Beyond mtDNA: nuclear gene flow suggests taxonomic oversplitting in the little brown bat (*Myotis lucifugus*)

C.L. Lausen, I. Delisle, R.M.R. Barclay, and C. Strobeck

Abstract: In southern Alberta and north-central Montana, there is substantial mtDNA sequence divergence between two groups of the little brown bat, *Myotis lucifugus* (LeConte, 1831), previously thought to be subspecies (*Myotis lucifugus lucifugus* and *Myotis lucifugus carissima*) but recently hypothesized to be species. We tested this hypothesis using population genetic techniques. Using nuclear microsatellite markers (10 loci), we found a lack of differentiation between these two groups of bats (ascribed based on mitochondrial hypervariable region II sequence), suggesting interbreeding was sympatric. ings add to the recent discovery that *M. l. lucifugus* haplotypes are found throughout the range previously thought to be only *M. l. carissima*, suggesting widespread sympatry and extensively mixed gene pools, thus refuting the cryptic species hypothesis. Clinal morphology and individual variation demonstrated the impossibility to differentiate groups based on original subspecies definitions. The lack of geographic and morphological boundaries, in addition to the likelihood that the interbreeding observed in this study is occurring across western North America, suggests that no line can be drawn between these two groups. We thus suggest that the *carissima* subspecies designation be dropped. This study highlights the importance of investigating nuclear gene flow in widely sympatric animals suspected of being cryptic genetic species, and has important implications for applications of the DNA Barcoding Project.

Résumé : Dans le sud de l'Alberta et le centre-nord du Montana, il existe une divergence importante dans les séquences de l'ADNmt entre deux groupes de murins bruns, *Myotis lucifugus* (LeConte, 1831), qu'on a cru antérieurement représenter des sous-espèces (*Myotis lucifugus lucifugus* et *Myotis lucifugus carissima*), mais qu'on pense maintenant former une seule espèce. Nous testons cette hypothèse à l'aide de techniques de génétique des populations. En utilisant des marqueurs microsatellites nucléaires (10 locus), nous ne trouvons aucune différenciation entre les deux groupes (assignés d'après la séquence de la région II hypervariable mitochondriale), ce qui indique qu'il y a des croisements en conditions de sympatrie. Nos résultats s'ajoutent à la découverte récente d'haplotypes de *M. l. lucifugus* dans toute l'aire de répartition qu'on croyait antérieurement ne contenir que des *M. l. carissima*; cela laisse croire qu'il y a une importante sympatrie et que les pools géniques sont fortement entremêlés, ce qui permet de rejeter l'hypothèse des espèces cryptiques. Les gradients morphologiques et la variation individuelle ne permettent pas de différencier les deux groupes d'après les définitions originales des sous-espèces. L'absence de frontières géographiques et morphologiques, de même que la probabilité que la reproduction croisée que nous observons s'étende à tout l'ouest de l'Amérique du Nord, font croire qu'il n'est pas possible de tirer une ligne entre les deux groupes. Nous suggérons donc d'abandonner la désignation de la sous-espèce *carissima*. Notre recherche met en évidence l'importance de l'étude du flux génique nucléaire chez des animaux à large répartition sympatrique que l'on soupçonne être des espèces génétiques cryptiques; elle a aussi des conséquences importantes sur les applications du projet des codes à barres de l'ADN.

[Traduit par la Rédaction]

Introduction

The increasing availability of genetic characters is bringing about revisions of the classification of many taxonomic groups (e.g., Thompson and McManus 2002; Gevers et al. 2005; Goncharov 2005). The current ease with which mitochondrial DNA (mtDNA) sequences are obtained, as well as the small sample sizes needed for phylogenetic studies, has resulted in an accumulation of mtDNA sequence data for a large number of species (GenBank 2007). Most recently, the "DNA Barcode of Life Project" has generated interest in acquiring cytochrome oxidase I (COI) mtDNA sequences for much of the world's biodiversity (Hebert et al. 2003). Mitochondrial gene sequences, such as cytochrome *b* (cyt*b*) or COI/II, can be informative for the reconstruction of phylogenetic histories, but because of the nature of mtDNA inheritance, contemporary gene flow is unlikely to be understood using this marker alone (Avise 2004). Whether a mitochondrial gene sequence can provide enough information to delineate new biological species is uncertain and debated (DNA barcoding, reviewed in Rubinoff et al. 2006). Furthermore, barcode identification of species depends on genetic diversity between species being higher than within, which is

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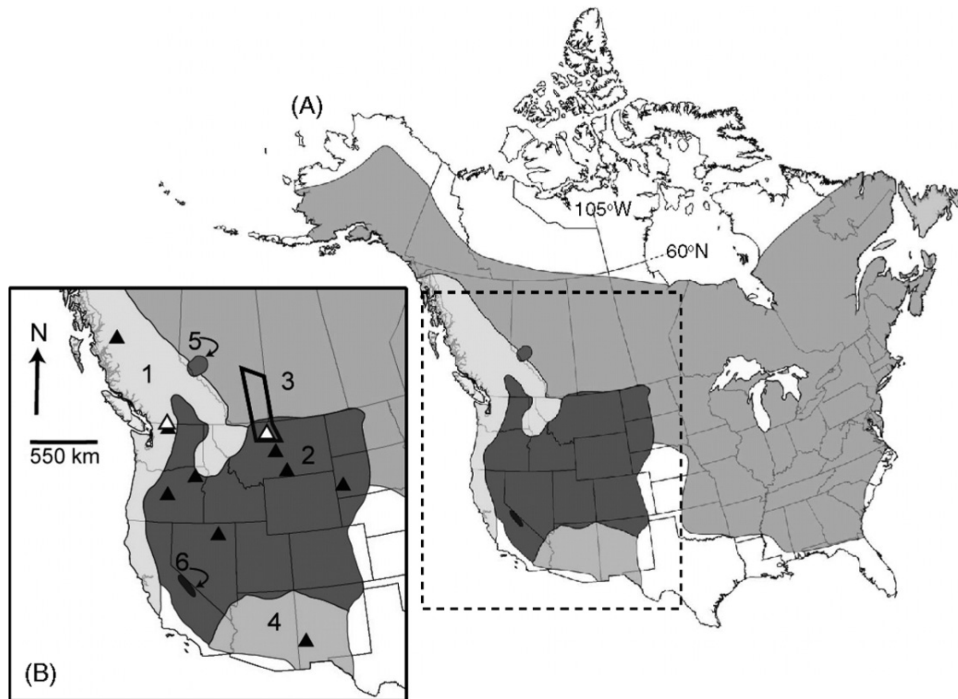
Fig. 1. (A) North American distribution of little brown bats (*Myotis lucifugus*). The delineated area is enlarged in B. Subspecies boundaries are as originally defined using morphology, adapted from Hall (1981): 1, *Myotis lucifugus alascensis*; 2, *Myotis lucifugus carissima*; 3, *Myotis lucifugus lucifugus*; 4, *Myotis lucifugus occultus*; 5, *Myotis lucifugus pernox*; 6, *Myotis lucifugus relictus*. Our study area is the polygon that spans the *M. l. lucifugus* and *M. l. carissima* ranges. Triangles are locations where at least one *M. l. lucifugus* haplotype has been located outside of its originally defined range; open triangles are from this study and solid triangles are from Dewey (2006).



not always the case (e.g., Elias et al. 2007). Conclusions based solely on mtDNA sequence data can be erroneous or misleading, with examination of nuclear DNA producing different conclusions (e.g., Cronin et al. 1991; Worthington-Wilmer et al. 1994 vs. 1999; Paetkau et al. 1998 vs. Talbot and Shields 1996; Elias et al. 2007).

