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Mitochondrial DNA phylogeography of European hedgehogs

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Abstract

European hedgehog populations belonging to *Erinaceus europaeus* and *E. concolor* have been investigated by mitochondrial DNA analysis. A 383 bp fragment of the cytochrome *b* gene has been sequenced and maximum parsimony and neighbour-joining trees of Tamura–Nei genetic distance values have been constructed. Similar topologies have been produced by both methods, showing a deep divergence between *E. europaeus* and *E. concolor* and a further subdivision of each species into a western and an eastern clade. A comparison with previously published allozyme data is made, and concordant and discordant patterns are discussed. The influence of Pleistocene glaciations on the observed pattern of divergence is inferred.

Keywords: cytochrome *b*, *Erinaceus*, hedgehogs, mtDNA, phylogeography, Pleistocene

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Introduction

The way in which genetic variation is partitioned within and between populations from different geographical areas is a product of the history and evolution of a species. Thus palaeogeographic events in Europe are expected to have had a great influence on today's species genetic structure. During major Pleistocene glaciations most European species were restricted to the south in parts of Iberia, Italy, the Balkans and the Caucasus. In these refugia, populations may well have been isolated for many thousands of years during each glacial period, and then expanded northwards following each climate amelioration (e.g. Hewitt 1993).

Phylogeographic studies look to find the relationships between present genetic structure of a species and its geographical origin and history, and molecular studies involving isozymes and latterly mitochondrial (mt)DNA analysis have played a significant role in this area (Avise *et al*. 1987; Avise 1994). Such studies on a number of organisms have provided much information on recent range contractions and expansions, as well as allopatric evolution associated with ice ages. Broad distributional DNA data that can be interpreted within the Pleistocene climatic history of Europe have been recently obtained for grasshoppers (Cooper *et al*. 1995), bees (Garnery *et al*. 1992, 1995), mice (Nachman *et al*. 1994; Boursot *et al*. 1996; Din *et al*. 1996), rabbits (Hardy *et al*. 1995) and bears (Taberlet & Bouvet 1994; Kohn *et al*. 1995). Some major effects on the genetic structure of such environmentally induced range changes are now appreciated (e.g. Avise 1989, 1994; Hewitt 1989, 1996) and a number of possible consequences have been outlined. For example, rapid expansion from refugial populations should involve serial bottlenecking with progressive loss of allelic diversity, so that populations in more recently colonized places will contain less genetic diversity. Conversely, it is expected that previously refugial areas will contain genetically rich populations. Such major range changes operate over large areas, so that allopatrically diverged genomes may be distributed in geographical patchworks with hybrid zones where they meet.

European hedgehogs belong to the genus *Erinaceus*, and are distributed across Europe to the Urals and from Scandinavia to Turkey and the near East. Two species are recognized, *E. europeaus* from western Europe, and *E. concolor* with an eastern distribution, and these appear to have distinct karyotypes (Geisler & Gropp 1967; Mandahl 1978). A north/south zone of overlap, and possible hybridization between the two species, exists from the Baltic to the border between Italy and Slovenia. Another such contact zone occurs from Lithuania east to around Kirov. It has long been recognized that both of these species are partitioned into several subspecies on the

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basis of morphological characters but all attempts to discretely define subspecies have failed (Corbet 1988; Reeve 1994). Therefore, according to Corbet (1988) none of the subspecific names proposed for *E. europaeus* and *E. concolor* can represent discrete definable units, and he recognized the need for a molecular-based investigation to solve the confusing taxonomy of hedgehogs. Recent studies have analysed genomic DNA utilizing restriction enzyme analysis (Bannikova *et al*. 1996) and microsatellite variation in populations (Becher & Griffiths 1997). However, both studies offer little insight into phylogeographic and taxonomic questions within *Erinaceus*. Filippucci & Simson (1996) have used autosomal allozyme polymorphism to investigate the distribution of genetic variation across the geographical distribution of *Erinaceus*. Their data indicate a considerable level of divergence between *E. concolor* from Turkey and Israel and *E. concolor* from the Balkans, leading them to assert that one of the previously described subspecies, *E. roumanicus*, is a separate species. Within *E. europaeus*, the Iberian populations appear quite distinct from the other European populations, suggesting that the taxon named *E. hispanicus* could also represent a distinct species, closely related to *E. europaeus*.

These findings clearly suggest a strong influence of Pleistocene isolation in southern refugia on the current genetic structure of populations. In this study we construct an mtDNA gene phylogeny to investigate genetic relationships among population groups and taxa, to illuminate the influence of Pleistocene range contraction/expansion, and to trace possible colonization routes for European hedgehogs.

Materials and methods

Collection of samples

Fifty-six hedgehogs from 14 and 12 different locations across the species ranges of *Erinaceus europaeus* and *E. concolor*, respectively, have been analysed (Fig. 1) (Table 1). In some cases individuals from within a 10 km radius were included as one location, although they may belong to different subpopulations. An additional hedgehog species from Asia minor, *Hemiechinus auritus*, was used as an outgroup. For each individual a piece of muscle tissue was stored either in 95% ethanol at 4 °C or frozen at – 80 °C. The majority of the samples have been generously provided by M. Filippucci (University of Rome, II), who used the same material for a previous allozyme study (Filippucci & Simson 1996). The French and Corsican samples were kindly provided by F. M. Catzeflis (USTL, Montpellier).

Fig. 1 Map of the geographical distribution of *Erinaceus europaeus* (vertical lines) and *E. concolor* (horizontal lines) showing the locations studied.

