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Phylogeography of the greater horseshoe bat, *Rhinolophus ferrumequinum*: contrasting results from mitochondrial and microsatellite data

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Abstract

Phylogeographical studies are typically based on haplotype data, occasionally on nuclear markers such as microsatellites, but rarely combine both. This is unfortunate because the use of markers with contrasting modes of inheritance and rates of evolution might provide a more accurate and comprehensive understanding of a species' history. Here we present a detailed study of the phylogeography of the greater horseshoe bat, *Rhinolophus ferrumequinum*, using 1098 bp of the mitochondrial *ND2* gene from 45 localities from across its Palaearctic range to infer population history. In addition, we re-analysed a large microsatellite data set available for this species and compared the results of both markers to infer population relationships and the historical processes influencing them. We show that mtDNA, the most popular marker in phylogeography studies, yielded a misleading result, and would have led us to conclude erroneously that a single expansion had taken place in Europe. Only by combining the mitochondrial and microsatellite data sets are we able to reconstruct the species' history and show two colonization events in Europe, one before the Last Glacial Maximum (LGM) and one after it. Combining markers also revealed the importance of Asia Minor as an ancient refugium for this species and a source population for the expansion of the greater horseshoe bat into Europe before the LGM.

Keywords: demographic history, glacial refugia, greater horseshoe bat, microsatellites, mitochondrial DNA, phylogeography

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Introduction

The majority of phylogeographical studies have been based on a single type of marker, usually a mitochondrial gene (Moore 1995; Avise 2000). Because different markers each have their own unique genealogy, which might deviate from the species' history (Hewitt 2004a), this approach can introduce problems. In particular, phenomena such as homoplasy, independent evolution, incomplete lineage sorting, biases caused by different modes of inheritance, effective population sizes and sex-biased dispersal can all lead to erroneous results and conclusions (Clutton-Brock 1989; Colbert *et al*. 2001). One way to minimize the impact of such problems is to combine markers with different modes of inheritance and rates of evolution (Hewitt 2004a). This allows us to look at the relative roles that historical and contemporary events have had on population structure with more confidence (Howes *et al*. 2006) as any potential ambiguity can be assessed and hopefully resolved according to the genes being used.

The choice of marker used in phylogeographical studies also has important implications for the timescale over which events can realistically be inferred. Hypervariable markers such as microsatellites, while useful for detecting contemporary gene flow, are widely considered less suitable

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for phylogeographical inference due to their tendency towards homoplasious mutations (Hewitt 2004b). This is also a problem associated with rapidly evolving parts of the mitochondrion such as the control region, for which homoplasy can confound genetic distance estimation and phylogenetic inference over timescales beyond tens of thousands of years (Avise 2000; Lloyd 2003). Conversely, markers with slower mutation rates (e.g. *ND1*, Lloyd 2003) might be better for characterizing ancient events, yet provide unsatisfactory resolution of patterns of intraspecific and phenotypic variation, which are necessary for revealing recent processes (Rokas *et al*. 2003).

The choice of marker should also be considered in the context of geographical scale. Phenomena such as isolation by distance, population bottlenecks and the balance between drift and gene flow will all be influenced by the rate and distance of dispersal events. Yet, many phylogeographical studies are based on restricted geographical sampling relative to the study species' natural range. This can be seen in Europe, where, even though many species range outside of Europe, sampling is often limited to European states. This reflects the focus in the literature on latitudinal postglacial movements and colonization patterns from southern refugia. By sampling beyond Europe and also by considering wider longitudinal patterns in genetic diversity (Rokas *et al*. 2003), it might be possible to gain a more comprehensive population history for an individual species.

One way to address these relative shortcomings is to apply markers with contrasting mutation rates and modes of inheritance to the same data set across a species' natural distribution. For example, by using both mitochondrial DNA (mtDNA) and microsatellite data, a number of studies have now been able to gain further insight into how pre- and postglacial dynamics have influenced current population structure in a range of species (Heckel *et al*. 2005; Howes *et al*. 2006; Jadwiszczak *et al*. 2006; Brito 2007).

Here we apply an analysis of mtDNA sequence variation to an extensive sample set from which phylogeographical history has already been inferred using microsatellites. Rossiter *et al*. (2007) examined the population history of the greater horseshoe bat (*Rhinolophus ferrumequinum*) across its Palaearctic range using microsatellite variability. A decline in allelic diversity from the Middle East to the UK, with patterns of isolation by distance and nested genetic structure, indicated a northwest expansion. Yet at the same time, broad subdivision within Europe supported at least two refugial populations, expanding from southwest and southeast Europe, with no evidence of gene flow from Asia Minor since the Last Glacial Maximum (LGM). To reconcile these apparently conflicting inferences, the authors concluded that the microsatellite data reflected two episodes of expansion, one before the LGM and one afterwards. Indeed, structure between the Middle East, Europe and west Russia also suggested non-European LGM refugia, and high allelic diversity in populations from northeast China and Japan pointed to additional East Asian refugia, although the presence of cryptic species was not discounted.

While demonstrating the potential utility of microsatellites in phylogeography, this earlier study also highlighted a need for additional genetic markers to test and confirm its conclusions of a complex history of multiple colonizations. mtDNA haplotype data, with its slower rate of mutation, can provide a means of recovering more ancient population splits and expansion events. To address these issues, here we present an analysis of mtDNA *ND2* gene sequences from individuals sampled from across the species' range. In addition, we undertake a detailed re-analysis of the microsatellite data set (see: Rossiter *et al*. 2007) in order to gain greater resolution of the hierarchical relationship among populations. We show that while both types of marker offer unique insights into population processes, it is only by combining data sets that we can gain a full and accurate picture of past events. In particular, we illustrate the potential pitfalls of using mtDNA haplotype data alone for assessing population expansions since the LGM.

Materials and methods

Sampling and DNA extraction

Tissue samples of greater horseshoe bats (*Rhinolophus ferrumequinum*) were collected or obtained from 45 localities in 17 different countries across the species' range (Table 1; Fig. 1). The number of samples analysed per locality varied due to sample availability and sequencing success. Samples comprised either 3 mm wing membrane biopsies obtained using a biopsy punch (Stiefel Laboratories; Worthington & Barratt 1996) or liver or muscle tissue from museum samples. All tissue samples were stored at –20 °C in 90% ethanol or 20% dimethyl-sulphoxide/saturated NaCl (~6 M). Genomic DNA was isolated from tissue by either a salt-chloroform extraction, or by using QIAGEN DNeasy Kits or Promega Wizard Purification Kits.

