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Mitochondrial DNA reveals a strong phylogeographic structure in the badger across Eurasia

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Abstract

The badger, *Meles meles*, is a widely distributed mustelid in Eurasia and shows large geographic variability in morphological characters whose evolutionary significance is unclear and needs to be contrasted with molecular data. We sequenced 512 bp of the mitochondrial DNA control region in 115 Eurasian badgers from 21 countries in order to test for the existence of structuring in their phylogeography, to describe the genetic relationships among their populations across its widespread geographic range, and to infer demographic and biogeographic processes. We found that the Eurasian badger is divided into four groups regarding their mitochondrial DNA: Europe, Southwest Asia, North and East Asia, and Japan. This result suggests that the separation of badgers into phylogeographic groups was influenced by cold Pleistocene glacial stages and permafrost boundaries in Eurasia, and by geographic barriers, such as mountains and deserts. Genetic variation within phylogeographic groups based on distances assuming the Tamura–Nei model with rate heterogeneity and invariable sites ($d_{T\text{-}N}$ range: 3.3–4.2) was much lower than among them ($d_{T\text{-}N}$ range: 10.7–38.0), and 80% of the variation could be attributed to differences among regions. Spatial analysis of molecular variance (SAMOVA), median-joining network, and Mantel test did not detect genetic structuring within any of the phylogeographic groups with the exception of Europe, where 50% of variation was explained by differences among groups of populations. Our data suggest that the European, Southwest Asian, and North and East Asian badgers evolved separately since the end of Pliocene, at the beginnings of glacial ages, whereas Japanese badgers separated from continental Asian badgers during the middle Pleistocene. Endangered badgers from Crete Island, classified as *Meles meles arcalus* subspecies, were closely related to badgers from Southwest Asia. We also detected sudden demographic growth in European and Southwest Asian badgers that occurred during the Middle Pleistocene.

Keywords: control region, Eurasia, *Meles*, mitochondrial DNA, phylogeography, postglacial colonization

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Introduction

The Eurasian badger, *Meles meles*, is classified within the mustelid subfamily Melinae (Wozencraft 1993; Macdonald 2001) where it is closely related to the hog badger, *Arctonyx collaris* (Bryant *et al*. 1993). During the Pliocene, the *Meles* lineage evolved in the temperate forests of Asia, spreading west into Europe between the Late Pliocene and the Early Pleistocene (Neal & Cheeseman 1996). Badger forms very similar to the modern Eurasian badger are found in the fossil record of Eurasia since the Middle Pleistocene (Kurten 1968; Petter 1971).

At the present time, the Eurasian badger is one of the most widely distributed mustelids. Its geographic range

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includes forested and steppe areas in the Palearctic region from the Iberian Peninsula in the west to the Japanese archipelago in the east; and from Scandinavia and west Siberia in the north to Palestine, Iran, south China and Tibet in the south (Heptner *et al*. 1967; Corbet 1978). Insular populations exist in Ireland, Britain, Sicily, Crete, Rhodes, and Japan (Corbet 1978). Considerable differences in size, coloration, and morphological characters are reported across its geographic range. Main morphological differences are found in dentition (e.g. the frequency of loss of first premolars, and the shape and proportions of first upper molar) and bones (e.g. skull and bacular structure) (Baryshnikov & Potapova 1990; Lynch *et al*. 1997; Abramov 2002).

Molecular data for badgers are scarce, but high levels of genetic differentiation across regions were found comparing complete cytocrome *b* sequences from 17 Japanese, one Siberian and two European badgers (Kurose *et al*. 2001). Locally, badger populations can show from moderate levels of genetic variability (Bijlsma *et al*. 2000, using microsatellites in The Netherlands and Denmark) to reduced variability (Pertoldi *et al*. 2000, in Denmark using allozymes; Domingo-Roura *et al*. 2003, in the UK using microsatellite markers).

The species has been killed for meat, fur, hair, and for being a wild host of *Mycobacterium bovis*, which causes bovine tuberculosis (Neal & Cheeseman 1996; Gallagher & Clifton-Hadley 2000). The Eurasian and hog badgers are classified as 'Least Concern' by the International Union for the Conservation of Nature and Natural Resources (IUCN). Where they are monitored, Eurasian badger populations appear to be either stable or increasing in Europe, although there are threats of local extinction in the Netherlands and Albania (Griffiths & Thomas 1997). Populations from the islands of Crete and Rhodes, which have been classified as different subspecies (*Meles meles arcalus* and *Meles meles rhodius*, respectively) from continental badgers, are considered 'Vulnerable' (Karandinos 1992). The badger is strictly protected and considered 'Endangered' in Albania (Zamir Dedej, personal communication). There is little information about the status of the species in most parts of continental Asia where, in general, it can be hunted.

The quantification and distribution of genetic variability, and the understanding of population history and structure, are crucial for improved management and conservation (Avise 1989). Extensive molecular data for Eurasian badger are needed to better understand the differences in morphological characters found in this species and to elucidate if differences described according to morphology correspond to genetic differences. In addition, the species shows variability in ecological adaptations, behaviour and social systems (Kruuk 1989) and the clarification of evolutionary relationships among badgers worldwide can offer insight into the link between ecological similarities and evolutionary relationships, as well as into the co-evolution of the species and its parasites (e.g. *Mycobacterium bovis*; biting louse, *Trichodectes melis*, or fleas, such as *Paraceas melis*) (Neal & Cheeseman 1996).

Phylogeographic studies of mammalian species across Eurasia have begun to emerge (for example grey wolf, *Canis lupus*, Vilà *et al*. 1999; red deer, *Cervus elaphus*, Mahmut *et al*. 2002 and Ludt *et al*. 2004; voles, *Microtus* sp., Jaarola & Searle 2002 and Brunhoff *et al*. 2003). Since the badger is extensively distributed across Eurasia, shows low dispersion rates, and its maternal philopatry has been repeatedly reported (Neal & Cheeseman 1996; Revilla & Palomares 2002) (the two latter characteristics promoting allele fixation and genetic drift (Melnick & Hoelzer 1992)), the regional structuring of its populations can offer insights into colonization routes, barriers to dispersal and glacial refuges at a continental level not hitherto much explored.

The objectives of this report are (i) to describe the genetic variation in badgers across their distribution; (ii) to define phylogeographic groups and check if their mitochondrial sequences are geographically structured and follow a pattern of isolation by distance; (iii) to explore geographic barriers to dispersal in Eurasia; and (iv) to draw inferences on past demographic and biogeographic processes, such as possible population expansions or contractions, of this philopatric and widely distributed carnivore.

Materials and methods

DNA extraction and sequencing

One hundred and fifteen (115) badger samples from 21 countries (considered here as populations) throughout Eurasia were kindly provided by collaborators listed in Table S1 (Supplementary material). Animals came mainly from road kills and were often museum specimens well preserved frozen or in ethanol (none was killed for this project). DNA was extracted from muscle, skin, ear, heart and blood tissues using standard phenol–chloroform protocols (Sambrook *et al*. 1989). Bone and teeth samples (13 out of 15 samples from the Siberian Zoological Museum collected between 1937 and 1974) were powdered in a coffee grinder, which was washed twice with lye and once with 96% ethanol between each sample. DNA from bone and teeth samples was extracted using DNeasy Tissue Extraction Kit (QIAGEN) according to modifications suggested by Iudica *et al*. (2001). To avoid contamination, extractions were done at a separate pre-PCR (polymerase chain reaction) room. Aerosol-barrier pipette tips were used. One sample was handled at a time and negative controls were included in the extraction procedure.