Table 1 Distribution of mtDNA haplotypes of *Erinaceus* among the studied locations. See Fig. 1 for geographical placement

Location	Haplotype
E. europaeus	
UK	9, 9, 9
East Anglia	
Germany	
Westphalia	
Bielefeld	6, 6, 6
Herford	7, 8
Wuppertal	7
France	
Les Matelles	9, 9, 10
Spain	
Almonte	11, 11
Italy	
Friuli	3, 3, 3, 3, 4
Tuscany	
Pisa	2
Grosseto	3, 3
Latium	2, 2, 2, 2, 3
Abruzzi	5, 5
Calabria	2, 2, 2, 5
Corsica	
Galeria	2
Sicily	
Ficuzza	12, 12, 12
E. concolor	
Italy	
Friuli	13, 14
Former Yugoslavia	
Zivogosce Murana	15
Rihpovec	16
Greece	
Igoumenitsa	15
Mt. Pangeon	17
Turkey	
Turkish Thrace	15, 15, 17
Anatolia	
Edremit	18
Ulu Dag Mts.	18
Ezine	18, 18
Acigöl	18
Israel	
Golan	19
Haifa	20, 20, 20, 21
H. auritus	
Egypt	22, 22

DNA extraction, PCR amplification and DNA sequencing

For each specimen a small piece of muscle tissue was ground in liquid nitrogen, to which 500 μL of extraction buffer was added (20 mM Tris, 10 mM EDTA, 0.5% SDS). This was digested overnight with 3 μL of proteinase K (25 μg/mL) at 45 °C. The DNA was then purified with Wizard minicolumns (Promega) following the manufacturer's recommendations.

Two primers for a relatively conserved region of the mammalian cytochrome *b* gene (*cyt-b*), L14724 (Irwin *et al*. 1991) and H15149 (Kocher *et al*. 1989), kindly provided by M. Bruford (Institute of Zoology, London), were used for PCR amplification. The 5' end of the first primer corresponds to position 14126 of the hedgehog mtDNA (Krettek *et al*. 1995), which is situated adjacent to the 5' end of the *cyt-b* gene. The 5' end of the second primer corresponds to position 14612. These primers produce a 486 bp fragment of the *cyt-b* gene.

Polymerase chain reaction (PCR) amplification was carried out using 1 unit of *Taq* DNA polymerase (Promega) in a Perkin Elmer DNA thermocycler 480. Reactions were carried out in 100 μL volumes including 5 μL of each 10 μM primer and 6 mM $MgCl_2$. Two microlitres of a 1/20 dilution of DNA extraction was used for amplification. The cycle profile was 94 °C denaturation (40 s), 49 °C annealing (60 s), and 72 °C extension (40 s) for 35 cycles. An initial cycle employed a 5 min denaturation at 94 °C and the final cycle had an extension step of 72 °C for 10 min. PCR products were purified using Wizard PCR Preps (Promega) following the manufacturer's recommendations and stored in TE at – 20 °C.

Sequencing was performed using the Thermo Sequenase cycle sequencing kit (Amersham) using 100–200 ng of PCR template. *Erinaceus*-specific internal fluorescent primers were developed for sequencing of both strands: HE-L14279, 5'-RRCTCCATCYAATATTTC-TTCT-3' (sense); and HE-B14570, 5'-CGTAACCCATAAA-AGCTGTAG-3' (antisense).

For the primer HE-L14279 the reaction conditions were 95 °C denaturation (45 s), 50 °C annealing (10 s), and 72 °C extension (30 s) for 20 cycles. An initial cycle employed a 4-min denaturation at 95 °C and after the completion of the cycles samples were stored at 4 °C. Conditions for reactions with the primer HE-B14570 differed by incorporating an annealing temperature of 53 °C.

Sequence analysis

Sequences were manually aligned to the *E. europaeus* mitochondrial DNA sequence of Krettek *et al*. (1995) and analysed using both parsimony and distance methods. An initial unweighted analysis of haplotypes was performed using the heuristic search option of PAUP 3.1 (Swofford 1993). All heuristic searches were done using the closest addition sequence holding 10 trees at each step, using the tree bisection-reconnection (TBR) branch-swapping algorithm, and swapping on minimal and nonminimal trees with the steepest descent option. Trees were rooted with *H. auritus*. Character weighting of codon positions was on

the basis of the frequency of change for each position within a codon for all taxa. The frequency of transversions relative to transitions was based on the frequencies of the two types of substitution calculated from the uniformly weighted tree using the 'state changes and stasis' option of MACCLADE version 3.03 (Maddison & Maddison 1992) in the chart menu. As this probably underestimates the ratio between closely related taxa, less conservative ratios were also examined in a parsimony analysis with transversions weighted relative to transitions.

The Tamura & Nei (1993) model of DNA sequence evolution was used to estimate the genetic distances between taxa. This measure has been found to be appropriate for analyses of mammalian *cyt-b* sequence data (Talbot & Shields 1996a) because it accounts for different rates of transition substitutions. Distance trees were generated using the computer program MEGA (version 1.01, Kumar *et al*. 1993). Trees were constructed using the neighbour-joining method of Saitou & Nei (1987).

Results

Nucleotide diversity

Cytochrome *b* sequence of 383 bp was obtained for all 56 *Erinaceus* analysed.

