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ORIGINAL PAPER

Population genetic structure of the Daubenton's bat (*Myotis daubentonii*) in western Europe and the associated occurrence of rabies

Helen Atterby · James N. Aegerter · Graham C. Smith · Christine M. Conyers · Theodore R. Allnutt · Manuel Ruedi · Alan D. MacNicoll

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Abstract The Daubenton's bat is widespread and common in the UK and countries bordering the English Channel and North Sea. European bat lyssavirus 2 (EBLV-2), a rabies virus, has been detected in Daubenton's bats in the UK and continental Europe. Investigating the relatedness of colonies and gene flow between these regions would allow regional estimates of the movement of Daubenton's bats and thus the potential for disease transmission. The genetic structure of the Daubenton's bat in western Europe was investigated by analysing variability at eight microsatellite loci. Genetic diversity was found to be high at all sites (H_E=0.73–0.84), with little differentiation between bats sampled in the UK and continental Europe. Mantel tests indicated a significant correlation between geographic distance and pair-wise F_{ST} (P=0.000), between colonies sampled in Scotland and northern England. However, this was not continuous throughout the sampled range, with evidence of panmixia within the area sampled in continental Europe. Assignment tests show no evidence that the (potential) EBLV-2 sero-positive and virus positive bats were more likely to have originated from the continental rather than UK populations. There is no sufficient significant genetic differentiation amongst most UK and continental colonies to conclude that EBLV-2 is maintained in the UK by immigration. Results show that it is likely to be maintained at a low endemic level within the UK. The relative genetic uniformity of UK and continental populations implies that there is no migration barrier to EBLV-2, between these regions.

Keywords Microsatellite · Bat · Europe · Gene flow · Migration · Lyssavirus · *Myotis daubentonii*

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Introduction

The Daubenton's bat, *Myotis daubentonii*, has been described as one of the most widely distributed mammals in Eurasia (Corbet 1978), common across its range and often associated with freshwater (Altringham 2003). It has a continuous transpalearctic distribution from Portugal and Ireland in the west to continental Kamchatka, Japan and China in the east and from the Mediterranean, northern India, southern China and Vietnam in the south to within a few hundred kilometres of the arctic circle (66.5°N; Bogdanowicz 1994; Mitchell-Jones et al. 1999). However, despite its widespread distribution, the Daubenton's bat has a poorly described spatial ecology.

Bats are reservoirs and vectors of serious zoonoses across the world (Biek et al. 2006; Calisher et al. 2006; Eaton et al. 2006; Fooks et al. 2003; Halpin et al. 2007; Wong et al. 2007). European bat lyssavirus (EBLV), a rabies virus, is widely associated with some species

(Whitby et al. 2000). There are two types: EBLV-1 and EBLV-2; both are poorly described, with the pathology, epidemiology and mode of transmission in their wildlife hosts being unknown (Vos et al. 2007). EBLV-2 appears rarely, with only 22 recorded cases across Europe since 1976 (McElhinney et al. 2008). With the exception of two human cases, all have been in either Daubenton's bats or Pond bats (*Myotis dasycneme*) suggesting that these are the normal maintenance hosts for the virus. Unstratified surveys for the disease (presence of serum antibody) in Scotland, England (Brookes et al. 2005; Harris et al. 2009) and The Netherlands (Van der Poel et al. 2005) indicated sero-prevalence rates of 2–4%. Associated work detected no live virus, via oropharyngeal swabs, in the UK. Stratified studies in Scotland have since found higher levels of antibody sero-prevalence in Daubenton's bats with rates of 32.9% in 2004 (Anonymous 2005), 15% in 2005, 8% in 2006 and 5.5% in 2007 (Ngamprasertwong et al. 2008), again with no live virus reported. The apparent peak and decline in sero-prevalence in Scotland is also associated with changing laboratories, techniques and cutoff levels used, thus increasing the difficulty in interpreting these combined studies of sero-prevalence to understand the dynamics of this disease in the wild.

During the summer months, Daubenton's are sedentary within their lowland, natal communities, moving relatively small distances, typically <3 km (Altringham 2003), to forage from smooth freshwater sites such as rivers, pools and lakes. However, they can show seasonal movements away from their summer landscapes during July to November. Ringing studies have commonly reported Daubenton's bats migrating distances of 100–150 km for many countries in Europe, with the longest recorded movements of 257 and 304 km (Hutterer et al. 2005). In the UK, published research investigating the seasonal movements of the Daubenton's bat is scarce. Parsons and Jones (2003) found maximum distances of Daubenton's movement in southern England of 35.1 km using ringing and 26.7 km using radio-tracking. The mating behaviour, referred to as 'swarming', is common amongst many temperate vespertilionid species, especially the myotids, and is thought to be a lekking behaviour which promotes outbreeding (Rivers et al. 2005). Numerous studies have identified swarming sites to be hotspots for gene flow between otherwise isolated bat communities (e.g. Glover and Altringham 2008). Both the summer natal colonies and autumnal swarming sites represent the times and locations when horizontal and vertical disease transmission appear most likely. Known natal colonies tend to number between tens and hundreds of bats, most of who will live in very close contact with each other in cavities in trees, buildings or bridges (Altringham 2003). The limited work on autumnal swarming sites suggests that hundreds to thousands of bats may use them over a few months, with chasing and mating behaviour thought common (Rivers et al. 2006). Although very little is known about how or where western European Daubenton's bats spend the rest of the year, it is likely that this is at low density or with limited bat to bat contact.

The use of population genetics to study the spatial structure of mammalian communities (e.g. Frantz et al. 2008; Pope et al. 2006; Rivers et al. 2005; Wandeler et al. 2003) provides a cost efficient, relatively non-invasive method for surveying large groups of otherwise elusive mammals. Population genetics and, in particular, microsatellite analysis can provide an insight into the largest distances and thus fastest rates of spread that zoonotic infections, such as EBLV-2, are likely to travel with their vectors. This will also provide information on the degree of interaction between bat communities and can begin to describe the geographical scales at which movement associated with mating is likely to occur (Burland et al. 1999; Petit and Mayer 1999).

In this paper, we contribute to the knowledge about the eco-geography of Daubenton's bats through analysis of microsatellite diversity. We aim to identify the presence, nature and geographical scale of population structure at the country/continental scale. This provides information on the origins of bats found with evidence of EBLV-2 and uses estimates of gene flow to infer the potential for the movement of this disease at a broad geographical scale.

Materials and methods

Sample collection

Wing biopsies (3 mm diameter, one from each wing) were taken from Daubenton's bats caught at summer roosts (mainly maternity colonies plus two bachelor roosts), autumnal swarming sites and occasional individuals in central and southern Scotland, Lancashire, Cumbria, Yorkshire and Middlesex, UK, between April 2003 and October 2006 (Fig. 1). Following capture, these individuals were ringed and sexed; saliva and blood samples were also taken and tested for EBLV-2, as part of an ongoing active surveillance programme (Brookes et al. 2005; Harris et al. 2009). All procedures were carried out in accordance with UK Home Office guidelines (Animals (Scientific Procedures) Act 1986) and under licence from the appropriate Statutory Nature Conservation Organisation.