Mitochondrial DNA amplification

The mitochondrial gene *ND2* was amplified by polymerase chain reaction (PCR) using the primers L5074.M (5′-CTGATAAAAGARTTACTTTGATAGAG-3′) (M. Sorenson, personal communication) and H6305 (5′-GGCTTTG-AAGGCYCTTGGTC-3′) (Sorenson *et al*. 1999). *ND2* was selected because, in terms of amino acid sequence, it is the third most variable gene in the mitochondria after *ATPase 8*, which is relatively short (~165–168 bp), and *ND6*, which is difficult to amplify due to its unusual base-pair composition close to the control region (Sorenson 2003). PCRs were performed in a 15-μL volume, containing approximately

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; SG, Suren Gazaryan; FL, François Leboulanger, GJ, Gareth Jones; GL, Gang Li; JF, Jon Flanders; JJ, Javier Juste; LE, Lazaro Echenique-Diaz, MS, Mozafar Sharifi; MZ, Maja Zagmajster; PB, Petr Benda; RA, Raphaël Arlettaz; SR, Stephen Rossiter; SZ, Shuyi Zhang

Location no.	Country	Locality	Easting	Northing	*n*	Collector/supplier
1	Bulgaria (N)	Nanin Kamak	E24 : 51 : 22	N43 : 37 : 37	5	CD
2	Bulgaria (N)	Samara Pestera	E25 : 29 : 58	N41 : 24 : 31	2	CD
3	Bulgaria (N)	Urushka Maara	E25 : 01 : 46	N43 : 14 : 44	1	CD
4	Bulgaria (SE)	Primorsko	E27 : 45 : 01	N42 : 17 : 15	1	CD
5	China (NE)	Beijing	E116 : 19 : 55	N39 : 54 : 25	12	GJ/SZ
6	China (NE)	Jilin	E126 : 29 : 56	N43 : 46 : 49	1	GJ/SZ
7	China (C)	Foping (Shaanxi)	E107 : 59 : 00	N33 : 34 : 00	1	PB
8	China (E)	Shenxian Cave (Henan)	E113 : 22 : 05	N34 : 41 : 49	9	GL/SZ
9	China (E)	Xiaya Cave (Shandong)	E118 : 07 : 01	N36 : 15 : 00	2	GL/SZ
10	China (E)	Wanfu Cave (Jiangxi)	E117 : 01 : 46	N28 : 05 : 47	1	GL/SZ
11	China (SE)	Youkuang Cave (Anhui)	E115 : 19 : 59	N31 : 10 : 01	1	GL/SZ
12	China (SW)	Nanton Village (Sichuan)	E102 : 13 : 52	N29 : 48 : 33	1	GJ/SR/SZ/GL
13	China (SW)	Emei Shan (Sichuan)	E103 : 16 : 92	N29 : 34 : 74	1	GJ/SR/SZ/GL
14	Cyprus	Cinarli	E33 : 46 : 00	N35 : 19 : 00	1	PB
15	Cyprus	Kalavasos	E33 : 16 : 00	N34 : 48 : 00	1	PB
16	England (SW)	Buckland	W04 : 09 : 57	N51 : 07 : 00	4	GJ
17	England (SW)	Chudleigh	W03 : 36 : 19	N50 : 36 : 15	3	GJ
18	England (SW)	Coomb Down	W02 : 21 : 21	N51 : 21 : 41	5	GJ
19	England (SW)	Corfe Castle, Dorset	W02 : 03 : 03	N50 : 37 : 59	2	GJ/JF
20	England (SW)	Golden Mill	W04 : 54 : 50	N50 : 16 : 55	5	GJ
21	England (SW)	Gunnislake	W04 : 12 : 33	N50 : 31 : 20	3	GJ
22	England (SW)	Woodchester	W02 : 16 : 39	N51 : 42 : 39	4	GJ/SR
23	France (N)	Normandy	W03 : 00 : 30	N48 : 10 : 56	13	FL
24	Greece (NE)	Koufovouno	E26 : 27 : 13	N41 : 21 : 10	2	CD
25	Iran (NW)	Ghalah Kord Cave (Zanjan)	E48 : 49 : 59	N35 : 45 : 00	5	MS
26	Iran (NW)	Kataleh Khor Cave (Zanjan)	E48 : 15 : 00	N35 : 40 : 01	1	MS
27	Italy (NW)	Giovo	E08 : 28 : 15	N44 : 25 : 49	8	DS
28	Italy (SW)	Campania	E14 : 41 : 39	N40 : 57 : 13	1	DR
29	Japan (NE)	Sendai, Honshu Island	E140 : 53 : 33	N38 : 15 : 03	15	LE
30	Morocco	Talkout, Oued Tessaout Valley	W07 : 17 : 00	N31 : 41 : 00	1	PB
31	Portugal	Exact locality unknown	W08 : 05 : 05	N39 : 23 : 10	3	CD
32	Russia (W)	Derbentskaya	E38 : 30 : 00	N44 : 46 : 00	8	EK/SG
33	Sardinia	Grotta di Monte Majore	E08 : 61 : 67	N40 : 05 : 00	1	CD
34	Slovenia (SE)	Kostanjeviska Jama	E15 : 43 : 84	N45 : 83 : 86	2	MZ/AP
35	Spain (SW)	Jerez de la Frontera, Cadiz	W06 : 08 : 14	N36 : 41 : 12	7	JJ
36	Switzerland	Vex	E7 : 23 : 56	N46 : 12 : 42	1	RA
37	Syria (N)	Qala'at Najm (Halab)	E38 : 16 : 0	N36 : 33 : 0	5	PB
38	Syria (N)	Qala'at Samaan (Halab)	E36 : 52 : 0	N36 : 18 : 25	3	PB
39	Syria (N)	Qala'at Salahadin (Lattaqia)	E36 : 3 : 0	N35 : 36 : 0	1	PB
40	Syria (N)	Qala'at Sheisar (Hama)	E36 : 34 : 0	N35 : 17 : 0	3	PB
41	Syria (N)	Qatura (Halab)	E36 : 50 : 0	N36 : 19 : 0	1	PB
42	Syria (E)	Dura Europos (Deir ez-Zur)	E40 : 43 : 0	N34 : 45 : 0	6	PB
43	Syria (S)	Bosra (Der'a)	E36 : 29 : 0	N32 : 32 : 0	2	PB
44	Turkey (NW)	Dupnisa Magarasi	E27 : 33 : 22	N41 : 50 : 27	1	CD
45	Turkey (SE)	Çevlik (Hatay)	E35 : 56 : 00	N36 : 08 : 00	5	PB

3–30 ng of genomic DNA, 0.67 μM of each primer, 0.33 μM of each dNTP, 1.33 μM $MgCl_2$ and 1.0 μM of *Taq* polymerase (Bioline) in the manufacturer's buffer. Reactions were performed on a DNA Engine Tetrad thermal cycler (MJ Research) under the following conditions: 95 °C for 15 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min. DNA sequencing was performed on an ABI 3700 DNA sequencer (Applied Biosystems). Chromatograms were edited and aligned using BioEdit version 7.0.5.3 (Hall 1999).