A total of 20 haplotypes have been identified (Fig. 2), with from 1 to 3 haplotypes per location (Table 1). Sequences have been deposited in GenBank with Accession nos AF051405–AF051424. The two individuals of *Hemiechinus auritus* possessed the same haplotype. Some haplotypes were observed in more than one specimen; therefore only one individual for each haplotype was included in subsequent analyses. One of the haplotypes from Italy was the most similar to the *E. europaeus* haplotype from Sweden, sequenced by Krettek *et al*. (1995), and therefore was numbered 2, considering the Swedish sequence that we used for alignment as haplotype 1. The distribution of haplotypes in the sampled localities and their number codes are presented in Table 1. Across all *Erinaceus* haplotypes 310 (81%) nucleotide positions were identical. Of the 73 variable sites, 11 were autapomorphies and 62 were phylogenetically informative. Of the phylogenetically informative sites 44 were at the third codon positions, 15 were at first positions and three were at second positions. The four nucleotides do not occur in equal frequency and, similar to other mammalian *cyt-b* sequences (Irwin *et al*. 1991; Groves & Shields 1996; Talbot & Shields 1996a, 1996b), there is a notable deficit of guanine nucleotides, particularly at third codon positions.

Haplotype distribution

In Italy we observed four haplotypes (2–5), among a total of 19 specimens. Haplotypes 2 and 3 were both present in central Italy, 3 and 4 in northeastern Italy, and 2 and 5 in the South. The single specimen from Corsica has the common Italian haplotype (2). The three samples from Sicily share a very distinct haplotype (12), more closely related to the second *E. europaeus* clade, formed by Spanish, French and English populations. The level of divergence within the mainland Italian haplotypes from 19 individuals is very low, with 1–3 substitutions distinguishing local haplotypes 3, 4 and 5 from the most widespread haplotype (2). A similar level of divergence was found among the three German haplotypes from the six samples from Westphalia (6–8). These are also very closely related to the Italian ones. The haplotypes found in Spain, France and the UK (9–11) are quite distinct from the Italian and German haplotypes, and cluster with the Sicilian haplotype. Within *E. concolor* a

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                 1111111111111111111111111111111111111111111111111111111111111111111111111111111
                 4444444444444444444444444444444444444444444444444444444444444444444444444444444
                 2222222222222222222233333333333333333333444444444444444444455555555555555555
                 0122334444566788899900001223444555677890000122223455566889000123334444667 7
                 3547092358109815815724587048124046517081478312584025624284349950140258362 5

E.eur  2 CTTTCAGATATTATACGCGCTCCGATTCTAGTCTTCTCCCTGCTCATCCAGCCTATCATTAAATTCATTGGTCA
E.eur  3 .......................C.....................................................
E.eur  4 .......................C.....................................................G..
E.eur  5 .....................................................................G..
E.eur  6 .........G...........T....C.....................................................
E.eur  7 ....................T....C.....................................................
E.eur  8 ....................T....CT....................................................
E.eur  9 T...T.A.C...........AT...C..C.A......TT..A.C..C.............G..CC....AA...
E.eur 10 T...T.A.............AT...C....AC.....TT..A.C..C....T........G..CC.G..A..T.
E.eur 11 T...T.A.............AT...C....AC.....TT..A.C..C.............G..CC.G..A..T.
E.eur 12 T...T.A..............TT.CC....A.TG...T...A..T.CT............G.G.C.G.......
E.con 13 T...TGA...C.G.T..TC..T.A.....TA..CCTAT.TCA......T.A.TCC.T..A.G.A.T..GA...G
E.con 14 T...TGA...CCG.T..TC..T.A.....TA...CTAT.TCA......TCA.TCC.T..A.G.A.T..AA.G.G
E.con 15 T...TGA...C.G.T..TC..T.A.....TA...CTAT.TCA......TCA.TCC.T..A.G.A.T..GA...G
E.con 16 T...TGA...C.GGT..TC..T.A.....TA...CTAT.TCA......TCA.TCC.T..A.G.A.T..GA...G
E.con 17 T...TGA...C.G.TT.TC..T.A.....TA...CTAT.TCA......TCA.TCC.T..A.G.A.T..GA...G
E.con 18 TC.CT.AG..C.GCC.ATCT.T.A.....TA....TAT.TCAT....T.CA.TCT.TGAA.GGA.T.C.A..TG
E.con 19 TC.CT.AG..C.GCC.ATCT.T.A.....TA.....AT.TCAT..G.T.CA.TCCCTGAA.GGA.T.C.A..TG
E.con 20 TC.CT.AG..C.GCC.ATCT.T.A....CTA.....AT.TCAT..G.T.CA.TCCCTGAA.GGA.T.C.A..TG
E.con 21 TC.CT.AG..C.GCC.ATCT.T.A.....TA.....AT.TCAT..G.T.CA.TCCCTGAA.GGA.T.C.A...G
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Fig. 2 DNA sequence variation among the sequenced part of the *cyt-b* gene for the 20 *Erinaceus* haplotypes identified in this study.

total of nine haplotypes were detected. Three (19–21) were from Israel, and one from Anatolia (18). Israeli and Anatolian haplotypes are closely related to each other but very distinct from the remaining six Balkan haplotypes. Two of these (15,17) are widespread in the Balkans, observed from Turkish Thrace to former Yugoslavia. Several other haplotypes have been observed in former Yugoslavia (16) and in northeastern Italy (13–14).