Wing biopsy samples were also obtained from one Irish and several maternity/swarming colonies in Belgium, France, Switzerland (Fig. 1) and Lake Baikal, Russia. This latter site, north of Mongolia, was a combination of

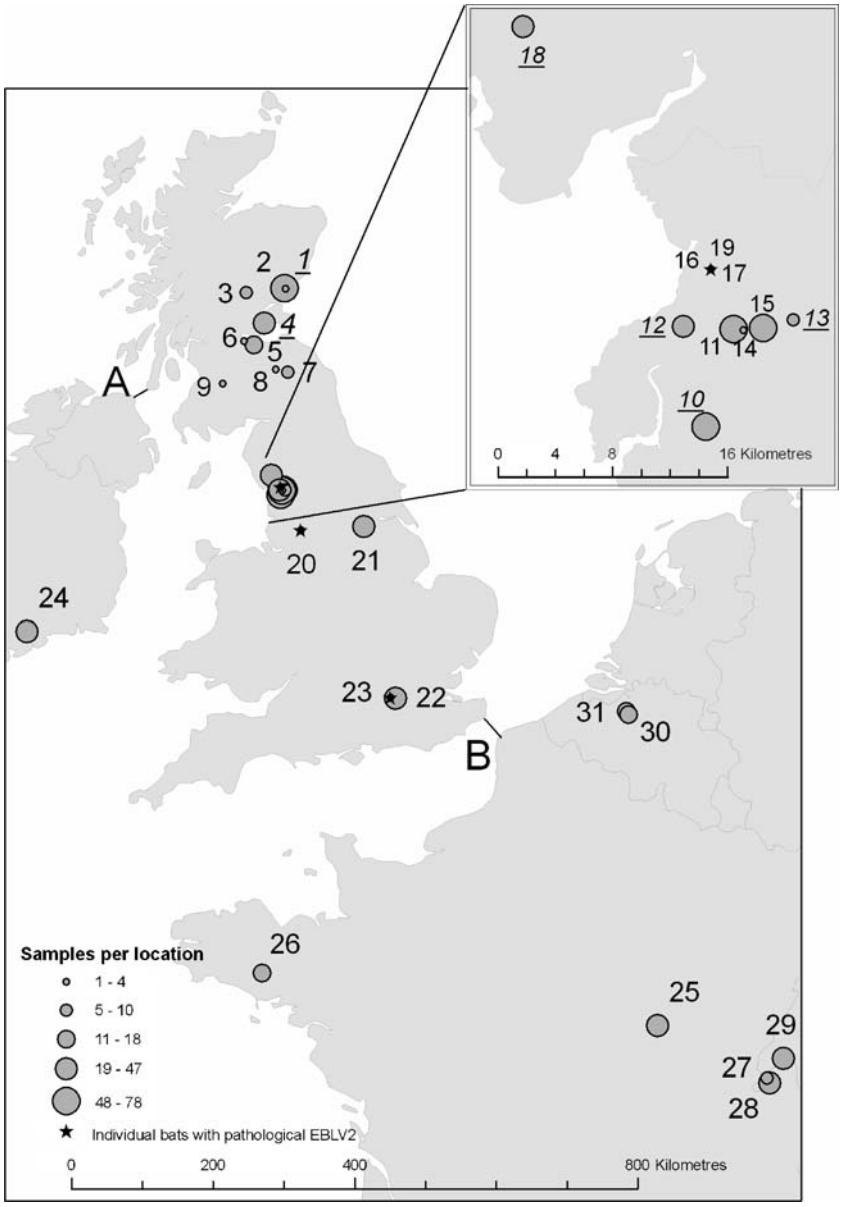

Fig. 1 Sampling locations of Daubenton's bats in the UK, Republic of Ireland and continental Europe. The minimum distances between the Mull of Kintyre and Northern Ireland (20 km, *A*) and Dover to Calais (33 km, *B*) are shown. Colonies with underscore are sites at which potential EBLV-2 sero-positive individuals were found

captures from three transient roosts within the town of Kultuk (51°43′ N, 103°41′ E). Upon collection, all wing biopsy samples were stored in individual tubes containing 70% ethanol (Fisher Scientific, Loughborough, UK) at 4°C, until analysis.

DNA was also obtained from the three Daubenton's individuals confirmed as virus positive for EBLV-2, found next to the Lancaster Canal, Lancashire, UK in 2002 (Johnson et al. 2003); Bury, Lancashire, UK in 2003 (Fooks et al. 2004b) and Staines, Surrey, UK in 2004 (Fooks et al. 2004a). In addition, 50 Daubenton's faecal pellets were collected from sheets laid under a roost site of a Daubenton's bat maternity colony in Brittany, France (Fig. 1) and stored in individual tubes containing silica gel, at room temperature (Puechmaille et al. 2007).

DNA extraction

DNA was extracted from each wing biopsy after first blotting the wing membrane dry on tissue paper to remove excess ethanol. A DNA extraction method for rapid isolation of nucleic acids from mammalian tissues (Sambrook and Russell 2001) was used, except that 10 M ammonium acetate (Sigma-Aldrich, Poole, Dorset, UK) was used for the protein precipitation stage, instead of potassium acetate. The DNA pellet was resuspended in 45 µl of 1× Tris–ethylenediaminetetraacetic acid buffer (Sigma-Aldrich) for microsatellite amplification.

DNA was extracted from Daubenton's faecal samples using an adaptation of the QIAamp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) as reported by

Puechmaille et al. (2007). A negative control was included with every batch of DNA extractions to check for cross contamination.

Microsatellite amplification

Polymerase chain reaction (PCR) reactions consisted of 10 µl JumpStart REDTaq ReadyMix PCR Reaction Mix (2× reaction concentrate; Sigma-Aldrich) containing 1.5 mM $MgCl_2$, 0.95 µM of each primer (Sigma-Aldrich/Applied Biosystems, Foster City, CA, USA), 2 µl of 1% dimethyl sulphoxide (Sigma-Aldrich) and 1.5 µl DNA template for wing biopsy extracts or 3 µl DNA template for faecal DNA extracts. Further, magnesium chloride (ABgene, Epsom, Surrey, UK) was added where required (Table 1) and the remaining volume was made up to 20 µl with de-ionised sterile water. DNA extracts were PCR amplified using eight microsatellite loci specific for myotid species (Table 1). PCR reactions were performed on a Hybaid Multiblock thermocycler (Thermo Life Sciences, Basingstoke, Hampshire, UK) and consisted of 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 1 min at the annealing temperature, 72°C for 1 min, 72°C for 5 min and 60°C for 1 h.

Repeat PCR amplifications of faecal DNA extracts were carried out according to the comparative multiple-tube approach developed by Frantz et al. (2003), to account for the high incidence of allelic dropout and/or false alleles obtained from faecal DNA extracts (Taberlet et al. 1999).

The accurate size of each microsatellite fragment was then determined using POP-7 polymer in a 36-cm capillary array, mounted on a 3130*xl* genetic analyser (Applied Biosystems), with Genescan Rox 350 size standard (Applied Biosystems), according to standard protocols.