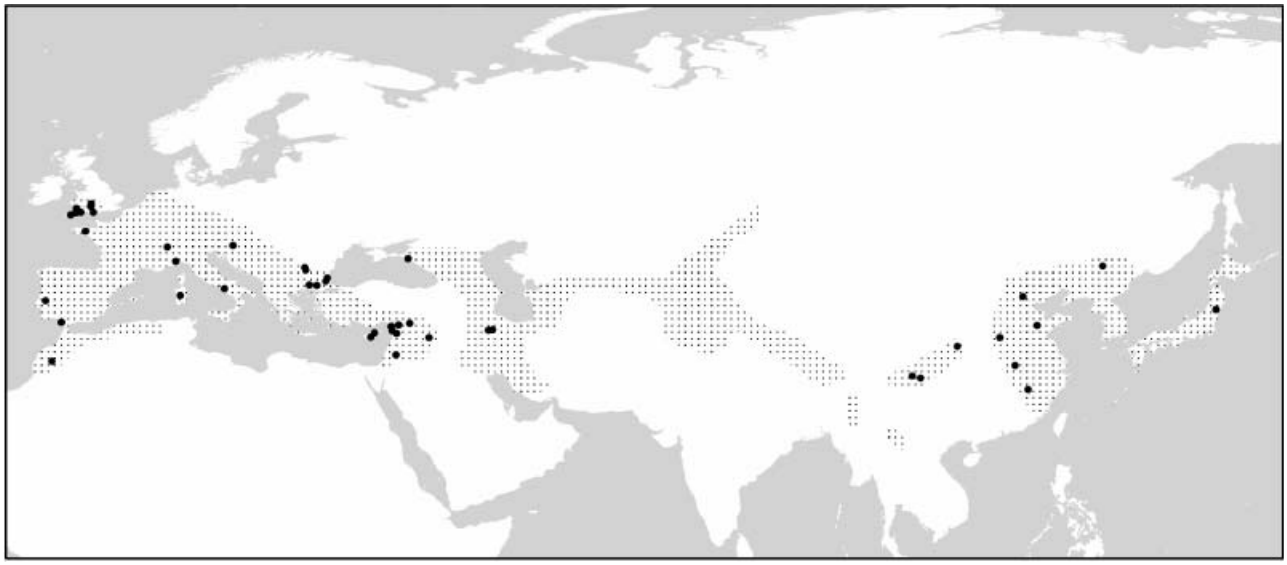

Fig. 1 Map of sampling localities across the entire range. Shading indicates the current known distribution of the greater horseshoe bat (*Rhinolophus ferrumequinum*) (adapted from Harris & Yalden 2008).

Genetic diversity

To calculate genetic diversity, we pooled neighbouring localities to give 25 populations. While populations broadly corresponded to countries, the Chinese localities were analysed separately, due to the large distances between them, and the samples from western and eastern Turkey were pooled with those from Bulgaria and Syria, respectively. Haplotype diversity (h) and nucleotide diversity (π) were calculated for all populations with sample sizes of two or more. Values for polymorphic sites and the mean number of pairwise differences were also estimated. All calculations were carried out using the software DnaSP, version 4.10.6 (Rozas *et al*. 2003).

Population structure

To test for geographical genetic structure, analyses of molecular variance (AMOVA) with 10 000 permutations (Excoffier *et al*. 1992; Excoffier 2003) were carried out in Arlequin version 3.1 (Schneider *et al*. 2000). Populations were grouped into five geographical clades based on the results of our phylogenetic analysis, and were assessed according to the degree of differentiation between regions (Φ_{CT}), between populations within regions (Φ_{SC}) and between all populations (Φ_{ST}).

We tested for isolation by distance among all localities with samples of two or more bats by regressing geographical distance against Slatkin's linearized Φ_{ST} (Slatkin 1993). Linear Euclidean distances (in kilometres) between samples were calculated from their metric easting and northing coordinates using the program Geographic Distance Matrix Generator (version 1.2.1) (Ersts Internet). A Mantel test (10 000 permutations) was performed with IBDWS version 3.14 (Isolation By Distance Web Service: http://ibdws.sdsu.edu/) (Jensen *et al*. 2005) to assess the statistical significance in correlation between genetic and the natural logarithm-transformed geographical distances.

Phylogenetic analysis

We undertook phylogenetic analyses of unique haplotypes using the neighbour-joining (NJ) algorithm in PAUP* version 4.0b10 (Swofford 2002) and Bayesian Inference (BI) in MrBayes version 3.1 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Statistical support for branching patterns was estimated by bootstrap replication (NJ: 1000 replicates). BI was run with four simultaneous chains, each of 1×10^6 generations, sampled every 100 generations and the first 25% of trees were discarded as 'burn-in'. We applied the Hasegawa–Kishino–Yano 85+ gamma model of DNA substitution, as determined by ModelTest version 3.7 (Posada & Crandall 1998) (base frequencies: A, 0.3480; C, 0.3201; G, 0.0961; and T, 0.2358; transition/transversion ratio = 30.0713; gamma distribution shape = 0.0174).

In addition, we constructed a minimum spanning haplotype network (MSN) (Templeton *et al*. 1992) using the program TCS version 1.21 (Clement *et al*. 2000). This method is particularly suited to the analysis of intraspecific gene genealogies, where ancestral and derived haplotypes might coexist in the population.