We amplified the complete mitochondrial DNA (mtDNA) control region in 16 samples using primers L15926 and H00651 (Kocher *et al*. 1989) and L-Pro and H-Phe (Mucci *et al*. 1999). We amplified seven more complete control regions with the pair L-Promel (5'-AATAGCCCCACCAT-

CAGCACCCAAAGC-3′; modified from L-Pro) and H-Phe. Using these 23 sequences, we designed a new pair of primers specific for Eurasian badger to amplify a fragment of 594 bp from the 5′ end of the control region which were named *Mel*CR1 (5′-AGCACCCAAAGCTGATATTCT-3′) and *Mel*CR6 (5′-CCATTGACTGAATTGCACCT-3′). We amplified three overlapping control region DNA fragments from 221 to 252 bp in bone and teeth samples using the following primer combinations: *Mel*CR1 and *Mel*CR2 (5′-CAAGGATTGATGGTTTCTCG-3′); *Mel*CR3 (5′-TGCATTTCACTTAGATCACGAG-3′) and *Mel*CR4 (5′-TACCAAATGCATGACACCAC-3′); and *Mel*CR5 (5′-TCTTCAAATGGGACATCTCG-3′) and *Mel*CR6. Thus, replication also was provided by the overlapping positions in the control region segments. PCRs contained between 100 and 400 ng of genomic DNA, 16.6 mM $(NH_4)_2SO_4$, 67.0 mM Tris-HCl (pH = 8.8), 0.01% Tween-20, 2.5 mM $MgCl_2$, 2.5 mM of each nucleotide, 4.25 pmol of each primer and 0.85 unit of *Taq* DNA polymerase (Ecogen). PCR programmes to amplify the complete control region started with a cycle of denaturing at 94 °C for 5 min, followed by 30 cycles divided in three steps of 1 min each (denaturing at 94 °C; annealing at 61 °C for L15926-H00651 and L-Pro–H-Phe, and at 68 °C for L-Promel–H-Phe; extension at 72 °C), and a final extension at 72 °C for 5 min. We used steps of 45 s and an annealing temperature of 59 °C for *Mel*CR1-*Mel*CR6. We also used 35–40 cycles of steps of 45 s and an annealing temperature of 55 °C to amplify the three short fragments in bone and teeth samples. A negative control was added in PCRs performed in these samples to detect possible contamination. PCR products were purified with Geneclean (Qbiogene), sequenced with BigDye Terminator Cycle Sequencing Kits (Applied Biosystems) and precipitated following the instructions of the manufacturer. Precipitates were run on an ABI3100™ automated DNA sequencer (Applied Biosystems).

Data analyses

Sequences were visualized with BIOEDIT Sequence Alignment Editor version 5.0.9 (Hall 1999), aligned with the CLUSTALW option included in this software and double-checked by eye. Number of polymorphic sites, transitions and transversions, and haplotype (h) and nucleotide (π) diversities were obtained with ARLEQUIN version 2.000 (Schneider *et al*. 2000). The Tamura–Nei model with rate heterogeneity and invariable sites ($\alpha = 0.8181$, $I = 0.7920$) was selected as the best-fit model of nucleotide substitution for the molecular data set by the Akaike information criteria approach using MODELTEST 3.6 (Posada & Crandall 1998). Therefore, we used this model and parameters for inferring distance matrices using PAUP* version 4.0b10 (Swofford 2002). To visualize differences among regional groups, we performed a two-dimensional scaling analysis (Kruskal & Wish 1977) with STATISTICA version 6.0 (StatSoft Inc.). This analysis is based on similarity, dissimilarity and correlation matrices extracted from the average genetic distance matrix between populations.

A median-joining network was performed to explore the phylogenetic relationships of control region haplotypes with NETWORK version 3.1.1.1 (Bandelt *et al*. 1999) downloaded from www.fluxus-engineering.com. Using the substitution model and parameters obtained with MODELTEST 3.6, we constructed maximum-likelihood tree with heuristic searches with 100 random addition replicates and tree-bisection–reconnection branch swapping using PAUP* version 4.0b10. Confidence in the resulting relationships was assessed using 1000 bootstrap replicates, tree-bisection–reconnection branch swapping, and one random addition replicate. We also performed a Bayesian phylogeny estimation using MRBAYES 3.0b4 (Huelsenbeck & Ronquist 2001). We run four chains simultaneously and each Markov chain was started from a random tree and run for 11×10^6 cycles, with sampling every 1000th cycle. Model parameters were treated as unknown variables and were estimated from the data. All sample points prior to reaching stationarity of the Markov chain (the first 1000 trees) were discarded as burn-in values. The whole procedure was repeated three times starting from different random trees and the tree topologies obtained were the same.

We defined groups of populations that are geographically homogeneous and maximally differentiated from each other, and identified genetic barriers between them using spatial analysis of molecular variance, SAMOVA, version 1.0 program (Dupanloup *et al*. 2002). We tested for three and four groups using the whole data set, and for two to seven groups using European samples. The number of initial conditions was 100 in all cases. To statistically test the existence of a pattern of isolation by distance, the correlation between geographic distances and mean genetic distances for each pair of populations was computed using Mantel test included in ARLEQUIN and performing 1000 permutations. Geographic distances were determined in kilometres from the latitudinal and longitudinal coordinates using Haversine geodesic distances (Sinnott 1984).

Mismatch distributions of the whole Eurasian sample and three of the regional groups detected — Europe, Southwest Asia (Crete, Israel, Georgia and Tajikistan) and North and East Asia (Russia, Kazakhstan and Mongolia) — were computed with ARLEQUIN using distances based on the Tamura–Nei model of substitution to detect past demographic expansions. The number of samples available precluded this analysis with Japanese badgers. We performed 1000 bootstrap replications to calculate standard errors. Fu's F_S statistic (Fu 1997) was computed to test for neutrality and demographic expansions with DNASP version 3.99.1 (Rozas & Rozas 1999). Significances for F_S statistics were obtained by means of coalescent simulations of a panmictic

population of constant size conditioning on the number of segregating sites. For each case, 1000 simulations were run in DNASP and the number of trees with values of interest statistic equal or more extreme than the observed value was recorded. The tau (τ) parameter, obtained from the mismatch distribution, is an estimate of time after expansion in mutational units. If the divergence rate per nucleotide and year ($m = 2\mu$, where μ is the substitution rate per lineage) and the number of nucleotides of the fragment analysed (l) are known, it is possible to calculate the age when the expansion occurred using the expression, $\tau = mlt$, modified from Harpending *et al*. (1993).

