

# Rangewide phylogeography in the greater horseshoe bat inferred from microsatellites: implications for population history, taxonomy and conservation

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## Abstract

The distribution of genetic variability across a species' range can provide valuable insights into colonization history. To assess the relative importance of European and Asian refugia in shaping current levels of genetic variation in the greater horseshoe bats, we applied a microsatellite-based approach to data collected from 56 localities ranging from the UK to Japan. A decline in allelic richness from west Asia to the UK and analyses of  $F_{ST}$  both imply a northwestward colonization across Europe. However, sharp discontinuities in gene frequencies within Europe and between the Balkans and west Asia (Syria/Russia) are consistent with suture zones following expansion from multiple refugia, and a lack of recent gene flow from Asia Minor. Together, these results suggest European populations originated from west Asia in the ancient past, and experienced a more recent range expansion since the Last Glacial Maximum. Current populations in central Europe appear to originate from the Balkans and those from west Europe from either Iberia and/or Italy. Comparisons of  $R_{ST}$  and  $F_{ST}$  suggest that stepwise mutation has contributed to differentiation between island and continental populations (France/UK and China/Japan) and also among distant samples. However, pairwise  $R_{ST}$  values between distant populations appear to be unreliable, probably due to size homoplasy. Our findings also highlight two priorities for conservation. First, stronger genetic subdivision within the UK than across 4000 km of continental Eurasia is most likely the result of population fragmentation and highlights the need to maintain gene flow in this species. Second, deep splits within China and between Europe and China are indicative of cryptic taxonomic divisions which need further investigation.

**Keywords:** genetic subdivision, genetic variation, glacial refugia, greater horseshoe bat, microsatellites, *Rhinolophus*

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## Introduction

The pervasive impact of past glaciations on current distributions of genetic and species diversity is well established (Taberlet *et al.* 1998; Hewitt 1999; 2000; 2004). In temperate latitudes, glacial cycles resulted in range contractions of many taxa, and subsequent recolonization and population

expansion are thought to have led to the loss of genetic diversity, as alleles were lost by repeated serial dilution of the gene pool (founder effects) (Hewitt 2000; Ibrahim *et al.* 1996). This process is thought to have been compounded by occasional long distance dispersal events, in which pioneer individuals founded new populations beyond the main population's leading edge, so hampering gene flow via density-dependent effects (Ibrahim *et al.* 1996).

During the Last Glacial Maximum (LGM, 23 000–18 000 years ago), the presence of ice and tundra over northern

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Europe forced temperate species into lower latitudes (Hewitt 1996). Comparative studies have been informative in illuminating glacial refugia of European biota and associated patterns of postglacial colonization (Taberlet *et al.* 1998; Hewitt 1999). Phylogeographical analyses indicate that most temperate European taxa have their origins in three refugial regions: Italy, Iberia and the Balkans (Taberlet *et al.* 1998; Hewitt 1999). Although the relative importance of these refugia differs among species (Taberlet *et al.* 1998), Hewitt (1999) recognized three broad routes of postglacial expansion, named after the grasshopper, hedgehog and bear. The rapid northward population expansion of many temperate taxa has left a latitudinal gradient in genetic diversity, characterized by southern richness and northern purity (Hewitt 1996).

A frequent limitation of studies that have identified glacial refugia of European biota has been one of restricted sampling. Many European species range beyond Europe to the Middle East, Africa, the Caucasus region and central Asia (Corbet 1978), yet their sampling is often confined to European states, inevitably leading to the identification of refugia within Europe. For such taxa, it is possible to envisage that areas such as the Balkans might in fact have served as a postglacial entry point into Europe, rather than a distinct glacial refugium. Under this scenario, latitudinal and longitudinal gradients in genetic diversity within Europe might be expected to continue into Asia. Indeed the relatively few studies of European biota that have considered population genetic structure and variability beyond Europe have revealed the importance of the Middle East and the Caucasus as centres of considerable genetic variation (Cooper *et al.* 1995; Hewitt 1999; Hampe *et al.* 2003; Dubey *et al.* 2006).

The greater horseshoe bat (*Rhinolophus ferrumequinum*) is distributed across the Palaearctic region from the UK to Japan (Csorba *et al.* 2003), broadly mirroring the wider distribution of many European taxa (Corbet 1978). During the last century, numbers declined sharply over much of northern Europe where the species is now considered rare or extinct (Ransome & Hutson 2000). In southern England and Wales, the crash is known to have resulted in the once widespread population becoming highly fragmented (Mitchell-Jones 1995). The current species range is thought to be continuous eastwards to Nepal, but becomes more disjunct in China with several known population centres (Csorba *et al.* 2003). The greater horseshoe bat has been the subject of several taxonomic revisions (Felten *et al.* 1977; Krystufek 1993). In the most recent and comprehensive of these, Thomas (1997) recognized seven subspecies based on morphological characters (*R. f. ferrumequinum* in Europe and northwest Africa, *R. f. creticum* in Crete, *R. f. irani* in Iran, Iraq and Turkmenistan, *R. f. proximus* from Afghanistan and Pakistan to India, *R. f. tragatus* in north India and China, *R. f. korai* in Korea and *R. f. nippon* in Japan)

although it is not known whether these subspecies are genetically distinct from each other. The greater horseshoe bat thus provides an opportunity to assess the relative contribution of European and Asian populations to the contemporary European gene pool, as well as to identify patterns of genetic structure that might be shared by other codistributed taxa.

The vast majority of attempts to characterize phylogeographical trends have relied on mitochondrial DNA and chloroplast DNA haplotype data (Avice 2000) rather than biparentally inherited markers such as microsatellites (Goldstein & Pollock 1997). Potential drawbacks of microsatellites for phylogeographical inference include their high mutation rate and stepwise mode of mutation, which together can lead to size homoplasy (Goldstein *et al.* 1997). Although size homoplasies appear to be widespread in microsatellites (e.g. van Oppen *et al.* 2000; Curtu *et al.* 2004), Estoup *et al.* (2002) concluded that in most cases, high allelic diversity and the use of multiple independent loci will compensate for homoplasious evolution. Microsatellite-derived data also have the advantage that they are less influenced by sex-biased dispersal than haplotype data, and by incorporating recombination, they also avoid the potential problem of discordance between gene and species histories (Nichols 2001). Several recent studies have recognized the potential application of microsatellites in phylogeography (Koskinen *et al.* 2002; Heuertz *et al.* 2004). In the greater horseshoe bat, limited dispersal and a generation time of around 3 years means that, at medium to large geographical scales, populations will have had insufficient time since the LGM to reach drift-flow equilibrium and thus patterns are expected to reflect past events (Barton 1992). Here we present a detailed microsatellite analysis of greater horseshoe bats sampled at three geographical scales: (i) within the UK, (ii) across mainland Europe and the Middle East, and (iii) the Palaearctic range. We hypothesize that, given the current range of this species and its apparent sensitivity to cold climate (Ransome & Hutson 2000), greater horseshoe bats survived the LGM in refugia in both Mediterranean Europe and west Asia. As a result, genetic diversity is predicted to decrease northwards across Europe, with most variable regions remaining in the Middle East.

## Methods

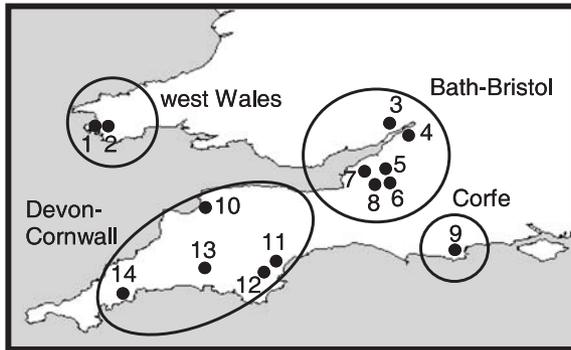
### *Tissue collection and DNA isolation*

Tissue samples were collected or obtained from 56 localities from across the species range (Table 1 and Fig. 1a, b). All tissue samples comprised either 3-mm wing membrane biopsies obtained using a biopsy punch (Stiefel Laboratories) or liver or muscle from museum specimens. All tissue was stored at  $-20^{\circ}\text{C}$  in either 90% ethanol or 20%

