorphic nuclear DNA loci on from little brown bat females capture at 14 maternity colonies in mainland Nova Scotia from 2010-2012.

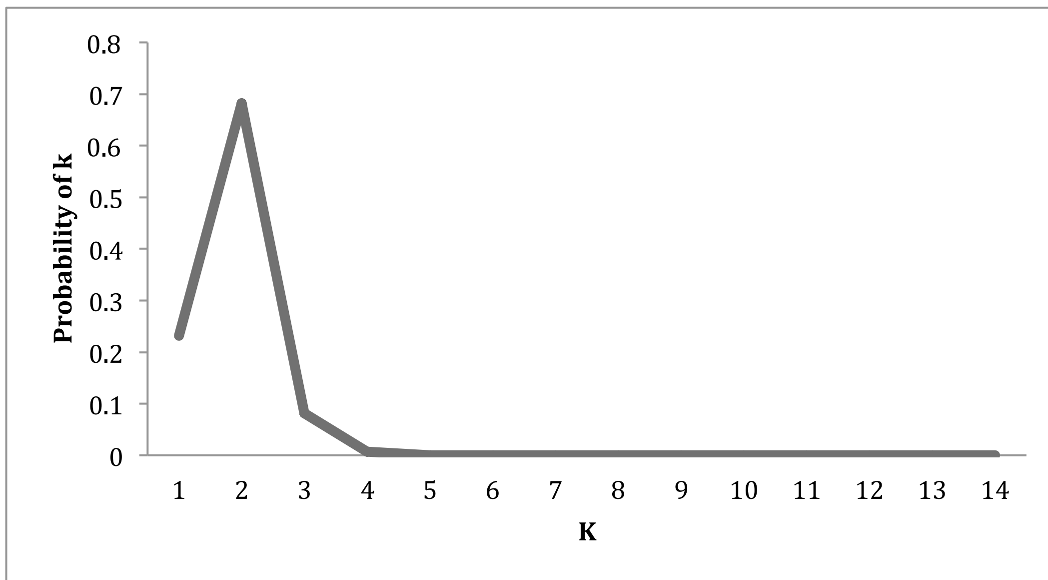

Figure 5: Cluster analysis. Probability of each k from k=1 to k=14 as calculated by the program Structurama based on the posterior probability of assigning individuals to each population for little brown bat females sampled at 14 maternity roosts in mainland Nova Scotia, Canada between 2010 and 2012.

Relatedness

The overall average colony relatedness was higher than expected and 2 of the 14 colonies were more related than expected by chance based on randomized groups formed from the sampled dataset. These colonies are Mineville and Renfrew (Table 7).

Table 7: Observed and expected R-values calculated for relatedness analysis using STORM for each of the 14 colonies. Bolded values represent significance with 1000 permutations.

Colony	Observed R-value	Expected R-value	p-value
Annapolis Royal	0.008	0.018	0.70
Caledonia	0.033	0.018	0.22
Mill Village	0.001	0.018	0.95
New Russell	-0.007	0.017	0.81
Martock	0.016	0.018	0.55
Center Rawdon	0.028	0.018	0.24
Upper Rawdon	0.037	0.018	0.10
Renfrew	**0.067**	**0.017**	**0.01**
Waverley	0.036	0.018	0.21
Mineville	**0.054**	**0.019**	**0.05**
West Chezzetcook	0.049	0.019	0.11
Tatamagouche	0.015	0.018	0.63
Earltown	0.035	0.018	0.29
Antigonish	0.008	0.018	0.74
Average	**0.027**	**0.018**	**0.01**

Discussion

In spite of a high degree of dispersal ability, weak but significant population level genetic structure at maternity colony levels was observed across mainland Nova Scotia, using molecular markers, this can provide insight into the life history of little brown bats. Mitochondrial DNA hypervariable region II had a higher degree of structuring than nuclear DNA microsatellite markers (Table 6) supporting the prediction that colonies may be structured by a propensity for females to be philopatric and male dispersal. In mammals, it is a common trait for females to remain within their natal colony while males will disperse (Greenwood 1980). The low nuclear structure found between maternity colonies indicates a high level of nuclear gene flow between colonies; this could be due to mating at swarming sites. Little brown bats are capable of long-distance sustained flight, mark recapture studies have found median distances between summering roosts as well as between summering roosts and hibernacula to be greater than 400 kilometers (Norquay *et al.* 2013). The distance between maternity colonies sampled in mainland Nova Scotia ranged between 7 and 295 kilometers (Table 3), therefore the distance between colonies is not large proportional to the potential for bat movement.