To estimate the effective maternal population size (N_e) we can assume a divergence rate per nucleotide and year (m) and apply the expression $\theta = N_e m$ modified from Nei (1987). Parameter θ was estimated using program FLUCTUATE version 1.4 (Kuhner *et al*. 1998) by the coalescent Metropolis–Hastings Markov chain (MHMC) method using genealogical relationships among haplotypes, empirical base frequencies, the transition/transversion rate obtained previously for each set of sequences, and assuming historically fluctuating population sizes. A generation time of 1 year was assumed (Neal & Cheeseman 1996).

We estimated a divergence rate from the weasel, *Mustela nivalis*, and ermine, *Mustela erminea*, split. The first extant members of lineages leading to these two species (*Mustela pliocaenica* and *Mustela plioerminea*, respectively) appeared in the Pliocene (about 4 million years ago or Ma) (Anderson 1989). The divergence rate estimated was based on the mean number of substitutions obtained by PAUP* version 4.0b10 (Swofford 2002) from published weasel (GenBank Accession nos: AB006717–28, AB049764–76) and ermine (AB006729–33, AB049777–78, 80, 82–88) control region sequences. The best-fit model of nucleotide substitution for these sequences was the transversional model with rate heterogeneity and invariable sites ($\alpha = 0.8181$, $I = 0.7920$) according to the Akaike information criteria approach using MODELTEST 3.6. The divergence rate estimated was of 1.92×10^{-8} substitutions per nucleotide per year (m_1). In addition, we used the divergence rate of 1.0×10^{-7} substitutions per nucleotide per year (m_2) estimated by Vilà *et al*. (1999) from the split between coyotes, *Canis latrans*, and grey wolves, *Canis lupus*. We also used these divergence rates to calculate divergence times among Eurasian badger phylogeographic groups.

		212234444555555561111111111111111122234444455																				
		002903670345678811112223333344589992281567890 0																				
		13470120478938853695652945272 5	Es	It	Ch	Lu	Uk	Nl	De	At	Pl	Se	No	Fi	Cr	Il	Ge	Tj	Kz	Ru	Mn	Jp
EUROPE	meles-1	ACTCGTGCTCCCCTCCAAAGGGATAGTTTTTGTCCAAAGCACACGGC		2	1	3	3	2	1	1	3											
	meles-2	.................G..............................	1		3		7				3											
	meles-3	.................G..............................			1			3	1	1												
	meles-4	.................G..............................						2	1													
	meles-5	........................C.....................								1												
	meles-6	....................................G.....			2																	
	meles-7	..................G...........C..............												1								
	meles-8	................G.G.......C..................												1								
	meles-9	................G.G.......C.....C..............												1								
	meles-10	................G.................G..........				2																
	meles-11	..........T..C..G..............................				1																
	meles-12	........C.......G.................G...G.....					2															
	meles-13	........C.......G.................G...GT.....				1							8									
	meles-14	........C.......G.........C.........G...G.....										1	1									
	meles-15	........C.......G.........C.........G..........	1			3																
	meles-16	........C.......G.........C........GG..........	4																			
	meles-17	........C.......G.........C.........G....T.....	1																			
	meles-18	........C.................C.........G....T.....			1																	
	meles-19	........C.................C.........G..........	2																			
	meles-20	........C.............................G..........	3																			
SOUTHWEST ASIA	meles-21	.TC.AC.T....G--.G..A.............T...A.G....AT													2							
	meles-22	.TC.AC.T....G--.G..AAA...........T...A.G....AT													1							
	meles-23	.TC.AC.T....G--.G..AA............T...A.G..T.AT													5							
	meles-24	.TC.AC.T....G--.G..AA............TG..A.G....AT														1						
	meles-25	.TC.AC.T....G--.G..AA............TG..A.....AT														1						
	meles-26	.TC.AC.T....G--.G..AA.G..........TG..A.G....AT														1						
	meles-27	.TC..C.T....G--.G..AA.G..........T...A.G....AT																1				
	meles-28	.TC..C.T....G--.G..AA.G..........T....TG....AT														1						
	meles-29	.TC.AC.T....G--.G..AA.G..........T...A.G....A.															1					
	meles-30	.TC.AC.T....G--.G...A.G..........T...A.G....A.														1						
NORTH AND EAST ASIA	meles-31	.TCT.CAT.T--G--.G..A.....C...C...T...C..G...A..																		2		
	meles-32	.TCT.CAT.T--G--.G..A.....C...C...T...C..G.G.A..																	1	4		
	meles-33	.TCT.CAT.T--G--.G..A.....C...C...T.G.C..G.G.A..																		1		
	meles-34	.TCT.CAT.T--G-.....A.....CC..C...T...C..G.G....																			1	
	meles-35	.TCT.CAT.T--G-.....A.....CC..C.......C..G.G....																			1	
	meles-36	.TCT.CAT.T--G--.G..A....GC...C...T...C.........																		2		
	meles-37	.TCT.CAT.T--G-.....A....GC...C...T...C.........																	1	1		
	meles-38	.TCT.CAT.T--G--.G..A...CGC...C...T...C.........																		4		
	meles-39	.TCT.CAT.T--G-.....A...CGC...C...T...C.........																	1	1		
JAPAN	meles-40	TTCT.CAT.T--A-.....A....GC..CC.A.....C.........																				1
	meles-41	TTCT.CAT.T--A-.....A....GC..CC.A.....C..G......																				1
	meles-42	TTCT.CAT.T--A-..G..A....GC..CC.A...G.C.........																				1
	meles-43	TTCT.CAT.T--A-.T...A....GCC.C..A.....C.........																				1

Fig. 1 Polymorphic sites, geographic distribution and frequency of the 43 mtDNA control region haplotypes found in the 115 Eurasian badgers. Dots (·) indicate nucleotide identity with the first sequence and hyphens (-) nucleotide deletions. Sequences are deposited in EMBL/GenBank database with Accession nos AJ563661–AJ563703. Geographic regional groups are indicated: Europe, Southwest Asia (Crete, Israel, Georgia, Tajikistan), North and East Asia (Russia, Kazakhstan and Mongolia), and Japan. Abbreviations: Es, Spain; It, Italy; Ch, Switzerland; Lu, Luxembourg; Uk, United Kingdom; Nl, The Netherlands; De, Germany; At, Austria; Pl, Poland; Se, Sweden; No, Norway; Fi, Finland; Cr, Crete, Il , Israel; Ge, Georgia; Tj, Tajikistan; Kz, Kazakhstan; Ru, Russia; Mn, Mongolia; Jp, Japan.

Results

Genetic variability, phylogeographic groups and population structure

The haplotype distribution (Fig. 1) and median-joining network (Fig. 2) clearly differentiated sequences from three geographic regions: (i) Europe, (ii) Southwest Asia (Crete, Israel, Georgia and Tajikistan), and (iii) North and East Asia (Russia, Kazakhstan and Mongolia) and Japan. Within the last group, Japanese sequences were also clearly, although not as deeply, separated from those of North and East Asia, suggesting a fourth phylogeographic group. The same structuring was also evident with the two-dimensional scaling analysis (Fig. 3), which showed a stress of 0.039, and also in the phylogenetic trees of haplotypes (Fig. 4). Some haplotypes were distributed across a wide geographic range within a single geographic region but none of them was shared across regions (Fig. 1). Twenty-two out of the 43 haplotypes encountered were restricted to a single individual.