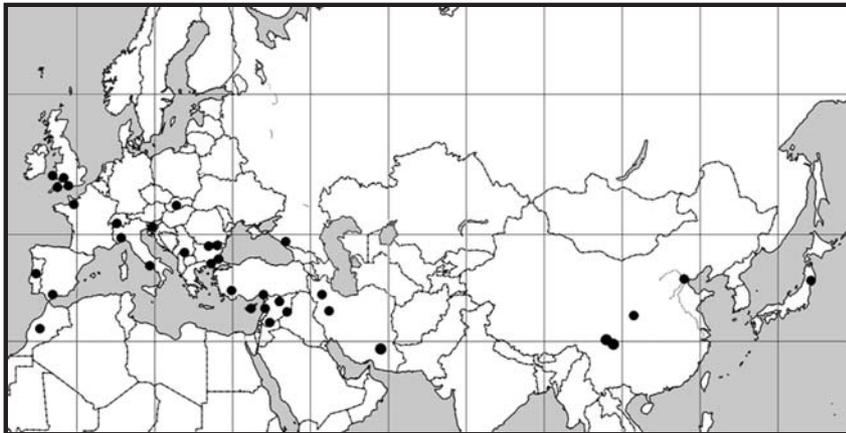
**Table 1** All samples analysed. Collectors/suppliers of samples are denoted by their initials as follows: AP, Alenka Petrinjak; CD, Christian Dietz; DR, Danilo Russo; DS, Dino Scaravelli; EK, Eugenia Kozhurina; FL, François Leboulanger; GJ, Gareth Jones; GL, Gang Li; JF, Jon Flanders; JJ, Javier Juste; LE, Lazaro Echenique-Diaz; MZ, Maja Zagmajster; PB, Petr Benda; RA, Raphaël Arletta; SR, Stephen Rossiter; SZ, Shuyi Zhang; RDR, Roger Ransome

Country	Locality/region	East	North	<i>n</i>	Collector/ supplier
Wales (SW)	Stackpole	4°54'46"W	51°37'52"N	10	GJ
Wales (SW)	Slebech	4°51'57"W	51°48'11"N	14	GJ
England (SW)	Dean Hall	2°28'47"W	51°49'09"N	18	GJ/RDR
England (SW)	Woodchester	2°16'39"W	51°42'39"N	29	GJ/SR/RDR
England (SW)	Brockley	2°46'13"W	51°23'42"N	19	GJ
England (SW)	Mells	2°23'43"W	51°14'38"N	21	GJ/RDR
England (SW)	Coomb Down	2°21'21"W	51°21'41"N	13	GJ/RDR
England (SW)	Iford	2°17'43"W	51°19'33"N	22	GJ
England (SW)	Corfe Castle, Dorset	2°03'03"W	50°37'59"N	15	JF
England (SW)	Golden Mill	4°54'50"W	50°16'55"N	11	GJ
England (SW)	Gunnislake	4°12'33"W	50°31'20"N	22	GJ
England (SW)	Buckland	4°09'57"W	51°07'00"N	22	GJ
England (SW)	Buckfastleigh	3°47'04"W	50°29'05"N	12	GJ
England (SW)	Chudleigh	3°36'19"W	50°36'15"N	21	GJ
France (N)	Normandy	3°00'30"W	48°10'56"N	19	FL
Portugal	Exact locality unknown	8°05'05"W	39°23'10"N	4	CD
Spain (SW)	Jerez de la Frontera, Cadiz	6°08'14"W	36°41'12"N	15	JJ
Morocco	Talkout, Oued Tessaout Valley	7°17'0"W	31°41'00"N	1	PB
Italy (NW)	Giovo	8°28'15"E	44°25'49"N	20	DS
Italy (SW)	Campania	14°41'39"E	40°57'13"N	5	DR
Sardinia	Grotta di Monte Majore	8°61'67"E	40°05'00"N	3	CD
Switzerland	Vex	7°23'56"E	46°12'42"N	20	RA
Slovenia (SE)	Kostanjevska Jama	15°43'84"E	45°83'86"N	13	MZ/AP
Kosovo	Mrasor	20°34'0"E	42°31'00"N	2	PB
Slovakia	Silica (Rožňava)	20°32'0"E	48°33'00"N	2	PB
Slovakia	Hodruša-Hámre (Žarnovica)	18°49'0"E	48°28'00"N	1	PB
Cyprus	Cinarli	33°46'00"E	35°19'00"N	2	PB
Cyprus	Kalavassos	33°16'00"E	34°48'00"N	3	PB
Bulgaria (SE)	Primorsko	27°45'01"E	42°17'15"N	10	CD
Greece (NE)	Koufovouno	26°27'13"E	41°21'10"N	10	CD
Bulgaria (N)	Samara Pestera	25°29'58"E	41°24'31"N	10	CD
Bulgaria (N)	Urushka Maara	25°01'46"E	43°14'44"N	9	CD
Bulgaria (N)	Nanin Kamak	24°51'22"E	43°37'37"N	11	CD
Bulgaria (N)	Mikrenskata Mandrata	24°31'29"E	43°03'45"N	2	CD
Turkey (NW)	Dupnisa Magarasi	27°33'22"E	41°50'27"N	1	CD
Turkey (SW)	Gündogan	27°20'48"E	37°06'46"N	2	CD
Turkey (SE)	Çevlik (Hatay)	35°56'00"E	36°08'00"N	9	PB
Russia (W)	Exact locality unknown	38°05'00"E	44°48'00"N	13	EK
Syria (E)	Dura Europos (Deir ez-Zur)	40°43'0"E	34°45'00"N	7	PB
Syria (N)	Qala'at Najm (Halab)	38°16'0"E	36°33'00"N	5	PB
Syria (N)	Qala'at Samaan (Halab)	36°52'0"E	36°18'25"N	4	PB
Syria (N)	Qatura (Halab)	36°50'0"E	36°19'00"N	2	PB
Syria (N)	Qala'at Sheisar (Hama)	36°34'0"E	35°17'00"N	7	PB
Syria (N)	Qala'at Salahadin (Lattaqia)	36°3'0"E	35°36'00"N	2	PB
Syria (S)	Bosra (Der'a)	36°29'0"E	32°32'00"N	5	PB
Israel/Syria (S)	Nimrod Fortress (Golan H)	35°43'0"E	33°15'00"N	1	PB
Iran (SE)	Deh Bakri (Kerman)	57°56'0"E	29°05'00"N	1	PB
Iran (SW)	Bisotun (Kermanshah)	47°26'00"E	34°24'00"N	1	PB
Iran (NW)	Choplu (W Azerbaijan)	47°02'00"E	36°28'00"N	1	PB
China (SW)	Nanton Village (Sichuan)	102°13'52"E	29°48'33"N	1	GJ/SR/SZ/GL
China (SW)	Emei Shan (Sichuan)	103°16'92"E	29°34'74"N	1	GJ/SR/SZ/GL
China (C)	Foping (Shaanxi)	107°59'00"E	33°34'00"N	2	PB
China (C)	Daguping (Shaanxi)	107°46'00"E	33°34'00"N	1	PB
China (NE)	Beijing (NW)	116°19'55"E	39°54'25"N	9	GJ/SZ
China (NE)	Beijing (NW)	116°19'55"E	39°54'25"N	12	GJ/SZ
Japan	Sendai, Honshu Island	140°53'33"E	38°15'03"N	18	LE

(a)



(b)



**Fig. 1** Map of sampling localities in (a) the UK showing the four main population centres, and (b) across the entire range. Numbers in Fig. 1 (a) correspond to the following nursery colonies: 1, Stackpole; 2, Slebech; 3, Little Dean; 4, Woodchester; 5, Coomb Down; 6, Iford; 7, Brockley; 8, Mells; 9, Corfe; 10, Buckland; 11, Chudleigh; 12, Buckfastleigh; 13, Gunnislake; 14, Golden Mill.

dimethyl-sulphoxide/saturated NaCl (~6 M). Genomic DNA was isolated from tissue by either a salt–chloroform extraction, or by using QIAGEN Dneasy Kits (QIAGEN) or Promega Wizard Purification Kits (Promega).

#### Microsatellite genotyping

Bats were genotyped at 17 polymorphic microsatellite loci (GenBank Accession nos AF160200, AF160202, AF160205, AF160207, AF160210, AF160211, AJ560694–AJ560698, AJ560702–AJ560704, AJ560708, AJ560710–AJ560713; (Rossiter *et al.* 1999; Dawson *et al.* 2004)). Bats from nine sites previously analysed at six loci (Rossiter *et al.* 2000) were genotyped at 10–11 additional loci for the purposes of this study. To ensure that scoring was consistent between studies, we reran individuals of known genotype as internal markers. All primers were labelled with 5'-Fluorescein bases (HEX, FAM or TAMRA) and 15  $\mu$ L PCRs contained AmpliTaq Gold Taq polymerase (PE Applied Biosystems) and final primer concentrations of 0.667  $\mu$ M. Reactions were performed on a DNA Engine Tetrad thermal cycler (MJ Research) with the following profile: 94 °C 2 min; 34 cycles of 94 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s; 72 °C for 3 min. PCR products were

visualized on an ABI 3700 Sequencer (PE Applied Biosystems) and analysed using GENOTYPER 3.6 NT software (PE Applied Biosystems).