In most animals, mitochondrial DNA acts as a single gene, and is passed on as one unit to the next generation only by females. Nuclear genes, on chromosomes, are shuffled each generation (Avise 2004). It is now well understood that examination of bi-parentally inherited nuclear markers, in addition to mtDNA, is necessary to get a full account of the history and contemporary breeding patterns of closely related taxonomic groups (Avise 2004). Taxonomic classifications must therefore be based on multiple lines of evidence, from molecular, morphological, ecological, and behavioural data (Cronin 1993; Mayer and von Helversen 2001).

In animals whose morphology may be highly constrained evolutionarily, convergent morphological traits have made taxonomy difficult (Lowe et al. 2004). Microchiropteran bats are a classic example of shared ecological constraints; flight and nocturnal behaviour are limiting forces on morphological variation (Norberg 1994; Ruedi and Mayer 2001; Kawai et al. 2003), and convergent evolutionary traits or phenotypic plasticity may influence traits that are often used to differentiate species (Miller and Allen 1928; van Zyll de Jong 1985). Genetics may allow differentiation of morphologically and ecologically similar species (e.g., Barratt et al. 1997).

As a widespread bat in North America (Wilson and Ruff 1999), the little brown bat, *Myotis lucifugus* (LeConte, 1831), has been the focus of much physiological, behavioural, and ecological research (Fenton and Barclay 1980; Kunz et al. 1998; Reynolds and Kunz 2000; Humphries et al. 2002). Six North American subspecies are generally recognized: *Myotis lucifugus lucifugus*, *Myotis lucifugus alascensis*, *Myotis lucifugus relictus*, *Myotis lucifugus carissima*, *Myotis lucifugus occultus*, and *Myotis lucifugus pernox* (Fenton and Barclay 1980). Original definitions were based mainly on pelage colour and forearm length (Miller and Allen 1928; Hall and Kelson 1959). Interestingly, these early subspecies definitions, with the exception of *M. l. occultus* (Barbour and Davis 1970; Findley and Jones 1967), have not been reviewed. There has been a longstanding recognition that "geographic variation in this species needs to be analyzed and the subspecies re-evaluated" (van Zyll de Jong 1985, p. 72); however, even today, field biologists assume subspecies designation based on the geographic location of capture in relation to the historic subspecies map (Fig. 1; Hall 1981). Recently, it has been determined that subspecies boundaries in *M. lucifugus* do not hold, with mtDNA haplotypes ascribed to *M. l. lucifugus* being found throughout the ranges of the other subspecies (Dewey 2006). Dewey (2006) has also suggested paraphyly within *M. lucifugus*, with the *M. l. lucifugus* lineage sister to a clade consisting of *M. l. carissima*, *M. l. alascensis*, *M. l. relictus*, and three long-eared *Myotis* species. This apparent paraphyly, obtained in a strongly supported phylogenetic analysis of cyt*b* sequences, led Dewey (2006) to suggest that these four subspecies of *M. lucifugus* may be distinct species. This recent suggestion questions the validity

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and comparability of previous research carried out on *M. lucifugus* (Dewey 2006).

Our goal was to evaluate this taxonomic recommendation for *M. l. carissima* and *M. l. lucifugus*, using population genetics methods. Substantial mtDNA differences are known to exist (Dewey 2006), reflecting historically separated maternal lineages. If these two haplogroups are behaving as sympatric species, there should be little gene flow between them. We therefore hypothesized that two separate populations would be detected through the investigation of bi-parentally inherited markers. Unlike conventional systematic approaches, population methods assess gene flow, evaluating whether, and to what extent, groups are interbreeding or not. We assessed nuclear gene flow in a region where these two putative subspecies (or species) are sympatric and found in relatively equal proportion. Because the *M. l. lucifugus* subspecies is now known to occur sympatrically across the full range of *M. l. carissima* (Fig. 1; Dewey 2006), evidence of gene flow in our study area would suggest widespread interbreeding between these two *M. lucifugus* groups across their sympatric range, homogenizing the nuclear gene pools, and making taxonomic distinction unnecessary. Alternatively, differentiated gene pools for each sympatric group may exist and may be reflected morphologically. We therefore also assessed morphology to determine whether forearm length and pelage colour (traits used in the original subspecies definitions; Miller and Allen 1928; Hall and Kelson 1959) could be used to reliably differentiate the two distinct mitochondrial groups of *M. lucifugus*.

Materials and methods

Study area and samples

We sampled *M. lucifugus* from Alberta and north-central Montana (Fig. 2), obtaining forearm measurements and tissue for genetic analysis (Table 1). The north-central Montana study sites, together with all southern Alberta sites, are part of the grasslands or Prairie Ecozone (Gauthier et al. 2003). Sites north of and including Dry Island Buffalo Jump are moister than the southern sites and are partially treed, falling within the Aspen Parkland Zone of the northern-most prairies. Sites north of Edmonton are well treed and belong to the Boreal Ecozone (Environment Canada 2003).

We caught bats each summer from 2001 to 2006. In some cases samples from a site were obtained over the course of 2 years, and therefore bats were banded to avoid sampling the same bat more than once. In the case of the Missouri River samples, all samples were acquired in 2003. Sex, species, and relative age (adult vs. juvenile; Anthony 1988) were recorded. Pelage colour was noted in individuals that were particularly dark or light. We measured forearm length of adult males and females using calipers. Tissue from the wing near the tibia was sampled using 2 mm diameter biopsy punches and stored in 90% ethanol for later extraction, amplification, mtDNA sequencing, and nuclear microsatellite genotyping. All bats used in genetic analyses were adults captured with mist nets between 2 June and 4 August of each year, with the exception of one volant juvenile male from Onefour, Alberta.

Eight locations make up the main study sites (Fig. 2) from which both mtDNA and microsatellite genotypes were obtained. At each of these main sites, ~30–50 male and female *M. lucifugus* were sampled in a small area (<10 km linear distance between mist-netting locations at each main study area). We collected fewer samples from the other sampling sites and therefore not all sites were used in all analyses. To examine forearm and genetic variations, we obtained forearm data from additional areas in Alberta (near Peace River (EMEND), Lac La Biche, and Drayton Valley; Table 1), and additional genetic samples from Alberta (Kananaskis, Rocky Mountains) and the Skagit Valley region of British Columbia and Washington (North Cascades National Park) (Table 1). All forearm analyses were done for males and females separately given that sexual dimorphism is common. All work conformed to the guidelines of the American Society of Mammalogists, as well as to the legal