Parsimony analysis

Sixteen most parsimonious (MP) trees (step length 112; CI, 0.705; RI, 0.911) were derived from a heuristic search with uniform weighting. A strict consensus tree resulted in polytomies among terminal lineages from close geographical proximity. The total number of steps at first, second, and third codon sites was reconstructed from the strict consensus tree giving a weighting ratio of 8:31:3. A transition/transversion ratio of 5:2 was estimated from substitutions on the strict consensus tree. We have adopted the suggestion of Fitch & Ye (1991) that an analysis incorporating both substitution and position weighting may describe a more accurate estimate of the true phylogeny. Eight MP trees were derived from a heuristic search with a transition:transversion weighting ratio of 2:5, and a codon position weighting ratio of 8:31:3 for first, second and third positions, respectively. Less conservative transition/transversion weighting ratios of 2:10 and 2:15 were also included in the analysis but did not affect the tree topology. A strict consensus of these eight trees (Fig. 3) differed from the unweighted strict consensus tree by the resolution of a single polytomy.

Distance analysis

The neighbour-joining algorithm was used to generate a tree from pairwise distance estimates calculated using the Tamura-Nei (1993) model (Fig. 4). This tree is in complete agreement with the parsimony tree (Fig. 3).

A comparison of our mtDNA phylogeny with a phylogeny derived from a recent study of *Erinaceus* using allozyme data (Filippucci & Simson 1996) is presented in Fig. 5. Branches containing taxa not in common in the allozyme and *cyt-b* trees have been pruned. The comparison reveals a high level of congruence between the allozyme and the *cyt-b* trees. The single major difference is the conflicting position of the Sicilian sample in the two trees.

Discussion

Genomic subdivision and divergence times

The analysis of the spatial genetic structure of these European hedgehogs revealed a clear geographical partitioning of the haplotypes with a considerable divergence between genomes occurring in different regions of the species range (cf. Figure 6). Maximum parsimony analysis and Tamura–Nei estimates of levels of genetic differentiation also show that *Erinaceus europaeus* and *E. concolor* are both subdivided into two major clades. Within the first clade samples from mainland Italy and Germany are clustered together, with a low level of divergence among haplotypes. The second *E. europaeus* clade includes samples from Sicily, Spain, France and the UK. Haplotypes in this clade present a higher level of differentiation than the first

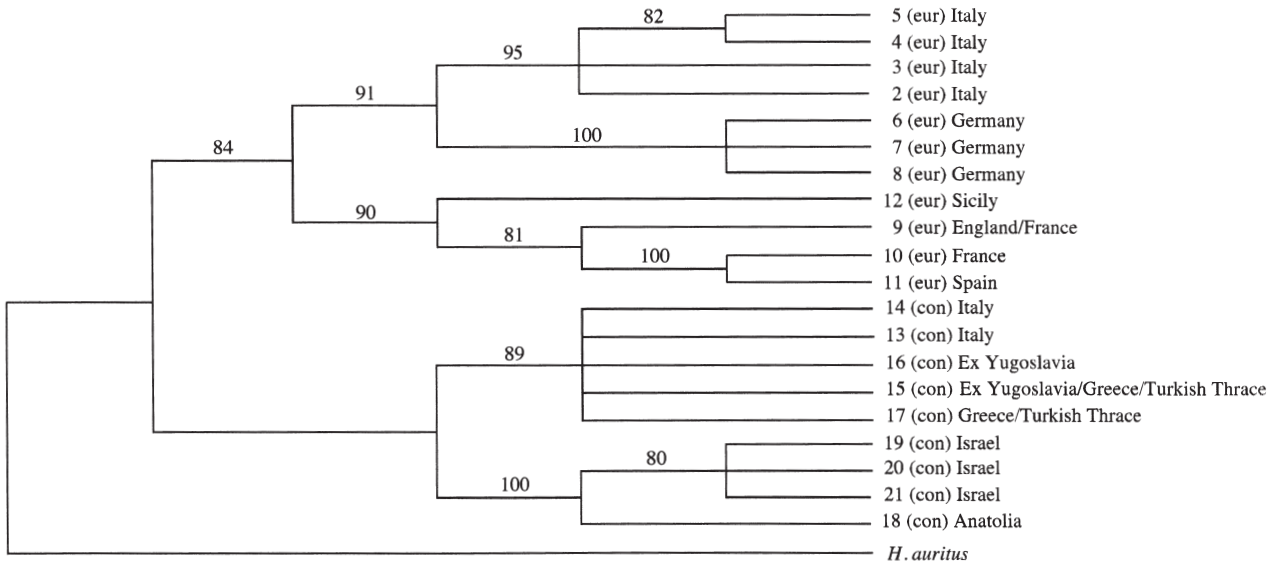

Fig. 3 Maximum parsimony tree for 21 hedgehog haplotytpes calculated from *cyt-b* sequences. Bootstrap values above 70% are given.