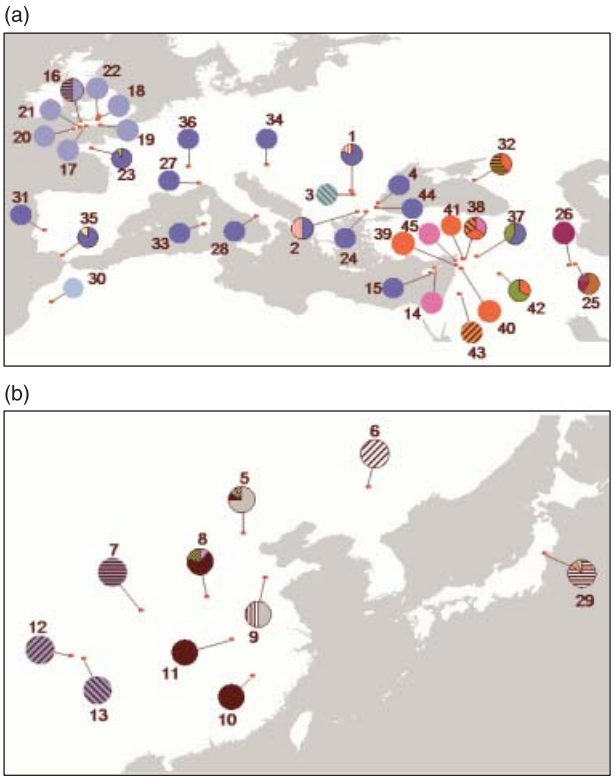

Fig. 2 Haplotype map based on greater horseshoe bats from sampling locations in (a) Europe and west Asia and (b) central and east China and Japan. Each colour/pattern combination represents a different haplotype. Pie charts show the proportion of different haplotypes found in each region. Numbers refer to sampling locations listed in Table 1.

Demographic analysis

We examined demographic history using mismatch distributions (Slatkin & Hudson 1991). Populations that have undergone a recent expansion typically show a smooth or unimodal distribution, whereas more stable populations show a ragged or multimodal distribution (Rogers & Harpending 1992). Distributions were generated for four regions (Europe, west Asia, east China and Japan). We also undertook a separate analysis for Russia, which showed evidence of isolation based on microsatellites. Significant difference from a model of sudden expansion was assessed using the sum of squared deviations (SSD) and raggedness index (r), and confidence intervals were generated with parametric bootstrapping (10 000 replicates) in Arlequin. Where expansions were detected, we estimated the time of expansion in generations (t) from $\tau = 2ut$. Where τ (tau) is calculated as the time to expansion in mutational units and u is the mutation rate per generation for the DNA sequence being studied. Mutation rates of 1.2% and 1.8% per million years were chosen as the upper and lower boundaries of the mutation rate for *ND2*, as previously calculated for the Philippine fruit bat (*Haplonycteris fischeri*; (Roberts 2006) and New Zealand short-tailed bat (*Mystacina tuberculata*; Lloyd 2003). The generation time was estimated to be 2 years, based on the earliest age at which this species can breed (Ransome 1995).

Microsatellite analysis

To compare patterns of structure obtained from our mtDNA haplotype data with those from multilocus markers, we re-analysed a data set of 516 bats genotyped at 16–17 microsatellite loci (see Rossiter *et al*. 2007 for details). These bats were sampled from across the range, but with less coverage in China and Iran than the present study. We repeated the Bayesian clustering analysis carried out by Rossiter *et al.* (2007) with STRUCTURE version 2.2 (Pritchard *et al*. 2000), but we extended this to force assignment of individuals to clusters at values of K beyond the number considered to maximize the posterior probability of the data $P(K|X)$. This approach can be used to reconstruct the hierarchical relationship among populations, as well as to distinguish between historical processes that are likely to shape this structure (Rosenberg *et al*. 2005; Wang *et al*. 2007). We undertook 10 replicate runs of STRUCTURE for each value of K, from $K = 2$ to 16. We applied the admixture model with a burn-in of 30 000 and a run length of 10^6. In order to sort our results, we derived symmetric similarity coefficients (SSC) among replicate runs within each value of K, following Wang *et al*. (2007). This was undertaken using the Greedy algorithm of CLUMPP (Jakobsson & Rosenberg 2007) in which groups of runs with an SSC ≥ 0.8 were identified and combined. Summary outputs for each value of K were then displayed graphically using the software DISTRUCT (Rosenberg 2004).

Results

Mitochondrial DNA gene diversity and population structure

In total, 31 unique haplotypes based on 1098 bp of the *ND2* gene (GenBank Accession nos FJ394385–FJ394545) were identified from 161 greater horseshoe bats (*Rhinolophus ferrumequinum*) sampled from 45 different locations (Table 1) A total of 109 polymorphic sites (9.9%) was recorded, of which 96 (8.7%) were parsimony-informative with a transition/transversion ratio of 30.07.

Haplotype diversity (h) showed clear differences among populations (Table 2). In Europe, no variability was seen in 7 of the 11 populations sampled (Table 2, Fig. 2a). European populations with more than one haplotype were England, France and Spain (2 haplotypes each) and Bulgaria (3 haplotypes). All haplotypes were separated by a single base pair and, with the exception of England, the main

Table 2 Genetic variability in 27 populations of *Rhinolophus ferrumequinum* based on 1098 bp of the mtDNA gene *ND2*. Sample size (*n*), number of haplotypes observed, number of polymorphic sites, mean number of pairwise differences among sequences, haplotype diversity (*h*) and nucleotide diversity (π) are shown

Population	*n*	Haplotypes observed	Polymorphic sites	Mean no. of pairwise differences	*h*	π
Japan	15	3	2	0.267	0.257	0.00024
Jilin (China)	1	1	—	—	—	—
Beijing China)	12	4	6	1.379	0.455	0.00126
Shenxian (China)	9	3	2	0.611	0.556	0.00056
Wanfu (China)	1	1	—	—	—	—
Xiaya (China)	2	2	20	20	1	0.01821
Youkuang (China)	1	1	—	—	—	—
Emei Shan (China)	1	1	—	—	—	—
Foping (China)	1	1	—	—	—	—
Nanton Village (China)	1	1	—	—	—	—
Bulgaria	9	3	2	0.611	0.556	0.00056
England	26	2	1	0.148	0.148	0.00013
France	13	2	1	0.154	0.154	0.00014
Greece	2	1	—	—	—	0
Italy	9	1	—	—	—	0
Portugal	3	1	—	—	—	0
Sardinia	1	1	—	—	—	—
Slovenia	2	1	—	—	—	0
Spain	7	2	1	0.286	0.286	0.00026
Switzerland	1	1	—	—	—	—
Turkey (NW)	1	1	—	—	—	—
Cyprus	2	2	11	11	1.000	0.01002
Iran	6	3	3	1.267	0.733	0.00115
Syria	21	6	15	3.638	0.776	0.00331
Turkey (SE)	5	1	—	—	—	0
Russia	8	2	1	0.536	0.536	0.00049
Morocco	1	1	—	—	—	—

European haplotype was found in each of these populations (Fig. 2a). In west Asia, 10 haplotypes were observed overall, with variability in 4 of the 5 populations, and most diversity recorded in Syria (6 haplotypes). In east Asia, populations were characterized by similar levels of diversity to those of west Asia. In China, 11 haplotypes were recorded in 10 populations, while in Japan, 3 haplotypes were found in the single population sampled (Fig. 2b). Similar trends were also seen for nucleotide diversity and numbers of pairwise differences (Table 2).

