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Spatial distribution of mitochondrial and microsatellite DNA variation in Daubenton's bat within Scotland

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Abstract

Daubenton's bat (*Myotis daubentonii*) is a known reservoir for European bat lyssavirus type 2 (EBLV-2). An appreciation of the potential for epidemiological spread and disease risk requires an understanding of the dispersal of the primary host, and any large-scale geographical barriers that may impede gene flow. The spatial pattern of microsatellite and mitochondrial DNA variation was examined to infer patterns of dispersal of bats among 35 populations across Scotland. DNA sequence variation at the mitochondrial control region and ND1 genes revealed two distinct phylogeographical clades, with generally non-overlapping geographical distributions except for a small number of populations where both matrilines were found in sympatry. Such discontinuity suggests that Scotland was recolonised twice following the retreat of the Pleistocene ice sheet with little subsequent matrilineal introgression. However, eight microsatellite loci showed low levels of genetic divergence among populations, even between populations from the two distinct mitochondrial DNA clades. An overall, macrogeographical genetic isolation-by-distance pattern was observed, with high levels of gene flow among local populations. Apparently contrasting patterns of mitochondrial and microsatellite divergence at different scales could be explained by sex-specific differences in gene flow at large scales.

Keywords: Daubenton's bat, EBLV-2, gene flow, *Myotis daubentonii*, phylogeography, sex-biased dispersal

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Introduction

The majority of pathogenic species causing infectious disease in humans are zoonotic pathogens which can be transmitted naturally between animals and humans (Taylor *et al*. 2001). Wildlife serves as a major reservoir of diseases transmittable to domestic animals and humans (Kruse *et al*. 2004). The mitigation of risk and control of disease can be informed by an understanding of the population structure and dispersal of host populations as their spatial distribution and probability of encounter are fundamentally important to the spread and dynamics of diseases (Ostfeld *et al*. 2005), as highlighted by examples such as West Nile virus in America (Bengis *et al*. 2004) and avian influenza H5N1 virus in Europe (Kilpatrick *et al*. 2006).

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European bat lyssavirus type 2 (EBLV-2) is one of seven genotypes of viruses in the genus *Lyssavirus* which cause rabies in humans and other mammals (Harris *et al*. 2006b). The principal reservoirs of EBLV-2 are Daubenton's bat *Myotis daubentonii* and the pond bat *Myotis dasycneme* (Amengual *et al*. 1997). Daubenton's bat is the indigenous reservoir of this virus in the UK as the pond bat is not present. EBLV-2 has been isolated from six individual Daubenton's bats in England and a man who died after being bitten by this bat species in Scotland (Racey & Fooks 2005; Fooks *et al*. 2006; Harris *et al*. 2006a; Bat Conservation Trust 2007). His death prompted active surveillance throughout the UK which revealed the prevalence of specific antibodies to EBLV-2 in Daubenton's bats reflecting past exposure to lyssavirus (Brookes *et al*. 2005). This indicates that EBLV-2 is currently present within the Daubenton's bat population in the UK and has been for some time.

Essential prerequisites for a study of the epidemiology of a zoonosis are a sound understanding of the rate and scale

of movement of those animals that carry the disease. The epidemiology of rabies clearly depends on the spread of viruses directly from infected to susceptible subjects (Kaplan 1985). Unlike other mammals, the ability to fly provides bats with high mobility, and potentially facilitates the widescale and rapid spread of bat rabies. Information on population isolation, site fidelity and dispersal among Daubenton's bat populations would facilitate the understanding of the epidemiology of EBLV-2 in the UK.

At a local scale, radio-tracking studies have revealed the faithfulness to roosting and foraging areas in Daubenton's bat during the summer (Rieger 1996; Senior *et al*. 2005; Ngamprasertwong 2008). No long-distance movement of Daubenton's bats between summer roosts and hibernacula has been observed in Scotland. Maximum distances of up to 27 km have been recorded between summer roosts and winter quarters in the UK (Parsons & Jones 2003), 104 km in Czechoslovakia (Gaisler & Hanák 1969) and 260 km in Poland (Urbanczyk 1990). Large numbers of Daubenton's bats have been found using the same hibernaculum in continental Europe, for example, Mønsted Limestone Cave in Denmark (> 1000 individuals; Baagøe *et al*. 1988) and Nietoperek Bat Reserve in Poland (> 10 000 individuals; Urbanczyk 1990), but not in Scotland. Up to 10 individuals have been found hibernating at the same underground site across Scotland (Herman & Smith 1992; Smith 2000).

Here, we use indirect estimates of dispersal based upon the levels and distribution of microsatellite and mitochondrial DNA variation. The spatial distribution of extant genetic diversity among natural populations is a consequence of both historical phylogeographical processes dictating overall levels of allelic variation, and contemporary ecological processes defining how microevolutionary forces such as random genetic drift and gene flow apportion this genetic diversity at a local scale (Hewitt & Butlin 1997). As such, surveys of genetic diversity using molecular markers such as microsatellite and mitochondrial DNA (mtDNA) polymorphisms can be used to make inferences about behavioural and population processes operating over different timescales that best explain the observed patterns of geographical variation. Several studies of bat population genetics have been carried out (Burland & Worthington Wilmer 2001), but no general pattern has emerged, which precludes inference of dispersal in other species. Although male-biased dispersal and strong female natal philopatry has been observed in many European bat species such as Bechstein's bat (*Myotis bechsteinii,* Kerth *et al*. 2002), the greater mouse-eared bat (*Myotis myotis*, Castella *et al*. 2001) and the noctule bat (*Nyctalus noctula,* Petit *et al*. 2001), natal philopatry in both sexes has been reported in the brown long-eared bat (*Plecotus auritus*, Burland *et al*. 1999; Entwistle *et al*. 2000). Female-biased dispersal has also been documented in the greater sac-winged bat in Costa Rica (*Saccopteryx bilineata,* Nagy *et al*. 2007).

Information on the population genetic structure of Daubenton's bat is scarce as few molecular studies have been carried out on this species. The studies that have been undertaken have focused on molecular systematics (Mayer & von Helversen 2001; Ruedi & Mayer 2001) and paternity (Senior *et al*. 2005). However, the phylogeographical relationships of Daubenton's bat based on sequence variations at ND1 gene demonstrated extensive genetic differentiation across the species range in mainland Europe (Mayer & von Helversen 2001), where four distinct mtDNA clades have been revealed: in Germany and Byelorussia, in Germany and Greece, in Spain, and in Germany alone. These haplogroups might represent the signature of range expansion of Daubenton's bat from several different refugia after the last glaciations with the admixture of lineages in Germany. Available information on the pattern of population genetic structure of Daubenton's bat at a smaller scale would determine the degree of gene flow among populations and reflect the pattern of dispersal in Daubenton's bat. Here, we resolve patterns of both phylogeographical and population genetic structure using microsatellite and mtDNA for Daubenton's bat populations in Scotland where active disease management and risk mitigation is underway. From such an approach, the extent to which historical evolutionary and contemporary ecological processes dictate the spatial distribution of extant genetic diversity can be established, and more accurate descriptors of dispersal and gene flow can be provided.

Materials and methods

Sampling and DNA extraction

Bats were caught by mist net or hand net at 35 sites throughout Scotland during the summers of 2003–2006 (Table 1; Fig. 1). All sampling was performed under licences issued by Scottish Natural Heritage. The maximum distance between sampling sites was approximately 345 km, and the minimum distance was 3 km. As radio-tracking studies have shown the faithfulness of *Myotis daubentonii* to its roosting area and foraging site (Ngamprasertwong 2008), bats captured from the same site were assumed to be from the same population. One or two 3-mm wing punches were collected from all bats (n = 1003) under Home Office licence, and preserved in 20% DMSO in 5 M NaCl, or 95% ethanol. Total genomic DNA was extracted from all samples using the QIAGEN DNeasy Tissue Kit according to the manufacturer's instructions, with elution to give a final concentration of approximately 5 ng/μL.