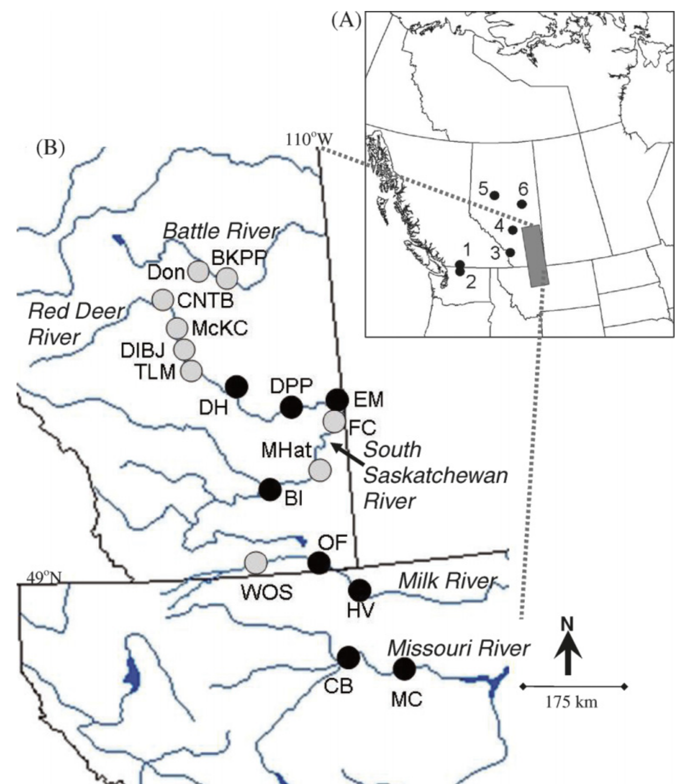

Fig. 2. Map of sample locations of little brown bats (*Myotis lucifugus*). (A) Samples or data collected by other researchers (solid circles): 1, Skagit Valley, British Columbia; 2, Skagit Valley, Washington (North Cascades National Park); 3, Sheep River, Alberta; 4, Drayton Valley, Alberta; 5, near Peace River, Alberta; 6, Lac La Biche, Alberta. The main study area is the shaded rectangle, which is expanded in B. (B) Shaded circles are areas where sequence data and (or) forearm measurements were collected; at locations marked by solid circles, microsatellite data were collected in addition to sequence data and (or) forearm measurements. BKPP, Big Knife Provincial Park; Don, Donalda; CNTB, Content Bridge; McKC, McKenzie Crossing; DIBJ, Dry Island Buffalo Jump; TLM, Tolman Bridge; DH, Drumheller; DPP, Dinosaur Provincial Park; EM, Empress; FC, Bindloss Ferry Crossing; MHat, Medicine Hat; BI, Bow Island; WOS, Writing on Stone Provincial Park; OF, Onefour; HV, Havre; CB, Coal Bank Landing; MC, McClelland Ferry Crossing.

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Table 1. UTM locations for sampling sites of little brown bats (*Myotis lucifugus*) in Fig. 2 are listed in descending order of latitude.

		UTM					
Location[a]	River	Zone	Easting	Northing	Type of data collected[b]	Sample sizes of msat, mtDNA, FA	Samples collected by
Inside of study area							
Near Peace River (EMEND), Alberta [5]	na	11	415986	6292662	FA	41	K. Patriquin
Lac La Biche, Alberta [6]	na	12	0372072–0500000	5983522–6096621	FA	28	L. Crampton
Drayton Valley, Alberta [4]	Near North Saskatchewan	11	634213	5874142	FA	29	Lippert 2001
Donalda, Alberta (on Meeting Creek) [Don]	Battle	12	395945	5826600	mtDNA, FA	2, 17	
Big Knife Provincial Park, Alberta [BKPP]	Battle	12	417028	5816359	mtDNA, FA	4, 12	
Content Bridge, Alberta [CNTB]	Red Deer	12	358373	5797285	FA	1, 14	
McKenzie Crossing, Alberta [McKC]	Red Deer	12	366277	5765649	FA	1, 1	
Dry Island Buffalo Jump Provincial Park, Alberta [DIBJ]	Red Deer	12	364705	5755366	mtDNA, FA	1, 6	
Tolman Bridge, Alberta [TLM]	Red Deer	12	361048	5744424	FA	27	
Drumheller, Alberta [DH]	Red Deer	12	387979	5697124	msat, mtDNA, FA	54, 12, 48	
Empress, Alberta [EM]	South Saskatchewan	12	571627	5640100	msat, mtDNA, FA	31, 8, 31	
Dinosaur Provincial Park, Alberta [DPP]	Red Deer	12	464336	5624406	msat, mtDNA, FA	49, 12, 47	
Bindloss Ferry Crossing, Alberta [FC]	South Saskatchewan	12	557276	5610380	FA	50	
Near Suffield Army Base, Alberta	South Saskatchewan	12	0543100–0565398	5601849–5620269	FA	7	Various locations between MHat and FC (not shown in Fig. 2)
Bow Island, Alberta [BI]	South Saskatchewan	12	478815	5542421	msat, mtDNA, FA	53, 11, 60	
Pinhorn Grazing Reserve, Alberta	Milk	12	507336	5441473	FA	19	~20 km west of OF on Milk River (not shown in Fig. 2)
Onefour, Alberta [OF]	Milk	12	538581	5440986	msat, mtDNA, FA	52, 12, 61	
Writing on Stone Provincial Park, Alberta [WOS]	Milk	12	454864	5436798	mtDNA, FA	2, 37	
Havre, Montana [HV]	Milk	12	588599	5373321	msat, mtDNA, FA	55, 15, 23	
Coal Bank, Montana [CB]	Missouri	12	558746	5320250	msat, mtDNA, FA	59, 17, 32	
McClelland Ferry, Montana [MC]	Missouri	12	620042	5284197	msat, mtDNA, FA	51, 18, 28	
Outside of study area and used for mtDNA comparison only							
Kananaskis, Rocky Mountains, Alberta [3]	Near Sheep	12	666127	5613540	mtDNA	1	D. Solick
Skagit Valley, British Columbia [1]	na	10	614611	5451864	mtDNA	1	T. Luszcz
North Cascades National Park, Washington [2]	na	10	639898	5452167	mtDNA	1	T. Luszcz
Total						404, 119, 618	

Note: The shaded rows refer to the eight main sampling areas mentioned in the text.

[a]Site number or abbreviation in brackets are from Fig. 2.

[b]msat, microsatellite genotypes; mtDNA, mitochondrial DNA sequences; FA, forearm lengths.

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requirements of Canada regarding conservation and animal welfare.

mtDNA sequences

We extracted DNA from wing tissue using the Qiagen DNeasy blood and tissue extraction kit (Alameda, California) using the spin-column protocol. We sequenced a region of approximately 250 base pairs (bp) of the hypervariable region II (HVII) of the control region of the mitochondrial genome. DNA was amplified as a large fragment of variable length (~1000 bp) using primers L16517 and sH651 (Fumagalli et al. 1996; Castella et al. 2001). Fragments were then sequenced unidirectionally using the L16517 primer only owing to the presence of the large repeats section (Fumagalli et al. 1996). Polymerase chain reactions (PCR) were performed in a 50 μL volume using 50–100 ng template, 1× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.8, 0.1% Triton), 2.5 mmol/L $MgCl_2$, 0.16 mmol/L dNTPs, 0.8 μmol/L each primer, and approximately 1–2 U (1 U ≈ 16.67 knat) *Taq* DNA polymerase (isolated as in Engelke et al. 1990) in the following cycle: initial 3 min at 94 °C followed by 25 cycles of 1 min at 94 °C, 1 min at 54 °C, 1.5 min at 72 °C. PCR product was purified using QIAquick gel extraction kit (Qiagen, Alameda, California) and sequenced using Big Dye® Terminator version 3.1 sequencing kit (Applied Biosystems, Foster City, California) according to manufacturer's directions. Sequencing products were resolved on ABI Prism® 3100 genetic analyzer (Foster City, California). We aligned sequences in Sequence Navigator version 1.0 (Applied Biosystems, Foster City, California).

In the 250 bp HVII fragment, we found 61 polymorphic sites and 26 haplotypes (GenBank accession nos. EF471399–EF471445), 24 of which were from the main study area, with the other 2 representing the Skagit samples. Using cyt*b* (Dewey 2006), 16S ribosomal subunit (Zinck et al. 2004), or COI (M. Vonhof, personal communication) markers, others have produced mtDNA sequences that define each of the two putative subspecies. All three mtDNA loci are able to resolve the putative subspecies of *M. lucifugus* to varying degrees, and generally produce congruent results (Dewey 2006). We calibrated our HVII markers to the putative subspecies definitions by having a subset of samples independently ascribed at one of the three above listed markers.