Analysis of microsatellite data

Microsatellite fragments were sized using GENEMAPPER 3.7 software (Applied Biosystems). The data were formatted using MICROSATELLITE TOOLKIT FOR EXCEL (http://oscar.gen.tcd.ie/~sdepark/ms-toolkit/index.php), which tests for invalid alleles and formats data for input into population genetics software programmes.

In order to estimate the minimum sample size (i.e. minimum number of individuals sampled per colony) required to produce an accurate estimate of the genetic variation within that colony, a re-sampling experiment was designed, using the colony with the largest sample size. The sum of squared differences (ssd) between the estimated and actual heterozygosity was calculated with increasing sample size (100 replicates per sample size).

Estimates of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were calculated using GENEPOP version 4.0 (Raymond and Rousset 1995). Exact tests of HWE were performed using a Markov chain method (dememorisation 1,000, batches 10,000, iterations per batch 1,000) (Guo and Thompson 1992). Observed and expected heterozygosities, mean number of alleles per colony, F_{IS} per colony and the overall estimate of G_{ST} for all populations were calculated using GENETIX version 4.05 (Belkhir et al. 2004). F_{IS} values per locus were estimated using FSTAT version 2.9.3.2 (Goudet 1995). Pair-wise F_{ST} estimates between populations (significance tested using a 1,000 replicate bootstrap) and the structure of the overall genetic variation were estimated using analysis of molecular variance (AMOVA) in ARLEQUIN version 3.1. (Excoffier et al. 2005). Colonies were split into geographic groupings based on sampling locations. A 1,000 replicate bootstrap was used to apportion variation amongst groups and roosts and to test the significance of each variation component. Mantel tests (1,000 replicates) were also performed between all pair-wise geographic and genetic distances (F_{ST}; Mantel 1967) and corresponding F_{ST} values for all roosts, using PopTools (http://www.cse.csiro.au/poptools/). Mantel tests were performed on all colonies and separately on northern UK colonies only (1–21).

Allele frequencies were analysed using STRUCTURE version 2.1 (http://pritch.bsd.uchicago.edu/structure.html; Pritchard et al. 2000), to estimate the number of clusters within the whole data set. Initially, each individual was assigned a probability of belonging to one of several clusters (K) based on the allele frequencies, using a Bayesian clustering methodology (Pritchard et al. 2000). The length of burn-in period was 10,000 iterations and number of Markov Chain Monte Carlo repetitions after burn-in was 1,000,000. The admixture ancestry model with correlated allele frequencies was used with default settings, assuming a uniform prior. No prior population information was included in the analysis. The optimum number of clusters was estimated by performing five independent runs at K=1–9. The arithmetic mean of all log-likelihood values at each K (log $P[X/K]$) were calculated, and these were used to estimate the posterior probability (log $P[K/X]$) at each K.

The probability of assignment of all 734 individuals to five populations (i.e. England, Scotland, Ireland, "continental" and Lake Baikal) was carried out using GENECLASS version 2 software (Piry et al. 2004). Preliminary analysis was used to determine the best model, based on the number of correctly assigned individuals and the "Quality Index" calculated by the software package. The best results were obtained using a Bayesian method (Baudouin and Lebrun 2001) and Monte Carlo re-sampling by the method of Cornuet et al. (1999). One thousand individuals were simulated.

Table 1 Sequences, PCR reaction conditions and allelic diversity of microsatellite primers used

Locus	Forward and reverse primer sequence 5′ to 3′	Microsatellite repeat	Magnesium concentration (mM)	Annealing temperature (°C)	Number of alleles	Allele size range (bp)	F_{IS} per locus	Reference
H19	F: 6-FAM-GGAATCCGAATCCCTGGC R: GTTTCTTGACATCCCCTCACCCCAAC	$(GT)_{18}CT(GT)_2$	1.5	60.8	19	82–120	0.006	Castella and Ruedi (2000)
H29	F: NED-TCAGGTGAGGATTGAAAACAC R: GTTTCTTGCTTTATTTAGCATTGGAGAGC	$(CA)_{21}$	1.5	46[b]	16	166–200	−0.000	Castella and Ruedi (2000)
C113	F: 6-FAM-ACCTCCCTGCCCTGCAC R: GTTTCTTGCAATGCTTCCTCCAAGTCC	$(ACC)_7$	1.5	60[b]	4	101–110	−0.024	Castella and Ruedi (2000)
G9	F: HEX-AGGGGACATACAAGAATCAACC R: GTTTCTTTAATTTCTCCACTGAACTCCCC	$(TC)_{19}$	1.5	51[b]	35	146–218	0.036	Castella and Ruedi (2000)
B15	F: NED-TAAGGTATAAAGAGAAATACC R: GTTTCTTAAAGGGTCTTGTTTAACTTT	$(CT)_X(CA)_Y$	1.5	46[b]	29	129–205	−0.033	Kerth et al. (2002)
D15	F: 6-FAM-GCTCTCTGAAGAGGCCCTG R: GTTTCTTATTCCAAGAGTGACAGCATCC	$(AC)_{17}$	3.0	62	22	100–154	0.066	Castella and Ruedi (2000)
B22	F: HEX-CTGATGCAAGACCCCTTACAAC R: GTTTCTTACGGCAGCAGTGAAATCAGA	$(TC)_x$	1.5	50[b]	24	148–200	0.019	Kerth et al. (2002)
E24	F: 6-FAM-GCAGGTTCAATCCCTGACC R: GTTTCTTAAAGCCAGACTCCAAATTCTG	$(TC)_{32}$[a]	1.5	61	35	208–266	−0.005	Castella and Ruedi (2000)

Leeds University kindly provided us with the primer sequences and optimised conditions for Daubenton's species. A 'pig-tailing' modification (GTTTCTT) was added to the 5′ end of each reverse primer to reduce non-templated addition of primarily adenosine nucleotides (Brownstein et al. 1996), which could otherwise lead to problems in accurate sizing of the DNA fragments. ABI dyes Hex, 6-Fam and Ned are indicated where present on the forward primer

[a] Indicates occasional single nucleotide repeats observed

[b] Indicates touchdown PCR. This consisted of an additional cycling stage, following the initial step of 94°C for 2 min. It comprised 20 cycles of 94°C for 1 min, 1 min at 10°C above the stated annealing temperature (which dropped −0.5°C per cycle) and 72°C for 1 min

Results

Samples collected

Of the individuals sampled from colonies where sample sizes were $n \geq 9$ (a total of 20 colonies), the proportion of females ranged from 0% to 97% with a mean of 53% (±7 standard error). Where the proportion of females sampled within colonies was below 40% (seven out of 19 colonies), these were either from summer roosts commonly frequented by adult males or because a significant proportion of the sampling at these roosts was during September or October, during pre-hibernation swarming. As juveniles were also sampled at all sites where possible, a significant proportion of males were juveniles, where this could be ascertained (33% [±9.0] in colonies of sample sizes where $n \geq 9$; a total of 14 colonies).