Mitochondrial DNA polymorphism

Control region variation. The 5′ hypervariable domain I of the mtDNA control region (692–935 bp) was amplified in

Table 1 Details of Daubenton's bat samples collected from 35 sampling sites across four geographical regions in Scotland

Site	Region	Males	Females	Total	Catching site
A1	North	1	24	25	Summer roost
A2	North	—	7	7	Summer roost
A3	North	6	11	17	Foraging area
B1	North East	15	42	57	Summer roost
B2	North East	39	20	59	Foraging area
B3	North East	14	5	19	Foraging area
B4	North East	2	5	7	Foraging area
B5	North East	4	10	14	Foraging area
B6	North East	13	50	63	Summer roost
B7	North East	1	1	2	Foraging area
B8	North East	2	1	3	Foraging area
B9	North East	1	—	1	Foraging area
B10	North East	2	—	2	Foraging area
B11	North East	2	—	2	Foraging area
B12	North East	39	6	45	Summer roost
B13	North East	17	26	43	Summer roost
C1	Central	8	51	59	Summer roost
C2	Central	12	29	41	Summer roost
C3	Central	29	9	38	Summer roost
C4	Central	7	31	38	Foraging area
C5	Central	5	10	15	Foraging area
C6	Central	19	37	56	Summer roost
C7	Central	2	14	16	Summer roost
C8	Central	8	7	15	Foraging area
C9	Central	20	5	25	Summer roost
C10	Central	27	31	58	Summer roost
C11	Central	—	1	1	Summer roost
C12	Central	18	24	42	Summer roost
C13	Central	17	3	20	Summer roost
C14	Central	9	11	20	Foraging area
D1	South	14	39	53	Foraging area
D2	South	16	—	16	Summer roost
D3	South	49	5	54	Foraging area
D4	South	15	39	54	Foraging area
D5	South	13	3	16	Summer roost

58 females from 23 Scottish bat populations using the polymerase chain reaction (PCR). Reactions were performed in a total volume of 25 μL in thin-walled 200-μL tubes using an MJ Research PTC-100 thermal cycler. The reaction mix contained approximately 25 ng of DNA, 2.5 mM $MgCl_2$, 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween-20, 0.2 mM of each nucleotide, 1 μM of each primer and 1 U of Bioline *Taq* polymerase. After an initial denaturation step of 3 min at 94 °C, 35 cycles of PCR were performed, each cycle consisting of a 45-s denaturation at 94 °C, 45-s annealing at 60 °C, and 45-s extension at 72 °C. The primers used in the reaction were P (5′-TCCTACCATCAGCACCCAAAGC-3′), annealing at position 15 975 in the human proline tRNA gene (Anderson *et al*. 1981), and E (5′-CCTGAAGTAGGAACCAGATG-3′), located in a conserved sequence region in the centre of the control region (Wilkinson & Chapman 1991). PCR products were purified using the QIAGEN PCR Purification Kit then sequenced using an ABI 3730 automated DNA sequencer.

ND1 gene variation. A portion of the mitochondrial ND1 gene (1330 bp) was PCR amplified in 27 female bats from 24 Scottish populations. Reactions were performed as described above, except the primers used in the reaction were L2985 (5′-CCTCGATGTTGGATCAGG-3′) and H4419 (5′-GTATGGGCCCGATAGCTT-3′) flanking the ND1 gene (A. Janke, personal communication cited in Petri *et al*. 1997). PCR products were purified using the QIAGEN PCR Purification Kit then sequenced using an ABI 3730 automated DNA sequencer. Primers L2985 and H4419 were used as sequencing primers.

A restricted fragment length polymorphism (RFLP) assay was developed for the ND1 DNA sequence data and used to examine the ND1 variation of most individuals from Scotland ($n = 945$) and samples from Norway ($n = 8$), the Netherlands ($n = 3$), Germany ($n = 16$), and Ireland ($n = 10$). From an initial sequence analysis of ND1 gene sequence variation, the program DNA-STRIDER (version 2.2, ABI Ltd) was used to identify any nucleotide polymorphisms present in restriction recognition sites. A *Hinf*1 recognition site was chosen with three variant restriction sites (Table 3b). The PCRs were performed using the same conditions as for DNA sequencing of the ND1 gene but in a total volume of 10 μL. After PCR, the reaction then mixed with 0.5 μL restricted enzyme *Hinf*1 (10 U/μL; Promega Corp), 2 μL 10× buffer and 7.5 μL AR H_2O. The mixtures were incubated at 37 °C overnight, and then run out at 5 V/cm on a 1% agarose minigel containing ethidium bromide. Restriction patterns were observed under ultraviolet light and described relative to a Hyperladder IV standard (Bioline Ltd) run concurrently.

Statistical analysis. Multiple sequence alignments at the control region and ND1 gene were made using MEGA version 3.1 (Kumar *et al*. 2004) with further modifications being made by eye. A repeated sequence of 81 bp (R1 repeat) within the control region, which varies in repeat copy number among individual bats in the subfamily Vespertilioninae (Wilkinson *et al*. 1997), was excluded from analysis.

The genetic relationship between unique control region and ND1 gene haplotypes was ascertained by constructing parsimony networks of all haplotypes based on the absolute number of nucleotide differences between them and the type of point mutation (synonymous or nonsynonymous). Phylogenetic trees of the unique haplotypes were constructed using maximum-likelihood (ML) approaches with PAUP (version 4.0b8; Swofford 1998). The optimal model of sequence evolution used in analysis was identified using the Akaike information criterion (AIC; Akaike 1974) from 56 candidate models by the program MODELTEST version 3.0 (Posada & Crandall 1998). The control region model

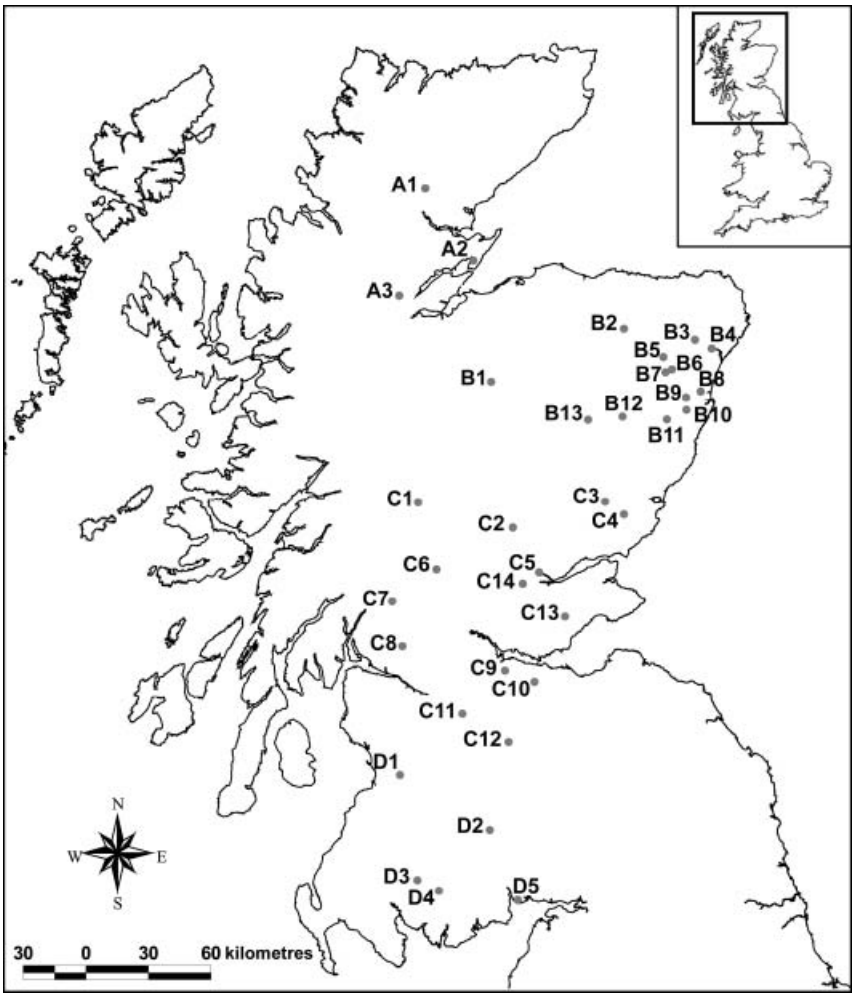

Fig. 1 Locations of all sampling sites in Scotland (details in Table 1).

was defined as: base frequencies of 0.3870, 0.2174, 0.1122 and 0.2834 for A, C, G and T nucleotides, respectively; transition/transversion ratio = 4.9960; a proportion of invariable sites = 0; and equal rates for all sites. The ND1 model had the following parameters: base frequencies of 0.3284, 0.2454, 0.1306 and 0.2956 for A, C, G and T nucleotides, respectively; transition/transversion ratio = 7.9262; a proportion of invariable sites = 0; and equal rates for all sites. The confidence in both control region and ND1 topologies were assessed using bootstrap analysis (Felsenstein 1985) based on 10 000 iterations.