Haplotype diversity was high within and across regions or phylogeographic groups, whereas nucleotide diversity within regions was one order of magnitude lower than in the whole Eurasian sample (Table 1). Genetic distances among regions (range: 10.7–38.0) were one order of magnitude higher than distances among samples within one region (range: 3.3–4.2, Table 2). We calculated that badgers from Europe and Southwest Asia diverged from those from North and East Asia and Japan between 2.87 and 0.55

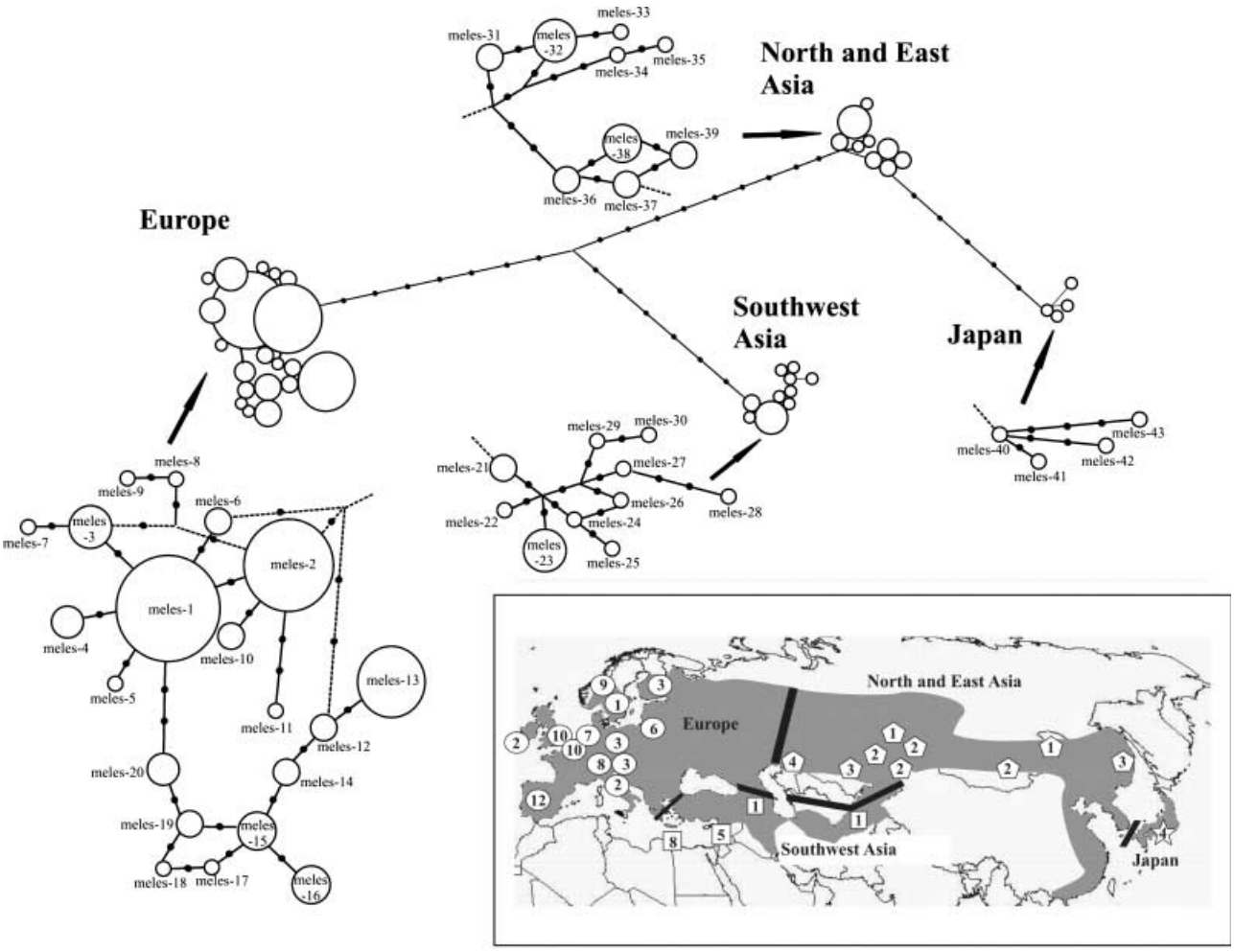

Fig. 2 Median-joining network showing the distribution of Eurasian badger control regions in phylogeographic groups. Circles represent haplotypes and their size is proportional to the frequency observed. Lines connecting circles are proportional to the number of mutations, indicated by black dots. The geographic distribution of Eurasian badger (in grey) is indicated in the map. Black bars indicate the hypothetical geographic boundaries of each phylogeographic group. Numbers within symbols indicate the sample size of localities within each phylogeographic group (Europe, within circles; Southwest Asia, within squares; North and East Asia, within penthagons; Japan, within star).

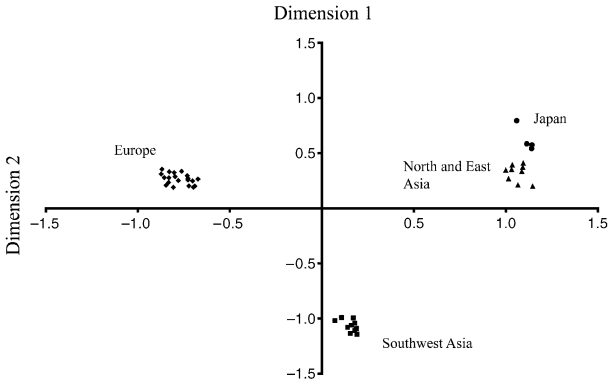

Fig. 3 Two-dimensional scaling analysis based on the distance matrix obtained assuming the Tamura–Nei model with rate heterogeneity and invariable sites ($\alpha = 0.8181$, $I = 0.7920$) between Eurasian badger populations from Europe (rhombuses), Southwest Asia (squares), North and East Asia (triangles) and Japan (circles).

Ma, based on $m_1 = 1.92 \times 10^{-8}$ and $m_2 = 1.0 \times 10^{-7}$ substitutions per nucleotide per year, respectively. Badgers from Southwest Asia diverged from European badgers between 2.37 and 0.45 Ma and Japanese and North and East Asian badgers diverged between 1.09 and 0.21 Ma.