Genotyping quality

Poor PCR amplification of the microsatellite target sequence was obtained from the faecal pellets. Thus, 32 faecal samples were excluded from the analysis and 18 gave amplification products at six or more loci, three of which were found to be identical matches. These three samples were therefore removed from the data set.

DNA extracts from all individuals or faecal samples, which produced clear amplification products at four or more loci, were included in the initial analysis (n=734). Of these, nine sites were from Scotland (n=157), 11 from England (n=363), one from southern Ireland (n=33), seven from continental Europe (n=146) and one from Lake Baikal, Russia (n=32). Also included were DNA extracts from the three virus positive individuals in Lancashire and Staines (Fig. 1). A total of 17 individuals, from which wing biopsies were taken (2% of total sampled), were excluded from the analysis due to poor genotyping quality.

F Statistics

In a re-sampling experiment using colony 15 (which contained the greatest number of biopsied individuals), the ssd, between estimated and actual heterozygosity, was plotted against sample size (100 replicates per sample size). This showed that the difference between estimated and actual heterozygosity levelled off at approximately ten samples per roost. A sample size of nine or more was thus considered sufficient to capture the variation in each population with a high degree of confidence. Therefore, colonies with fewer than nine samples and the three individuals confirmed positive for EBLV-2 were excluded from HWE and linkage disequilibrium tests, observed and expected heterozygosities, F_{IS} and pair-wise F_{ST} calculations. However, these data were included in STRUCTURE analysis (see below) where colony assignments were disregarded.

Analysis of HWE (for all colonies where $n \geq 9$ [21 in total]) indicated a lack of equilibrium for the overall probability at eight loci for all populations. This was primarily the result of a significant heterozygote deficit at four loci (H19, G9, B22 and E24) in the Lake Baikal samples. A substantial heterozygote deficit was found at several other colonies; however, in all cases, it was restricted to just one locus per colony. This was therefore unlikely to be the result of null amplification, and as such assumed to be of little significance. Similarly, a heterozygote excess was present within three colonies; however, this was again restricted to just one locus. This was confirmed by a P value of 0.135 [(±0.006) where H1 = heterozygote deficit] and 0.860 [(±0.006) where H1 = heterozygote excess], for the global test for all loci and all populations, when the Lake Baikal samples were excluded from the analysis. Tests for linkage disequilibrium across all populations indicated linkage between six pairs of loci out of a total of 28 possible pairings. Nevertheless, this was a maximum of two out of 21 colonies per loci pair, and it was therefore considered to be of no overall significance.

F_{IS} estimates per locus (calculated for all 734 samples), excluding D15, did not differ significantly from zero, the latter one being slightly above the threshold of 0.05 (Table 1). Therefore, although it is possible that this locus had a higher frequency of null amplification, it is unlikely to significantly bias the results. The observed heterozygosity (H_O), expected heterozygosity (H_E) and mean number of alleles per colony (AC) were all high (0.65 to 0.88, 0.73 to 0.84 and 7.38 to 12.88, respectively), which suggests a high level of genetic diversity within colonies (Table 2). F_{IS} estimates for all western European colonies (except colony 3) did not differ significantly from zero (0.04 to −0.042), indicating that there was no significant inbreeding within these colonies. The significantly positive F_{IS} value obtained with colony 3 of 0.172 (95% confidence interval (CI)=−0.021 to 0.224) indicates the presence of inbreeding at this roost. This colony also had the lowest diversity of those sampled (H_E=0.73). The Lake Baikal samples had a significantly positive F_{IS} of 0.164 (95% CI=0.071 to 0.201), which is likely to be the result of sub-structuring within this sample set, this being a combination of three separate transient roosts.

Pair-wise F_{ST} values between all UK and continental European colonies sampled were low (0.000–0.050; Table 3). The greatest genetic differentiation was observed between the Lake Baikal samples and all west European colonies (pair-wise F_{ST} range=0.074–0.151). Significant genetic differentiation was also present between the southern Irish colony and all other colonies sampled. A dendrogram,

Table 2 Genetic variability within the colonies sampled (where $n \geq 9$)

Colony ID	Number of samples analysed	H_O	H_E	A	F_{IS}
1	71	0.83	0.81	11.63	−0.023
3	10	0.65	0.73	7.38	0.172
4	38	0.83	0.80	11.38	−0.022
5	18	0.85	0.84	10.50	0.020
7	9	0.88	0.79	7.50	−0.042
10	59	0.83	0.82	12.75	−0.001
11	53	0.83	0.83	12.50	0.005
12	33	0.84	0.81	11.88	−0.011
15	78	0.81	0.81	12.75	0.010
18	47	0.82	0.82	11.13	0.011
21	33	0.85	0.81	12.00	−0.026
22	44	0.81	0.81	12.63	0.009
24	33	0.79	0.81	11.00	0.036
25	31	0.87	0.84	12.00	−0.018
26[a]	15	0.84	0.80	9.00	−0.006
27	9	0.80	0.79	8.75	0.040
28	31	0.81	0.83	12.88	0.035
29	31	0.86	0.84	11.88	0.002
30	14	0.83	0.82	10.00	0.030
31	15	0.87	0.80	9.75	−0.046
32	32	0.65	0.77	10.13	0.164

The colony identification number relates to its geographical position in Fig. 1

H_O observed heterozygosity, H_E expected heterozygosity, A mean number of alleles per locus, F_{IS} inbreeding coefficient

[a] Faecal samples

based on pair-wise F_{ST} values, indicated four sub-groups within the European samples: (a) Scotland, roosts 1, 3, 4; (b) England, roosts 10, 11, 12, 15, 21 and 22; (c) the remaining English, Scottish and continental roosts and (d) southern Irish roost, 24 (Fig. 2). Two Scottish (5 and 7) and one English (18) colony were therefore not distinguishable from the continental roosts sampled. Amongst other continental roosts, there was no clear genetic geographical distinction. Colony 3 was distinct within the Scottish group. Out of all the UK colonies sampled, the Cumbria (18) and southern Scotland sites (5 and 7) appeared most closely related to the Irish colony. However, there was no discernable difference in these pair-wise F_{ST} values and those between continental colonies and the Irish site. P values for each F_{ST} reflect these groupings. Several pair-wise F_{ST} values within each group were not significant (Table 3). This indicates a high degree of gene flow and lack of population disjunction within these groups. However, even amongst groups, the F_{ST} values were low, with only colony 24 exhibiting appreciable differentiation (harmonic mean pair-wise F_{ST} [excluding Lake Baikal]=0.054).

In order to analyse the overall genetic variation using AMOVA, colonies were placed into one of three groups: Scotland, England and continental Europe. The southern Irish roost was not included in the group structure, but was included in the population analysis. The Lake Baikal roosts were excluded from this analysis. The proportion of variation amongst colonies was generally low (Φ_{ST}=0.012) and lower amongst groups (Φ_{ST}=0.056), but both proportions were significant, P=0.00 and 0.01, respectively (where P is probability of a higher or equal proportion being observed by chance). This again indicates a high level of gene flow and low genetic subdivision between colonies and groups of colonies.