Microsatellite DNA polymorphism

A total of 671 individuals from 27 populations, with a sample size greater than 10 bats (Table 4) were screened at eight hypervariable microsatellite DNA loci (Table 2). PCR amplifications were performed in a total volume of 10 μL using an MJ Research PTC-100 thermal cycler. Each reaction mix contained 10 ng of template DNA, 1.5–2.5 mM $MgCl_2$ depending on locus (see Table 2), 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween-20, 0.2 mM of each nucleotide, 1 μM of each primer and 0.5 U of Bioline *Taq* polymerase. The forward primer of each primer set was labelled at the 5′ end with a fluorescent tag to allow genotyping on an automated DNA sequencer. After an initial denaturation step of 3 min at 94 °C, 35 cycles of PCR were performed, each cycle consisting of a 45-s denaturation at 94 °C and 45-s annealing at primer-specific annealing temperature (Table 2). Touchdown (Don *et al*. 1991) profiles was used with some primers, whereby after an initial denaturation step, 20 cycles of PCR were performed, each cycle consisting of 30-s denaturation at 91 °C, and 30 s of annealing starting at 60 °C and dropping by 0.5 °C per cycle. A further 25 cycles were then performed with 30-s denaturation at 91 °C and 30-s annealing at 50 °C. No extension steps were included in any programme, apart from a 5-min period at 72 °C following the final annealing step. Up to three microsatellite PCR fragments labelled with different fluorescent tags, that is FAM, NED, and VIC

Table 2 Characteristics of eight microsatellite loci for Daubenton's bat. Primer sequences are given together with allele number and size ranges

Locus	Sequences (5′–3′)*	Array	$MgCl_2$ conc. (μM)	Annealing temp. (°C)	Fluorescent tag	Mix	No. of alleles	Allele size range (bp)
MM5 (F1R2)†	F: TTCATCCAGTTCTGG R: ACCTAGGGTTGATTTAACATGC	$(GT)_n$	2.5	60–50 TD	NED	1	13	128–152
MM1‡	F: GTTAATGACTGTAGC R: TTGCCCTTCCTGTCCTTTAA	$(GT)_n$	2.5	60–50 TD	FAM	1	12	161–183
H29§	F: TCAGGTGAGGATTGAAAACAC R: GCTTTATTTAGCATTGGAGAGC	$(CA)_n$	2.5	63	VIC	1	12	160–190
PAUR6¶	F: GATCAGATTTCCAAACAGAG R: AGGTTCTTTCTTCAGCTATG	$(AC)_n(AG)_n$	2.5	58	NED	2	13	141–173
E24§	F: GCAGGTTCAATCCCTGACC R: AAAGCCAGACTCCAAATTCTG	$(TC)_n$	2.0	60–50TD	FAM	2	22	220–264
G9§	F: AGGGGACATACAAGAATCAACC R: TAATTTCTCCACTGAACTCCCC	$(TC)_n$	1.5	55	NED	3	27	150–212
C113§	F: ACCTCCCTGCCCTGCAC R: GCAATGCTTCCTCCAAGTCC	$(ACC)_n$	1.5	60	FAM	3	4	93–102
D9§	F: TCTTTCCTCCCCTGTGCTC R: TCTGGACCCAAAATGCAGG	$(CT)_n$	2.0	60	HEX	3	24	112–164

*F = forward primer; R = reverse primer; †Modified from Petri *et al*. 1997; ‡Petri *et al*. 1997; §Castella & Ruedi 2001; ¶Burland *et al*. 1998; TDTouchdown program of decreasing annealing temperatures.

or HEX, were mixed together in equal volumes, diluted 1 in 10 and genotyped using an ABI 3730 automated DNA sequencer according to manufacturer's instructions.

Statistical analysis. Microsatellite genotype data were scored in GENEMARKER software version 1.4 (SoftGenetics Inc.). Tests for linkage disequilibrium between loci for each population were performed by generating exact probabilities of type I error for the null hypothesis that a pair of loci is unlinked. The test was performed for all locus pairs within each population using GENEPOP version 1.2 (Raymond & Rousset 1995).

Genetic diversity for each population was estimated by observed heterozygosity (H_O), number of alleles and allelic richness. Multilocus F_{IS} was calculated for each population, and then deviation from Hardy–Weinberg expectation was tested by permutation using FSTAT version 2.9.3 (Goudet 2001).

Microsatellite population structure was quantified by estimating differentiation between populations with *F*-statistics (Wright 1978). Pairwise F_{ST} were computed according to Weir & Cockerham (1984) and population differentiation was tested by randomising (7020 permutations) multilocus genotypes between each pair of samples with FSTAT version 2.9.3. F_{ST} estimates were then used to test for isolation by distance using the Mantel test in GENEPOP version 1.2, following the recommendations of Rousset (1997), whereby correlation is examined between $F_{ST}/(1 - F_{ST})$ and the logarithm of Euclidean geographical distance between populations in kilometres.

To detect sex-biased dispersal, pairwise F_{ST} values and the mean of corrected assignment indices obtained from each sex were compared by the randomisation procedure using FSTAT version 2.9.3. Assignment tests implemented within Genetic Analysis in Excel (GENALEX; Peakall & Smouse 2006) version 6 were used to examine the proportion of individuals reassigned to their source population and mtDNA clade.

An analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) was performed using ARLEQUIN version 3.1 (Excoffier *et al*. 2005) to examine the levels of microsatellite-estimated differentiation between bat populations. Such an analysis permits a hierarchical examination of structure, partitioning the variance: (i) within populations, (ii) between populations within mtDNA clades, and (iii) between mtDNA clades. As such, the relative contributions of within and between population structure to overall patterns of genetic divergence within Scotland can be ascertained.

Results

Mitochondrial DNA polymorphism — control region

A total of 40 of 449 nucleotide positions were variable over the 23 populations screened (after exclusion of the R1 repeat region; Table 3a), defining nine unique control region haplotypes. Only seven of the 40 (17.5%) variable nucleotide positions involved transversion mutations (A↔C, A↔T, G↔C, or G↔T). The evolutionary relationships between these individual haplotypes are displayed as a

Table 3 Nucleotide variations at (a) the control region and (b) the ND1 gene among Daubenton's bat populations throughout Scotland. The 40 variable nucleotide positions of 189 bp and 260 bp segments of the control region observed among 58 female bats from 23 populations. A 189-bp segment is located between tRNA Pro and R1 repeats and a 260-bp segment located between the R1 repeat and the conserved sequence region in the centre of the control region. The 35 variable positions of a 1330-bp segment of the ND1 gene observed among 27 female bats from 24 populations. Diagnostic RFLP sites are highlighted by grey shading. The populations from which each haplotype was detected are provided.