SAMOVA was also consistent with the regional subdivision of samples in four groups (Europe, Southwest Asia, North and East Asia, and Japan) as suggested by the median-joining network, the two-dimensional scaling and the phylogenetic trees. Most probable phylogeographic structures were those with maximum and statistically significant percentages of variation explained by differences among groups (Table 3). This value was maximum when sequences from Europe, Southwest Asia, North and East Asia, and Japan were separated in four different groups (83.18% in partition number 1 of Table 3). In this case, as in partition number 2, percentages of variation explained

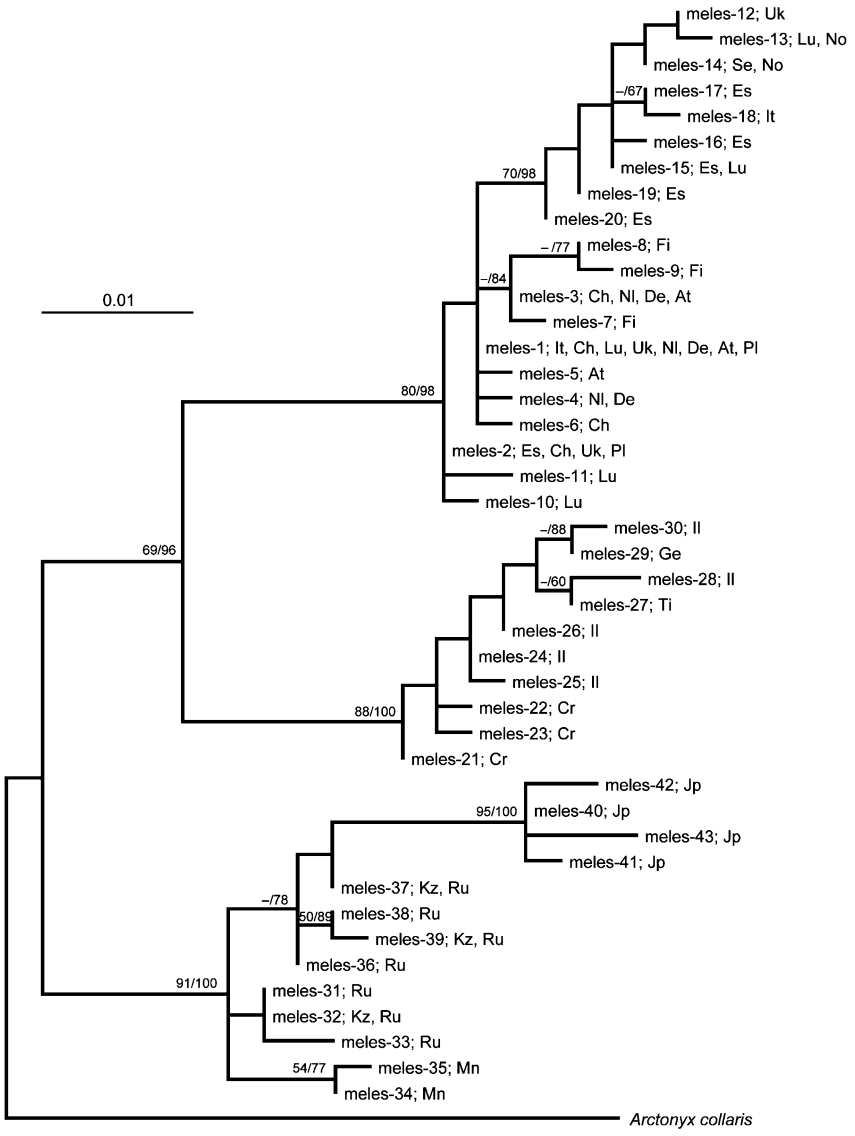

Fig. 4 Maximum-likelihood tree of haplotypes assuming the Tamura–Nei model with rate heterogeneity and invariable sites ($\alpha = 0.8181$, $I = 0.7920$). The same topology was obtained with the Bayesian analyses. The outgroup is *Arctonyx collaris*, the hog badger. Bootstrap and posterior probabilities values higher than 50% are indicated at nodes separated by a slash. The geographic distribution of each haplotype has been included (see Fig. 1 legend for abbreviation and country correspondences).

Table 1 Molecular diversity indices for the 512-bp control region fragment for all Eurasia and for subsets belonging to regional divisions inferred from the data (standard deviations are in parentheses)

	n	No. of haplotypes	Haplotype diversity (*h*)	No. of polymorphic sites	Nucleotide diversity (π)
Eurasia	115	43	0.952 (0.09)	47	0.046 (0.023)
Europe	76	20	0.900 (0.02)	14	0.008 (0.004)
Southwest Asia	15	10	0.895 (0.07)	11	0.005 (0.003)
North and East Asia	20	9	0.905 (0.03)	9	0.007 (0.004)
Japan	4	4	1.000 (0.17)	6	0.006 (0.005)

Table 2 Matrix of distances between the four phylogeographic groups inferred from the data (below diagonal) and their standard errors (above diagonal) assuming a Tamura–Nei model with rate heterogeneity and invariable sites ($\alpha = 0.8181$, $I = 0.7920$). Values within regions and their standard errors, in parenthesis, are shown in the diagonal. All values have been multiplied by sequence length (*l*)

	Europe	Southwest Asia	North and East Asia	Japan
Europe	4.2 (1.9)	3.5	4.3	4.7
Southwest Asia	23.3	3.4 (1.4)	2.8	2.8
North and East Asia	31.4	25.1	4.0 (2.3)	2.9
Japan	38.0	34.0	10.7	3.3 (1.7)

Table 3 Population structures inferred by spatial analysis of molecular variance (SAMOVA). Percentages of variation explained by the groupings are indicated. Statistical significance is indicated with asterisks (**$P < 0.01$, *$P < 0.05$). Values of most probable population structures are indicated in bold. Four and three partitions were tested for all data (1 and 2) and two to seven partitions were tested within Europe (3–8)

Partition		Among groups	Among populations within groups	Within populations
1.	[Europe] [Crete, Israel, Georgia, Tajikistan] [Russia, Kazakhstan, Mongolia] [Japan]	**83.18****	**6.86****	**9.96****
2.	[Europe] [Crete, Israel, Georgia, Tajikistan] [Russia, Kazakhstan, Mongolia, Japan]	**81.45****	**8.64****	**9.91****
3.	[Spain, Ireland, Great Britain, Italy, Switzerland, Germany, Luxembourg, the Netherlands, Austria, Finland, Poland] [Norway, Sweden]	48.08**	18.09**	33.83*
4.	[Ireland, Great Britain, Italy, Switzerland, Germany, Luxembourg, the Netherlands, Austria, Finland, Poland] [Spain] [Norway, Sweden]	50.54**	9.86**	39.59**
5.	[Spain] [Norway, Sweden] [Ireland, Great Britain, Italy, Switzerland, Germany, Luxembourg, the Netherlands, Austria, Poland] [Finland]	51.91**	7.22**	40.87**
6.	[Ireland, Great Britain, Italy, Switzerland, Germany, Luxembourg, the Netherlands, Austria, Poland] [Spain] [Sweden] [Norway] [Finland]	52.33**	6.79**	40.87**
7.	[the Netherlands] [Spain] [Norway, Sweden] [Ireland, Great Britain, Switzerland, Luxembourg, Poland] [Italy, Germany, Austria] [Finland]	51.04**	1.64**	47.31**
8.	[Ireland, Great Britain] [Germany, the Netherlands, Austria] [Spain] [Norway, Sweden] [Italy, Switzerland, Poland] [Finland] [Luxembourg]	52.92**	–4.06**	51.14**

by differences among populations within regional groups and among individuals within populations were very low, although statistically significant. Within Europe we detected lower population structuring among groups. In all these cases (partition numbers 3–8) percentages of variation explained by differences among groups were around 50%. Percentages of variation explained by differences among individuals within populations were moderate (between 30% and 50%) in this continent. The lowest genetic differentiation was found among populations within groups in all cases.