We examined relationships among HVII haplotypes using the program TCS (Clement et al. 2000). This program generates a network representation of haplotype divergence using the method of Templeton et al. (1992). Population distances based on haplotypes were measured as Φ_{ST} linearized following Slatkin (1995), calculated in ARLEQUIN version 3.1 (Excoffier et al. 1992, 2006). These Φ_{ST} distances were used to generate a neighbour-joining (NJ) tree of population structure in PAUP* (Swofford 2002). The analysis was repeated on 100 bootstrap replicates generated by resampling the original data, and a 50%-majority-rule consensus tree was constructed using PAUP*.

Microsatellite genotyping

We genotyped individuals from the 8 main sampling sites at 11 microsatellite loci. The following sets of primers were used: MME24, MMG9, MMH19, MMD9, MMD19, MMH29, MMF19 (Castella and Ruedi 2000), MYBE22 (Kerth et al. 2002), EF21, EF5, and EF6 (Vonhof et al. 2002). We used a PCR volume of 15 μL containing 1× PCR buffer, 0.8–1.5 mmol/L $MgCl_2$, 0.12 mmol/L dNTPs, 0.2–0.27 mmol/L of each primer, 0.4 units of *Taq* DNA polymerase, and 2 μL (~100 ng) DNA template. Cycling was performed under the following conditions: 1 min at 94 °C, three cycles of 30 s at 94 °C, 20 s at 47 °C, and 5 s at 72 °C, 33 cycles of 15 s at 94 °C, 20 s at 47 °C, and 1 s at 72 °C, followed by final extension at 72 °C for 30 min. PCR products were resolved on model 377 ABI sequencers (Applied Biosystems, Foster City, California), and analysed using GENESCAN version 3.1 and GENOTYPER version 2.0 softwares (Applied Biosystems, Foster City, California).

We checked all loci in all populations for deviations from Hardy–Weinberg equilibrium (Hardy–Weinberg probability test, GENEPOP version 3.4; Raymond and Rousset 1995) and adjusted our type I error rate for multiple comparisons using the Bonferroni method (Sokal and Rohlf 1995). Because of heterozygosity deficiencies at some loci, loci were further examined for null alleles using Micro-checker (Van Oosterhout et al. 2004). The null frequency for each locus was estimated using the software FreeNA, and an adjusted allele frequency data set was produced (Chapuis and Estoup 2007). Chapuis and Estoup (2007) caution that their software, FreeNA, makes the unrealistic assumption of a single null allele common to all populations; as such, they strongly recommend that the performance of their adjusted data sets be carefully tested. We have therefore performed all of our analyses on three separate data sets (see Results), one of which was corrected for null alleles using FreeNA.

We created NJ trees of population structure. While F_{ST} has been shown to perform reasonably well in producing population genetic trees using microsatellite data (e.g., Pérez-Lezaun et al. 1996), other genetic differentiation measures, such as the Cavalli-Sforza and Edwards distance (D_c; Cavalli-Sforza and Edwards 1967), are more likely to accurately resolve population relationships, although not necessarily produce reliable tree branch lengths (Takezaki and Nei 1996). Chapuis and Estoup (2007) additionally recommend D_c, having shown this genetic distance to perform well on data sets corrected for null alleles and is overall less affected by the presence of null alleles than several other genetic distances. We calculated pairwise D_c and F_{ST} (using the unbiased ENA method; Chapuis and Estoup 2007) for all eight populations using the FreeNA-adjusted allele data set and created NJ trees (PAUP*; Swofford 2002) for comparison with the mtDNA NJ tree (Φ_{ST}). The program Populations version 1.2.30 (Langella 1999) was used to generate 1000 bootstrapped data sets across loci. Global and pairwise population F_{ST} values were also calculated for the data set consisting of only loci not found to contain null alleles ("small data set"; see Results) using FSTAT version 2.9.3 (Goudet 2001); exact tests (loci weighted for polymorphism) were performed to determine significant pairwise population differentiation (Goudet 2001).

To determine whether individuals from the putative subspecies interbreed, we tested for the presence of genetic structure within *M. lucifugus* along an approximately 100 km (river distance) stretch of the Missouri River, where

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M. l. lucifugus and *M. l. carissima* haplotypes occur at an approximately 1:1 ratio (see below). We predicted that if these groups were independent breeding units, they should have different allelic frequencies and possess unique microsatellite alleles. This would be detected as genetic structure and would support the hypothesis that *M. l. lucifugus* and *M. l. carissima* are different species, and thus separate breeding populations along the Missouri River. We estimated population structure within the Missouri River using the program STRUCTURE (version 2.1; Pritchard et al. 2000), which is well-suited to this type of analysis (Latch et al. 2006) because it makes no a priori assumptions of group membership and uses a model-based clustering method to infer population structure and assign individuals to breeding groups. A collection of the highest individual-assignment probabilities occurs when the appropriate number of groups (k) has been identified. We ran STRUCTURE for five competing models, $k = 1$ through 5, with 10 iterations of each. We used a burn-in period of 250 000 with the same number of Monte Carlo Markov chain repetitions after burn-in. All combinations of Admixture and No Admixture models of ancestry with correlated and independent allele frequencies were used as parameter sets. We calculated the best model from posterior probabilities using Bayes' rule (Sokal and Rohlf 1995).

Forearm length analysis

We analyzed forearm lengths of adult males and females separately owing to probable sexual dimorphism. We used analysis of variance (ANOVA) and Scheffé's pairwise comparisons in Stata version 9.0 (StataCorp LP, College Station, Texas). When assumptions of normality were not met, we performed a nonparametric test using ANOVA of ranked forearm measurements and calculated a χ^2 equivalent (Sokal and Rohlf 1995). We conducted nonparametric pairwise comparisons using Mann–Whitney tests (two-sample Wilcoxon rank-sum; Stata version 9.0) and adjusted the alpha value for multiple comparisons using the Bonferroni method. When sample sizes allowed, areas were tested separately and individuals from neighbouring areas that did not differ significantly were pooled. All values are presented as means ± SE.

Results

From 2001 to 2006, we captured 1039 *M. lucifugus*, and used subsets of these for various analyses. We obtained microsatellite genotypes for 404 individuals from the main study area, and produced HVII sequences for 91 of these plus an additional 3 samples from British Columbia, Washington, and Sheep River, Alberta. Forty-four of the HVII sequenced *M. lucifugus* and an additional 25 were sequenced at one or both of the cyt*b* and 16S loci of the mtDNA to determine correspondence with subspecies designations as defined by mtDNA and geography (Dewey 2006). Thirty-three (46%) were sequenced at both the cyt*b* and 16S loci because of an inability of the cyt*b* sequence to differentiate *M. lucifugus* from *Myotis volans* (H. Allen, 1866) in some (11, 31%; field identification verified using 16S instead) cases, and an inability of 16S sequence to fully resolve *M. l. carissima*. Species identifications were verified by calibration with sequences from voucher specimens collected by Dewey (2006).