Global mitochondrial F_{ST} values among maternity roosts in mainland Nova Scotia were higher than those of a similar study conducted in the midwestern United States using Cytochrome b markers (Dixon 2011). However, population level structure is expected to be correlated with molecular marker substitution rates. Markers with low substitution rate are most often used for species identification,

cytochrome b is a protein-coding gene; therefore substitutions in this region are less frequent than in a non-coding region due to selection pressure. The control region includes sections of highly polymorphic hypervariable DNA and is hypothesized to be responsible for control of transcription and replication. It often includes a section of known short tandem repeats that are conserved in position across mammalian species, which are thought to be involved in the termination of transcription (Lunt *et al.* 1998). The global F_{ST} value of 0.114 ($p<0.001$) using the hypervariable region II found in this study is lower than the F_{ST} value found in the Bechstein's bat of 0.68 ($p < 0.001$) using mitochondrial control region R1 repeat copy number (Kerth & Mayer 2000). The mitochondrial control region R1 has less variation than the hypervariable region; therefore we expect to see a lower degree of structure using this marker. The high degree of structure at Bechstein's bat maternity colonies corresponds to the closed groups observed during observational studies, where no movement between groups have been recorded over a three-year period (Kerth & Mayer 2000). A population genetic structure study of big brown bat (*Eptesicus fuscus)* females roosting in buildings spanning two states, found that while some females are philopatric, all colonies sampled were formed of between 5-15 matrilines and that female dispersal between colonies was a main factor in population level structuring (Vonhof *et al.* 2008). We can see a varying level of philopatry and female dispersal found between bat species using molecular techniques.

Pairwise mitochondrial F_{ST} values for all colonies sampled in mainland Nova Scotia ranged from 0 to 0.38 (Table 2). Low F_{ST} values are an indication that within

group variation is consistent with population-wide variation, suggesting no genetic structure. F_{ST} values tend to be low when groups exchange members. In this study, 65 of 91 pairwise comparisons were not significantly different from 0 suggesting that individuals from these roosts were genetically similar to each other. When F_{ST} values are high, it is an indication of genetic differentiation between groups. This can occur when individuals are philopatric to their natal colonies and are sampled within these groups. The remaining 26 mitochondrial DNA pairwise comparisons were significantly different from 0 (Table 2), suggesting that female dispersal from these colonies is low.

If the main reason for genetic differentiation of colonies were geographic distance, I would expect that genetic differentiation among colonies would be proportional to geographic distance between them. This was not the pattern observed across mainland Nova Scotia (Figures 2& 3). However, as discussed earlier, bats are capable of long-distance sustained flight (Norquay *et al.* 2013) and the geographic distances between colonies are not large relative to the potential movement distances of the bats.

Global nuclear F_{ST} was much lower at 0.0034 ($p=0.0014$, Table 6) than mitochondrial DNA at 0.114 ($p < 0.001$), yet still significantly different from zero. If some females are coming back to the same colony each year, this may explain the low level of nuclear structure found at maternity colonies. As nuclear DNA microsatellites are bi-parentally inherited they are most useful for studies of population history and parentage. Pairwise nuclear DNA F_{ST} ranged from 0 to 0.014 (Table 2), with none being significantly different from zero after Bonferoni

correction. This indicates that nuclear gene flow remains high between these colonies. Research suggests that when F_{ST} values are greater than 0.05, a sample size of 20 individuals from each population is sufficient, yet when F_{ST} is lower (0.01), sample size must increase to 100 individuals to detect genetic structuring accurately below this level (Kalinowski 2004). However, it is unlikely that the differentiation of an F_{ST} value of 0.01 or lower would be biologically meaningful. With our sample size, ranging from 13-92 individuals per colony, weak nuclear structure was found (Global F_{ST}=0.0034, p=0.0014), indicating that nuclear gene flow is high between all colonies; therefore increasing sample size to infer a lower F_{ST} value would not be beneficial.

For each colony, observed nuclear DNA heterozygosity was 3-17% lower than expected (Table 5), resulting in a high F_{IS} value (Table 6). This is expected when roosts consist of maternal relatives. One study of the greater horseshoe bat (*Rhinolophus ferrumequinum*) observed pairwise relatedness values for some pairs of bats within maternity roosts to range between 0.17 to 0.62, yet the remaining pairwise value were close to 0 for other pairs (Rossiter *et al.* 2002). This suggests that while some individuals within these colonies are related, many matrilines are roosting together.

Nuclear genetic clustering analysis for my study suggests that *M. lucifugus* females on mainland Nova Scotia form 1-2 genetic populations (Figures 4 & 5) suggesting there is nuclear gene flow among colonies. This could be a result of individuals mating at hibernacula where individuals come together from many

summering sites, including unrelated males. Arrival at the hibernacula and swarming behavior (mating) begins in late summer (Fenton 1969). In *Myotis sodalis* (the Indiana bat) the sex-ratio at swarming has male-biased activity at the beginning and the end of this period, this is thought to be due to males mating with both the early and late arriving females (Cope & Humphrey 1977).