(a)

		Nucleotide position in a 189-bp segment																									Nucleotide position in a 260-bp segment															
Clade	Haplotype	14	16	17	30	31	42	46	48	50	63	71	78	86	101	133	136	145	148	160	167	169	175	179	182	185	13	40	50	74	94	95	99	101	102	104	131	152	209	220	236	Populations
A	H1	A	A	T	C	C	T	T	A	G	T	T	C	C	A	G	A	T	G	T	A	A	T	C	G	T	C	C	C	G	A	G	A	G	T	A	A	T	C	-	A	B1, B2, B3, B4, B5, B6, B7, B13
	H2	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	-	.	B1
	H3	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	-	.	B1, C2, C4, C5, C6, C7, C10, C14
	H4	.	C	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	-	.	C1
	H5	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C	.	-	.	C6, C8, C11, C12
B	H6	C	C	C	T	T	C	C	G	A	C	A	T	A	G	A	G	C	A	C	G	G	C	T	A	C	A	.	T	A	G	A	T	A	C	C	.	C	T	A	G	A1
	H7	C	C	C	T	T	C	C	G	A	C	A	T	A	G	A	G	C	A	C	G	G	C	T	A	C	A	.	T	A	G	A	T	A	C	C	.	C	T	A	.	A1, A2
	H8	C	C	C	T	T	C	C	G	A	C	A	T	A	G	A	G	C	A	C	G	G	C	T	A	C	A	T	T	A	G	A	T	A	C	C	.	C	T	A	.	C3
	H9	C	C	C	T	T	C	C	G	A	.	A	T	A	G	A	G	C	A	C	G	.	C	T	A	C	A	.	T	A	G	A	T	A	C	C	.	C	T	A	.	D4

(b)

		Nucleotide position																																		
Clade	Haplotype	132	151	157	259	272	344	419	423	425	449	458	463	473	494	500	512	563	584	692	725	845	854	867	923	926	929	938	950	998	1007	1027	1059	1070	1154	1268	Populations
A	H1	T	C	T	C	C	A	T	C	A	T	G	T	C	T	T	G	T	A	G	T	G	C	G	C	C	C	A	T	T	G	C	T	T	A	G	B1, B2, B3, B4, B7, B12, B13, C1, C2, C4, C5, C6, C7, C10, C14
	H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	C3
B	H3	C	.	C	T	T	G	C	T	G	A	A	C	T	C	C	A	C	G	A	C	A	T	.	T	T	T	G	C	C	A	G	A	C	G	T	A1, A2, A3
	H4	C	T	C	T	T	G	C	T	G	A	A	.	T	C	C	A	C	G	A	C	A	T	.	T	T	T	G	C	C	A	G	A	C	G	T	C2, C3
	H5	C	.	C	T	T	G	C	T	G	A	A	.	T	C	C	A	C	G	A	C	A	T	.	T	T	T	G	C	C	A	G	A	C	G	T	A1, D1, D3, D4

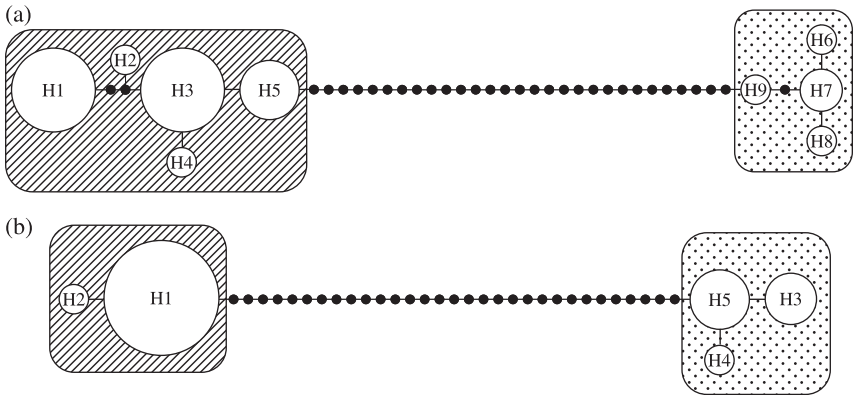

Fig. 2 Minimum-spanning networks derived from: (a) the nine control region haplotypes excluding R1 repeats and (b) the five mitochondrial ND1 gene haplotypes resolved among the samples of Daubenton's bat across Scotland. The circle area is proportional to the relative frequency of that haplotype/type among all populations. Each line within the network represents a single mutational change. Black dots represent inferred interior nodes that were not represented among the samples. Those haplotypes/types found in mtDNA clade A and clade B are clustered by the cross-hatched and speckled areas, respectively.

minimum-spanning network in Fig. 2a, and as a maximum-likelihood phylogeny in Fig. 3a. Two major clades, A and B, were distinguished by 30 variable positions including a 1-bp insertion/deletion. With the exception of population B1, the three most common haplotypes (H1, H3 and H5) were found only in North-east Scotland, upper Central Scotland and lower Central Scotland, respectively. Six other haplotypes (H2, H4, H6, H7, H8 and H9) were found only in one or two adjacent populations. All control region haplotypes have been deposited in GenBank under accession nos EU447261–EU447269.

Mitochondrial DNA polymorphism — ND1 gene

Thirty-five variable nucleotide positions (31 transition and four transversion point mutations) were found among the 1330 bp of ND1 gene examined. These defined five ND1 haplotypes among the 24 Scottish populations screened (Table 3b). Two distinct clades were separated by 32 variable positions which corresponded exactly with the two clades observed from the control region data (100% bootstrap support; Fig. 3). The evolutionary relationships between these individual haplotypes are shown as a minimum-spanning network in Fig. 2b and a maximum-likelihood phylogenetic tree in Fig. 3b. All ND1 haplotypes have been deposited in GenBank under accession nos EU447270–EU447274.

Using the combination of RFLP and mtDNA sequencing, the total of 945 individuals from 35 populations were classified as mtDNA clades A or B (Fig. 4). All bats from North Scotland and most bats (97%) from four populations in South Scotland were clade B. All bats from North-east Scotland with the exception of one male from population B1 were clade A. Co-existence of both mtDNA clades was found in 11 populations mainly in Central Scotland, but most bats (95%) in Central Scotland were clade A. Among these populations, bats from the geographically 'wrong' clade were both males (n = 13 bats from 10 populations) and females (n = 18 bats from 6 populations). Most of these populations were located at the edge of the distribution range of each clade, except for a few where only males were identified as having the 'wrong' mtDNA haplotype.

If females are considered alone, strong matrilineal structure is apparent (Fig. 5). Among 31 female bat populations, six of them consisted of both clades. These were located at the northern and western part of Central Scotland indicating a suture zone between two clades. Bats from the different clades were usually the minority of that group (10–20%, or 1–5 of 6–51 bats), except population C3 where three bats from clade B were found among a total of seven females.

All samples from Norway (n = 8) and the Netherlands (n = 3) clustered with mtDNA clade A, whereas all samples form Ireland (n = 10) clustered with mtDNA clade B. The samples from Germany consisted of bats from clade A (n = 12) and clade B (n = 4).

Microsatellite DNA polymorphism

No evidence could be found for a high incidence of null alleles at any locus, nor genotypic linkage disequilibrium between loci within population samples. Of the 561 Fisher exact probability tests undertaken, only 20 were significant ($P < 0.05$), and these were scattered randomly across locus pairs. It is therefore valid to utilise the data generated using these markers to assess levels of genetic structure between bat populations.

Allele frequencies for eight microsatellite loci in each of the 27 Scottish bat populations are provided in Appendix 1. Three estimates of genetic diversity — observed heterozygosity, number of alleles and allelic richness — for each population are presented in Table 4. Observed heterozygosity for each population ranged from 0.64 to 0.84. Number of alleles and allelic richness, on average across all loci, were between 5.5–10.5 and 1.73–1.82, respectively. Deviations from Hardy–Weinberg expectation are also provided in Table 4 as Wright's F_{IS}. Across all loci, significant deviations from Hardy–Weinberg expectations were