Mantel test revealed a significant positive correlation between genetic and geographic distances when all populations were considered ($r = 0.77$, $P < 0.0001$) indicating a

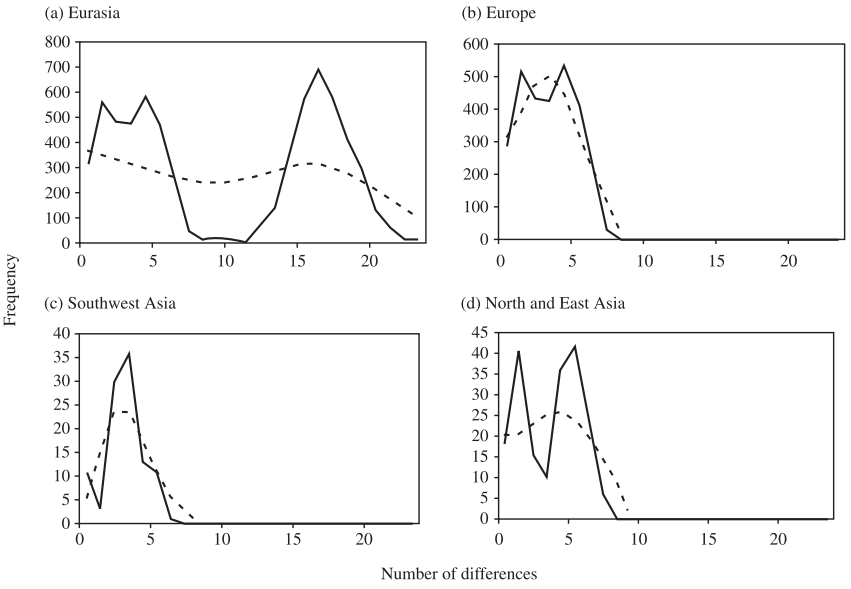

Fig. 5 Mismatch distributions based on Tamura–Nei genetic distances for badgers of the whole Eurasia (a), Europe (b), Southwest Asia (c), and North and East Asia (d). Solid lines represent the observed distribution and dashed lines represent the expected distribution according to the sudden expansion model.

Table 4 Tau (τ) parameter obtained from mismatch distributions, Fu's (F_S) values, and estimates of theta (θ_F) parameter based on coalescent Metropolis–Hastings Markov chain method, using genealogical relationships among haplotypes and assuming historically variable population sizes. The current effective number of females (N_e) estimated from θ_F for all samples and for each regional group is also indicated. Due to low sample size, estimates for the Japanese population have not been obtained

		Tau (τ)		Fu's F_S			N_e	
	N	Estimate	95% confidence interval	F_S	P	θ_F	1.92×10^{-8}	1.0×10^{-7}
Eurasia	115	17.5	9.1–27.4	–10.24	0.02	0.063	3.2×10^6	6.3×10^5
Europe	76	3.9	1.5–6.8	–7.17	0.01	0.039	2.0×10^6	3.9×10^5
Southwest Asia	15	3.0	0.9–4.4	–4.62	0.02	0.058	3.0×10^6	5.8×10^5
North and East Asia	20	5.2	1.8–9.1	–1.68	0.20	0.010	5.2×10^5	1.0×10^5

pattern of isolation by distance across Eurasia. Little, but significant, correlation was found within Europe ($r = 0.3$, $P = 0.03$). However, values of correlation coefficient decreased to nonsignificance when the analysis was executed among populations within Southwest Asia ($r = -0.27$, $P = 0.67$) and North and East Asia ($r = -0.11$, $P = 0.58$) — indicating a lack of structuring within these phylogeographic groups.

Mismatch distributions and demographic fluctuations

When the mismatch distribution was estimated with all samples (Fig. 5a) two frequency waves were detected. Within the European (Fig. 5b) and the Southwest Asian (Fig. 5c) regional groups the negative and statistically significant values of Fu's statistic (Table 4) and the bell-shaped mismatch distributions are indicative of population expansions in the past. Using the 95% confidence interval around τ, we estimated that the population expansion occurred in Europe between 0.15 and 0.69 Ma for m_1 and between 0.030 and 0.134 Ma for m_2. In the Southwest Asia group, the expansion occurred between 0.09 and 0.45 Ma for m_1 and between 0.017 and 0.086 Ma for m_2. The North and East Asia group showed a bimodal mismatch distribution (Fig. 5d) that could indicate the admixture of two expanding populations, or that populations could be stationary in the past as also suggested by the non-significant result of Fu's test (Table 4). Reduced sample numbers could also be responsible for not detecting a population expansion; thus, a larger number of samples is needed to clarify the demographic history of this group. Estimates based on parameter θ showed that the current worldwide population of Eurasian badgers (MHMC $\theta_F = 0.063$) is 30 times more diverse than the initial population represented by $\theta_O = 0.002$, in the mismatch distribution. In addition, these estimates show that European and Southwest Asian badgers are more diverse and have effective numbers of females six times greater than North and East Asian badgers (Table 4).

Discussion

Genetic variation in the mtDNA control region of the Eurasian badger

The statement that Eurasian badgers are genetically depauperate, while true at a local scale (Burke *et al*. 1996; Pertoldi *et al*. 2000; Domingo-Roura *et al*. 2003) does not stand when considering the species as a whole. The nucleotide diversity found in the Eurasian badger mtDNA control region ($\pi = 4.6\%$, Table 1) is higher than the value reported in the same locus for the grey wolf, *Canis lupus*, a widely distributed and highly mobile species ($\pi = 2.6\%$, Vilà *et al*. 1999). However, values within geographic regions were much lower ($\pi = 0.5–0.8\%$) and comparable to those reported for control regions of mustelids that have been classified as endangered, such as the European polecat, *Mustela putorius*, $\pi = 0.9\%$ (Davison *et al*. 2001) and the European mink, *Mustela lutreola*, $\pi = 0.12–1.2\%$ (Michaux *et al*. 2005); but still higher than those of the Eurasian otter, *Lutra lutra*, $\pi = 0.06\%$ (Ferrando *et al*. 2004); and the sea otter, *Enhydra lutris*, $\pi = 0.09\%$ (Larson *et al*. 2002). The disparity between species-wide and regional nucleotide diversities is a consequence of the extreme geographic structuring of the genetic variation in the badger, as discussed below. Eurasian and regional haplotype diversities found in our study were high, around $h = 0.9$ (Table 1), and comparable to values reported in mtDNA control regions of mammalian species with no known bottlenecks (see Larson *et al*. 2002). Within Europe, moderate levels of haplotype diversity have been reported in other mustelids, such as the pine marten, *Martes martes* ($h = 0.76$, calculated from sequences of Davison *et al*. (2001) and the endangered European mink ($h = 0.47–0.94$, Michaux *et al*. 2005). However, for instance the Eurasian otter shows considerably lower levels of haplotype diversity ($h = 0.16$, Ferrando *et al*. 2004) in the same geographic range.