#### Genetic diversity and differentiation

Genetic diversity was assessed for each sample comprising five or more individuals, by calculated expected heterozygosity ( $H_E$ ), observed heterozygosity ( $H_O$ ) and average allelic richness ( $R_s$ ).  $R_s$  is a rarefaction-derived estimate of allelic diversity that is adjusted for sample size ( $N$ ), thus allowing meaningful comparisons among samples that would otherwise be heavily biased by  $N$ . To test for deviation from Hardy–Weinberg equilibrium (HWE) both within each population and locus, we calculated  $F_{IS}$  using the software FSTAT version 2.9.3 (Goudet 1995). Significance of  $F_{IS}$  values was assessed by comparing the observed values against distributions obtained by randomization (1000 times), and adjusting for multiple tests using Bonferroni correction.

Genetic differentiation among samples was quantified using  $F_{ST}$  values calculated in GENETIX 4.02 (Belkhir *et al.* 2004) and  $R_{ST}$  values (Michalakis & Excoffier 1996) calculated in SPAGED1 (Hardy & Vekemans 2002). The analogue

$R_{ST}$  accounts for the stepwise mode of mutation that characterizes microsatellite loci and thus a comparison of corresponding  $F_{ST}$  and  $R_{ST}$  values can shed light on the relative importance of drift and mutation underpinning genetic differentiation. These estimators are expected to be similar when drift is most important, while  $R_{ST}$  should increase relative to  $F_{ST}$  as the contribution of stepwise mutation to differentiation increases. We also compared observed  $R_{ST}$  values to expected values ( ${}_pR_{ST}$ ) based on 1000 permutations of allele size, following Hardy *et al.* (2003) with SPAGED1. The  ${}_pR_{ST}$  estimator thus represents a recalculated  $R_{ST}$  in which allele size is not important. Consequently, where  $R_{ST}$  is significantly larger than  ${}_pR_{ST}$ , we can infer that stepwise mutation is contributing to differentiation (Hardy & Vekemans 2002), whereas a nonsignificant difference suggests  $F_{ST}$  is a more suitable estimator.

#### *Isolation by distance and neighbour-joining trees*

Isolation by distance was assessed by plotting pairwise  $F_{ST}$  values against corresponding natural logarithm-transformed geographical distances (Rousset 1997). We tested for significance of the coefficients of determination ( $R^2$ ) by permuting locations (10 000 times) and estimated the regression slope ( $b$ ) and associated standard error by jackknifing (Sokal & Rohlf 1995), implemented in SPAGED1. To depict the overall relationship among all localities, we applied derived pairwise values of  $F_{ST}$  and  $R_{ST}$  to construct neighbour-joining trees with the program NEIGHBOUR in PHYLIP (Felsenstein 2004).

#### *Cluster and correspondence analyses*

We also conducted two additional approaches to describe the pattern of genetic structure among samples within the UK, across Europe and the Middle East and among all samples. Both methods are based on individuals and therefore samples from all localities were used irrespective of sample size. First, factorial correspondence analyses (FCA) were undertaken at multiple scales, using the software GENETIX 4.02. An FCA allows the synthetic graphical display of the genetic similarity among samples in a multidimensional graphical space. Axes are derived that are independent of each other and ranked according to how much variance they explained. Unlike the dendrogram, the FCA is not limited to a two-dimensional branching pattern of a single tree, which can sometimes oversimplify inter-population relationships, especially where these result from recurrent gene flow as well as splitting (Lowe *et al.* 2004).

Second, we used the Bayesian clustering approach (Pritchard *et al.* 2000; Falush *et al.* 2003), implemented in the software STRUCTURE 2.0, to estimate the number of clusters present in the data without reference to assumed

population membership. This method, which assumes Hardy–Weinberg equilibrium and linkage equilibrium within populations, estimates the posterior probability for different values of  $K$  (the number of populations). As such, it can be useful for identifying discrepancies between geographically and genetically defined population structure, the source of which can include historical factors. We used a burn-in length of 30 000 and a run length of  $10^6$  without prior population information. Ten independent runs were undertaken for each value of  $K$ , which was set at values suspected to bracket the true number of populations in the data. The number of clusters present was considered to be the value of  $K$  that maximized the posterior probability of the data, expressed as  $P(K|X)$ . In addition, where  $L(K)$  increased monotonically, we derived  $\Delta K$ , defined as the mean of the absolute value of the second order rate of change of  $L(K)$  with respect to  $K$  divided by the standard deviation of  $L(K)$ . This value has been shown to detect successfully the uppermost hierarchical level of structure present in the data (Evanno *et al.* 2005). Finally, we displayed structure graphically based on the maximum value of  $P(K|X)$  for several regional scales using the software DISTRUCT (Rosenberg 2004).

## Results

To examine the range-wide distribution of genetic variation and population structure in *Rhinolophus ferrumequinum*, we genotyped 516 bats at 16–17 polymorphic microsatellite loci sampled from 56 localities across Europe and Asia (Table 1). Bats from Japan and China did not amplify at marker AJ560708. Observed heterozygosity values and  $F_{IS}$  were derived for all samples comprising five or more individuals and showed no consistent deviation from Hardy–Weinberg equilibrium (Table 2). Out of 612 marker-locality estimates just three (0.5%) were significant by permutation following Bonferroni correction.

#### *Genetic structure*

*Genetic differentiation and isolation by distance.* Calculation of  $F_{ST}$  was undertaken for all samples of five or more bats. Within the UK, significant genetic subdivision was detected among sampling localities (global  $F_{ST} = 0.051$ ,  $P < 0.001$ ). Colonies broadly fell into four geographical areas [Bath-Bristol area, west Wales, Devon-Cornwall area, and Corfe (Dorset) area], which also correspond to the main population centres (see Fig. 1a). Pairwise  $F_{ST}$  values among colonies ranged from  $-0.001$  (not significant) for Buckfastleigh/Chudleigh to 0.153 ( $P < 0.001$ ) for Slebech/Woodchester. Out of 91 pairwise values, just 8 showed no significant differentiation, all of these corresponding to neighbouring colonies. On the other hand, many neighbouring sites did show marked subdivision, and significant  $F_{ST}$  values

Country	Locality/region	$H_E$	$H_O$	$F_{IS}$	$A$	$R_s$
Wales (SW)	Stackpole	0.47	0.40	0.16	3.06	n/a
Wales (SW)	Slebech	0.48	0.41	0.15	3.44	2.86
England (SW)	Dean Hall	0.55	0.56	-0.01	4.06	2.99
England (SW)	Woodchester	0.54	0.53	0.01	4.41	2.83
England (SW)	Brockley	0.56	0.58	-0.03	4.35	3.07
England (SW)	Mells	0.60	0.58	0.03	4.53	3.27
England (SW)	Coomb Down	0.63	0.63	0.00	4.35	3.37
England (SW)	Iford	0.57	0.57	0.00	4.65	3.12
England (SW)	Corfe Castle, Dorset	0.63	0.61	0.02	4.76	3.41
England (SW)	Golden Mill	0.62	0.69	-0.11	4.41	3.40
England (SW)	Gunnislake	0.64	0.58	0.10	5.29	3.62
England (SW)	Buckland	0.64	0.63	0.02	5.00	3.45
England (SW)	Buckfastleigh	0.62	0.58	0.07	4.44	3.49
England (SW)	Chudleigh	0.63	0.62	0.01	5.41	3.53
France (N)	Normandy	0.72	0.65	0.10	6.76	4.23
Spain (SW)	Jerez de la Frontera, Cadiz	0.75	0.76	-0.02	7.00	4.55
Italy (NW)	Giovo	0.75	0.75	-0.01	7.18	4.50
Italy (SW)	Campania	0.69	0.74	-0.08	4.06	4.13
Switzerland	Vex	0.70	0.70	0.01	7.06	4.05
Slovenia (SE)	Kostanjevska Jama	0.74	0.70	0.06	6.24	4.39
Bulgaria (SE)	Primorsko	0.78	0.70	0.10	6.53	4.80
Greece (NE)	Koufovouno	0.79	0.70	0.11	7.06	5.12
Bulgaria (N)	Samara Pester	0.75	0.69	0.07	6.53	4.68
Bulgaria (N)	Urushka Maara	0.79	0.78	0.01	6.47	5.04
Bulgaria (N)	Nanin Kamak	0.77	0.73	0.06	6.88	4.82
Turkey (SE)	Çevlik (Hatay)	0.80	0.76	0.06	7.00	5.35
Russia (W)	Exact locality unknown	0.71	0.67	0.06	6.71	4.56
Syria (E)	Dura Europos (Deir ez-Zur)	0.80	0.79	0.01	6.41	5.29
Syria (N)	Qala'at Najm (Halab)	0.82	0.73	0.14	4.12	5.13
Syria (N)	Qala'at Sheisar (Hama)	0.74	0.74	0.00	5.35	5.10
Syria (S)	Bosra (Der'a)	0.76	0.74	0.02	4.88	4.81
China (NE)	Beijing (NW)	0.67	0.62	0.07	4.81	4.51
China (NE)	Beijing (NW)	0.67	0.58	0.14	5.56	4.76
Japan	Sendai, Honshu Island	0.65	0.63	0.04	6.94	5.04