Results of Mantel tests (for colonies where $n \geq 9$) indicated no significant overall correlation between pair-wise F_{ST} and geographic distance (P=0.392), i.e. 39.2% of randomly re-sampled matrices' correlations were higher than the observed correlation between geographic distance and F_{ST}. However, when the Mantel test was performed separately on the colonies situated in the northern UK (1–21 only), a significant relationship was found between F_{ST} and geographic distance (P=0.000; Fig. 3). When pair-wise F_{ST} values for colony 3 are disregarded, the remaining values are within a similar range to those calculated for the colonies sampled in continental Europe. There was no apparent correlation between pair-wise F_{ST} and geographic distance for the continental colonies sampled (P=0.822).

Structure analysis

STRUCTURE analysis of all 734 individuals (which produced amplification products at four or more loci) indicated best fit of K=5 (Table 4; Fig. 4). This analysis supports the pair-wise F_{ST} data in suggesting that the Lake

Table 3 Pair-wise F_{ST} values (below the diagonal) and their significance (above the diagonal, as P values) for colonies where $n \geq 9$

Colony ID	1	3	4	5	7	10	11	12	15	18	21	22	24	25	26[a]	27	28	29	30	31	32
1		0.005	0.047	0.001	0.015	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.049	0.000	*0.998*	0.013	*0.616*	0.000
3	0.021		*0.106*	0.013	0.005	0.000	0.000	0.000	0.000	0.000	0.006	0.003	0.000	0.001	0.003	0.035	0.000	*0.057*	0.012	*0.192*	0.000
4	0.003	*0.009*		*0.259*	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010	*0.138*	0.000	*0.109*	0.024	*0.434*	0.000
5	0.015	0.026	*0.001*		*0.762*	0.001	0.043	*0.083*	0.002	*0.611*	0.000	0.000	0.000	*0.339*	*0.302*	*0.185*	*0.214*	*0.998*	*0.912*	*0.919*	0.000
7	0.015	0.041	0.019	*0.000*		0.008	*0.060*	0.038	0.004	*0.265*	0.024	0.001	0.000	0.027	*0.076*	*0.153*	*0.138*	*0.771*	*0.442*	*0.389*	0.000
10	0.032	0.041	0.018	0.015	0.017		0.002	*0.404*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.038	0.048	0.032	0.000
11	0.025	0.030	0.016	0.007	*0.010*	0.006		*0.258*	0.014	*0.057*	*0.117*	0.002	0.000	0.000	0.001	0.002	0.000	*0.753*	0.014	*0.278*	0.000
12	0.030	**0.050**	0.025	*0.006*	0.013	*0.000*	*0.001*		*0.103*	0.000	0.002	0.000	0.000	0.000	0.000	0.009	0.000	0.002	0.006	0.016	0.000
15	0.032	0.041	0.018	0.015	0.024	0.013	0.004	*0.003*		0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.041	0.000	*0.147*	0.000
18	0.026	0.035	0.017	*0.000*	*0.002*	0.015	*0.003*	0.014	0.017		0.007	0.000	0.000	0.000	*0.109*	0.000	0.004	*0.987*	*0.093*	0.011	0.000
21	0.034	0.021	0.016	0.015	0.015	0.015	*0.002*	0.011	0.010	0.008		*0.402*	0.000	0.000	0.000	0.000	0.001	*0.753*	0.008	*0.449*	0.000
22	0.029	0.024	0.013	0.022	0.028	0.021	0.006	0.017	0.010	0.017	*0.000*		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
24	**0.062**	**0.097**	**0.069**	**0.046**	0.041	**0.074**	**0.065**	**0.066**	**0.084**	**0.056**	**0.074**	**0.087**		0.002	0.000	0.000	0.002	*0.998*	0.015	*0.326*	0.000
25	0.017	0.035	0.013	*0.000*	0.013	0.019	0.011	0.014	0.014	0.013	0.015	0.009	**0.052**		0.001	0.019	0.024	*0.998*	0.031	*0.823*	0.000
26[a]	0.017	0.042	0.011	*0.003*	*0.011*	0.027	0.021	0.031	0.035	*0.006*	0.033	0.031	0.036	0.017		*0.775*	*0.096*	*0.239*	*0.051*	0.047	0.000
27	0.012	0.037	*0.007*	*0.008*	*0.010*	0.017	0.024	0.021	0.041	0.028	0.036	**0.049**	0.041	0.018	*0.000*		0.023	*0.647*	*0.377*	*0.265*	0.000
28	0.017	0.036	0.015	*0.003*	*0.007*	0.024	0.014	0.021	0.024	0.010	0.019	0.011	**0.054**	0.007	*0.007*	0.020		*0.970*	*0.093*	0.043	0.000
29	*0.000*	*0.012*	*0.002*	*0.000*	*0.000*	0.005	*0.000*	0.009	0.004	*0.000*	*0.000*	0.000	*0.028*	*0.000*	*0.001*	*0.000*	*0.009*		*0.715*	0.024	0.000
30	0.011	0.033	0.010	*0.000*	*0.000*	0.009	0.011	0.015	0.024	*0.006*	0.014	0.012	**0.051**	0.010	*0.012*	*0.002*	*0.008*	*0.000*		0.030	0.000
31	*0.000*	*0.009*	*0.000*	*0.000*	*0.000*	0.009	*0.001*	0.011	*0.004*	0.013	*0.000*	0.001	*0.052*	*0.000*	0.012	*0.003*	0.010	0.009	0.014		0.000
32	**0.107**	**0.151**	**0.111**	**0.098**	**0.100**	**0.098**	**0.100**	**0.094**	**0.120**	**0.119**	**0.118**	**0.127**	**0.093**	**0.091**	**0.096**	**0.091**	**0.095**	**0.074**	**0.083**	**0.110**	

The colony identification number relates to its geographical position in Fig. 1. Significant F_{ST} values are represented by a P value of<0.05. Non-significant P values and the corresponding F_{ST} are indicated in italics. High F_{ST} values ≥0.05 are indicated in bold

[a] Faecal samples

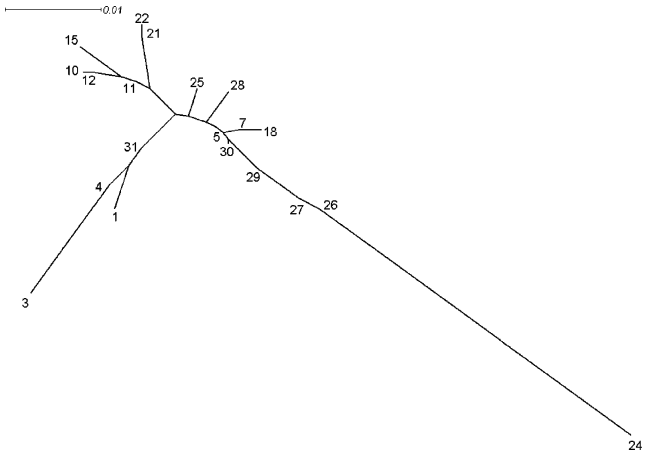

Fig. 2 Pair-wise F_{ST} dendrogram of colonies sampled in western Europe. The *numbers* represent the colony locations as shown in Fig. 1. Colonies only included where nine or more individuals produced amplification products at four or more loci

Baikal and Ireland colonies appear to be two very discrete populations, as the two locations are represented by two discrete clusters. The majority of individuals within Scottish sample sites (1–9) have a high probability of belonging to a single cluster. However, substantially more individuals within southern Scottish locations 4–9 (23%) assign to a different cluster at $P \geq 0.4$ than those at the more northerly locations of 1–3 (8%). An abrupt change in the assignment of individuals occurs between locations 9 (Scottish) and 10 (English). This may indicate some genetic separation by distance within the northern part of the UK.