Phylogeographic groups

Median-joining network (Fig. 2), two-dimensional scaling analysis (Fig. 3) and the highest percentage of genetic variation explained by differences among regional groups in partition number 1 of SAMOVA (Table 3) clearly confirm the existence of only four phylogeographic groups in the Eurasian badger. Although the phylogeographic pattern found in continental areas is robust, further research would be rewarding in zones of secondary contact or geographic boundaries suggested by our findings. Our limited sampling might have prevented us from detecting ongoing gene flow in intermediate populations.

It has been proposed that at the beginning of the last glacial maximum (LGM), little phylogeographic structuring existed in European mammals and phylogeographic patterns found nowadays are transient relics of the last glaciation and do not represent long-term environmental adaptations (Hofreiter *et al*. 2004). Thus, the association between phylogenetic structure and geography would not necessarily imply long-term genetic isolation (Leonard *et al*. 2000). However, our results support the separation of Eurasian badgers in three largely allopatric groups — Europe, Southwest Asia, and North and East Asia — since the end of Pliocene, at the beginnings of glacial ages, 2.9 Ma, many hundred thousands of years before the LGM. The current genetic divergences between clades of related mtDNA sequences likely include polymorphisms that existed in the ancestral population at least since the early Pleistocene. Mitochondrial allopatric subdivisions are maintained by barriers to gene flow and low dispersal rates promoted by maternal philopatry and population isolation as has been proposed for bears (Barnes *et al*. 2002).

Eurasian badgers might define main geographic barriers in Eurasia

The female philopatry and restricted movement shown by badgers (Neal & Cheeseman 1996) explain the presence of signs of past geographic separation in their genomes. Thus, badgers might retain in their genomes the effect of past or current geographic barriers that have been erased in other species such as the grey wolf that do not show geographic differentiation across Eurasia. Possibly, this is a result of multiple expansions and contractions experienced by wolf populations during glacial ages, together with the change in distribution of suitable habitats and the high mobility of this species (Vilà *et al*. 1999).

Although it will be desirable to confirm our results using nuclear data and further studies in other species, the comparison of our findings with current knowledge on historical geographic processes, and results from other species, can help to define where the main barriers to gene flow might have been located across Eurasia. The Volga River and the Ural Mountains have been proposed as the geographic boundaries between European badgers and continental Asian badgers from Siberia, Kazakhstan, Mongolia, China and Korea on the basis of morphological relationships (Ognev 1931; Heptner *et al*. 1967). During glacial ages, badgers inhabited latitudes south of the permafrost where prey survival was possible. During glacial periods, southernmost permafrost limits expanded down to the north of Black and Caspian Seas (Williams *et al*. 1998), isolating European and Asian badger populations for thousands of years. In this scenario, the Ural Mountains and Volga River would have a secondary and minor effect and their current role in the separation of badger regional groups should be further studied.

Badgers distributed in Southwest Asia were presumably isolated from badgers of the other two regions by strong biogeographic barriers such as the Black and Caspian

Seas, as well as the Caucasus Mountains in the west. In Turkmenistan and Uzbekistan, the Kopet Dagh and Hindu Kush Mountains, together with Kara-Kum and Kizil-Kum large sandy deserts, could determine the geographic border, whereas in Central Asia the Pamiro-Alai and Tien Shan Mountains would promote the separation. This interpretation is supported by the fact that since the end of the early Pleistocene the formation of deserts had started in the Tarim Basin, and by the middle Pleistocene the Tien Shan Mountains were covered with glacial sheets (Wen 1994). A boundary between western Tien Shan and eastern Tien Shan mtDNA lineages has also been reported in the red deer, *Cervus elaphus*, in this Central Asian region (Mahmut *et al*. 2002). However, in the badger, our results should be taken with caution since they are based on few individuals from this area. In addition, in the foothills of western and central Tien Shan, Southwest and Northeast Asian badgers nowadays are sympatric (Alexey Abramov, personal communication), prompting the need of further research to evaluate the current degree of intermixing between Southwest and North and East Asian badgers.

We estimated that Japanese badgers diverged from continental Asian badgers between 0.21 and 1.09 Ma during the Riss glacial stage. Sea levels decreased during glacial stages and land bridges connecting Japanese islands with the continent were formed (Emery *et al*. 1971). One of these land bridges connected the Korean Peninsula and Japan and was used, for instance, by the Japanese macaque, *Macaca fuscata*, ancestors to colonize the Japanese archipelago (Kamei 1969). The fact that nowadays no Eurasian badger occurs in the northern island of Hokkaido and those fossils of Japanese badgers of about 0.20 Ma have been excavated in southern Japan (Kawamura *et al*. 1989) may indicate that Japanese badgers and Japanese macaques had similar colonization patterns. On the contrary, another mustelid, the sable, *Martes zibellina*, colonized Japan through the Kuril Islands from Kamchatka and Sakhalin before spreading from Hokkaido to the southern islands of Honshu, Shikoku and Kyushu and giving rise to the Japanese marten, *Martes melampus*. Nowadays the Japanese marten and the sable are separated by the Tsugaru Strait (Anderson 1994).

Isolation by distance

Mantel test showed a strong positive correlation between genetic and geographic distances when all populations were considered, indicating a clear pattern of isolation by distance across Eurasia. This result is consistent with a species with low dispersal rates like is the Eurasian badger. While excursions, and interbreeding, between neighbouring social groups of badgers can be common, Macdonald *et al*.'s (submitted) analysis of 17 years of data on one marked population revealed that 35% of individuals never dispersed from their natal range, and of those that did the sex ratio was close to 1:1 and dispersal distances rarely exceeded two or three home range diameters. Coefficient correlation among genetic and geographic distances was low but significant within Europe. Microsatellite DNA, in some cases using the same badger samples, showed a clear genetic structuring within Europe, indicating also a pattern of isolation by distance and that no significant barriers to gene flow have existed within this region (Pope *et al*., 2006). The use of a large number of microsatellite markers with high mutation rates might be responsible for the stronger genetic structure provided by microsatellites than mtDNA control region.

Mantel test correlation values were negative and nonsignificant within Southwest Asia and North and East Asia. However, 22 of 43 haplotypes were restricted to a single individual indicating that more individuals should be investigated to fully explore genetic variation within these two groups.

Glacial refuges and postglacial colonizations

The high levels of haplotype diversity and low levels of nucleotide diversity found (Table 1) may suggest rapid demographic expansions from small effective population sizes, multiple refuges and secondary contact of haplotypes from different refuges within each Eurasian badger phylogeographic group. The evidence of population structuring found within Europe may be also a consequence of a postglacial recolonization from more than one refuge with posterior intermixing. The geographic distribution of some haplotypes (Fig. 1) agrees with this last possibility. For instance, the geographic distribution of haplotypes 'meles-1', 'meles-2' and 'meles-15' could represent postglacial recolonization routes from the Italian and Iberian refuges to Central and Eastern Europe and the British Isles. A recolonization route from Iberia is also supported by the robustness of the cluster formed by haplotypes from Spain and Central and East Europe in the phylogenetic tree (Fig. 4). No haplotype from Finland was found in the rest of Europe, suggesting that Eastern Fennoscandia was recolonized from an undetermined eastern refuge from which no other part of Europe was recolonized. This is supported by the fact that SAMOVA partitions suggest a weak genetic isolation of Iberian and Fennoscandian populations. On the contrary, Norway shared one haplotype (meles-13) with Luxembourg indicating that Western Fennoscandia could be recolonized via Central Europe. A similar pattern of postglacial recolonization in Fennoscandia has been found in other mammals, such as the brown bear, *Ursus arctos* (Taberlet & Bouvet 1994) and the root vole, *Microtus oeconomus* (Brunhoff *et al*. 2003).