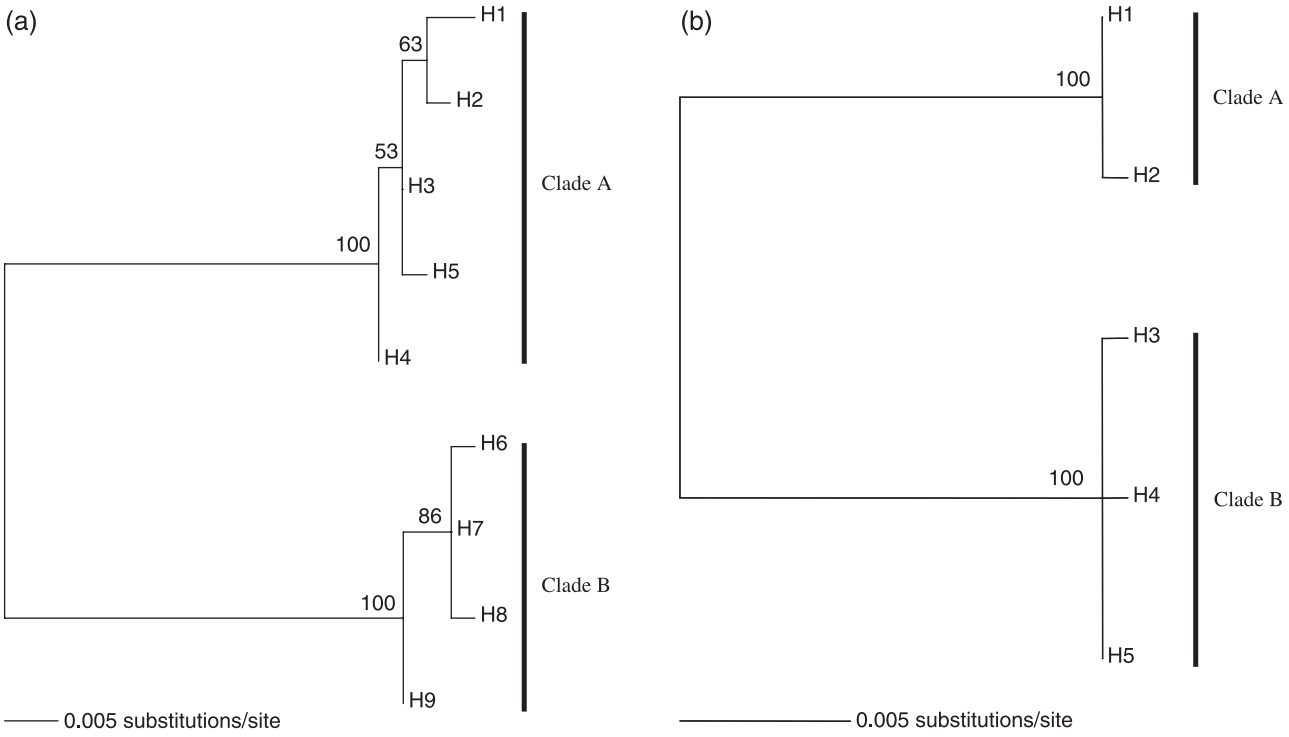

Fig. 3 Phylogenetic trees obtained from a maximum-likelihood analysis of sequence variation at: (a) the control region excluding R1 repeats, and (b) the ND1 gene. The scale bar represents 0.005 nucleotide substitution per site. Values at nodes are bootstrap values derived from 10 000 iterations (maximum parsimony analysis). Only bootstrap proportions above 50% are shown.

observed in three populations at $P < 0.05$, although after sequential Bonferroni correction, these values were deemed nonsignificant. Moreover, none of these three populations showed significant F_{IS} estimates at more than one locus.

Considerable levels of population differentiation were apparent. Multilocus Weir & Cockerham (1984) estimators of Wright's F_{ST} are given in Table 5. Values ranged from –0.007 to 0.061, with a large proportion of these values being deemed significant by permutation testing at $P < 0.05$ (231 of 351; 66%). The global F_{ST} value over all populations was 0.017. Although the mean F_{ST} value and the mean corrected assignment index (*mAIc*) obtained for females ($F_{ST} = 0.023$; *mAIc* = 0.095) were higher than males ($F_{ST} = 0.017$; *mAIc* = –0.115), no significant difference between sexes was apparent ($P = 0.071$ for F_{ST}; $P = 0.090$ for *mAIc*; 1000 randomisations).

From individual assignment tests, only 85 individuals (13%) were assigned to their sampled populations, whereas the majority (87%) were assigned to other populations. The proportion of individuals correctly re-assigned to their sampled populations was different between the sexes ($\chi^2 = 8.66$, d.f. = 1, $P = 0.003$), with the average percentage of females assigned to their sampled populations being significantly greater than for males (16.8% vs. 6.2%; Mann–Whitney *U*-test, $U = 195.5$, $P = 0.004$). However, there was no difference between sexes when individuals were assigned to their mtDNA clade ($\chi^2 = 0.49$, d.f. = 1, $P = 0.484$). Among those individuals that were not assigned to their original population, more than 60% of both males and females were assigned to other populations located within 100 km from the original population.

Genetic divergence at microsatellite DNA loci was more apparent at a regional scale. Overall, levels of gene flow maintain genetic homogeneity over the scale of a few tens of kilometres. No significant population divergence was found between populations located less than 25 km apart. The pairwise F_{ST} between populations within the same region were mostly nonsignificant (100% of pairwise population comparisons in the North; 71% in the North-east; 62% in the Central; 90% in the South), whereas only 17% of pairwise F_{ST} between populations in different regions were nonsignificant. When $F_{ST}/(1 - F_{ST})$ is plotted against the logarithm of Euclidean geographical distance between populations in kilometres, a significant, positive correlation was observed (Fig. 6; Mantel test, $P < 0.001$; $R^2 = 0.3782$). When the two sexes were considered separately, similar significant correlations between genetic and geographical distance (Mantel test: $P = 0.005$ for female, $P < 0.001$ for male) were also detected, but with females

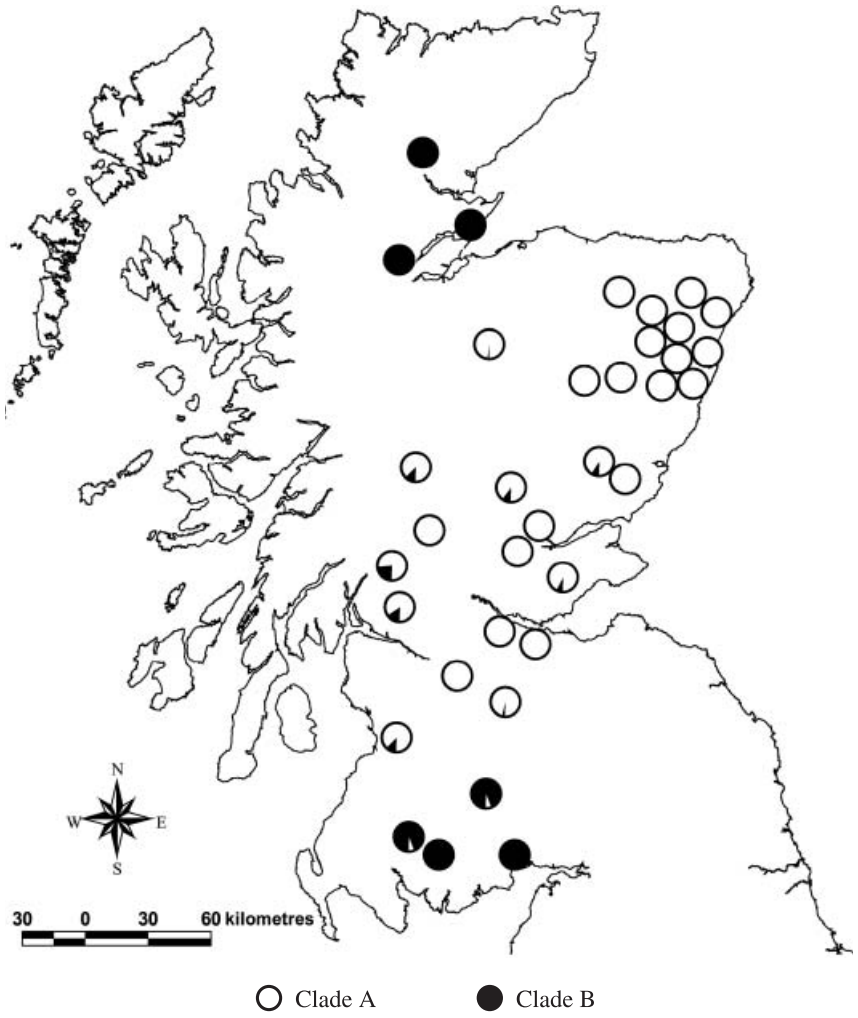

Fig. 4 Distribution of mitochondrial DNA clades A and B among all Scottish populations. Each pie diagram shows the relative frequency of each mtDNA clade in the population.

showing a higher degree of isolation by distance than males, as indicated by a steeper gradient to the regression line and a higher associated R^2 value ($R^2 = 0.0643$ for females, $R^2 = 0.0599$ for males).