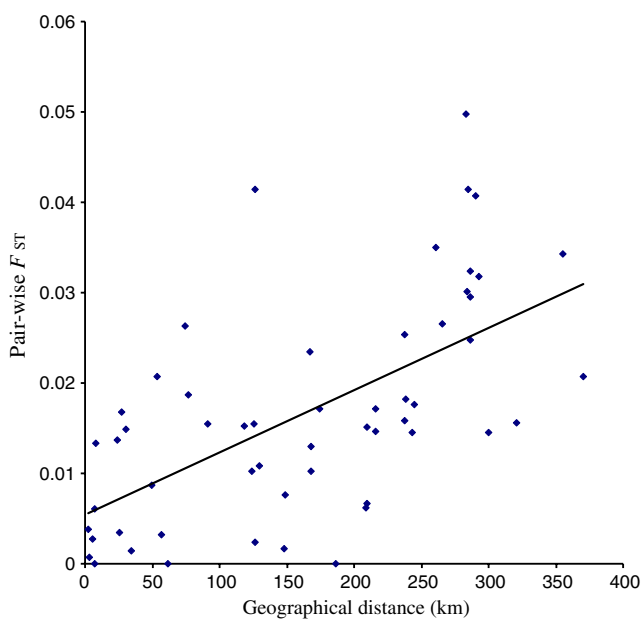

Fig. 3 A scatterplot of pair-wise F_{ST} versus geographic distance, for all colonies sampled ($n \geq 9$) in Scotland and northern England. $R^2=0.46$; Mantel $P=0.0002$

Table 4 STRUCTURE analysis average log-likelihood values and estimated posterior probabilities for all individuals scored at four or more loci ($n=734$) at $K=1$–9 populations

K populations	Log *P* (*X*/*K*)	Log *P*(*K*/*X*)[a]
1	−26,070.5	0.00000
2	−25,647.9	0.00000
3	−25,046.5	0.00000
4	−24,952.1	0.00000
5	−24,819.0	0.98365
6	−24,874.0	0.00000
7	−24,829.0	0.00004
8	−24,823.1	0.01630
9	−24,869.4	0.00000

See Fig. 4 for associated histogram plot of $K=5$

[a] The posterior probabilities calculated are an approximate prediction of the most appropriate model for the data and not a precise estimate of probability

The majority of individuals at the Lancashire (10–17) sample sites have a high probability of assigning to two clusters. There appears to be little substructure detectable using this method between the Lancashire, Yorkshire and Middlesex colonies. The France, Switzerland and Belgian colonies appear to have a similar clustering profile, which is different from the clustering pattern in the UK. Colony 18 in Cumbria, the most northwesterly sample site in England, appeared to be the most closely related to the Irish colony of those sampled, with 49% of individuals assigning to the Irish cluster at $P=\geq 0.4$.

Assignment tests

For individual assignment tests, UK and continental colonies were placed into groups according to the geographic origin of the sample site: Scottish, English or continental. These were plotted as graphs (Figs. 5a, b and 6a, b) of probability of assignment of each individual to two locations. Any points falling on the diagonal line on the graph indicates that the probabilities of assignment to those two locations were the same. Points below the line relate to individuals assigned to their sampled location as indicated on the ordinate axis. A total of 76% of all individuals correctly assigned to the location from which they were sampled. All potential sero-positive individuals ($n=13$; filled circles, Fig. 5a, b) sampled in England had a greater probability of assignment to the English rather than the Scottish group. Eleven possible sero-positive individuals showed a higher assignment to the English rather than continental group (Fig. 5b). The two remaining potential sero-positives had an equal probability of assignment to both England and the continent. One of the three virus-positive individuals (a juvenile found in Lancashire in

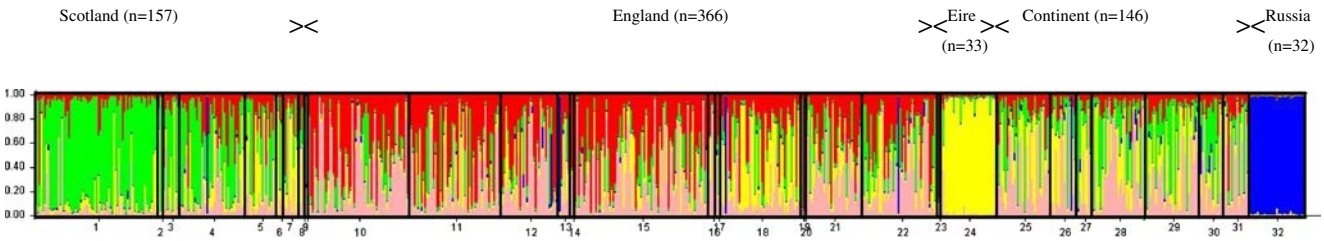

Fig. 4 STRUCTURE plot of assignment of all samples (n=734), with no prior population information, at K=5. Each individual is represented by a *vertical line* on the x-axis split into K *shaded segments*, which represent the estimated membership coefficient for each individual within each cluster. The *numbers* represent the colony locations as shown in Fig. 1

2002; Johnson et al. 2003; filled squares, Fig. 5a, b) had a greater probability of assignment to the Scottish or continental rather than the English group. All potential sero-positive individuals (n=16; filled circles, Fig. 6a, b) sampled in Scotland had a greater probability of assignment to the Scottish rather than English group. However, four possible sero-positive individuals had a greater probability of assignment to the continental rather than Scottish or English group. The blood samples from these individuals were tested within pools of blood from several bats due to limited blood volume, and therefore, it is impossible to determine which individual was actually sero-positive. At least some of the other individuals within these pooled samples assigned more closely to the Scottish rather than continental group, and therefore, no specific assignments can be made regarding the likely origins of these potential sero-positive bats.