AMOVA revealed significant genetic variance at all three hierarchical levels tested (among regions, among populations/within regions and within populations) ($P < 0.001$), although greatest variation was seen among regions (93.44%) (Table 3). Pairwise genetic distance plotted against geographical distance indicated significant isolation by distance across the species' range (Mantel test: $b = 23.29 \pm 3.90$, $R^2 = 0.241$, $P < 0.0001$), with a sharp increase in gradient attributable to comparisons among localities from the main geographical regions (Europe, west Asia, central China, east China and Japan) (Fig. S1, Supporting information).

Phylogenetic and demographic analysis

Neighbour-joining (Fig. 3) and Bayesian analyses (not shown) produced highly concordant trees each revealing that greater horseshoe bats form a monophyletic lineage with respect to the congeneric species *R. cornutus* (GenBank Accession no. AB061526). In both trees, haplotypes grouped into two major clades (Europe/Morocco with west Asia vs. China and Japan). Within these clades, there was high bootstrap support (> 87%) for five main lineages: Europe/Africa, west Asia, east China, Japan (with two samples from east China) and central China.

Demographic analyses carried out for each geographical region revealed contrasting population histories. Separate mismatch distributions based on west Asia and east China were characterized by multiple peaks, indicative of stable populations. In contrast, separate mismatch distributions for Europe, Japan and Russia (see Fig. S2, Supporting information) failed to reject the model of a population expansion ($P_{SSD} > 0.05$) based on the sum of squared deviations (SSD). Russia and Japan were also rejected based on the raggedness index ($P_R > 0.05$). Timings of expansion

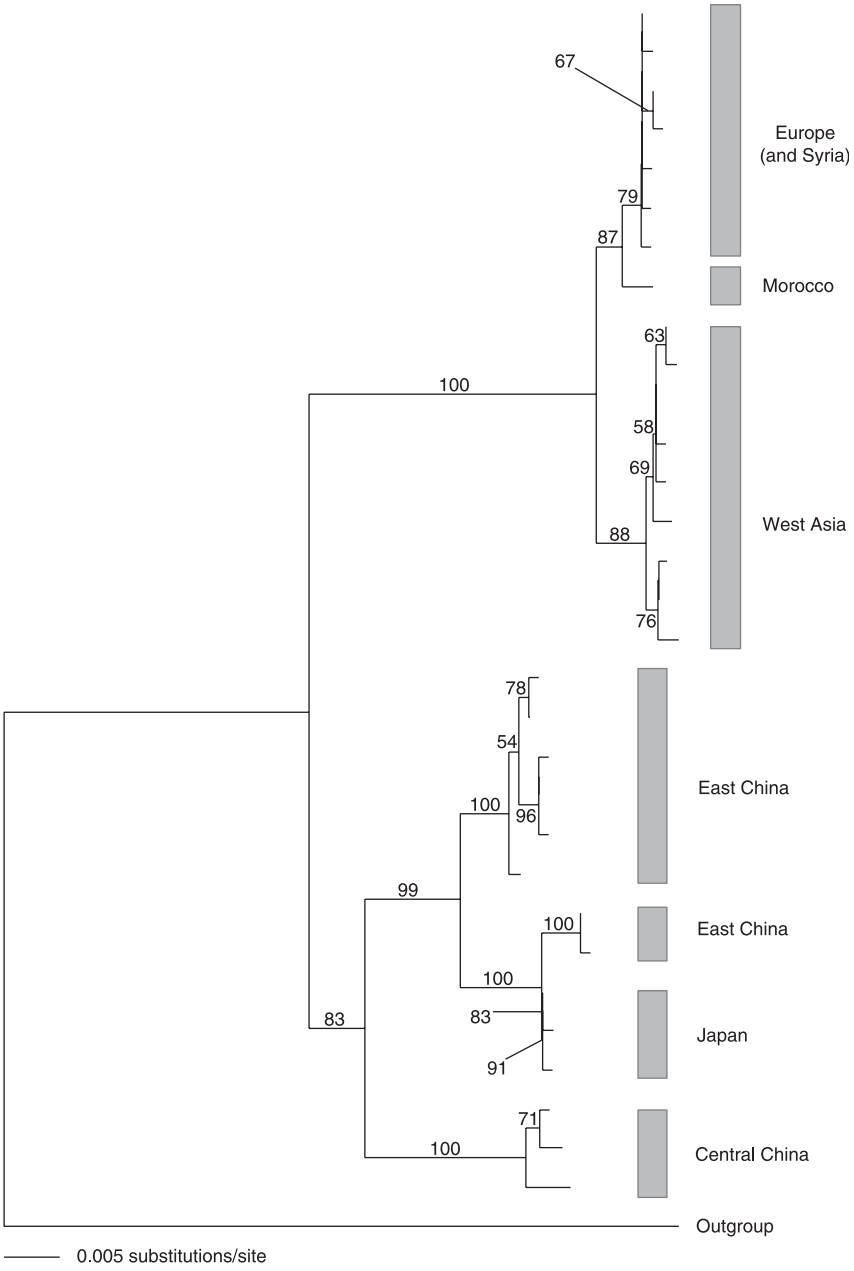

Fig. 3 Neighbour-joining tree of greater horseshoe bat *ND2* haplotypes. Numbers on branches indicate the percentage bootstrap support. The outgroup is *Rhinolophus cornutus pumilus*.

Table 3 Hierarchical analysis of molecular variance (AMOVA) among mtDNA *ND2* gene region of greater horseshoe bats with different geographical groupings. Regions chosen: Europe, west Asia, central China, east China and Japan

Structure	Source of variation	Variation (%)	Fixation indices	*P* value
Five major regions	Among regions	93.44	Φ_{CT} 0.680	< 0.001
	Among populations/within regions	4.46	Φ_{SC} 0.979	< 0.001
	Within populations	2.10	Φ_{ST} 0.934	< 0.001

calculated from these distributions were *c*. 40 000–60 000 years before present (BP; 95% CI 24 000–101 000 BP) for Europe, *c*. 14 000–81 000 BP (0–154 000) for Russia and 127 000–191 000 BP (22 000–265 000) for Japan.