**Table 2** Summary of multilocus genetic variation for all samples comprising five or more individuals.  $H_{E'}$ , expected heterozygosity;  $H_E$ , observed heterozygosity;  $F_{IS}$ , inbreeding coefficient;  $A$ , mean number of alleles per locus;  $R_s$ , allelic richness corrected for sample size

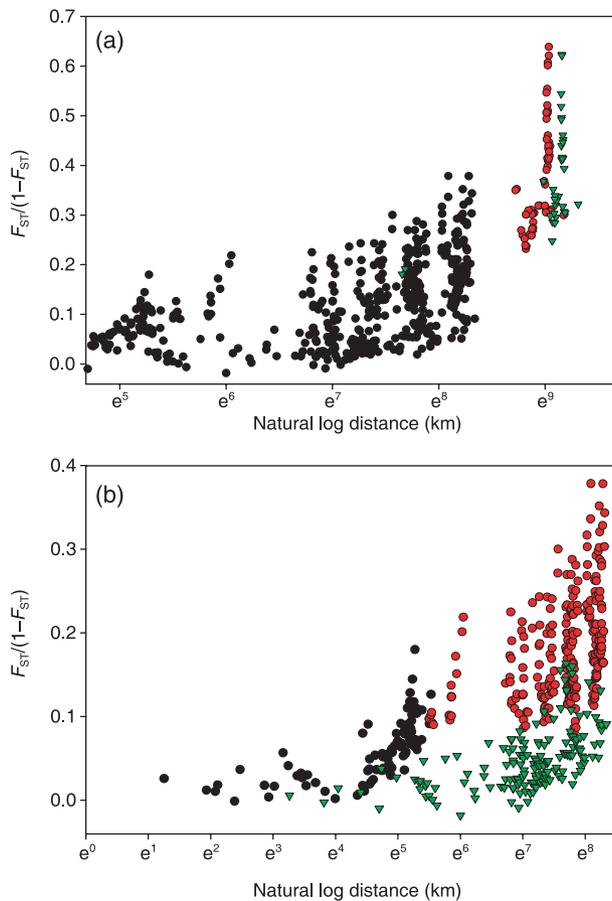
among colonies were recorded in two of the three population centres tested (Bath-Bristol area  $F_{ST} = 0.026$ ,  $P < 0.001$ ; west Wales  $F_{ST} = 0.0179$  ( $P > 0.05$ ); Devon-Cornwall area  $F_{ST} = 0.014$ ,  $P < 0.001$ ).

Genetic differentiation among samples from across mainland Europe and the Middle East was on average lower (global  $F_{ST} = 0.043$ ,  $P < 0.001$ ) than within the UK. Pairwise  $F_{ST}$  values ranged from  $-0.0185$  ( $P > 0.05$ ) between some Syrian colonies to  $0.099$  ( $P < 0.001$ ) between samples from southwest Italy and Syria. In the whole data set, the highest pairwise differentiation was recorded between colonies from the UK and Japan (mean  $F_{ST} = 0.320$ ,  $n = 14$ ,  $P < 0.001$ ) from the opposite geographical limits of the species range. High levels of differentiation were also recorded between Japanese bats and all other samples (mean  $F_{ST} = 0.277$ ,  $n = 30$ ,  $P < 0.001$ ) and between northeast Chinese bats and all other samples (mean  $F_{ST} = 0.266$ ,  $n = 65$ ,  $P < 0.001$ , respectively). Marked genetic division

was also recorded between island populations and continental counterparts (Japan/east China,  $F_{ST} = 0.157$ ,  $P < 0.001$  and UK/north France,  $F_{ST} = 0.112$ ,  $P < 0.001$ ).

Significant isolation by distance was detected across the species range ( $b = 0.057 \pm 0.012$ ,  $R^2 = 0.49$ ,  $P < 0.001$  by permutation) in which a sharp increase in gradient corresponding to the greatest distances can be attributed to comparisons that include either east China or Japan (see Fig. 2a). Figure 2(b) reveals that isolation by distance also occurs in the UK ( $b = 0.023 \pm 0.005$ ,  $R^2 = 0.42$ ,  $P < 0.001$ ) and that this trend is steeper (with slightly overlapping standard errors) than the one observed across continental Europe and west Asia ( $b = 0.016 \pm 0.003$ ,  $R^2 = 0.35$ ,  $P < 0.001$ ).

To assess the relative importance of drift and mutation in the observed genetic subdivision, we repeated the analysis of genetic differentiation using  $R_{ST}$  and derived permuted values ( ${}_pR_{ST}$ ) expected if stepwise mutation was not important. Across the whole data set, pairwise values of estimators



**Fig. 2** Isolation-by-distance plots based on samples comprising at least five individuals for (a) the species range and (b) Europe and west Asia. For (a) black circles, comparisons within Europe and west Asia; red circles, comparisons between northeast China and non-northeast China samples; green triangle, comparisons between Japan and non-Japan samples. For (b) black circles, comparisons within the UK; red circles, comparisons between the UK and non-UK samples; green triangle, comparisons among non-UK samples.

were strongly correlated with  $F_{ST}$  (Pearson's correlation coefficient  $R = 0.88$ ,  $P < 0.001$  by randomization); however, the extent to which the two markers differed varied at different spatial scales. Within the UK,  $R_{ST}$  among sampling localities was significant but was slightly smaller than its  $F_{ST}$  equivalent (global  $R_{ST} = 0.041$ ,  $P < 0.001$ ). Moreover,  $R_{ST}$  did not differ significantly from the expected  $pR_{ST}$  based on random permutations of allele size. Pairwise  $R_{ST}$  values ranged from  $-0.04$  (Stackpole/Chudleigh) to  $0.20$  (Slebech/Corfe) but were only different ( $P < 0.05$ ) from the corresponding pairwise  $pR_{ST}$  values in three out of 91 tests before Bonferonni correction (Corfe/Buckland, Corfe/Golden Mill and Buckland/Gunnislake) and in no cases afterwards.

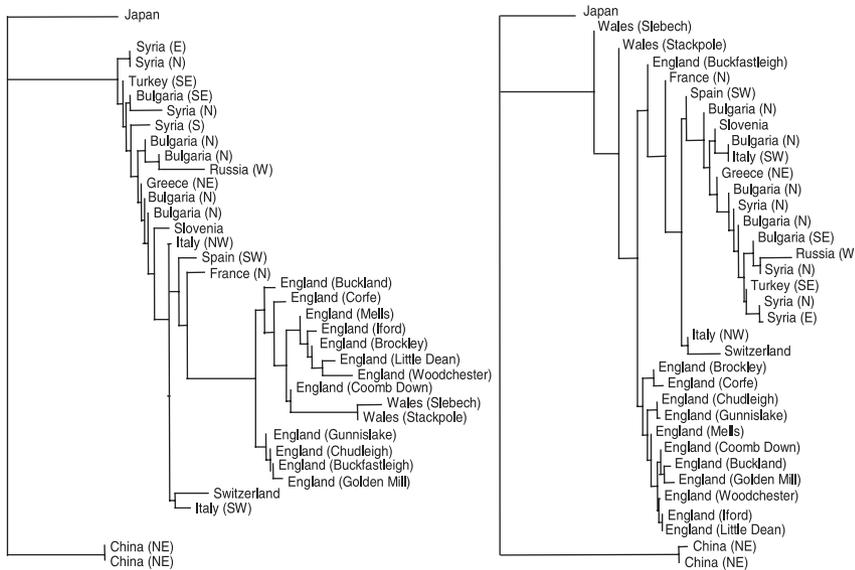
In agreement with the  $F_{ST}$  results,  $R_{ST}$  analysis revealed weaker structure across mainland Europe and the Middle East (global  $R_{ST} = 0.021$ ) than within the UK. Once again, the global  $R_{ST}$  was smaller than the  $F_{ST}$  value and did not differ significantly from equivalent  $pR_{ST}$  ( $0.076$ ). Pairwise values ranged from  $-0.108$  [Turkey/Syria (Qatura)] to  $0.18$  [Switzerland/Bulgaria (Samara Pester)]. Observed  $R_{ST}$  and  $pR_{ST}$  values differed ( $P < 0.05$ ) in nine out of 120 cases, four of which involved the Swiss sample. No comparison remained significant after Bonferonni correction.