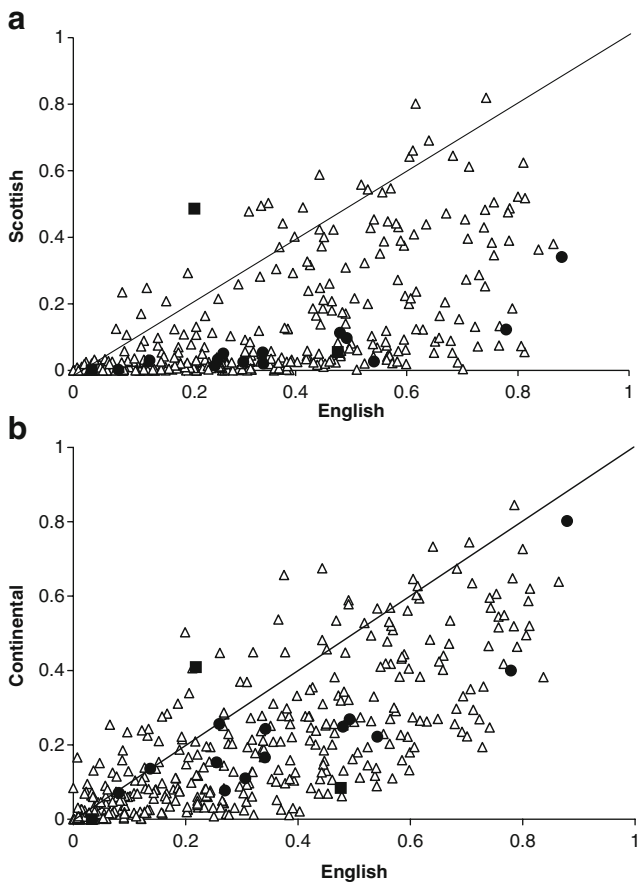

Fig. 5 Assignment of individual genotypes of Daubenton's bats, sampled in England, using GENECLASS software (Piry et al. 2004). **a** England and Scotland populations. **b** England and continental populations. The units of both axes are probability. The three EBLV-2 virus-positive bats are shown as *filled squares*, the 13 potential EBLV-2 sero-positive bats are shown as *filled circles* and the remaining 350 individuals are shown as *open triangles*

Discussion

Genetic diversity

Genetic diversity within colonies was high and similar to that reported in other studies on European myotid bat species, for example, Daubenton's bat (Ngamprasertwong et al. 2008), Natterer's bat (*Myotis nattereri*; Rivers et al. 2005) and greater mouse-eared bat (*Myotis myotis*; Ruedi and Castella 2003). Exceptions to this were the Lake Baikal colonies with a low observed and expected heterozygosity and significant positive F_{IS}. Scottish colony 3 also had a low H_O and H_E, as well as a significant positive F_{IS}, which suggests a degree of inbreeding at this roost. Pair-wise F_{ST} values indicated that this colony was distinct within the Scottish group (as visible in Fig. 2). Within the Scottish sites sampled, colony 3 was the only one located within the mountainous regions of central Scotland. All other colonies were in lowland areas of Scotland, with no obvious geographical barriers to gene flow. Colony 3 was situated in a narrow river valley surrounded by a large expanse of treeless uplands, a landscape which could be considered geographically restricted for the Daubenton's bat. Considering both the geographic and genetic data, it is therefore likely that this colony is at least partially isolated from the other Scottish colonies sampled, and it may therefore be evidence of the presence of discrete populations of Daubenton's bats existing within the mountainous regions

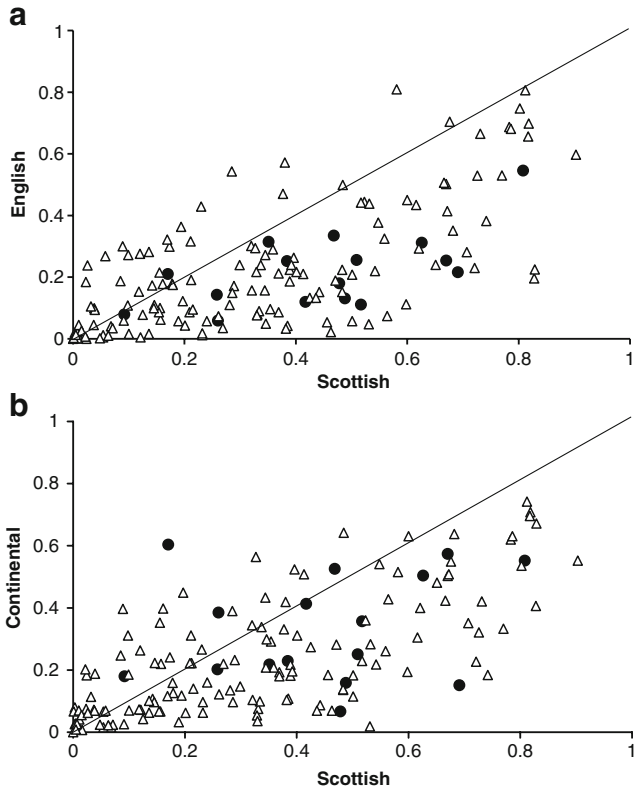

Fig. 6 Assignment of individual genotypes of Daubenton's bats, sampled in Scotland, using GENECLASS software (Piry et al. 2004). **a** Scotland and England populations. **b** Scotland and continental populations. The units of both axes are probability. The 16 potential EBLV-2 sero-positive bats are shown as *filled circles* and the remaining 141 individuals are shown as *open triangles*

of central Scotland. Ngamprasertwong et al. (2008) reported the existence of a macro-geographic structure within the Daubenton's bat population in Scotland, with two mitochondrial DNA clades apparent—one covering the extreme north western region of Scotland and the southern borders with England and the second in central and north eastern Scotland. They suggested that the latter clade is confined by the mountainous regions directly to its north and south. Little indication of local structure (<25 km) was evident from microsatellite analysis (possibly due to the mating of many local colonies at one or two swarming sites). However, few colonies appear to have been sampled in the mountainous region, pertaining to colony 3, in our study.

F Statistics

This is the first study to report the wider population genetic structure of the Daubenton's bat in the UK and western continental Europe. As a consequence, there are few direct comparisons to draw on from other published articles. Low pair-wise F_{ST} values and a low proportion of variation between roosts throughout the study range are indicative of substantial inter-colony gene flow, as expected with a highly mobile mammalian species, such as the Daubenton's bat, which has a common and widespread distribution within the sampled area. However, although the overall value of F_{ST} was low, it was still significant, therefore suggesting the presence of some overall population structure. The range of pair-wise F_{ST} values (0.000–0.050) obtained within Europe (excluding Ireland) is largely consistent with those obtained for other species of vespertilionid bats covering similar geographical areas, for example, *Nyctalus noctula* (Petit and Mayer 1999) *M. myotis* (Castella et al. 2001), *M. nattereri* (Rivers et al. 2005) and *M. daubentonii* (Ngamprasertwong et al. 2008; also reviewed by Burland and Worthington Wilmer 2001).

Genetic isolation by distance

Sufficient density of sampling was achieved in Scotland and northwestern England to reveal any marked genetic subdivision between localities. Evidence of genetic isolation by distance, from central Scotland through to northern England, is apparent in the STRUCTURE analysis at $K=5$, Mantel tests and by the sub-groups obtained with pair-wise F_{ST} values. However, with only one colony sampled in southern England, there is insufficient data to provide evidence for a continuation of this trend. This pattern of genetic isolation by distance within Daubenton's bats in northern Britain is also supported by Ngamprasertwong et al. (2008), at a macro-geographic scale in Scotland.