Our minimum spanning network analyses yielded three subnetworks (Europe/west Asia/Morocco, central China and east China/Japan) based on a 95% statistical parsimony threshold. Reducing this threshold to 25% resulted in a single

network containing two main clades (Europe/Morocco/west Asia and east Asia), which were separated by > 50 mutational steps (Fig. 4). Large numbers of mutational steps were recorded between east China and central China (≥ 30 steps) and between Europe and west Asia (9 steps). We also found evidence of additional deep structure within China; the haplotype from Foping (Shaanxi province) was separated from haplotypes from Emei Shan (Sichuan province) and Nanton (Sichuan province) by 6 and 7 steps, respectively. Haplotypes from Japan showed the same unexpected affiliations with two haplotypes from east China (Xiaya and Jilin), as also found in the phylogenetic trees.

Microsatellite analysis

Forced clustering of individuals based on their microsatellite multilocus genotypes revealed substantial hierarchical structure among populations across the range (summarized in Fig. S3, Supporting information). At $K = 2$, clustering recovered two major groups (Europe/Morocco/west Asia and east Asia), which also corresponded to the two main subnetworks of the haplotype network (Fig. 4a). At $K = 3$, the UK formed an additional distinct cluster from mainland Europe/west Asia. Mainland Europe was observed to separate into two clusters at $K = 4$, represented by west Europe and east Europe/west Asia, with evidence of a cline in membership between these clusters. An additional division was detected between east Europe and west Asia at $K = 5$, although most individuals were seen to have partial membership of both of these groups. Individuals from Russia showed least similarity with the east European individuals, which was further supported by the separation of the Russian population into its own cluster at $K = 6$. Subsequent increments in the number of clusters recovered structure between central China and the rest of east Asia ($K = 7$), the separation of Switzerland within Europe ($K = 8$), and the separation of the Dura Europos region of Syria ($K = 9$). Patterns of clustering for runs of K between 10 and 14 were generally uninformative. At $K = 15$ a new cluster emerged within the UK, corresponding to the samples from Wales that were previously shown to be isolated from other colonies in the UK (Rossiter *et al*. 2007).

To compare population structure between the two markers, the results of the microsatellite clustering analysis for different values of K were combined with the mtDNA haplotype network (Fig. 4). Circles representing individuals with the same haplotype were also colour-coded based on the proportional membership of those individuals to the microsatellite clusters. This was repeated for increasing values of K to reveal differences in population structure as defined by the two markers.

Discussion

Most large-scale phylogeographical studies are based on a single mtDNA gene (Moore 1995; Avise 2000), with hypervariable markers often considered undesirable due to higher levels of homoplasy (Hewitt 2004a). To reconstruct the phylogeographical history of the greater horseshoe bat, we sequenced mitochondrial haplotypes from individuals sampled across the species' range. We then compared our results to those that we obtained from spatially matched microsatellite genotypes. We show that combining both data sets provides a unique insight into this species' history, and that without this multigene approach, valuable information would have been lost.

Population history inferred from mtDNA

Phylogenetic and demographic analyses of European and west Asian mtDNA sequences revealed that Europe is mostly made up of a single haplotype, and this haplotype spread throughout Europe around 40 000–60 000 years ago by a single population expansion from a west Asian refugium. This expansion thus took place before the Last Glacial Maximum (LGM) and corresponds to the Middle Pleniglacial (29 000–60 000 BP), one of the warmest periods of the Last Pleniglacial (15 000–72 000 BP) (Guiter *et al*. 2003). High nucleotide and allelic diversity, and mismatch analysis, all point towards there also being one or several refugia in west Asia, from which an initial population expansion occurred.

Estimated divergence times and evidence of a rapid demographic expansion in the Russian sample indicate that populations in west Asian might also have seeded the colonization of populations outside of Europe, supporting the emerging view that Asia Minor and eastern Europe served as ancient glacial refugia and reservoirs of genetic and species diversity (Taberlet & Bouvet 1994; Bilton *et al*. 1998; Taberlet *et al*. 1998; Jaarola & Searle 2002; Durka *et al*. 2005; Bilgin *et al*. 2006). However, the limited sample size prevents a precise timing of this split (*c*. 14 000–81 000 BP), and thus further sampling is needed before this expansion can be reliably placed before or after the LGM.

In comparison to Europe and America, relatively little is known about the extent to which parts of east Asia acted as refugia for temperate species during episodes of glaciation. However, our findings of high nucleotide diversity and marked genetic subdivision do point to multiple glacial refugia in this region. Indeed, while broad-scale sampling precludes the accurate identification of the number and location of east Asian refugial populations, deep divergence between central China and east China/Japan points to at least two refugia. Our mismatch analysis indicates that the colonization of Japan by greater horseshoe bats was the result of single population expansion from the