Despite strong mitochondrial DNA structure, the level of genetic divergence of microsatellite variation between the two mtDNA clades was very weak. An analysis of molecular variance (AMOVA) highlighting low but significant microsatellite variation ($P < 0.001$) was attributable to differences among populations within each mtDNA clade and between mtDNA clades. Almost 98% was attributable to differences between individuals within populations (Table 6).

Discussion

This study has highlighted considerable mitochondrial DNA structure of Daubenton's bat at a broad geographical scale, with apparently low levels of microsatellite DNA population structure at finer geographical scales.

The patterns of large-scale structure are indicative of the effects of historical phylogeographical processes. Sequence variation at both the mitochondrial ND1 and control region loci consistently shows two deep, distinct intraspecific clades. Principally, clade A was found in North-east and Central Scotland, and clade B was found in North and South Scotland. A previous pan-European study of ND1 sequence variation revealed four distinct mtDNA clades in Daubenton's bat (Mayer & von Helversen 2001). The mtDNA clades A and B found in Scotland in the present study are consistent with the most derived and ancestral clades in their phylogenetic tree, respectively. The levels of genetic divergence between these clades (2.6% sequence divergence; this study) are in the intraspecific range, being five times less than the sequence divergence (13.6% over 957 bp) observed between *Myotis daubentonii* (this study) and its sister taxon *Myotis petax* (Kawai *et al*. 2003; Matveev *et al*. 2005). These mtDNA clades are likely to represent the signature of recolonisation from different glacial refugia in Europe after the retreat of the Pleistocene ice sheet. As

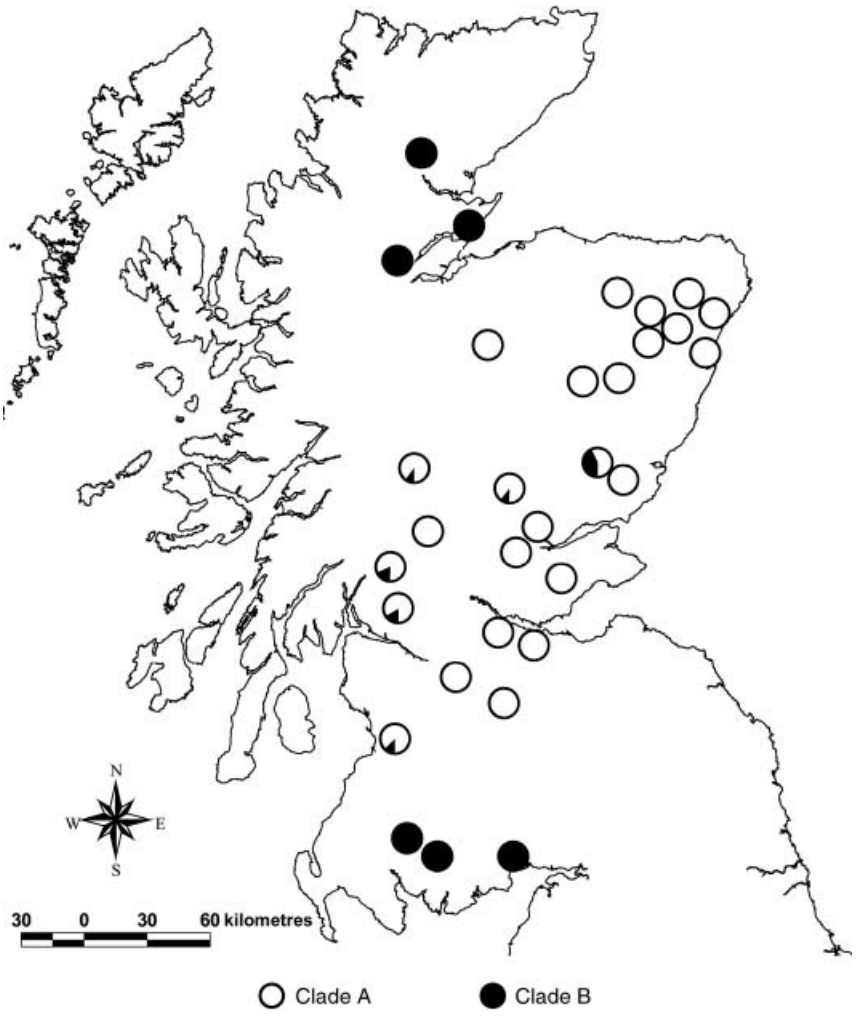

Fig. 5 Distribution of mitochondrial DNA clades A and B among female bat populations. Each pie diagram shows the relative frequency of each mtDNA clade in the population.

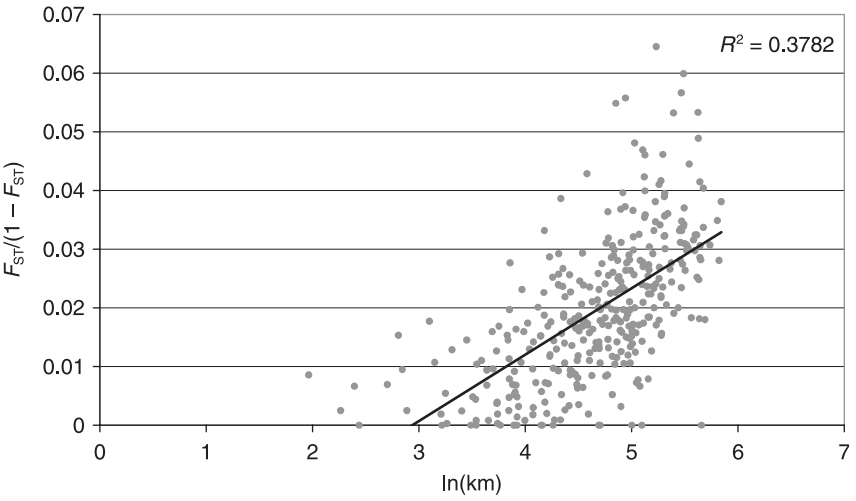

Fig. 6 Scatter plot of the logarithm of Euclidean geographical (km) vs. genetic distance between Daubenton's bat populations. The line represents optimal linear regression.

such, the distinct patterns of mtDNA structure observed in Scotland are consistent with the effects of multiple post-glacial recolonisation events, from source populations that were previously sufficiently isolated to accrue relatively large levels of genetic divergence. Such patterns of multiple recolonisation are not uncommon for the UK. A similar situation was described in the water vole *Arvicola terrestris*, with major differences between Scottish haplotypes and

Table 4 Levels of genetic diversity and Wright's F_{IS} estimated from microsatellite DNA makers for the Scottish Daubenton's bat populations. Values are averaged across loci. Significant F_{IS} estimates ($P < 0.05$) are highlighted by asterisks.