When range-wide samples were analysed, global  $R_{ST}$  was similar to  $F_{ST}$  but, on this occasion, was significantly larger than global  $pR_{ST}$  ( $0.38$  and  $0.09$ , respectively,  $P < 0.001$ ). Moreover, pairwise values of  $R_{ST}$  and  $pR_{ST}$  were significantly different in 44.5% (265/595) of tests carried out. The majority of these significant results corresponded to comparisons between China and other samples (mean  $R_{ST} = 0.85$  vs.  $pR_{ST} = 0.14$ ,  $n = 65$ ,  $P < 0.001$ ), Japan and other samples (mean  $R_{ST} = 0.71$  vs.  $pR_{ST} = 0.13$ ,  $n = 30$ ,  $P < 0.001$ ), and the UK and other samples (mean  $R_{ST} = 0.26$  vs.  $pR_{ST} = 0.10$ ,  $n = 265$ ,  $P < 0.001$ ). Also significant was the Japan/east China comparison ( $R_{ST} = 0.81$  vs.  $pR_{ST} = 0.12$ ,  $n = 2$ ,  $P < 0.001$ ). After Bonferonni correction, 121 comparisons remained significant at the  $P < 0.05$  level, comprising the same spread.

*Neighbour-joining trees.* The overall relationship among populations, as depicted by neighbour-joining trees, differed depending on the measure of genetic distance used. The tree based on pairwise  $F_{ST}$  values (Fig. 3a) showed all samples from the UK clustering together, and nested within samples from mainland Europe, which are nested within samples from Asia. In contrast, in the tree based on pairwise  $R_{ST}$  (Fig. 3b), samples from Wales were basal to a clade containing all other samples from Europe and West Asia. An  $R_{ST}$  tree based on a reduced data set that does not include samples from China and Japan recovers the same relationship among populations as the  $F_{ST}$ -based tree (figure not shown).

*Factorial correspondence analysis.* An FCA based on matrices of allele frequency data was undertaken for three geographical scales. An analysis based solely on individuals sampled from the UK revealed considerable genetic subdivision. Axes 1, 2 and 3 together explain 46.7% of the total inertia (respectively, 20.9%, 15.4% and 10.4%) (Fig. 4a). Axis 1 reveals differences between colonies from the Cornwall-Devon area and the rest of the range. Similarly, clear differentiation is also evident between the Welsh colonies and the other sites, and between Corfe in Dorset and the other sites, as shown by Axes 2 and 3, respectively.

This analysis was repeated for just Europe and west Asian samples, thus excluding individuals from China, Japan and Morocco (Fig. 4b). The first three axes account



**Fig. 3** Neighbour-joining trees for 35 samples based on (a) pairwise  $F_{ST}$  values and (b) pairwise  $R_{ST}$  values. Both trees were rooted with the sample from Japan.

for 30% of the total inertia (axes 1, 2 and 3 explain, respectively, 18.8%, 6.5% and 5.0% of the total inertia), with axis 1 describing the cline of genetic differentiation corresponding to longitude. Axes 2 and 3 both illustrate clear differences between the relatively isolated Russian bats and those from other localities.

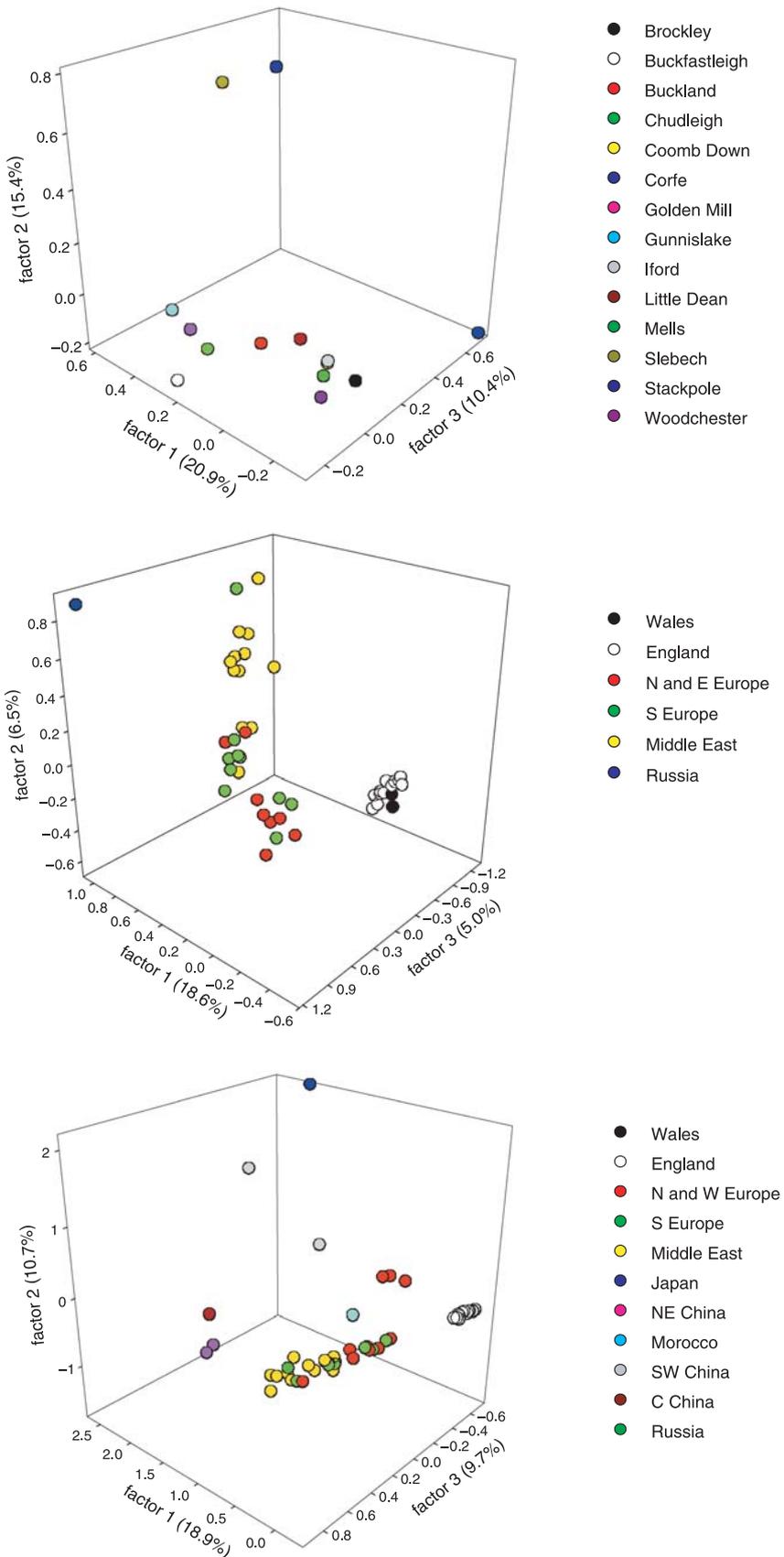
The range-wide analysis, in which the first three axes together explained 39% of the total inertia (respectively 18.9%, 10.7 and 9.7% of the total inertia), revealed multiple clouds of points (Fig. 4c). Axis 1 broadly separates populations from Europe, Morocco and the Middle East from those sampled of China and Japan. Axis 2 illustrates clear differentiation between bats from the relatively nearby areas of Northeast China and Japan. Axis 3 reveals differences within Europe and the Middle East, and shows a general cline corresponding to longitude.