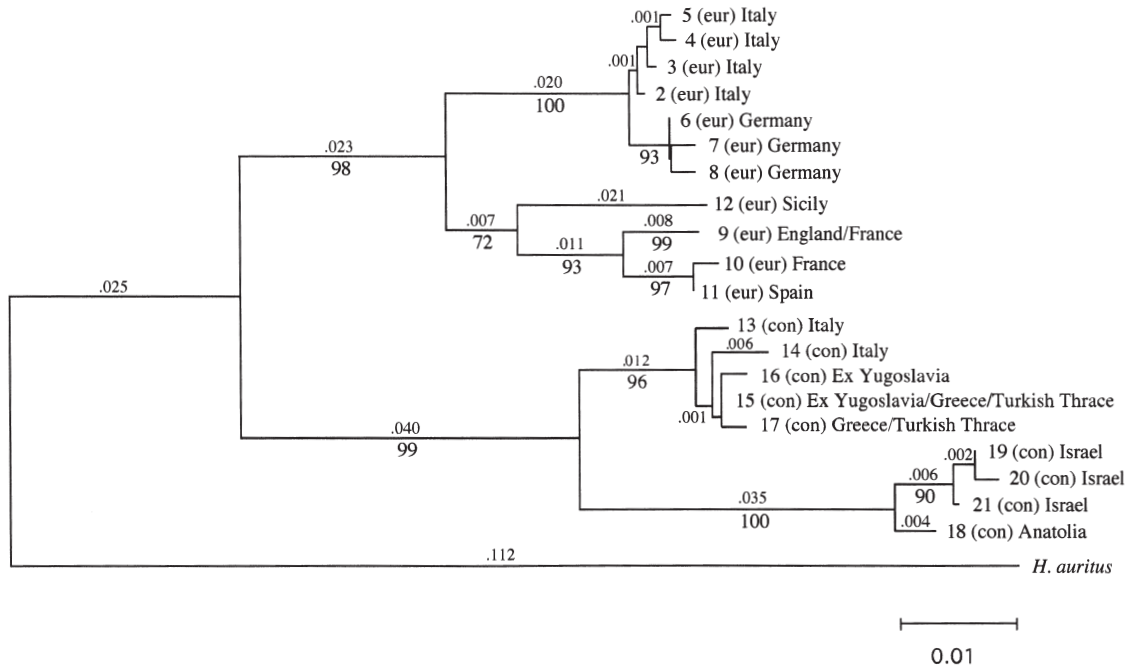

Fig. 4 Neighbour-joining tree of Tamura & Nei (1993) genetic distance values (above) for 21 hedgehog haplotypes calculated from *cyt-b* sequences, also showing bootstrap values above 70% (below).

clade, with branch nodes also well supported by bootstrap analysis (Figs 3 and 4).

Samples from the Balkans, Greece and from northeastern Italy form the first *E. concolor* clade. The second *E. concolor* clade includes haplotypes from Turkey and Israel and is clearly divergent from the first one with high bootstrap values and branch lengths. These findings amply support the general view of Filippucci & Simson (1996), that there is a high level of divergence within each of the two species.

We have applied the standard mtDNA time calibration (Wilson *et al*. 1985) to Tamura–Nei and Kimura 2-parameter genetic distance values, and obtained similar estimates. The time estimates reported here refer to the Tamura–Nei distance values. Similar values have also been obtained for Tamura–Nei distances calculated from third codon positions only, using the time calibration on Ursid fossil data proposed by Talbot & Shields (1996b).

We place an age estimate of ≈ 5.8 Ma for the split between *E. europaeus* and *E. concolor*. Within *E. europaeus*

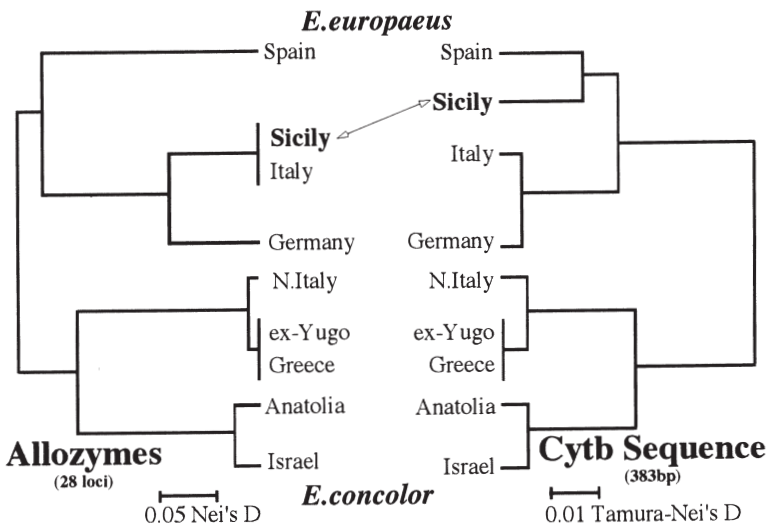

Fig. 5 Congruence of UPGMA trees for allozyme data (Filippucci & Simson 1996) and *cyt-b* data. The *cyt-b* tree is presented as a UPGMA tree for comparison with the 1996 allozyme analysis.