		Observed heterozygosity		Number of alleles		Allelic richness		
Population	*n*	Mean	SD	Mean	SD	Mean	SD	F_{IS}
A1	24	0.74	0.24	7.63	3.85	1.74	0.24	0.006
A3	17	0.70	0.26	6.63	3.16	1.75	0.24	0.083
B1	26	0.76	0.26	9.50	3.93	1.75	0.23	–0.01
B2	37	0.77	0.18	9.38	3.34	1.77	0.16	0.005
B3	18	0.64	0.23	6.38	2.20	1.73	0.20	0.125*
B5	14	0.79	0.29	5.50	2.33	1.74	0.24	–0.073
B6	56	0.79	0.12	10.25	3.65	1.78	0.13	–0.006
B12	45	0.75	0.17	10.13	3.94	1.77	0.16	0.019
B13	25	0.83	0.14	8.75	3.49	1.78	0.13	–0.065*
C1	26	0.79	0.15	9.75	3.06	1.80	0.17	0.016
C2	36	0.77	0.16	9.50	3.59	1.79	0.15	0.027
C3	36	0.80	0.09	10.50	4.17	1.80	0.13	–0.011
C4	25	0.78	0.14	9.13	3.09	1.78	0.16	–0.007
C5	15	0.75	0.28	8.25	2.92	1.77	0.21	0.021
C6	25	0.83	0.15	10.50	3.96	1.82	0.15	–0.022
C7	15	0.80	0.21	8.75	2.92	1.80	0.17	–0.001
C8	15	0.84	0.13	8.25	2.49	1.81	0.11	–0.034
C9	22	0.80	0.15	10.00	3.16	1.81	0.14	0.016
C10	26	0.83	0.13	9.00	3.07	1.81	0.15	–0.025
C12	21	0.83	0.11	8.13	2.23	1.81	0.14	–0.025
C13	20	0.81	0.13	9.38	3.29	1.81	0.15	–0.004
C14	20	0.84	0.21	7.63	2.97	1.78	0.19	–0.082*
D1	25	0.83	0.13	9.38	2.33	1.82	0.11	–0.012
D2	16	0.80	0.19	8.50	3.16	1.80	0.16	0.002
D3	25	0.80	0.18	9.25	3.45	1.80	0.18	–0.002
D4	25	0.79	0.21	8.25	2.49	1.78	0.18	–0.009
D5	16	0.76	0.14	9.50	3.59	1.82	0.15	0.01

English/Welsh haplotypes (Piertney *et al*. 2005), and the common shrew *Sorex araneus*, with considerable karyotypic and allozymic differences being apparent between Scottish and Welsh populations with English populations (Searle & Wilkinson 1987).

Two distinct mtDNA clades based on sequence variations at cytochrome *b* gene have been recently revealed in Daubenton's bats within the Iberian Peninsula separating *Myotis daubentonii nathalinae* from *Myotis daubentonii daubentonii* (Simões *et al*. 2007). By comparison with sequences from GenBank (Ruedi & Mayer 2001; *M. d. daubentonii*: GenBank Accession nos AF376847 for cytochrome *b* gene and AY033985 for ND1 gene, *M. d. nathalinae*: GenBank Accession nos AF376862 for cytochrome *b* gene and AY033954 for ND1 gene), mtDNA clade B in the present study is equivalent to *M. d. daubentonii* group but clade A is not equivalent to *M. d. nathalinae* group in the study by Simões *et al*. (2007). Moreover, phylogenetic distance between clade A and B is much greater than those between clade A and *M. d. nathalinae* group. As such, mtDNA clade B is not only found in the UK (Scotland and England), Ireland, Sweden and Germany, but also in Portugal suggesting the Lusitanian connection of Daubenton's bat populations during the last glacial maxima. The Lusitanian influences have recently been suggested, based on mtDNA control region variations, in the Leisler's bat *Nyctalus leisleri* within Europe where one of the lineages, including the closely related species *Nyctalus azoreum* endemic to the Azores Islands, is restricted to Ireland and the Azores (Boston *et al*. 2007). However, small sample size and limited distribution of Daubenton's bat samples outside Scotland precludes any firm conclusions being drawn.

Spatial distributions of clades A and B in Scotland are consistent with the high mountain ranges in northern Scotland and the hills of southern Scotland acting as barriers. Only a few populations had both matrilines present, and these were found along the edge of the clade distribution range indicating that females only occasionally move between populations in different clades at the suture zone.

Strong genetic discontinuity across the geographical range has also been found in other bat species such as

Table 5 Microsatellite DNA derived estimates of pairwise genetic divergence measured from F_{ST} averaged across eight loci between 27 Scottish populations. The significant values are underlined ($P < 0.01$) and highlighted by grey shading ($P < 0.05$)

	North Scotland		North East Scotland							Central Scotland													South Scotland				
	A1	A3	B1	B2	B3	B5	B6	B12	B13	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C12	C13	C14	D1	D2	D3	D4	D5
A1	0	0.0226	0.017	0.03	0.0164	0.053	0.026	0.028	0.023	0.029	0.038	0.018	0.02	0.039	0.032	0.023	0.051	0.03	0.033	0.029	0.024	0.04	0.028	0.03	0.034	0.027	0.037
A3		0	0.0072	0.013	0.012	0.027	0.0156	0.022	0.025	0.016	0.0116	0.036	0.035	–0.007	0.0226	0.013	0.044	0.0168	0.0151	0.0187	0.0078	0.0169	0.0196	0.018	0.0178	–0.002	0.018
B1			0	0.012	0.0109	0.022	0.018	0.018	0.016	0.017	0.025	0.021	0.018	0.014	0.017	0.024	0.036	0.023	0.021	0.0248	0.0085	0.017	0.033	0.027	0.033	0.026	0.03
B2				0	–0.003	0.0106	0.0024	0.0003	0.0078	0.011	0.014	0.017	0.011	0.0052	0.011	0.023	0.034	0.022	0.026	0.0201	0.018	0.025	0.027	0.029	0.03	0.027	0.024
B3					0	0.0094	0.0025	–0.004	–0.001	0.012	0.018	0.0069	0.0035	0.0032	0.017	0.018	0.0345	0.023	0.028	0.0259	0.019	0.019	0.029	0.043	0.039	0.033	0.04
B5						0	0.0085	0.01	0.0193	0.038	0.023	0.028	0.0231	0.013	0.016	0.03	0.061	0.035	0.034	0.044	0.016	0.052	0.054	0.057	0.051	0.047	0.031
B6							0	–0.003	0.0112	0.019	0.021	0.01	0.0128	0.0083	0.015	0.027	0.037	0.021	0.014	0.0216	0.014	0.028	0.027	0.034	0.031	0.025	0.031
B12								0	0.0151	0.014	0.023	0.013	0.0047	0.0091	0.018	0.027	0.046	0.03	0.024	0.0263	0.023	0.029	0.034	0.035	0.036	0.03	0.032
B13									0	0.016	0.015	0.016	0.0069	0.005	0.008	0.019	0.026	0.01	0.018	0.0315	0.018	0.0134	0.026	0.038	0.032	0.023	0.032
C1										0	0.0025	0.008	0.0019	0.002	0.005	0.005	0.017	0.016	0.021	0.0194	0.0063	–0.001	0.018	0.027	0.023	0.024	0.015
C2											0	0.015	0.016	–0.003	0.0037	0.007	0.026	0.012	0.025	0.0161	0.0056	0.0127	0.024	0.027	0.018	0.022	0.013
C3												0	0.0066	0.0143	0.009	0.017	0.0167	0.017	0.02	0.0254	0.0095	0.0171	0.023	0.041	0.031	0.029	0.021
C4													0	0.0091	0.0064	0.014	0.03	0.023	0.024	0.0278	0.013	0.014	0.027	0.045	0.038	0.031	0.024
C5														0	–0.002	0.0009	0.0092	0.007	0.0106	0.025	0.0019	0.0025	0.02	0.029	0.017	0.0066	0.008
C6															0	0.0003	0.0095	0.0018	0.014	0.0117	0.0039	0.0039	0.012	0.022	0.014	0.016	–0.003
C7																0	0.0174	0.0057	0.019	0.0163	0.009	0.015	0.009	0.025	0.012	0.015	0.0072
C8																	0	0.001	0.032	0.0279	0.0105	0.022	0.02	0.041	0.027	0.035	0.0173
C9																		0	0.0069	0.0044	0.0008	0.017	-1E-04	0.015	-1E-04	0.0105	–0.003
C10																			0	0.0143	0.0008	0.027	0.018	0.023	0.019	0.018	0.013
C12																				0	0.0072	0.0372	0.0008	-3E-04	0.0026	0.0138	0.0009
C13																					0	0.0054	0.013	0.02	0.015	0.017	0.01
C14																						0	0.02	0.031	0.031	0.02	0.025
D1																							0	0.0048	0.0032	0.003	0.0033
D2																								0	0.0019	0.0093	0.0109
D3																									0	–0.001	–0.002
D4																										0	0.007
D5																											0

Source of variation	Sum of squares	Variance components	Percentage of variation
Among clades	11.542	0.03674	1.14793*
Among populations within clades	64.752	0.03990	1.24670*
Within populations	1925.814	3.12386	97.60537*
Total	2002.108	3.20050	

Table 6 Analysis of molecular variance (AMOVA) for microsatellite-derived genetic divergence within populations, between populations within mtDNA clades and between mtDNA clades. Asterisks highlight which hierarchical levels explain a significant proportion of the overall variance ($P < 0.001$).