Sequence haplotypes

Relationships among haplotypes are shown in Fig. 3. Three distinct clusters were apparent; by comparing the cyt*b*/16S sequences with our HVII haplotypes, it was determined that all of our samples ascribing to *M. l. carissima* clustered together (MYLU.CA), while the *M. l. lucifugus* samples clustered together and into two sub-haplogroups (MYLU.LU-A and MYLU.LU-B). In other words, each HVII haplotype could be assigned a "subspecies" designation, consistent with Dewey (2006), allowing all individual HVII sequences to be assigned to one of the two subspecies groups. The MYLU.CA haplotypes cluster (mean (SE) sequence divergence within MYLU.CA is 2.5 ± 0.4 bp, 1.0%, range 1–4 bases) was distant from the two MYLU.LU haplogroups, which were more closely related to each other (mean (SE) sequence divergence within MYLU.LU was 7.0 ± 0.3 bp, 2.8%, range 1–15 bases, and between MYLU.LU-A and MYLU.LU-B was 11.7 ± 0.2 bp, 4.7%, range 7–15 bases). The MYLU.CA and MYLU.LU haplogroups differed by a mean (SE) sequence divergence of 36.3 ± 0.3 bp (14.5%, range 31–39 bases), which was 5–14 times the divergence level found within each group. The Skagit samples, haplotypes 20 and 22 (not in Fig. 3), fell between the haplogroups but clustered with MYLU.CA and MYLU.LU, respectively; the fact that they did not cluster tightly within each group likely reflects their distance (~800 km) from the rest of the samples (Fig. 2). These outlier samples were sequenced at the cyt*b*/16S to verify their subspecies designations. Because MYLU.LU-A and MYLU.LU-B subgroups did not correspond to previous definitions of subspecies and were widespread across all sampling areas, we dropped this A/B distinction from further analyses, using MYLU.LU as one haplogroup only.

Myotis lucifugus lucifugus haplotypes were found at all locations where genetic samples were obtained (Table 2). In Alberta, *M. l. carissima* haplotypes were found only along the Milk River. There, both putative subspecies were found in the same maternity colony at Onefour (1 male (juvenile) and 1 female MYLU.CA, and 6 female MYLU.LU). Similarly, in Montana, both subspecies were found along the Missouri River, with mixed subspecies in a maternity roost at Coal Bank (3 female MYLU.CA and 1 female MYLU.LU). At a maternity colony in Havre, only *M. l. lucifugus* was found (6 females sequenced).

We calculated pairwise population distances between the eight main sampling sites using the haplotype data and constructed a NJ tree (Fig. 4A). The three study sites where MYLU.CA haplotypes were present are separated from the other sites with a distance relative to the frequency of these haplotypes in the population. This tree is in stark contrast to its equivalent using microsatellite data (see below; Figs. 4B, 4C).

Microsatellite genotypes

A subsample of captured individuals ($n = 404$) were genotyped at 11 microsatellite loci. Number of alleles per locus ranged from 19 to 49 (mean (SE) 32.2 ± 3.7) and mean expected heterozygosity (He) in the eight main sampling

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Fig. 3. Haplotype networks for little brown bats (*Myotis lucifugus*) generated using TCS (Clement et al. 2000). HVII haplotypes (24) for *M. lucifugus* formed two main haplogroups: MYLU.CA corresponding to specimens identified as *Myotis lucifugus carissima* and found exclusively on the Milk and Missouri rivers, and MYLU.LU consisted of *Myotis lucifugus lucifugus*. MYLU.LU was further subdivided into two subgroups (MYLU.LU-A and MYLU.LU-B) for which no geographic, morphological, or other correlate could be found. Each circular node signifies 1 bp change. Lengths of the connection lines are not drawn proportional to divergence distance.

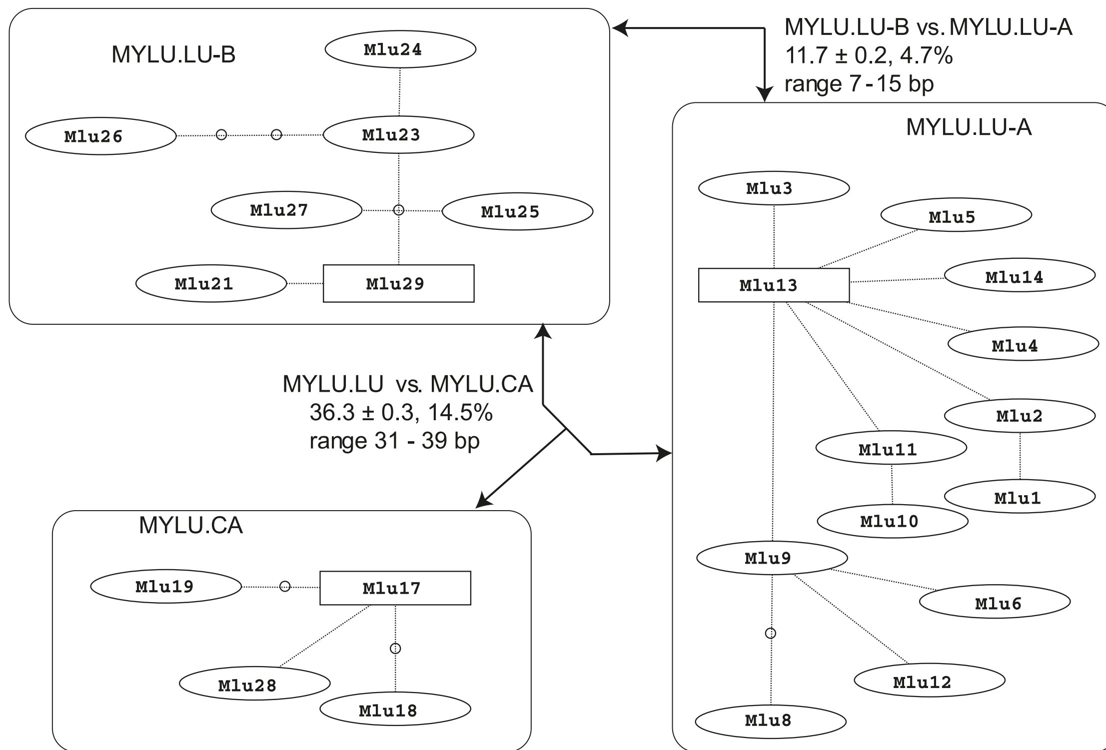

Table 2. Subspecies designations, *Myotis lucifugus carissima* (MYLU.CA) and *Myotis lucifugus lucifugus* (MYLU.LU), of sequenced male and female little brown bats (*Myotis lucifugus*).

		MYLU.LU		MYLU.CA		Percentage of MYLU.CA	
River	Site	Male	Female	Male	Female		
Battle	Don	1	1	0	0		0
Battle	BKPP	2	2	0	0		
Red Deer	Northmost, which includes CNTB, McKC, and DIBJ	3	0	0	0		0
Red Deer	DH	5	7	0	0		
Red Deer	DPP	11	1	0	0		
South Saskatchewan	EM	4	4	0	0		0
South Saskatchewan	BI	4	7	0	0		
Milk	WOS	1	1	0	0		7
Milk	OF	4	6	1*	1	17	
Milk	HV	8	7	0	0		
Missouri	CB	7	5	2	3	29	43
Missouri	MC	4	4	4	6	56	
Outside study area	Skagit, British Columbia [1]	0	0	0	1		na
Outside study area	Skagit, Washington [2]	0	1	0	0		na
Outside study area	Alberta Rocky Mountains [3]	0	1	0	0		na
Total		54	47	7	11		

Note: Sites sampled by authors are shaded and are listed in order from north to south. Site abbreviations are found in Fig. 2; where abbreviations are not used, site numbers are provided in brackets. Sample sizes are in Table 1.

*Juvenile.