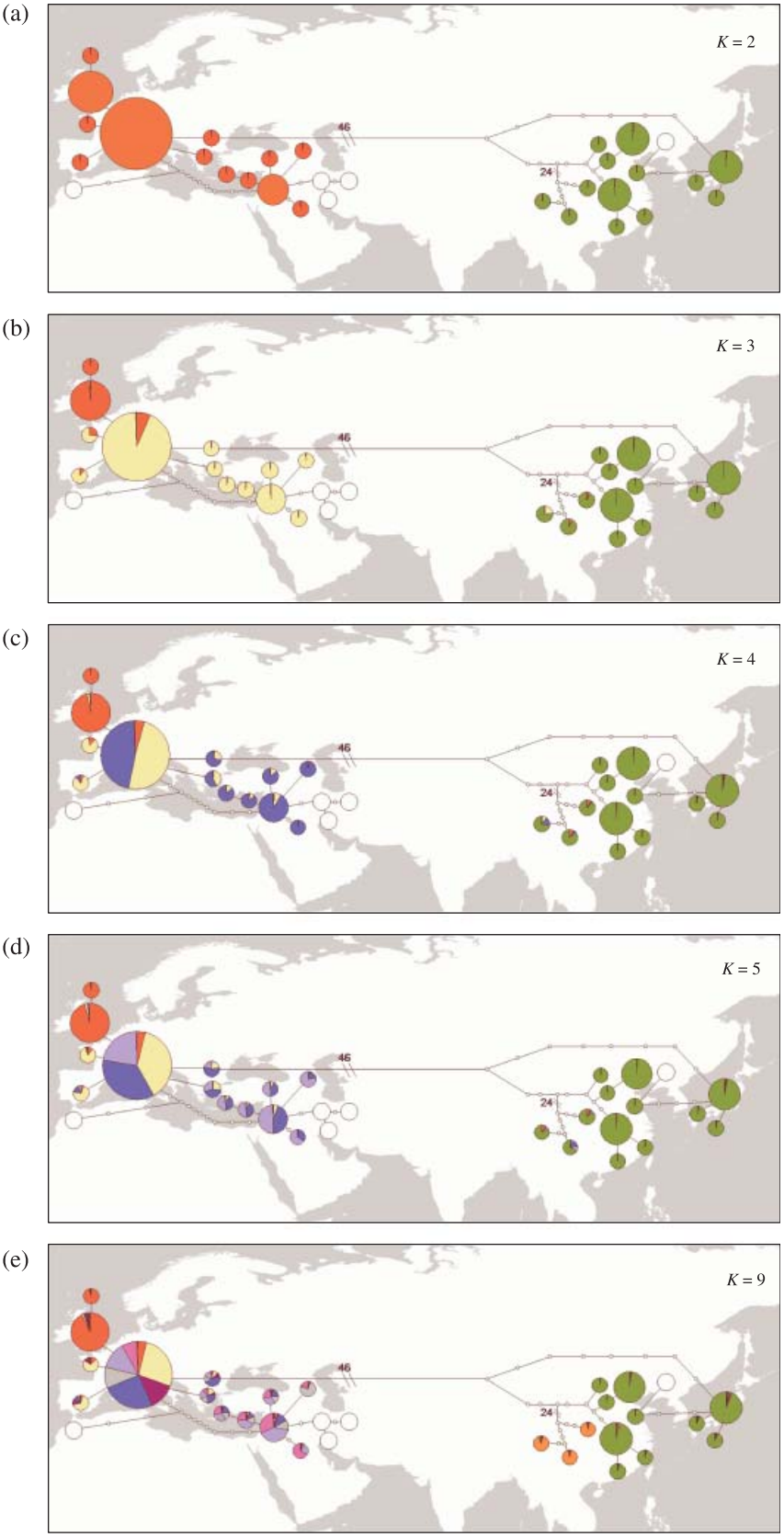

Fig. 4 Combined minimum spanning network (MSN) for 161 greater horseshoe bats based on 1096 bp of ND2. Circle size represents the frequency of haplotype, and circles are placed over the main region where the individuals were sampled. The presence of the main European haplotype in four individuals from syria is not shown. Each circle is colour-coded to represent the proportional membership of individuals sampled at the same locality to microsatellite based clusters inferred from STRUCTURE for (a) $K = 2$, (b) $K = 3$ (c) $K = 4$, (d) $K = 5$, and (e) $K = 9$. Unfilled circles were not sampled in the microsatellite analysis.

Table 4 Pairwise point estimations of genetic divergence between monophyletic groups of greater horseshoe bats based on 1096 bp of *ND2*. The numbering of two individuals from east China (9 and 6) refers to their sampling localities (Table 1, Fig. 2b)

Monophyletic group comparison	Divergence (%)
West vs. east	5.35
Europe + Morocco vs. west Asia	1.16
East China + east China/Japan vs. central China	3.45
East China vs. east China/Japan	1.64
East China (9 + 6) vs. Japan	0.47

comparatively more stable populations of east China (127 000–191 000 BP). This appears to have occurred before the colonization of Europe and Russia and corresponds roughly to the Eemian interglacial (116 000–130 000 BP) (Kukla *et al*. 2002), when the climate was similar to present-day conditions. Our findings also support the theory that Japan was not connected to mainland China during the LGM (Park *et al*. 2000) and acted as a separate refugium during this time. These results therefore appear to support those of Park *et al*. (2000), who argued that Japan was not connected to mainland China during the LGM on the basis of radiocarbon dating of sediments.

In spite of these results, the clustering of two haplotypes from east China with those from Japan, in both the neighbour-joining tree and haplotype network, raises questions on the taxonomic distinctiveness and isolation of these populations. Indeed, the sequence divergence between the two populations (0.47%), compared to the divergence between west Asia and Europe (1.16%) (Table 4), suggests that some recolonization has occurred since the Eemian, and possibly since the LGM. If recolonization has taken place, then the large distance between Japan and east China (presently *c.* 800 miles) would have precluded direct dispersal without a land bridge. A more likely route would have been via South Korea (currently *c.* 160 miles from Japan), possibly assisted by natural events such as a typhoons, which are common in the region. Further sampling is needed in northeast China and Korea before colonization patterns in this area can be fully resolved, as well as rule out other explanations such as incomplete lineage sorting since their separation, or, given the ancient timing of these events, homoplasious mutations. However, limited phenotypic data do also appear to support an expansion from Japan to east China. Specifically, the bat sampled in Jilin in east China was larger than the other Chinese bats and echo-located with a dominant call frequency of approximately 65 kHz, 10 kHz lower than observed for other Chinese bats in the area (G. Jones, unpublished data) but similar to individuals of the subspecies *R. f. nippon* recorded from Japan (Taniguchi 1985; Fukui *et al*. 2004).

Comparison of mtDNA with microsatellite data

Large-scale population structure based on mtDNA haplotypes, with clear genetic divisions among the regions of Europe, west Asia, central China, east China and Japan, broadly agreed with the results of an earlier microsatellite study (Rossiter *et al*. 2007) as well as our re-analyses of this data set. Our comparisons show that the deepest phylogenetic split among the haplotypes, evident from both the neighbour-joining tree and haplotype analyses, corresponds exactly to microsatellite-based structure when genotypes are forced into two clusters (Fig. 4a). Both markers thus support the scenario that European and west Asian populations share a common history.

However, when these regions were considered separately, we found clear differences in the phylogeographical signal obtained from the two sets of markers. For example, while the large-scale separation of the east Asia vs. other populations is maintained following the introduction of a third microsatellite-based cluster, concordance between the markers is seen to breakdown within the Europe/west Asia clade. These similarities and discrepancies between the data sets together clarify the history of this species in Europe and elsewhere. Indeed, at $K = 3$, bats in this clade become separated into two geographically defined clusters (Fig. 4b), the first of which contains populations from continental Europe and west Asia, while the second contains the populations from the UK (Fig. S3). Despite this clear division, however, the haplotypes of these bats were only separated by one, or in a few cases, two mutational steps. Together, these apparently contradictory results provide good evidence that the post-LGM arrival of this species in the region that is now the UK resulted in a founder effect, which has probably been compounded by subsequent genetic drift in isolation following the establishment of the English Channel.