For nuclear genetic flow to remain high between maternity colonies, females from a colony must have the opportunity to mate with genetically different males. This could occur if individuals mate at hibernacula during swarming, where individuals come together from a larger geographic range, including many males from many different areas (Kerth *et al.* 2003). Little brown bats follow a similar pattern to that of the Indiana bats, where males arrive before females, and are more active in the early swarming period. One little brown bat study observed mating activity at hibernacula beginning in mid- to late-August, yet individuals started arriving at the site as early as July (Thomas *et al.* 1979).

Mitochondrial DNA cluster analysis separated the roost sites into 2 distinct populations (Table 4) based on genetic composition and geographical locations. Given the distribution of these clusters, one containing the18 individual sampled at West Chezzetcook and the second being all remaining individuals it is unlikely that this is a true representation of the groups. One issue with the program used for this analysis is that it is unable to calculate the likelihood of the samples representing 1 genetic cluster of individuals. It was predicted that mitochondrial DNA structure would infer a higher level of clustering than nuclear DNA; each one may correspond to a group of colonies, and therefore separate lineages.

Weak but significant population genetic structure was found for the 14 *Myotis lucifugus* maternity colonies sampled on mainland Nova Scotia. Mitochondrial DNA structure was found to be higher than nuclear DNA structure. This pattern of higher mtDNA structure than nuclear DNA structure is consistent with other mammalian species that exhibit female philopatry and male dispersal. For mitochondrial DNA structure to remain high between colonies, one hypothesis is that a matrilines must be roosting together as mitochondrial DNA is maternally inherited. Relatedness analysis supports this interpretation, the average relatedness value within all colonies was higher than expected if these colonies were formed by chance, as well two colonies, Renfrew and Mineville had higher relatedness values than expected. Relatedness values are higher than expected if the colonies were formed by chance, yet if all individuals in a colony were related we would expect a much higher relatedness value. This suggests that multiple matrilines are roosting together, instead of one single matriline. For nuclear DNA population genetic differentiation to remain at the level found in this study, females from a matriline must be able to mate with genetically different males. This is most likely to occur at swarming sites, where many individuals come together from summering areas to mate followed by a period of hibernation within hibernacula.

Bat species are currently facing declines due to 2 factors, a fungal pathogen and wind turbines (Kunz *et al.* 2007; Puechmaille *et al.* 2010). The fungal pathogen, *Pseudogymnoascus destructans* has already caused significant regional declines in little brown bat populations in the north-eastern United States with a high possibility of regional extirpation predicted for the area (Frick *et al.* 2010).

Characterization of the movement dynamics of the little brown bat at a regional scale both between maternity colonies, and within the population as a whole is important as the spread of *Pseudogymnoascus destructans* is facilitated by the movement of individuals (Frick *et al.* 2010).

A low global nuclear F_{ST} suggests that females within a maternity colony are mating with different and unrelated males, allowing for high gene flow. Females in one colony may not travel to the same swarming site and/or hibernacula with the remaining females in her roost. If individuals are regularly switching roosts, the movement may allow for the transmission of fungal spores between sites. As the fungus requires the cold temperature of the hibernacula for growth, the effect of this movement may not be observed until swarming behavior and hibernation. If individuals are regularly switching swarming sites for increased access to mates, they may be transferring the spores between sites.

Recent increases in the number of wind energy farms may also have an impact on bat species. Since 2003, the reported number of bat fatalities at wind farms in North America has dramatically increased, yet this number could be an under-representation of the actual fatalities due to low frequency monitoring and scavengers at the sites (Cryan & Barclay 2009). Bats most often affected by wind turbines are long-range migratory species, such as hoary (*Lasiurus cinereus*), silver-haired (*Lasionycteris noctivagans)* and red bats (*Lasiurus boreali)s*, yet little brown bats account for approximately 5% of wind turbine fatalities reported in the United States (Kunz *et al.* 2007). It is known that some fatalities are caused by the rotating

blades of the wind turbines, yet the reasons is currently unknown (Kunz *et al.* 2007; Arnett *et al.* 2008). With only 28% of the pairwise F_{ST} values showing significance, this is an indication that there is movement between the remaining roost sites. If wind turbine development were to occur in the movement path between any of the roost sites, this may have an increased risk of mortality to individuals.

Using two molecular markers, I found weak population level structuring at maternity colonies in mainland Nova Scotia. Mitochondrial DNA was expected to show a higher level of structuring and clustering than that of nuclear DNA. Mitochondrial structuring was a hundredfold higher than that of nuclear DNA structuring, as indicated by F_{ST} values. While clustering analysis for both markers separated the colonies into 1-2 genetic clusters, not a higher level of mitochondrial DNA clustering than nuclear DNA clustering. These patterns are consistent with a degree of female philopatry and male dispersal, where some females will return to their natal colonies while others will disperse. This is also supported by relatedness analysis results, where on average, females roosting at colonies were related than expected had they been formed by chance.

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