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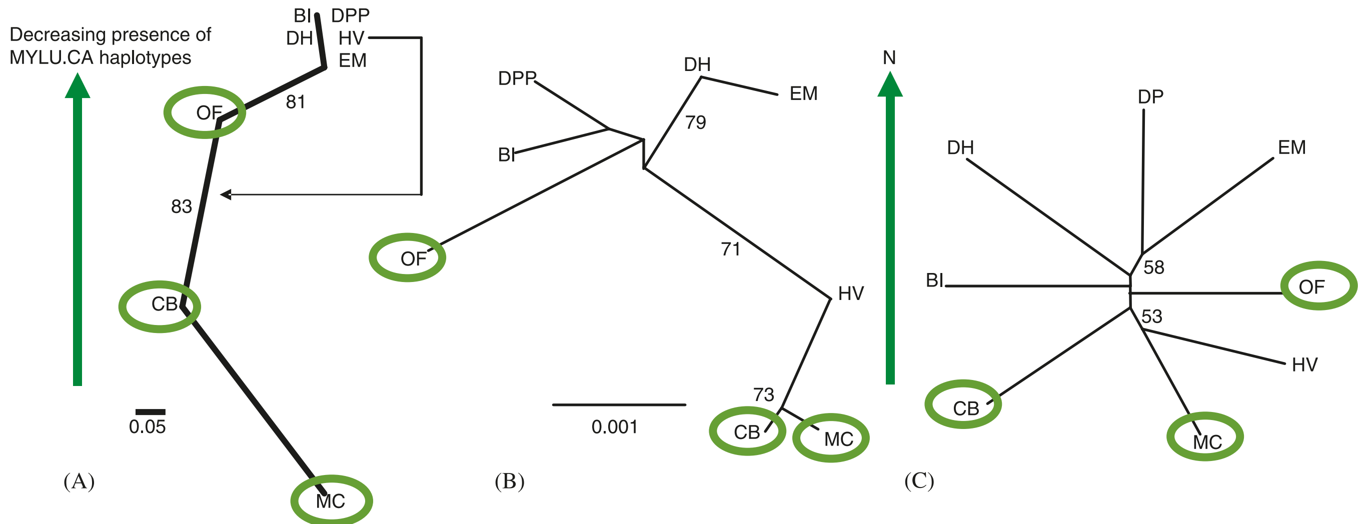
Fig. 4. Neighbour-joining trees (with bootstrap values >50%) showing population structure of little brown bats (*Myotis lucifugus*) within the main study area based on HVII haplotypes (A; Tamura–Nei model, Slatkin's linearized Φ_{ST}) and microsatellite genotypes (B, C; F_{ST}). The right arrow in A shows the displacement of the Havre study site from its geographic position in the tree. Sites containing MYLU.CA haplotypes are circled. The nuclear DNA based population structure (B) resembles the actual geographic orientation of the sites (see Fig. 2). F_{ST} (B) and the Cavalli-Sforza and Edwards genetic distance (C) were calculated using the null-allele-adjusted data set (ENA for F_{ST}), as recommended by Chapuis and Estoup (2007). Branch lengths are not shown for C based on findings of Takezaki and Nei (1996).



sites ranged from 0.86 to 0.95 (mean (SE) total He 0.92 ± 0.03). Using GENEPOP, we found that 5 (EF5, EF6, MMH29, MMD9, MYBE22) of the 11 loci were not in Hardy–Weinberg equilibrium. Using Micro-checker, we determined that these five loci contained null alleles. We estimated null-allele frequencies using FreeNA, producing a corrected data set of allele frequencies for each locus. Using GENEPOP on this corrected data set, we found all loci to be in Hardy–Weinberg equilibrium, except MMH29, which we subsequently dropped from the data set. Based on these results, we created three data sets: a "small data set" containing only the 6 loci not found to contain null alleles, a "large uncorrected data set" containing all 11 loci with no allele frequency adjustments, and a "large corrected data set" of 10 loci with the null-allele frequencies estimated and all allele frequencies corrected. Because of the uncertainty whether the corrected data set accurately reflects allele frequencies (Chapuis and Estoup 2007), and because there is a tendency for genetic structure to be overestimated when null alleles are present in a data set (Chapuis and Estoup 2007), we performed all analyses with all data sets; conclusions were the same in all cases.

When microsatellite alleles of individuals for which subspecies designation was known (i.e., those sequenced) were compared, 2.3% (9) were found only in MYLU.CA individuals (n = 16) and 32% (124) were found in the larger number of sequenced MYLU.LU (n = 80) only. However, when the comparison was expanded to include a larger data set of individuals from the northern part of the study area where no MYLU.CA haplotypes were found, all MYLU.CA alleles were also found in MYLU.LU individuals, suggesting no unique microsatellite alleles exist within *M. l. carissima*.

F_{ST} values were low across the entire study area, with only 7 (of 28) pairwise comparisons yielding significant differentiation after Bonferroni correction (global F_{ST} = 0.003, maximum = 0.009, minimum = –0.001; Table 3). On the Missouri River, the two sampling locations were not significantly differentiated (F_{ST} = 0.002, p > 0.0018; Goudet 2001). Unlike the NJ tree using mtDNA (Φ_{ST}; Fig. 4A), those based on microsatellites (Figs. 4B, 4C) showed concordance with geography. In particular, the 71 km straight-line distance between the two Missouri river sites was associated with a D_c of 0.286 (Table 3), while the mean (SE) genetic distance for all sampling locations was 0.311 ± 0.003; the maximum genetic distance of 0.359 was between two sites on opposite ends of our study area (MC and EM; 360 km straight-line distance), and the minimum genetic distance of 0.275 was between the two closest sites (HV and CB; 60 km).

Using the program STRUCTURE, we found no population structure along the Missouri River (CB and MC comparison), meaning that the genotype distribution is best explained by all samples coming from one population (k = 1). We anticipated the Admixture model to reveal structure between the two haplogroups if these groups were of mixed ancestry but experiencing no or limited gene flow, and the Correlated allele frequencies model to best differentiate groups in the presence of some gene flow (Falush et al. 2003). However, given that ancestry and degree of gene flow between the groups was unknown, we tested all combinations of parameters (No Admixture/Admixture models and Correlated/Independent allele frequencies). We also tested all three data sets. The conclusion was the same regardless of data set or parameter set: we found no evidence of structure associated with the putative subspecies and concluded k = 1 (one population) to be the most probable model. For example, 10 replicates of each of k = 1 through 5 using the Admixture/Correlated-alleles model produced a mean $\ln \Pr(X|K)$ for k = 1 of –3563, –6736, and –6665 for the small, large corrected, and large uncorrected data sets,

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Table 3. Pairwise population F_{ST} (lower triangle) and D_c (upper triangle) for all eight main sampling sites of little brown bats (*Myotis lucifugus*).

	Sampling location							
Sampling location	DH	DP	EM	BI	OF	HV	MC	CB
DH	0	0.307	0.309	0.317	0.295	0.300	0.318	0.293
DP	0.002	0	0.321	0.326	0.331	0.313	0.310	0.313
EM	0.000	0.004	0	0.334	0.324	0.324	0.359	0.335
BI	0.001	0.001	0.006*	0	0.314	0.299	0.331	0.297
OF	0.003	0.003*	0.009*	0.004	0	0.299	0.310	0.282
HV	–0.001	0.002	0.004	0.003*	0.002	0	0.281	0.275
MC	0.002	0.001	0.006*	0.005*	0.004	0.000	0	0.286
CB	0.004	0.001	0.008*	0.003	0.003	0.002	0.002	0

Note: Percentages of MYLU.CA are 17%, 56%, and 29% for OF, MC, and CB locations, respectively. Site abbreviations are found in Fig. 2.