Low inter-colony F_{ST} values in the Lancashire and Cumbria area indicate a high degree of mixing. These findings are partially supported by the results of Ngamprasertwong et al. (2008) and Senior et al. (2005), which showed high levels of gene flow in Daubenton's colonies within Scotland and the Yorkshire Dales, respectively. However, these colonies appear to belong to a panmictic population, which does not appear evident at a much larger scale, between northern England and Scotland, according to our results. It is possible that the swarming of Daubenton's bats from several local maternity colonies (up to 32 km in the Yorkshire Dales; Glover and Altringham 2008) promotes genetic diversity and low differentiation observed between geographically close colonies, as suggested for the Natterer's bat (*M. nattereri*; Rivers et al. 2005). However, the genetic isolation by distance observed could be the result of communities of bats remaining faithful to a particular cluster of swarming sites, but having substantially less interaction with swarming sites from a more geographically distant area. Nevertheless, this effect is likely to have little impact on EBLV-2 transmission, due to the lack of any significant barriers to gene flow and low genetic differentiation throughout the study range.

Continental versus British Isles population structure

The sample sizes obtained from the seven continental colonies provided sufficient data for a comparison of the wider population structure. Here, a lack of any apparent sub-division between Daubenton's roosts in France, Belgium and Switzerland, as indicated by STRUCTURE analysis, is evident. These findings are supported by very low non-significant pair-wise F_{ST} values obtained between these continental colonies and a non-significant Mantel test. This provides a strong indicator of panmixia within the continental Daubenton's populations studied, with little genetic, behavioural or geographical barriers to gene flow. Although panmixia is present within the sampled area of continental Europe, this does not appear to extend to the area sampled in the UK, as although there are high levels of gene flow and little genetic subdivision especially at a local level within the UK colonies sampled, there appears to be some genetic isolation by distance between Scotland and northwestern England. The geographical distances between colonies sampled on the continent and those in the UK are comparatively similar, and therefore, it is likely that dispersal for mating and/or migration tends to be within a smaller geographical area for northern UK than in continental Europe. Low pair-wise F_{ST}s, high intra-colony diversity and no abrupt changes in allele frequency between regions provide evidence to indicate that few geographical barriers to gene flow exist within the UK population. Therefore, the isolation by distance observed is likely to be the result of a balance between gene flow and allelic drift since the last ice age (Beebee and Rowe 2004). The longer distances recorded for Daubenton's bat migrations in continental Europe (Hutterer et al. 2005) than those reported in the UK (Glover and Altringham 2008; Parsons and Jones 2003) provide further evidence to support our findings. A possible suggestion as to why these two contrasting patterns of gene flow appear to exist within the areas sampled could be that there is a greater cost associated with longer dispersal distances at high latitudes in northwestern England and Scotland than the warmer drier climates of regions sampled in continental Europe. In order to further elucidate these findings, future work should include microsatellite and mtDNA analysis of Daubenton's populations in Denmark, southern Norway and southern Sweden, which are on a similar latitude to the Daubenton's populations in the north of the UK, as well as further sites in southeastern England.

The low genetic differentiation observed between the UK and continental Europe samples also indicates that the English Channel provides little, if any, geographic barrier to gene flow and therefore transmission of EBLV-2. However, it is therefore interesting to note that EBLV-2 cases in Europe are clearly clustered (in Denmark, The Netherlands and Switzerland), with relatively few in Germany and none in Belgium, Poland or France (Smith et al. 2006).

Unfortunately, as only one colony was sampled in southern Ireland, few conclusions can be drawn from this data set. However, high pair-wise F_{ST} values and a discrete Irish cluster within the STRUCTURE analysis indicate a significant degree of genetic isolation between the Irish colony and any other western European colony sampled. High levels of within colony heterozygosity and non-significant F_{IS} indicate a lack of inbreeding within the Irish colony. It is likely that the Irish population is derived from a location genetically distinct from those UK and continental European colonies included in this study and that insufficient gene flow has occurred to eradicate this distinction since this colonisation occurred. It also appears, according to our data, that the Irish Sea presents a substantially greater barrier to gene flow than the English Channel. This is despite the fact that distances between the Mull of Kintyre and Northern Ireland of 20 km (labelled A on Fig. 1) and Dover to Calais of 33 km (labelled B on Fig. 1) are remarkably similar. Clearly, factors influencing the wider genetic structure amongst bat species will be related to physical and behavioural characteristics, as well as migration rates and the population size in any given region. Furthermore, within species, behavioural characteristics related to mating may vary according to wider environmental factors such topography.

Both pair-wise F_{ST} values and STRUCTURE analysis indicate that the Irish samples appear more closely related to colonies sampled in Cumbria, southern Scotland and the continent rather than further south in Lancashire. This may be evidence consistent with the possibility of a post-glacial colonisation, via a fleeting land bridge between Scotland and Ireland (Devoy 1985). An alternative suggestion is that species survived within the unglaciated region of southern Ireland during the last ice age (Yalden 1982).

The clear genetic distinction between the Irish and UK/continental colonies may be sufficient to have important consequences for past and future EBLV-2 transmission. It is therefore important to our understanding of Daubenton's ecology and disease transmission to conduct further work in northern as well as southern Ireland and Wales.

Lake Baikal samples

Daubenton's sampled from Lake Baikal were clearly separated from the western European populations, as indicated by high pair-wise F_{ST} values, a discrete cluster within STRUCTURE analysis. Results of cytochrome b sequencing (unpublished data) are in agreement with those obtained for Daubenton's sampled in Novosibisk, Russia and Hokkaido, Japan (Kawai et al. 2003) and bear little genetic similarity to Daubenton's of European origin.

Moreover, it has recently been suggested that the eastern transpalearctic range of the Daubenton's bat should be a distinct species, *Myotis petax*, considering both the morphological and genetic differences (Matveev et al. 2005). This cytochrome b analysis of the Lake Baikal samples, indicating sub-specific or specific differences, supports the removal of these individuals from some of the analyses.

Assignment tests

Results of the assignment tests indicate that there is no evidence that potential sero-positive and virus-positive individuals sampled are more likely to have arisen from the continental rather than the UK populations. There is insufficient significant genetic differentiation amongst most UK and continental colonies to imply that EBLV-2 is maintained in the UK by immigration. Results show that EBLV-2 is likely to be maintained at a low endemic level within the UK. The relative genetic uniformity of UK and continental populations implies that there is no migration barrier to EBLV-2, between these regions. It is also possible that the isolation of some colonies, in particular, the region sampled in southern Ireland and possibly others, may be sufficient to prevent EBLV-2 from spreading to these localities.

As highlighted by Ngamprasertwong et al. (2008), further analysis of both the nuclear and mitochondrial population genetic structure of the Daubenton's bat in Europe would improve our knowledge of the broad-scale dispersal patterns of this species in relation to EBLV-2 transmission. As female Daubenton's bats display a significantly higher degree of natal philopatry compared to males (Senior et al. 2005), analysis of mtDNA haplotypes may reveal another extent of population structure not evident through microsatellite analysis, as shown in Ngamprasertwong et al. (2008). However, as males are the predominant sex responsible for gene flow in the Daubenton's bat, further analysis of nuclear and possibly Y-chromosome population genetics are more likely to reveal the fundamental genetic discontinuities between geographic areas, which may therefore have consequences for EBLV-2 transmission.

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