We found one main lineage and we detected a late Pleistocene population expansion in badgers from Southwest Asia (Table 4 and Fig. 5c). For many small mammals, such

as the field vole, *Microtus agrestis*, showing widely ranging phylogeographic groups, it has been proposed that huge areas of Eurasia might have been recolonized by derivatives of a single lineage (Jaarola & Searle 2002). Our results are in agreement with this extensive colonization of Southwest Asia by a single lineage and provide preliminary evidence to extend this pattern of colonization of Eurasia to median-sized mammals. Pollen data suggest that main glacial refuges were located in western coastal regions of Turkey and Near East (Brown & Gibson 1983). On the other hand, our results clearly suggest that neither European nor Russian regions were recolonized by Southwest Asian badgers, and this is consistent with findings for other mammalian species, such as the yellow-necked fieldmouse, *Apodemus flavicollis* (Michaux *et al*. 2004).

Western Caucasus, the coastal zone of the Black Sea, Southwestern Ural and western Altay Mountains have been proposed as glacial refuges in the remaining parts of Asia (see Tarasov *et al*. 2000). We are not able to infer glacial refuges and recolonization routes in North and East Asia phylogeographic group because of its large geographic distribution and the limited sampling performed in this region. However, the moderately supported little groups of haplotypes within North and East Asian cluster (Fig. 4) may indicate the existence of several refuges for this species in this region during glacial stages. Further research including a large number of samples is needed to elucidate the recolonization history of this phylogeographic group.

Demographic history within regions

The hypotheses of past population expansions in Europe and Southwest Asia are supported by mismatch distributions and, statistically, by the values of Fu's index. However, it is important to notice that an episode of positive selection could lead to similar results (Fu 1997). The bimodal mismatch distribution suggested that no demographic fluctuation occurred in the North and East Asia group since the recent past, although the admixture of two expanding populations could also lead to a similar result. Population expansions are characterized by low and negative values of Fu's test and even if our data for North and East Asia followed this tendency ($F_S = -1.68$), they were not statistically significantly different from zero ($P = 0.2$) and a larger sample size would be required to confirm one of these two possibilities. Of course, there are no prehistorical data on badger demography, but lessons from recent studies reveal density dependence in contemporary populations (Macdonald *et al*. 2002) and trebling of numbers within two decades (Macdonald & Newman 2002). The latter was attributed to the impact of climate change on cub recruitment and winter survival, as has been recent expansion in the species' geographic range. Clearly, such mechanisms could have led to similarly rapid and substantial demographic changes prehistorically. Macdonald & Newman (2002) also argue that badger numbers have increased in historical times through human activities such as the increase of land under pasture and the removal of wolves, although these factors have been counteracted by people hunting badgers.

Management implications

The phylogeographic groups described are robust and consistent with relevant morphological differences found among European, Southwest Asian, Siberian and Japanese badgers (Abramov 2002, 2003). Several nucleotide combinations are specific of the different phylogeographic groups found and the assignment of badger samples to each one of these groups for research or forensic purposes is possible using any molecular technique of single nucleotide polymorphisms (SNPs) detection (see Domingo-Roura *et al*. 2006 for an example).

Our study is based on a single female-inherited marker that would need to be complemented with other studies based on nuclear and/or ecological or morphological data reflecting potential adaptive differences between populations and considering recent as well as historical processes before management recommendations can be drawn (Crandall *et al*. 2000; Fraser & Bernatchez 2001). Information on nuclear genes representing male genomes is especially pertinent for species showing female philopatry. The misidentification of populations that are linked by nuclear gene but not organelle gene flow should be avoided (Moritz 1994). Several species, for instance macaques, show mtDNA divergence in presence of nuclear gene flow (Melnick & Hoelzer 1992). Nevertheless, the level of sequence divergence obtained among the four main phylogeographic groups typically would require several hundred thousands to millions of years of historical separation (Fraser & Bernatchez 2001), and we believe these groups are likely to be confirmed with nuclear data in the future to guarantee the use of these divisions in wildlife management.

The confirmation of our results would have important implications for the management of endangered Eurasian badger populations. This is especially relevant for the badgers from Crete that have classically been classified as *Meles meles arcalus*, Miller 1907, and recommended special protection (Griffiths & Thomas 1997). We found that Cretan badgers are closely related to Southwest Asian badgers (Figs 2 and 3 and Table 3). However, Crete was not connected with the continent by land bridges during the Pleistocene, and the presence of badgers in Crete, like other mustelids except otters whose presence is supported by palaeontological evidence, would be related to human migrations from Asia Minor (Masseti 1995). Threats of badger local extinction also exist in some parts of continental Europe, such as in the Netherlands (Griffiths & Thomas 1997). Our results suggest that Dutch populations are

genetically similar in their mtDNA to populations from the rest of Europe and the British Isles. However, it is well documented that Eurasian badgers show ecological differences, for instance in sociability (Neal & Cheeseman 1996) and diet (Goszczynski *et al*. 2000) throughout Europe, and ecological data should be taken into account before taking drastic measures, such as translocating badgers into Europe.

Nowadays, the demographic status of the badger within Europe is relatively well documented, and in general populations are stable or increasing (Griffiths & Thomas 1997). However, the status of Asian populations is widely unknown. The North and East Asia group showed a female effective population size (N_e) remarkably lower than the value estimated for Europe and Southwest Asia (Table 4). This could be a first indication of declining badger populations in this region, or, at least, given the large number of assumptions included in this estimate, a reason to focus conservation interest in the subject.

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Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2747/MEC2747sm.htm

Table S1 List of origins and collectors of Eurasian badger samples used in this study. We have also included the sample code of samples coming from museums and of samples from our collection from haplotype sequences were initially isolated (IRTA-T followed by a number). The haplotype codes and their EMBL/GenBank Accession Numbers (Acc Num) are also included for these samples. See Figure 1 for remaining correspondences.

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This work is part of the research conducted in mammalian conservation genetics and evolution by the group led by Xavier Domingo-Roura established as Conservation Genetics Unit at the Institut de Recerca i Tecnologia Agroalimentàries where Francesc López-Giráldez is conducting his doctoral studies. Josep Marmi is now working in conservation genetics of endangered animal domestic breeds in the Departament de Ciència Animal i dels Aliments at the Universitat Autònoma de Barcelona. Francesc Calafell is a senior researcher specialized in genetic data analyses within the Departament de Ciències Experimentals at the Universitat Pompeu Fabra. The Conservation Genetics Unit collaborates with the Wildlife Conservation Research Unit, University of Oxford, led by David W. Macdonald. Elena Zholnerovskaya is a curator of the mammal collection at the Siberian Zoological Museum of the Russian Academy of Sciences.