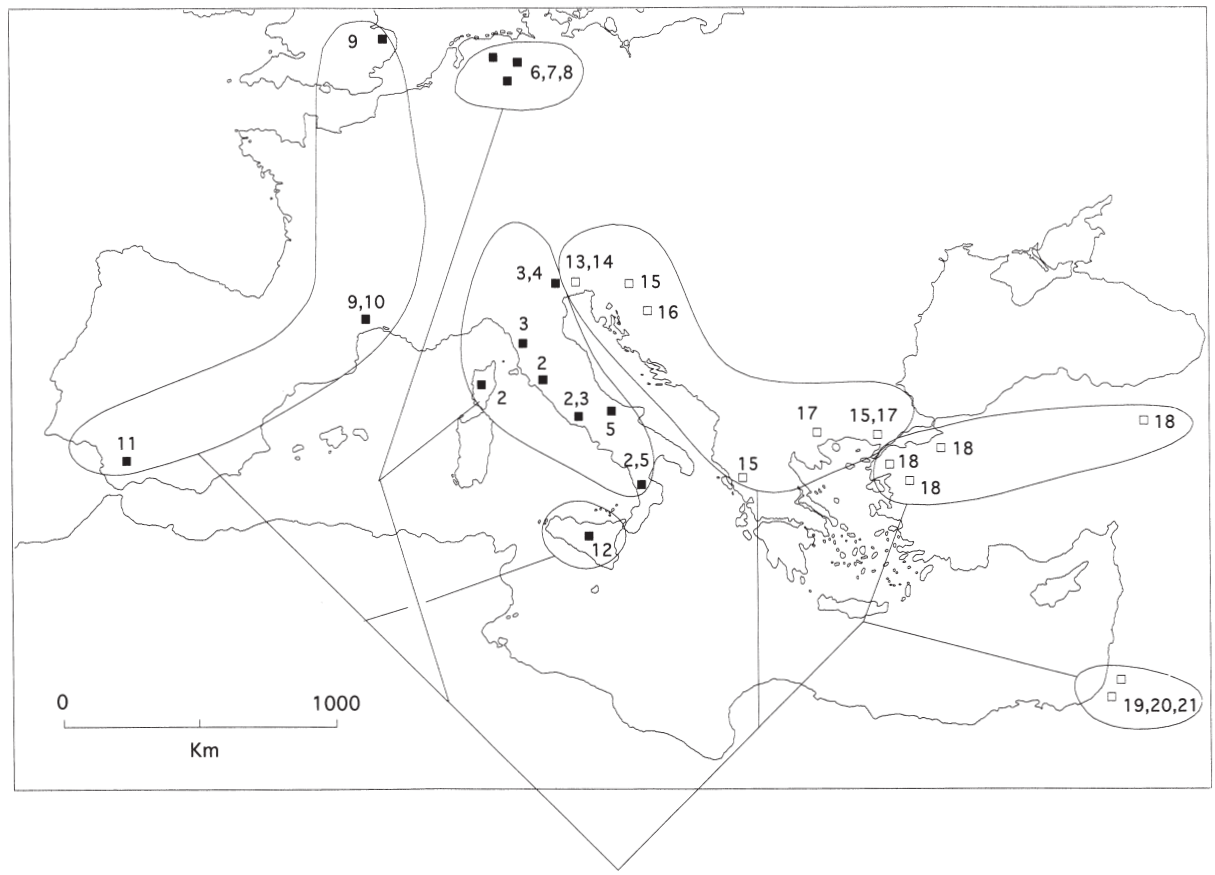

Fig. 6 Phylogeography of the different haplotypes emphasizing the east/west division of *Erinaceus europaeus* and *E. concolor* and the more recent dichotomies in each of them.

we estimate the divergence of the two major clades to have occurred ≈ 2.7 Ma. The divergence estimate for the two *concolor* clades is similar to that for the major division of the *E. europaeus* clade with a timing of ≈ 3.0 Ma.

In addition to the major mtDNA division between *E. europaeus* and *E. concolor*, another feature is the demonstration that the haplotypes of the French and UK samples are closely related to the Spanish sample. These data, together with the low level of divergence of haplotypes belonging to the second major *E. europaeus* clade, including Italy, Corsica, Germany and Sweden (the last one obtained from the sequence published by Krettek *et al*. 1995), indicate an east–west geographical partitioning down central Europe between France and Germany. Similarly, in *E. concolor* the Balkan samples form a clade distinct from the Anatolian and Israeli samples, and these two clades show a partitioning at the Bosphorus which forms a long-standing natural divide.

A number of species show hybrid zones between races or subspecies running north–south down central Europe from the Baltic to the Alps, separating divergent western and eastern genomes, e.g. house mouse (*Mus domesticus* and *M. musculus*; Ferris *et al*. 1983; Dallas *et al*. 1995; Boursot *et al*. 1996), yellow-bellied and fire-bellied toads (*Bombina variegata* and *B. bombina*; Szymura 1993), crows (*Corvus corone* and *C. cornix*; Mayr 1963), grass snakes (*Natrix natrix*; Thorpe 1984), oaks (*Quercus robur* group; Ferris *et al*. 1993), and shrews (*Sorex araneus* group; Taberlet *et al*. 1994). These are best explained as contacts between the postglacial expansions from western and eastern refugia. Hybrid zones in other parts of Europe, such as the Alps, Pyrenees and central Sweden (Jaarola & Tegelstræm 1995; Hewitt 1996), are also explicable in terms of postglacial expansions and secondary contacts. The hedgehog mtDNA phylogeography indicates a major genomic division between western and eastern Europe (*E. europaeus* and *E. concolor*) with a contact zone running in the same general region as for many other organisms, and within each of these there is a further subdivision with contact zones between the lesser diverged clades (Fig. 6). It is interesting to note that the karyotypes from the UK, western Europe and eastern Europe are distinct (Mandahl 1978; Searle & Erskine 1985; Sokolov *et al*. 1991), which would suggest that perhaps hedgehog genomes are similarly subdivided by karyotypes.

Sicilian intrigue

Paradoxically, the small Sicilian sample which is similar to the Italian populations on the basis of allozyme data shows a *cyt-b* haplotype more closely related to the 'Spanish/French/UK group' than to the 'Italian/German/Swedish' group. The effects of the long-term isolation of Sicily and southern Calabria from the rest of Italy (caused by the presence of marine-flooded grabens in central Calabria for most of the Pleistocene) are apparent from a number of plant and animal distributions, with endemic forms from Sicily and southern Calabria (Malatesta 1985; Caloi *et al*. 1989; Santucci *et al*. 1996). The lack of congruence between allozyme and mtDNA data for the Sicilian hedgehogs may be explained by: (i) a lower resolution of allozyme analysis compared to mtDNA; (ii) a possible colonization of Sicily by the Italian form, with a subsequent partial replacement of Sicilian nuclear alleles with the Italian ones, but maintaining the original mtDNA; (iii) human transport of animals between Italy and Sicily. The first hypothesis does not fit with the high congruence of allozyme and mtDNA for all the remaining common samples. The second possibility seems more probable, given also the evidence of late Pleistocene contacts between Sicily and peninsular Italy. Either unidirectional hybridization, or stochastic factors, or selective pressure on nuclear loci could be responsible for the observed pattern. Of course the limited number of Sicilian samples included in both studies may also have caused the discrepancy between the protein and the DNA trees.