Myotis myotis where the Alps acts as a geographical barrier to dispersal for both females and males (Castella *et al*. 2001; Ruedi & Castella 2003). In Daubenton's bat, it appears that male and female bats differ in their ability to cope with high altitude. Surveys at higher elevations found a predominance of males (Russo 2002; Senior *et al*. 2005), and it is suggested that females are confined to lower elevations where insects are more abundant. As such, the high mountain ranges in Scotland may represent barriers to dispersal for females but not males, which would account for the discontinuities in the distribution of clades A and B over Scotland.

Sex-specific differences in gene flow in Daubenton's bat are also supported by low levels of microsatellite DNA structure contrasting with the strong mtDNA structure. The global F_{ST} value over all populations was low, and AMOVA analysis for microsatellite-derived genetic divergence also indicated low genetic differentiation among populations and more pertinently between mtDNA clades. Low levels of microsatellite genetic differentiation have also been established in many European bat species such as *Plecotus auritus* (Burland *et al*. 1999; Veith *et al*. 2004), *Myotis bechsteinii* (Kerth *et al*. 2002), *M. myotis* (Castella *et al*. 2001) and *Nyctalus noctula* (Petit & Mayer 1999) and may have been attained either by male natal dispersal or extra-colony fertilisation.

Although an association between genetic and geographical distance was apparent in Daubenton's bat, genetic divergences of microsatellite DNA between most populations within the same localised region were nonsignificant. Indeed, no significant F_{ST} value was found among populations located less than 25 km apart. Moreover, assignment analyses reassigned only a few individuals to their original sampled population and most misassigned individuals were assigned to other local populations, less than 100 km away. These indicate considerable levels of gene flow among local bat populations which may be the consequence of mating behaviour or individual dispersal among local colonies. This finding is consistent with the autosomal genetic structure in *M. bechsteinii* in Central Europe. Within areas of up to 150 km in diameter, Bechstein's bat colonies were significantly differentiated from each other (Kerth *et al*. 2002), but such differentiation was not related to intercolony distance (Kerth & Petit 2005).

One possible explanation for the lack of localised structure is the aggregation of individuals from different natal groups at caves or underground sites (swarming behaviour). Large numbers of Daubenton's bats, with a pronounced male bias, were reported to visit the same swarming site during late summer and autumn (Parsons *et al*. 2003). Swarming probably has an important reproductive function as most males are reproductively active during this time (Parsons *et al*. 2003; Encarnação *et al*. 2004), and it is suspected swarming may be important for the avoidance of inbreeding. High genetic diversity at swarming sites indicating a mixing of multiple populations has been found in other bat species such as *Myotis nattereri* (Rivers *et al*. 2005), *M. bechsteinii* (Kerth *et al*. 2003) and *P. auritus* (Veith *et al*. 2004). As in the present study, no isolation by distance or genetic substructure was detected among local colonies of *M. nattereri* visiting the same swarming area (Rivers *et al*. 2005).

Male-biased dispersal is commonly observed in mammalian species, including bats (Greenwood 1980; Dobson 1982; Castella *et al*. 2001; Petit *et al*. 2001; Kerth *et al*. 2002). Results from the present study suggest male-biased dispersal also occurs in Daubenton's bats in Scotland. Mitochondrial DNA variation at the ND1 gene based on RFLP data revealed the presence of male bats from the geographically 'wrong' clade in broadly distributed multiple populations indicating a propensity for long-distance dispersal in males. Conversely, spatially clustered distributions of control region haplotypes suggest no long-distance dispersal of females within clades. However, the patterns of local structure are possibly consistent with concurrent male and female mediated gene flow. Randomisation procedures based on microsatellite DNA polymorphism showed no significant difference between male and female dispersal suggesting that strong male-biased dispersal is not evident in Daubenton's bat. The power to detect sex bias using this method decreases significantly if its intensity drops below 80 : 20, that is less than 80% of dispersers are one sex and more than 20% are another (Goudet *et al*. 2002). Extreme sex-biased dispersal was found in some bat species such as *M. bechsteinii* (Kerth *et al*. 2002), *M. myotis* (Castella *et al*. 2001) and *N. noctula* (Petit *et al*. 2001) where more than 70% of the dispersing individuals are males. Although extreme sex-biased dispersal is unlikely to be found in Daubenton's

bat, lower F_{ST} values, lower mean assignment indices and lower proportions of individuals correctly re-assigned to their sampled populations suggest that male dispersal is more prevalent.

Two complementary evolutionary factors, that is, inbreeding avoidance and competitive interaction, have been suggested as the potential explanations for sex-biased dispersal (Greenwood 1980; Wolff 1994; Lambin *et al*. 2001). Although swarming behaviour in bats probably has an important reproductive function as mixing of multiple populations has been found at swarming sites (Kerth *et al*. 2003; Veith *et al*. 2004; Rivers *et al*. 2005; Senior *et al*. 2005), a paternity study in Daubenton's bat suggested that resident males have a high probability of successful mating with resident females (Senior *et al*. 2005). Male-biased natal dispersal would therefore have the benefit in inbreeding avoidance for this bat species. Substantial levels of intrasexual segregation in male Daubenton's bats indicating the competition for resources and reproductive enhancement (Russo 2002; Senior *et al*. 2005) would also provide a considerable explanation for the prevalence of male-biased dispersal.

Contrasting patterns of mitochondrial and nuclear DNA divergence have been reported in many European bat species which are consistent with male-biased dispersal (Castella *et al*. 2001; Kerth *et al*. 2002). Although matrilineal structure can be apparent in bat colonies as a consequence of female philopatry, low colony relatedness and low levels of genetic differentiation among adjacent colonies have been identified in many bat species (Burland & Worthington Wilmer 2001). By using indirect genetic methods, it is important to use the combination of different types of marker at different geographical scales to understand dispersal in natural populations.

Dispersal among Daubenton's bat populations, especially among males, is relatively high, and sufficient to homogenise nuclear genetic structure between two distinct mitochondrial clades. Much evidence indicates that bats may survive rabies infection with possible long-term maintenance of the virus in infected but apparently healthy bats (Wellenberg *et al*. 2002; Shankar *et al*. 2004; Amengual *et al*. 2007). This is important in respect of EBLV-2 epidemiology as it indicates a high potential for the spread of disease.

Although the presence of EBLV-2 has been detected in only six Daubenton's bats and one human, those incidents were distributed across the UK. It is unclear how rabies virus is transmitted between individuals within and among bat colonies. However, close proximity obviously increases the risk of infection and direct contact such as biting and grooming is considered the most likely route of transmission of EBLV-2 between bat individuals. In Scotland, the incidence of antibodies to EBLV-2 in Daubenton's bats, indicating previous exposure to lyssavirus, has varied between 0.05–3.8% in 2003 (Brookes *et al*. 2005), 15% in 2005, 8% in 2006 and 5.5% in 2007 (R. Raynor, Scottish Natural Heritage, personal communication), possibly reflecting the dynamics of EBLV-2 infection in bats. Periodic oscillations of EBLV-1 inflection in the number of susceptible, immune and infected bats have been found in spatially discrete subpopulations of *M. myotis* in Spain without any significant increase in associated mortality, and viral transmission from infective bats of different colonies possibly cause the recurrent epidemics (Amengual *et al*. 2007). More research is clearly needed on the epidemiology of EBLV-2 in order to plan surveillance strategies and for the management and control of bat rabies in the UK.

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This study formed part of Thongchai Ngamprasertwong's PhD thesis examining how the behaviour, ecology and population structure of Daubenton's bat could influence the epidemiology of European Bat Lyssavirus type 2. Paul Racey and Iain Mackie are bat biologists with broad interests in the ecology and conservation biology of temperate and tropical bats. Stuart Piertney is a molecular ecologist with a focus on understanding the causes and consequences of variation in levels of genetic diversity among natural populations.