Additional discordance at $K = 4$ provides valuable insight into broader patterns of postglacial colonization across continental Europe. Here, a lack of variation and structure in the mtDNA sequence data, together with the wide distribution of a haplotype also seen in Syria, support a rapid expansion from a single refugium in west Asia, rather than from within Europe itself. At the same time, however, bats in Europe found to share the same haplotype also fall into two major microsatellite clusters, one from west and east Europe (Fig. 4c and Fig. S3). Thus, bats with the same haplotype appeared to have retreated into separate eastern and western European refugial populations, before postglacial expansion led to the formation of a suture zone in central Europe (see Rossiter *et al*. 2007). These results strongly support two expansion events. The first, evident from the low mtDNA diversity, was an ancient event that occurred during the interglacial period when global warming led to range expansions for many taxa (for a review of climatic changes in western Europe see Guiter *et al*. 2003).

The timing of this expansion was also supported by our estimate from the mismatch distributions. More recently, when most of Europe was covered by ice and tundra, greater horseshoe bat populations were forced into at least two refugial regions, in southwest Europe (Iberia and/or Italy) and southeast Europe (Balkans/Greece) (Hewitt 1999), before re-expanding since the LGM. Thus, the mtDNA haplotype homogeneity in this species in Europe reflects a common ancestry that predates the LGM, and thus contrasts with numerous other taxa in which refugial areas and postglacial colonization routes have been inferred from deep structure in mtDNA within Europe (Hewitt 1999). In fact, although mtDNA sequence data provide limited information on the re-colonization of Europe by greater horseshoe bats after the LGM, microsatellite loci show that this followed a similar trend of other European biota (Rossiter *et al*. 2007).

At high values of *K*, additional new clusters were recovered within Europe and west Asia, corresponding to Switzerland, Russia, Dura Europos and, at $K = 15$, Wales (Fig. 4e and Fig. S3). Given that each of these clusters appears to represent a discrete discontinuous jump across a relatively small geographical distance, and with little evidence of a cline in cluster membership, it seems likely that such population subdivision has arisen from either the presence of a geographical barrier to dispersal (see Rosenberg *et al*. 2005) or via a bottleneck that has led to accelerated drift (Rossiter *et al*. 2007).

In east Asia, comparisons of structure defined by haplotype and microsatellite data showed further discordance. In particular, we found that in spite of several mutational steps among haplotypes recorded in this region, all samples from east China and Japan fell into the same microsatellite cluster, even at high values of *K* (Fig. 4 and Fig. S3). Although Rossiter *et al*. (2007) reported probable homoplasy at microsatellite loci sampled from Japan and mainland China, it seems unlikely that this could account for the extent of the apparent homogeneity. Indeed, no clustering was found at relatively large geographical distances, contrasting with deep structure recorded within Europe and west Asia. One plausible explanation for this lack of structure is that the greater horseshoe bats in this region belong to a population that was historically very large, and thus insufficient time has passed for drift to take place since these populations became isolated from each other. The main exceptions to this pattern were several bats sampled in central China, which formed their own cluster at $K = 7$ and also showed a high number of mutational steps from haplotypes distributed further east (Fig. 4). This concordance appears to provide good evidence of at least two refugial populations in east Asia.

An alternative mtDNA marker that might have proven more useful for reconstructing postglacial history is the control region, which contains hypervariable (HV) domains characterized by more rapid rates of mutation than *ND2*. Although widely used (Petit *et al*. 1999; Van Hooft *et al*. 2002; Ruedi & Castella 2003; Durka *et al*. 2005; Bilgin *et al*. 2006), the control region also has limitations, such as R2 repeats (Fumagalli *et al*. 1996) that can reduce the length of the comparable sequences. For example, a study of the impact of ice ages on the greater mouse-eared bat (*Myotis myotis*) was only able to use 307 bp of the HVII control region due to R2 repeats (Ruedi & Castella 2003). It is also doubtful that HV domains would have resolved population substructure in greater horseshoe bats to the same degree as microsatellite markers. Notably, Ruedi & Castella (2003) found the same haplotype in 207 (43%) bats sampled across Europe, despite intensive sampling, and speculated that the Middle East was a source population for eastern Europe. Our study, which is based on greater geographical coverage but less intensive sampling (for examples of similar sampling regimes based on fishes, see Kotlík & Berrebi 2001; Culling *et al*. 2006), was able to confirm the importance of west Asian populations. Ruedi & Castella (2003) also found low microsatellite differentiation among their samples (based on 10 markers), which they attributed to contemporary gene flow, and concluded that mtDNA better reflect past events than microsatellites. While our findings show that this mtDNA certainly does better reveal ancient events, microsatellites certainly do offer distinct advantages for examining the genetic consequences of the relatively recent LGM. Indeed, previous studies have also shown that, due to their high levels of polymorphism, microsatellites are able to resolve in detail recent colonization histories in Europe, whereas mtDNA and allozymes were unable to do so (e.g. Queney *et al*. 2001).

Conclusions

Our results have important implications for phylogeographical studies. We show that patterns of structure described by mtDNA and microsatellite data can yield conflicting phylogeographical signals. In our study, only by combining the data sets were we able to reconstruct the species' history through multiple glacial periods. Furthermore, by analysing samples from Europe, we are able to assess the relative importance of European vs. Asian refugia. With growing numbers of phylogeographical studies underway, our findings therefore serve as an important cautionary reminder of the limitations of only using one genetic marker or markers with similar rates of mutation and modes of inheritance. Our results also support other studies (e.g. Rosenberg *et al*. 2005) that have demonstrated that the value and power of clustering programs such as STRUCTURE in analysing population relationships go beyond searching for the maximum posterior probability of the data. These approaches can add to a general understanding of the demographic history of a species, which can then be teased apart and tested with

other methods such as mtDNA sequencing. Finally, the wide distribution of the greater horseshoe bat is broadly mirrored by many other European taxa (Corbet 1978). We look forward to wide-scale phylogeographical studies of these codistributed taxa to determine whether the trends reported in this study hold more widely.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Fig. S1 Plot of genetic vs. geographical distance for pairwise population comparisons using Slatkin's linearized Φ_{ST} to calculate genetic distance on population sizes > 2.

Fig. S2 Mismatch distribution of *Rhinolophus ferrumequinum* for different geographical groupings (a) east China (b) Japan (c) west Asia and (d) Europe. Solid black lines indicate the observed frequency of pairwise distributions, dashed black lines indicate the expected Poisson distribution under a model of population expansion and the solid grey lines indicate the upper and lower confidence intervals for the expected. Russia is not shown as it has too few pairwise differences.

Fig. S3 Estimated population structure. Each individual is represented by a vertical line which is partitioned into K coloured segments, the length of each colour being proportional to the estimated membership coefficient (Q). Black lines separate individuals of different populations as indicated by the labels at the bottom of the figure. Labels above the figure indicate the regional affiliation of each population.

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