Pleistocene range changes

From a review of all the available data, Butler (1988) considers that hedgehogs entered Europe from Asia, and fossil species related to current *Erinaceus* have been found across Europe from the Pliocene. The degree of genetic differentiation observed across the sampled geographical range combined with growing evidence of the extent of glacial ice sheets and palaeoclimates allows some inference about the evolutionary history of these hedgehogs. The genetic distance between *E. europaeus* and *E. concolor* suggests that their original divergence may be in the late Miocene to early Pliocene (11.7% – ≈ 5.8 Ma). This period saw important climatic changes with much evidence of increased glaciation around 6 Ma, followed by a warm period in the mid-Pliocene, which preceded the onset of the Pleistocene glaciations around 3.5 Ma (Eyles 1993). It is possible, therefore, that this deep divergence between the two species was instigated by this early Pliocene cold period, when the original population was restricted to east and west refugia.

The next divergence within each of *E. europaeus* and *E. concolor* (5.1% and 6.0%) would seem to have been around 2.7 Ma and 3.0 Ma, respectively, when in *E. europaeus* the line giving rise to the Sicilian, Spanish, French and UK populations was separated from that producing the Italian and German populations, while in *E. concolor* the Balkan lineages separated from the Anatolian and Israeli ones. There was an intensification of glaciation at this time with the establishment of the great northern ice sheets in America and in Europe. It is tempting to suggest that the proto-*europaeus* was forced into refugia in Iberia and Italy, and the proto-*concolor* into the southern Balkans and Turkey/near-East (e.g. Lebanon, Israel, Jordan), respectively. The succession of Pleistocene ice ages and interglacial warm periods on an approximately 100 000-year cycle would have allowed the hedgehogs to expand from these refugia only to be restricted again with each ice age. While the details of each interglacial expansion may have been different, the geographical structuring of the four lineages from west to east across Europe implies that the southern peninsulas have acted as continual refugia (cf. Hewitt 1993).

The present data also indicate a more recent divergence around 0.5 Ma, within the eastern clade of *E. europaeus* between the Italian and German samples, and similarly one in the eastern clade of *E. concolor* between the Anatolian and Israeli samples. Obviously, more detailed sampling is required, but these data suggest further geographical substructuring in the extant and refugial populations of hedgehogs, so that in the last few ice ages German populations may have had a separate refugium in perhaps the south of France or possibly northern Italy. The same reasoning applies to Turkish and Israeli lineages, with possible recent refugia in northern Turkey and the near-East. Further sampling across northern Europe is also required to determine the routes of postglacial expansion of these hedgehogs, particularly those in Eastern Europe which one would predict come from populations in the Balkans. This analysis would also provide a test of whether the postglacial expansion involved loss of allelic variation through colonization bottlenecking (Hewitt 1996; Ibrahim *et al*. 1996).

The divergence of the Sicilian mitochondrial haplotype from the Italian ones, and its affinity to the Spanish haplotypes, may be explained as residues of one of the more ancient expansions surviving the ice ages in southern Spain and Sicily. The nuclear genome, as identified by allozymes, may have invaded recently from the Italian mainland, carried by the males, with the Sicilian females retaining their ancestral mitochondria.

The times of the various divergence events for hedgehogs may be compared with a few other species for which such data are available. Taberlet *et al*. (1998) were able to estimate the depth of divergence between populations from refugial areas in Iberia, Italy and the Balkans for *Ursus arctos*, *Crocidura suavolens* and *Arvicola* spp. at 2%, 6.4% and 7.6%, respectively, for *cyt-b* gene sequences. These approximate to 1 Ma, 3.1 Ma and 3.8 Ma. These are all more recent

than the hedgehog divergence, and those for the shrew *Crocidura* and the water vole *Arvicola* would approximately coincide with the onset of the Pleistocene glaciations. In comparison with these species the hedgehogs have thus retained quite an ancient European genome divergence.

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References

Avise JC (1989) Gene trees and organismal histories: a phylogenetic approach to population biology. *Evolution*, **43**, 1192–1208.

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Avise JC, Arnold RM, Ball M *et al.* (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics*, **18**, 489–522.

Bannikova AA, Dolgov VA, Fedorova LV *et al.* (1996) Taxonomic relationships among hedgehogs of the subfamily Erinaceinae (Mammalia, Insectivora) determined based on the data of restriction-endonuclease analysis of total DNA. *Zoologichesky Zhurnal*, **74**, 95–106.

Becher SA, Griffiths R (1997) Isolation and characterization of six polymorphic microsatellite loci in the European hedgehog *Erinaceus europaeus* L. *Molecular Ecology*, **6**, 91–92.

Boursot P, Din W, Anand R *et al.* (1996) Origin and radiation of the house mouse: mitochondrial DNA phylogeny. *Journal of Evolutionary Biology*, **9**, 391–415.

Butler PM (1988) Phylogeny of the insectivores. In: *The Phylogeny and Classification of the Tetrapods*, Vol. 2, *Mammals* (ed. Benton MJ), pp. 117–141. Clarendon Press, Oxford.

Caloi LA, Malatesta A, Palombo MR (1989) Biogeografia della Calabria meridionale durante il Quaternario. *Atti della Accademia Peloritana dei Pericolanti*. Classe I Scienze Fis. Mat. e Nat. **LXVII**, 261–278.