*An exact test, weighting loci based on polymorphism (Goudet 2001), was used to determine significant population differentiation after Bonferroni correction (α = 0.05).

Table 4. Forearm comparisons of adult male and female *Myotis lucifugus* from three main areas (north, central, and south study regions).

Forearm comparison (mm).

	Male			Female			Both sexes		
Region	Mean ± SE	Range	*n*	Mean ± SE	Range	*n*	Mean ± SE	Range	*n*
North	38.8±0.2	34.5–41.6	69	38.9±0.1	37.0–41.2	58	38.8±0.1	34.5–41.2	127
Central	37.6±0.1	37.0–40.0	119	38.1±0.1	32.0–40.4	112	37.9±0.08	37.0–40.4	231
South	37.0±0.1	32.1–39.9	120	37.4±0.1	32.3–40.3	140	37.3±0.07	32.1–40.3	260

Statistical results.

Comparison	*z*	*p*
North region		
Male vs. female	0.119	0.91
Central region		
Male vs. female	3.494	<0.001*
South region		
Male vs. female	3.603	<0.001*
Male		
North vs. central	6.181	<0.0001*
Cental vs. south	4.366	<0.0001*
North vs. south	8.912	<0.0001*
Female		
North vs. central	3.649	<0.0001*
Central vs. south	4.347	<0.0001*
North vs. south	6.903	<0.0001*

Note: North region includes samples from the Battle River, Drayton Valley, near Peace River, and Lac LaBiche; central region includes the Red Deer River sampling sites; south region includes the Milk and Missouri rivers and Bow Island of the South Saskatchewan River. Analysis of variance was non-parametric. The asterisks indicate significant results (ANOVA — sex: χ^2 = 20.9, df = 1, p < 0.001; region: χ^2 = 139.8, df = 2, p < 0.001).

respectively; in each data set this corresponded to a 99.99% probability for k = 1 being the best estimate of the number of populations.

Forearm lengths of *M. lucifugus* from the south, central, and north parts of our study area differed significantly (Table 4; n = 618), with forearms getting longer from south to north (farthest straight-line distance 730 km). Males had significantly shorter forearms than females in the south and central regions (p < 0.001), but not in the north region (p = 0.91) where males and females had similar means; males compared across the three areas differed significantly, as did females. The south study region contained all Milk and Missouri river samples and was therefore the only area known to contain *M. l. carissima* samples, although it is possible that *M. l. carissima* individuals may occur farther north (Smith and Schowalter 1979) but were rare and undetected in the genetic analysis. When forearm lengths were regressed against UTM coordinate northings, the regressions for males and females were significant (Fig. 5; p < 0.001; males, $F_{[1,307]}$ = 107.3, p < 0.001, R^2 = 0.26, $y = 22.8 \pm 1.4 + (2.64 \times 10^{-6} \pm 2.6 \times 10^{-7})x$; females, $F_{[1,309]}$ = 46.6, p < 0.001, R^2 = 0.13, $y = 26.6 \pm 1.7 + (2.01 \times 10^{-6} \pm 3.0 \times 10^{-7})x$).

At the same sampling site on the same night, we captured *M. lucifugus* that ranged in pelage colour from dark brown to light blonde. We genetically sampled a dark morph and blonde morph from each of the Milk and Missouri rivers. Because *M. l. carissima* is described as the palest of all of the subspecies (Miller and Allen 1928; van Zyll de Jong

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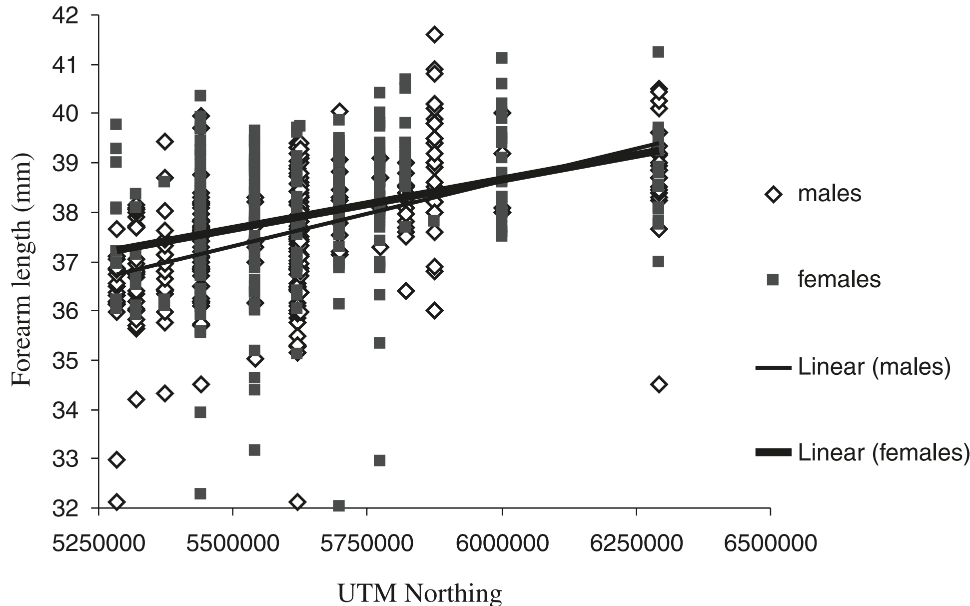
Fig. 5. Regression of male and female forearm lengths of *Myotis lucifugus* on UTM northings. For sample sizes refer to Table 4.



1985), we hypothesized that the blonde individuals from each river would be *M. l. carissima* and the dark individuals would be *M. l. lucifugus*. However, the mtDNA sequences of both samples from the Milk River were that of *M. l. lucifugus*, and both Missouri River samples were that of *M. l. carissima*. Along the Milk River, we occasionally observed individuals with white outlines to their wing margins, a trait thought to characterize *M. l. carissima* (Miller and Allen 1928); however, a *M. l. lucifugus* (based on genotype) with this white wing margin was also found north of Edmonton, Alberta (collected by B. McClymont, Alberta Fish and Wildlife, Edmonton; deposited at Royal Ontario Museum by C.L.L., ROM collection no. F54056; COI sequence provided by Biodiversity Institute of Ontario Barcodes of Life Database; subspecies ID provided by M. Vonhof, unpublished data), suggesting that this trait also does not distinguish these two subspecies.

Discussion

As predicted based on preliminary findings of Dewey (2006), *M. lucifugus* in southern Alberta and north-central Montana consisted of two different mitochondrial groups (14.5% uncorrected mean sequence divergence HVII), which corresponded to the cyt*b*-based subspecies designations of *M. l. carissima* and *M. l. lucifugus*. The two groups showed a full range of mixed light and dark brown pelage colouration, had smaller forearm lengths than individuals from more northerly regions of Alberta, and were found roosting together.