**Cluster analysis.** A model-based clustering method applied to all bats from the UK revealed the most likely number of clusters (the value of  $K$  with the highest posterior probability) to be four ( $K = 4$ ,  $P(K|X) = 0.993$ , see Figs 5(a) and 6(a)) broadly corresponding to the four population centres described (Fig. 1a). Colonies located at the edges of main population centres, including Buckland and Coomb Down, comprised individuals assigned to multiple clusters. In a range-wide analysis, eight clusters were found ( $K = 8$ ,  $P(K|X) > 0.999$ , see Figs 5(b) and 6(b)). Representative colonies of the four UK clusters were allocated to a single group, which was separate from individuals from mainland Europe. Bats from France, Spain, Portugal and Italy formed a single cluster, but appeared different to those from Switzerland, which formed a separate cluster. Bats from Slovenia and Slovakia to southeast Europe all showed a similar signature of

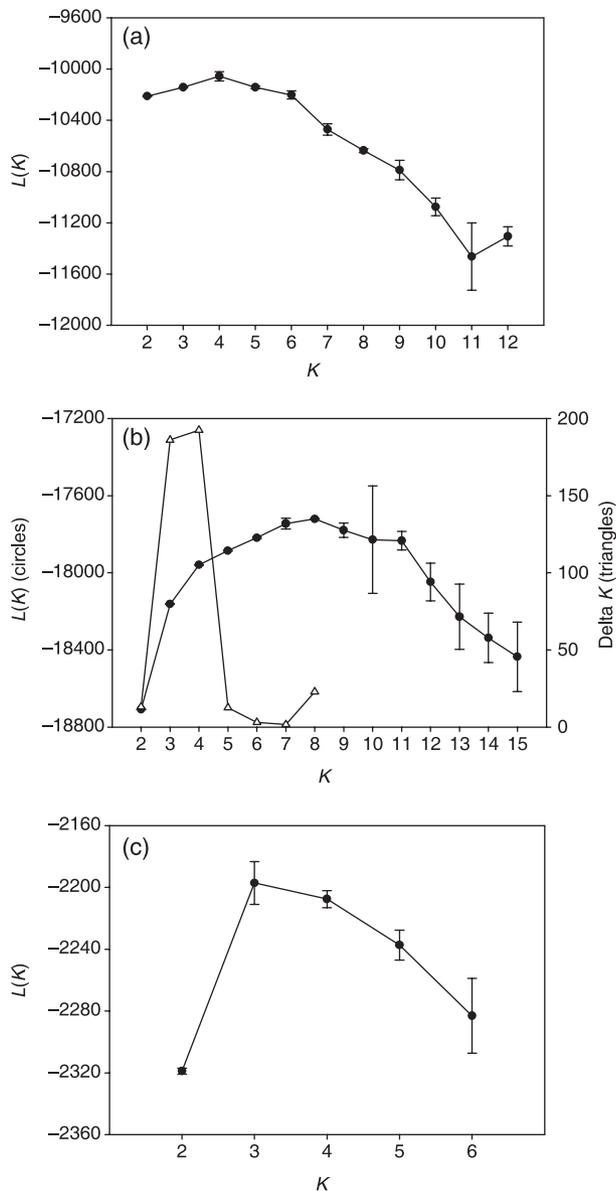
cluster membership, although this changed between Turkey to Iran, and bats from Russia formed a private cluster, although with some similarities to some individuals from eastern Syria. Bats from southwest and central China grouped together, as did bats from east China and Japan. Values of  $\Delta K$ , calculated for where  $L(K)$  increased monotonically with  $K$ , were highest at  $K = 3$ ,  $K = 4$  and, to a lesser degree,  $K = 8$  (see Fig. 5b). Of these,  $K = 3$  recovered broad structure between the UK, mainland Europe/west Asia and China/Japan, while at  $K = 4$  a cluster comprising samples from Spain, France, Italy and Switzerland was also identified. A separate analysis of just bats from China and Japan revealed that Japan and China represented different clusters ( $K = 3$ ,  $P(K|X) = 0.858$ , Figs 5(c) and 6(c)).

### Genetic diversity

Allelic richness per locus and population was calculated for every sample for which at least five individuals were typed at every locus (Table 2). Markers 11 and 12 were excluded from Europe and Far East samples, respectively. Mean allelic richness ( $R_s$ ) ranged from 2.83 for Woodchester, Southwest England to 5.35 for Çevlik, Southeast Turkey. Allelic diversity was strongly positively correlated with longitude across Europe and the Middle East (Pearson's correlation coefficient = 0.87,  $P < 0.001$ ) and the entire range (Pearson's correlation coefficient = 0.59,  $P < 0.001$ ) (Fig. 7a). The Middle East was the centre of allelic diversity with populations both west and farther east showing lower values. Allelic richness was also strongly negatively correlated with latitude across all samples (Pearson's correlation coefficient = -0.89,  $P < 0.001$ ) (Fig. 7b), a trend which persisted within Europe (Pearson correlation



**Fig. 4** Three-dimensional plots of the first three axes based on factorial correspondence analysis, undertaken for samples from (a) the UK, (b) Europe and west Asia, and (c) across the species range. Percentage of variance explained by each axis is given in parentheses.



**Fig. 5** Results of cluster analyses showing log probability of the data  $L(K)$  (left Y-axis) as a function of  $K$  (mean values averaged over 10 replicates  $\pm$  SD) for (a) the UK, (b) across the species range, and (c) China and Japan. For plot (b), values of  $\Delta K$  calculated according to Evanno *et al.* (2005) are also given on a right-hand Y axis (see Methods).

coefficient =  $-0.87$ ,  $P < 0.001$ ). The significant correlations between  $R_s$  and both longitude and latitude result reflect the fact that these two aspects of sampling location are themselves correlated (Pearson's correlation coefficient =  $0.61$ ,  $P < 0.001$ ). However, samples from Spain, east China and Japan showed unusually large standardized residuals from a best-fit line of this trend (data not shown). Japanese and Chinese samples had low  $R_s$  values for their longitude,

while Spain (leftmost open circle in Fig 7a, b) had a high value of  $R_s$  for its longitude (compared to samples from the UK) and a low  $R_s$  for its latitude (compared to the Middle East).

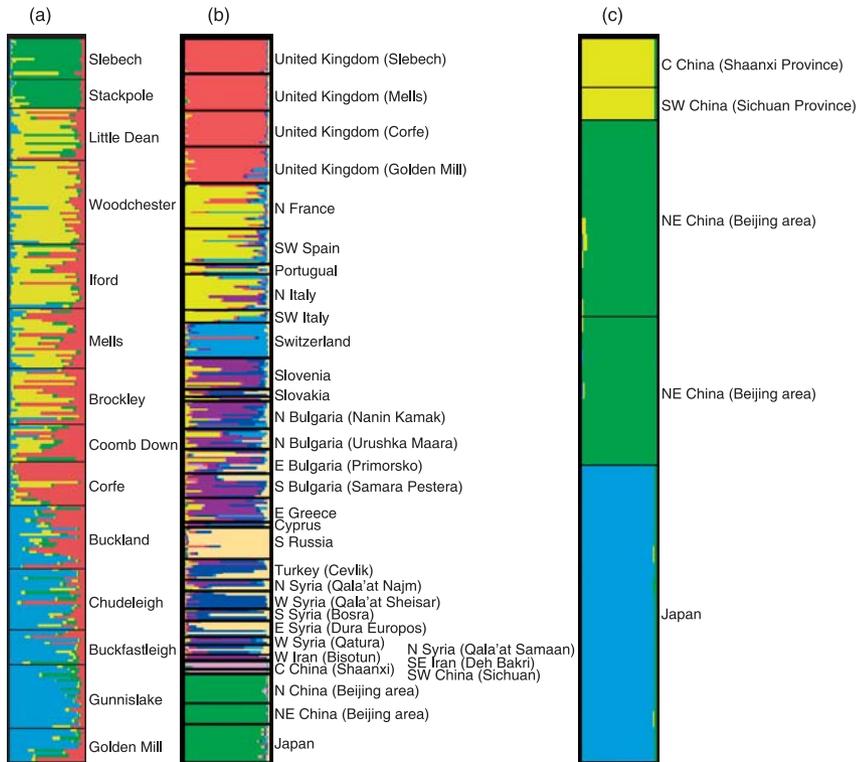
## Discussion

In spite of their popularity in population genetics, the suitability of microsatellites for phylogeographical inference has been questioned (Paetkau *et al.* 1997). High rates of stepwise mutation are often considered to result in unacceptable levels of size homoplasy, potentially obscuring true relationships among deeply diverged populations (Estoup *et al.* 2002). Yet in several cases, microsatellites have been successfully applied to resolve population histories (Koskinen *et al.* 2002; Marshall *et al.* 2002) highlighting the need for more studies. Here we analyse microsatellite variation in greater horseshoe bats sampled from across Europe and Asia to shed light on the consequences and routes of postglacial colonization. Although we find evidence of drift, stepwise mutation and homoplasy operating at different geographical scales, distribution of allele frequencies are consistent with postglacial population expansions from multiple refugia.