Using cyt*b* sequences, Bradley and Baker (2001) found, on average, 3.0% intraspecific variation in bats, providing support for Dewey (2006) who suggested that the 4.5% variation found between *M. l. lucifugus* and *M. l. carissima* (Dewey et al. 2006) may represent cryptic species. We found, as have others (e.g., Elias et al. 2007), that nuclear DNA data produced very different conclusions about species differentiation than mtDNA. Using microsatellites, we found overall low pairwise F_{ST} for *M. lucifugus* across our entire study area (Table 3), and an isolation-by-distance pattern (Lausen 2007). F_{ST} was low between the two Missouri sites in particular, with no significant population differentiation, suggesting gene flow between them. Low F_{ST} does not always equate with high gene flow owing to its inability to account for individual alleles (Hedrick 2005); however, the relatively low genetic distance (D_c) between these two sites, which does account for individual alleles (Cavalli-Sforza and Edwards 1967), suggests relatively low genetic differentiation and high levels of gene flow between the two Missouri sites. Our STRUCTURE results from the Missouri River further supports our hypothesis of local gene flow among *M. lucifugus* on this river. In particular, these results are consistent with substantial nuclear gene flow between *M. l. lucifugus* and *M. l. carissima* on the Missouri River, where *M. l. lucifugus* and *M. l. carissima* haplotypes occur in nearly equal frequency and are sympatric. We therefore conclude that individuals of the two haplogroups are interbreeding on the Missouri River. These findings go against the hypothesis that the two haplogroups correspond to separate species in this region.

Extreme differences among haplogroups suggest that *M. l. lucifugus* and *M. l. carissima* were historically separate races that interbred after they became sympatric; the same may also be true of the two distinct haplogroups MYLU.LU-A and MYLU.LU-B. A hybrid zone for two divergent mitochondrial clades of big brown bat (*Eptesicus fuscus* (Beauvois, 1796)) was described by Neubaum et al. (2007). Two subspecies, *Eptesicus fuscus pallidus* and *Eptesicus fuscus fuscus*, had been described for Colorado (reviewed in Neubaum et al. 2007), and they suggest that landscape changes since the last glaciation together with human influences have facilitated secondary contact and subsequent interbreeding in the prairies of these two separated lineages. The two *E. fuscus* haplogroups roost together with no morphological or ecological factors to distinguish the two groups. We propose that a similar situation of sec-

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ondary contact has occurred with *M. lucifugus* in the prairies and western North America. Our study area is in a suspected region of overlap for these two putative subspecies according to the original subspecies definitions (Hall 1981), and we are thus documenting one hybrid zone. Because it is now known (Dewey 2006; this study) that the two mtDNA forms are fully sympatric across the range previously thought to be that of *M. l. carissima* only (after Hall 1981) (Fig. 1), interbreeding is likely widespread, with these two groups of *M. lucifugus* composing one large nuclear gene pool showing isolation by distance across North America. What taxonomic ranking this equates to is a matter of definition (e.g., Edwards 1954; Avise and Ball 1990; Cronin 1997; Baker and Bradley 2006*a*). Because a "barcode-based species concept has not yet been explicitly articulated or tested" (Rubinoff et al. 2006, p. 589), and because there are many technical problems associated with defining a species using mtDNA sequence, we suggest these two groups of bats should be considered simply one taxonomic unit made up of several diverse haplogroups. We therefore reject the hypothesis of cryptic species, and suggest dropping the "*carissima*" subspecies designation based on widespread sympatry, lack of nuclear differentiation consistent with interbreeding, mixed maternity colonies, and the absence of differences in morphological traits.

With the exception of a morphological review of *M. l. occultus* (Findley and Jones 1967; Barbour and Davis 1970), the original subspecies definitions (Thomas 1904; Hollister 1909, 1911; Miller and Allen 1928; Harris 1974) and geography for *M. lucifugus* have not been reviewed (van Zyll de Jong 1985). Subspecies identification in the field has been based on historical geography according to the map by Hall (1981). *Myotis lucifugus carissima* supposedly has the lightest pelage colour of any subspecies, and shorter forearms than *M. l. lucifugus* (Miller and Allen 1928). However, we found that colour was not diagnostic, corroborating results about colour morphs in other genetic studies of bats (e.g., Jacobs et al. 2004). Forearm length was clinal, increasing from south to north across our study area and farther into northern Alberta. Similar colour variation and clinal forearm patterns also exist for *E. fuscus* and western small-footed bats (*Myotis ciliolabrum* (Merriam, 1886)) in our study area (Lausen 2007), and do not correspond to any subspecies boundaries (van Zyll de Jong 1985); this suggests that these morphological variations are environmental, not taxonomic, traits. This corroborates findings of clinal bat morphology from other studies (Findley and Jones 1967; Burnett 1983; Bogdanowicz 1990; Patriquin 2001). An extensive re-evaluation of morphology of Europe's most widespread bat, the Daubenton's bat (*Myotis daubentonii* (Kuhl, 1817)), showed an increase in body size from south to north and phenetic overlap of morphological measures between subspecies; this resulted in the proposal to drop two of the four recognized subspecies (Bogdanowicz 1990; Kruskop 2004). A snapshot of the smaller *M. lucifugus* forearms in the southern portion of Alberta in relation to the larger ones in the northern part, together with darker pelage of individuals in moister northern Alberta, prompted Smith and Schowalter (1979) to propose that the range of *M. l. carissima* should include all *M. lucifugus* of the Alberta prairies, rather than just the small southeastern corner of the province as was proposed by Hall and Kelson (1959). This is further evidence that environmental influences on colour and morphology have added to the confusion surrounding the taxonomy of this species.

In animals such as bats, whose morphology is highly constrained evolutionarily (Norberg 1994), convergent morphological traits may falsely group unlike individuals (Ruedi and Mayer 2001; Dewey 2006; Stadelmann et al. 2007), necessitating consideration of ecological, morphological, and genetic factors over a broad geographic range (Cronin 1993). Sequence divergence may be indicative of morphologically cryptic species (Baker and Bradley 2006*a*), and morphologically cryptic genetic species may be prevalent within Chiroptera (Baker and Bradley 2006*b*; Jacobs et al. 2006); this was the case for European pipistrelle bats (Barratt et al. 1997). However, as we have shown, mtDNA on its own can be misleading. In some cases, its discordance with morphological and geographic divisions among well-defined species (e.g., genus *Sorex* L., 1758, Demboski and Cook 2001; *Eptesicus* spp., Mayer and von Helversen 2001; butterflies, Elias et al. 2007), together with its inability to represent contemporary gene flow, serves caution to those interpreting mtDNA sequence divergence in a taxonomic context.

Substantial genetic, protein, and morphological data have been collected for another *M. lucifugus* subspecies, *M. [lucifugus] occultus*. There is much disagreement over the specific status of this little brown bat (reviewed in Piaggio et al. 2002); its taxonomic state has changed several times in response to discoveries of clinal traits (Findley and Jones 1967; Barbour and Davis 1970), of extensive gene flow with other little brown subspecies (allozymes; Valdez et al. 1999), and of contradictory mtDNA sequence divergence data (cyt*b* and COII, 5%–6% sequence divergence; Piaggio et al. 2002). Most recently, finer scale sampling has produced similar or identical mtDNA sequences between *M. [l.] occultus* and other *M. lucifugus* subspecies (Dewey 2006; Stadelmann et al. 2007). Investigation of the nuclear relationships among *M. lucifugus* subspecies and other closely related *Myotis* species (e.g., long-eared species; Dewey 2006) may be informative.

Our study highlights the importance of investigating contemporary gene flow in widely sympatric animals suspected of being cryptic species and has important implications for application of the DNA Barcoding Project (Hebert et al. 2003) to mammalian taxonomy. An increasing number of "new species" are being described based on mtDNA (COI) sequence only (reviewed in Prendini 2005; Rubinoff et al. 2006), including >6 new bat species in Guyana (Clare et al. 2007). High intraspecific mtDNA sequence divergence (>2.5%) in 6 of 87 species of bats examined prompted the suggestion of cryptic species complexes (Clare et al. 2007). Based on our findings, we suggest that an investigation of nuclear gene flow and extent of sympatry is warranted to determine whether these potential new species are indeed biologically relevant or merely a signature of remnant mtDNA variation.

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