### Population history

At the time of the LGM, the presence of ice and tundra over much of northern Europe forced temperate species into lower latitudes. Our finding of a decline in genetic diversity from the Middle East and southeast Europe to the UK is consistent with a northwest population expansion from this region into newly suitable habitats following climate warming. Recent population declines and fragmentation in northern Europe might also have contributed to the overall cline; however, stepwise colonization is also supported by a pattern of isolation by distance across continental Europe and a neighbour-joining tree based on pairwise  $F_{ST}$  values (Fig. 3a), in which populations from UK are nested within those from northern Europe, which are in turn nested within southeast Europe and west Asia.

At apparent odds with a simple northwestward expansion, Bayesian and correspondence analyses both revealed several abrupt discontinuities in gene frequencies within continental Europe. The detection of a single cluster comprising distant colonies from France, Portugal, Spain and Italy suggests that these populations share a common history and thus likely originate from the same wave of colonization. Similar allelic diversity levels among these samples preclude definitive identification of an Iberian or Italian refugium, although our results do suggest that gene flow has not been hindered by the Pyrenees. On the other hand, a distinct boundary was identified between this cluster and more easterly colonies, with Slovenia appearing



**Fig. 6** Graphical representation of clusters for samples from (a) the UK, (b) across the species range, and (c) China and Japan. Each individual is depicted by a horizontal line that is partitioned into  $K$  coloured sections, with the length of each section proportional to the estimated membership coefficient ( $Q$ ) of the bat to each cluster.

to represent the westernmost sampled colony from a population stretching from central to southeast Europe. We speculate that this observed genetic structure denotes an area of contact between major refugial populations expanding from southwest (Iberia and/or Italy) and southeast Europe (Balkans/Greece). A suture zone in the Alps delineating east and west genomes has previously been reported in a range of taxa (Taberlet *et al.* 1998). In light of this structure, the overall northwest decline in allelic richness across Europe is probably a consequence of a Balkans expansion augmented by northward expansion(s) from west Europe, given the correlation between sample longitude and latitude in this study. However, the nested relationship of colonies from northwest Europe in those from the southeast, and their relatively lower levels of allelic diversity, would both appear to reflect a more ancient expansion event presumably following an earlier period of glaciation.

The relationship between the two main European clusters and Switzerland, which formed its own cluster, was unexpected. Lower allelic diversity in the Swiss bats than in all other continental colonies suggests that observed differentiation is attributable to genetic drift effects, resulting from either a founder event/bottleneck or long-term isolation. Our failure to detect a bottleneck (data not shown), as well as the isolated valley location of the Swiss colony, both favour the second explanation, although more sampling in the area is needed to rule out a founder effect, which in bats

could occur either during the original colonization wave or more recently as part of recurrent metapopulation dynamics.

To date, comparative Europe-wide phylogeographical studies have only been undertaken for a handful of bat species. Like the greater horseshoe bat, the noctule *Nyctalus noctula* (Petit *et al.* 1999) and the mouse-eared bat *Myotis myotis* (Ruedi & Castella 2003) both show broad-scale genetic structure indicative of postglacial colonization from multiple refugia. However, contrary to our findings and classical expectations, genetic variation was not found to decrease with latitude. The apparent absence of recurrent bottlenecks during colonization of both species was attributed in part to the high mobility, which offset the effects of genetic drift and, in the case of the noctule, allowed for a rapid range shift (Petit *et al.* 1999). We previously showed that in greater horseshoe bats, gene flow can be restricted over relatively short distance (Rossiter *et al.* 2000), and we propose that this apparent difference with the other bat species studied might also account for the contrasting patterns of structure detected. Deep divergence among lineages of other bat genera, including *Plecotus* (Juste *et al.* 2004; Ibanez *et al.* 2006), *Pipistrellus* (Hulva *et al.* 2004; Ibanez *et al.* 2006) and *Eptesicus* (Ibanez *et al.* 2006) have also been attributed to historical isolation of populations in different Mediterranean and north African refugia.

Cluster and correspondence analyses revealed additional subdivision between southeast Europe and Asia Minor, suggesting no genetic exchange has occurred between

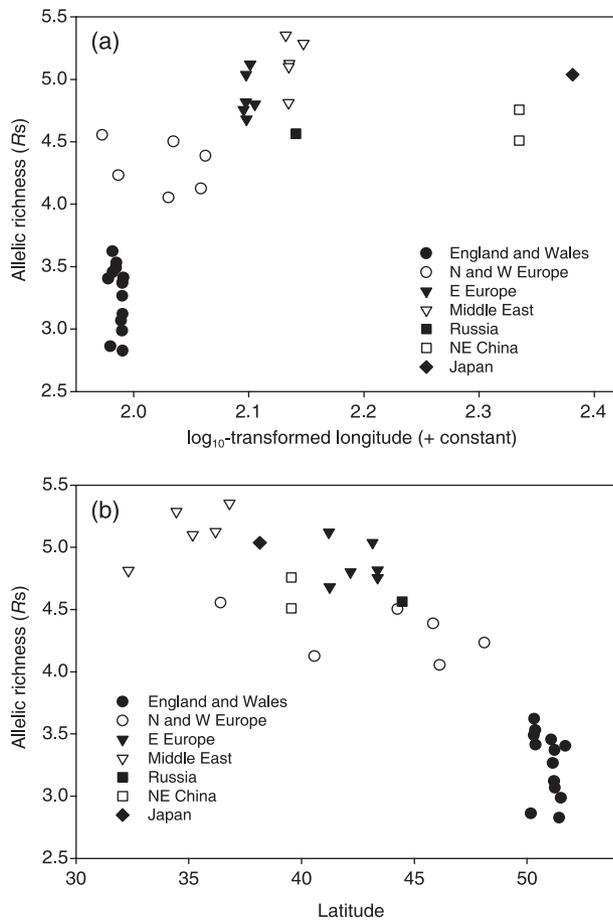


Fig. 7 Relationship between allelic richness ( $R_s$ ) vs. (a) longitude and (b) latitude, based on samples of five or more individuals.

these regions since the LGM (Fig. 8). Once again, this would suggest that the relationship between these populations identified by  $F_{ST}$  analyses reflects an earlier period of gene flow. Hotspots of genetic variability in west Asia have been reported in a range of other European taxa, including plants (e.g. alder buckthorn tree, *Frangula alnus*, Hampe *et al.* 2003), invertebrates (Cooper *et al.* 1995), and mammals (e.g. field vole, *Microtus agrestis*, Jaarola & Searle 2002; bent-winged bat, *Miniopterus schreibersii*, Bilgin *et al.* 2006; and Eurasian shrew, *Crocidura suaveolens*, Dubey *et al.* 2006). Bilton *et al.* (1998) examined small mammal data and highlighted the importance of west Asian refugia in repopulating Europe, a trend also proposed for the oak gallwasp, *Andricus quercustozae* (Rokas *et al.* 2003), and the spined loach, *Cobitis taenia* (Culling *et al.* 2006). On the other hand, several studies have shown that based on haplotype distributions and sequence divergence levels, genetically rich non-European populations are frequently not the source of European postglacial populations (Bilton *et al.* 1998; Jaarola & Searle 2002; Hampe *et al.* 2003; Michaux *et al.* 2004). Our results support this emerging trend, at least for the LGM,

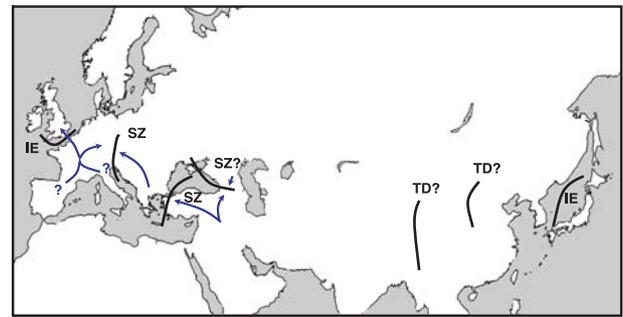


Fig. 8 Map showing the main zones of genetic subdivision detected across the species range with proposed colonization routes in Europe after the Last Glacial Maximum. Genetic structure is attributed to (i) genetic isolation and/or founder effects associated with island effects (IE) (ii) suture zones between different refugial populations (SZ), and (iii) possible cryptic taxonomic divisions (TD). Cases of IE might also represent TD.

and we suggest the exchange between Asia Minor and Europe might have been blocked by the Marmara Sea as well as population growth in both refugia. Sequence divergence data would provide a means to estimate the timing of these splits, and so test this hypothesis.

The sample of bats from southwest Russia formed a further distinct cluster from populations to the south in Syria and Turkey (Figs 4b and 6b). A refugium north of the Caucasus Mountains has been postulated for some tree species (Demesure *et al.* 1996) as well as small mammals such as the field vole, *M. agrestis* (Jaarola & Searle 2002), and Eurasian shrew *C. suaveolens* (Dubey *et al.* 2006). The role of the Caucasus Mountains and the Black Sea in impeding genetic homogenization with more southerly populations is evident from deep sequence divergence in the white-breasted hedgehog, *Erinaceus concolor* (Seddon *et al.* 2002), as well as taxonomic divisions in the goat *Capra* spp. (Manceau *et al.* 1999) and mouse *Mus musculus* (Boursot *et al.* 1996). In our study, the inclusion of too few samples from this region means we cannot confirm a refugium north of the Caucasus for greater horseshoe bats. Although this is a possibility, drift and associated loss of allelic richness relative to other west Asia samples could also have resulted from more recent phenomena such as a bottleneck or founder event.

Few studies of Eurasian taxa have examined genetic diversity in populations south and east of Asia Minor. We found high allelic richness in Syrian greater horseshoe bats, supporting the Levant region as a refugial region, although most samples from Syria, Iran, central and west China were too few to assess genetic diversity and too sparse for meaningful interpretation of structure. In contrast, plots based on correspondence analyses were more informative and showed that Syrian and Iranian bats fell broadly in line with the trend observed across Europe and the Middle

East, while samples from southwest and central China fell out of this axis and were also markedly different from each other (Fig. 4c). More studies are needed to identify the probable glacial refugial areas of temperate species from this region, which during the LGM was covered by polar desert.

Comparisons of genetic distances ( $F_{ST}$ ,  $R_{ST}$  and  $pR_{ST}$ ) between the most distantly sampled populations (Japan and China vs. Europe) showed evidence that stepwise mutation has contributed to divergence. However, a neighbour-joining tree based on  $R_{ST}$  was not supported by the  $F_{ST}$  topology, with the Welsh samples moving to a more basal position. These differences support previous studies that found distance estimators based on stepwise differences to be especially prone to the effects of allele size homoplasy, particularly for divergent populations (Goldstein *et al.* 1997; van Oppen *et al.* 2000; Queney *et al.* 2001). Indeed, due to mutational constraints that operate at microsatellite loci (Queney *et al.* 2001; van Oppen *et al.* 2000) the genetic distances reported between bats from Europe and east Asia should be considered as probable underestimates.

In addition to structure among continental populations, clear discontinuities in gene frequencies were also detected between the UK and France and also between China and Japan. In the case of the UK, inhospitable conditions during the LGM means that low allelic richness here compared with mainland European populations almost certainly resulted from founder effects during colonization across the temporary land bridge combined with long-term genetic isolation following the formation of the English Channel. It is also possible that colonization effects have contributed to the cline in diversity within the UK, from Cornwall to Wales; however, differentiation among four identified population centres probably reflects the discontinuous species range, a result of population and habitat fragmentation (Rossiter *et al.* 2000). The situation for Japan and northeast China appears to be different, both of which show higher allelic diversity than west Europe. Reconstructions of vegetation maps during the LGM indicate that Japan and parts of eastern China were covered by open woodland, raising the possibility that greater horseshoe bats persisted in east Asian refugia (Adams & Faure 1997). Moreover, analyses of river deposits suggest that Japan was not connected to mainland China during the LGM (Park *et al.* 2000), in which case the Japanese population must predate this period. Similar inferred long histories have also recently reported for the related Japanese endemic *Rhinolophus cornutus* (Li *et al.* 2006) as well as the Taiwanese endemic *Rhinolophus monoceros* (Chen *et al.* 2006).

#### Implications for taxonomy

Thomas (1997) recognized seven subspecies of greater horseshoe bat based on morphology but was unable to

consider genetic evidence due to low sample sizes. A number of aspects of the results presented here add to the current knowledge of the species taxonomy. First, genetic subdivision between the UK and continental Europe requires further investigation to establish whether the populations of the former justify recognition of an island subspecies. Second, bats across Europe represented as a single cloud in an FCA (see Fig. 4b) corroborates earlier findings that this is one subspecies (Thomas 1997), thereby contradicting earlier claims that multiple subspecies occur within mainland Europe (see discussion in (Csorba *et al.* 2003). Third, no obvious differences between bats from Asia Minor and Iran yet substantial subdivision among bats from across China (currently all lumped together as *R. f. tragatus*) point to a need for taxonomic re-evaluation of Asian subspecies (Fig. 8). Finally, the taxonomic status of *R. ferrumequinum* on Japan (*R. f. nippon*) has been questioned (Ellerman & Morrison-Scott 1951; Thomas 1997; Csorba *et al.* 2003) and is currently recognized as a subspecies or a potentially separate species (Thomas 1997). The failure of both east China and Japan samples to amplify at one marker, as well as the similar level of differentiation as between colonies from the UK and southeast Europe, both suggest a recent common ancestry. Other currently recognized subspecies from Crete (*R. f. creticum*), Korea (*R. f. korai*) and Afghanistan/Pakistan (*R. f. proximus*) were not analysed due to difficulties in obtaining samples.

Patterns of echolocation call variation among populations of greater horseshoe bats show some concordance with the genetic discontinuities described here, and so appear to support our suggestions of cryptic diversity in oriental populations. Heller & von Helversen (1989) reported higher values in France and Germany (80–81 kHz) than in Greece and Asia Minor (77–81 kHz) and suggested that in Europe there appears to be cline from the northwest to the southeast, which they speculated might continue farther eastwards. However, our recordings show that while bats from southwest China could in theory fit with an extrapolation of this trend (72–73.9 kHz for bats from Yunnan and Sichuan, respectively), those from east China are higher again (males 74.8–76.8 kHz; females 76.4–77.1 kHz) (G. Jones, unpublished data). Moreover, bats from Japan show a further decrease (~65 kHz) (Taniguchi 1985; Fukui *et al.* 2004).

#### Implications for conservation

The greater horseshoe bat is a protected species over much of Europe and is the subject of numerous conservation initiatives (Ransome & Hutson 2000). Evidence of inbreeding depression (Rossiter *et al.* 2001) and limited immigration (Rossiter *et al.* 2006) in this species highlight the importance of gene flow in maintaining population health. The results presented here provide a number of useful insights for conservation management. First, the finding that  $F_{ST}$

values among some colonies within the UK exceed values between all mainland Europe/west Asia populations, including ones separated by 4000 km, indicates that populations of greater horseshoe bats are likely to be especially vulnerable to small population effects and fragmentation. On the other hand, clustering-based analyses show that several UK colonies represent important sites of genetic exchange among different populations (shown to contain many individuals assigned to more than one cluster), indicative of genetic mixing and a reflection of the geographical location of these colonies between clusters. Protection of these and neighbouring colonies should help to maximize gene flow among clusters. We also confirm the relative isolation of colonies in west Wales (Rossiter *et al.* 2000), although some movement between this area and the nearest known maternity roost (Little Dean) is suggested for a few individuals (shown as green in Fig. 6a).

In continental Europe, although structure between distant sampling sites probably reflects both older history and isolation, the Swiss colony stands out as warranting further study. Additional sampling in this region would help to delineate the identified gene pool, and so determine whether it could benefit from intervention to promote mixing from the considerable surrounding genetic diversity. Populations farther east, in China and Japan, probably represent separate subspecies or species and are in need of further study to refine their conservation priorities.

## Conclusions

Our study is one of the first to use microsatellites to examine phylogeographical trends across a species' range. Observed patterns of genetic diversity, structure and inferred suture zones all show that the greater horseshoe bat probably survived the LGM in several refugia, located in southwest and southeast Europe in addition to areas of west and east Asia. The inferred route of recent postglacial colonization of Europe shows similarities to the hedgehog paradigm with independent refugia in west and southeast Europe (Hewitt 1999), but does not appear to involve genetically diverse populations in the Middle East (Fig. 8). Sequence data will provide valuable additional information on the approximate timing of population separations as well as help to resolve apparent the taxonomic divisions identified here. At a smaller geographical scale, we detected strong differentiation within the UK, a pattern that is most likely attributable to population fragmentation and underlines the need for careful conservation management of this protected species.

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