Cooper SJB, Ibrahim KM, Hewitt GM (1995) Postglacial expansion and genome subdivision in the European grasshopper *Chorthippus parallelus*. *Molecular Ecology*, **4**, 49–60.

Corbet (1988) The family Erinaceidae: a synthesis of its taxonomy, phylogeny, ecology and zoogeography. *Mammal Review*, **18**, 117–172.

Dallas JF, Dod B, Boursot P *et al.* (1995) Population subdivision and gene flow in Danish house mice. *Molecular Ecology*, **4**, 311–320.

Din W, Anand R, Boursot P *et al.* (1996) Origin and radiation of the house mouse: clues from nuclear genes. *Journal of Evolutionary Biology*, **9**, 519–539.

Eyles N (1993) Earth's glacial record and its tectonic setting. *Earth-Science Reviews*, **35**, 1–248.

Ferris C, Oliver RP, Davy AJ *et al.* (1993) Native oak chloroplasts reveal an ancient divide across Europe. *Molecular Ecology*, **2**, 337–344.

Ferris SD, Sage RD, Prager EM *et al.* (1983) Flow of mitochondrial DNA across a species boundary. *Proceedings of the National Academy of Sciences of the USA*, **80**, 2290–2294.

Filippucci MG, Simson S (1996) Allozyme variation and divergence in Erinaceidae (Mammalia, Insectivora). *Israel Journal of Zoology*, **42**, 335–345.

Fitch WM, Ye J (1991) Weighted parsimony: Does it work? In: *Phylogenetic Analysis of DNA Sequences* (eds Miyamoto MM, Cracraft J), pp. 147–154. Oxford University Press, New York, Oxford.

Garnery L, Cornuet JM, Solignac M (1992) Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. *Molecular Ecology*, **1**, 49–60.

Garnery L, Mosshine EH, Oldroyd BP *et al.* (1995) Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. *Molecular Ecology*, **4**, 465–477.

Geisler M, Gropp A (1967) Chromosome polymorphism in the European hedgehog *Erinaceus europaeus* (Insectivora). *Nature*, **214**, 396–397.

Groves P, Shields GF (1996) Phylogenetics of the Caprinae based on cytochrome *b* Sequence. *Molecular Phylogenetics and Evolution*, **5**, 467–476.

Hardy C, Callou C, Vigne JD *et al.* (1995) Rabbit mitochondrial DNA diversity from prehistoric to modern times. *Journal of Molecular Evolution*, **40**, 227–237.

Hewitt GM (1989) The subdivision of species by hybrid zones. In: *Speciation and its Consequences* (eds Otte D, Endler J), pp. 85–110. Sinauer Associates, Sunderland, MA.

Hewitt GM (1993) Postglacial distribution and species substructure: lessons from pollen, insects and hybrid zones. In: *Evolutionary Patterns and Processes* (eds Leeds DR, Edwards D), pp. 97–123. Linnean Society Symposium Series 14. Academic Press, London.

Hewitt GM (1996) Some genetic consequences of ice ages. *Biological Journal of the Linnean Society*, **58**, 247–276.

Ibrahim KM, Nichols RA, Hewitt GM (1996) Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity*, **77**, 282–291.

Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome *b* gene of mammals. *Journal of Molecular Evolution*, **32**, 128–144.

Jaarola M, Tegelström H (1995) Colonization history of north European field voles (*Microtus agrestis*) revealed by mitochondrial DNA. *Molecular Ecology*, **4**, 299–310.

Kocher TD, Thomas WK, Meyer A *et al.* (1989) Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA*, **86**, 196–200.

Kohn M, Knauer F, Stoffella A *et al.* (1995) Conservation genetics of the European brown bear – a study using excremental PCR of nuclear and mitochondrial sequences. *Molecular Ecology*, **4**, 95–103.

Krettek A, Gullberg A, Arnason U (1995) Sequence analysis of the complete mitochondrial molecule of the hedgehog, *Erinaceus europaeus*, and the phylogenetic position of the Erinaceidae. *Journal of Molecular Evolution*, **41**, 952–957.

Kumar S, Tamura K, Nei M (1993) *MEGA*: *Molecular Evolutionary Genetics Analysis*, Version 1.01. The Pennsylvania State University, PA.

Maddison WP, Maddison DR (1992) *MacClade* v3.03. Sinauer Associates, Sunderland, MA.

Malatesta A (1985) Cause e meccanismi delle variazioni di taglia e delle modificazioni strutturali nei mammiferi quaternari delle isole mediterranee. *Bollettino della Societa Paleontogica Italiana*, **24**, 195–199.

Mandahl N (1978) Variation in C-stained chromosome regions in European hedgehogs (Insectivora, Mammalia). *Hereditas*, **89**, 107–128.

Mayr E (1963) *Animal Species and Evolution*. Harward University Press, Cambridge, MA.

Nachman MW, Boyer SN, Searle JB *et al*. (1994) Mitochondrial DNA variation and the evolution of Robertsonian chromosomal races of house mice, *Mus domesticus*. *Genetics*, **136**, 1105–1120.

Reeve N (1994) *Hedgehogs*. T & AD Poyser Ltd, London.

Saitou M, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **10**, 512–526.

Santucci F, Nascetti G, Bullini L (1996) Hybrid zones between two genetically differentiated forms of the pond frog *Rana lessonae* in southern Italy. *Journal of Evolutionary Biology*, **9**, 429–450.

Searle JB, Erskine I (1985) Evidence for a widespread karyotypic race of hedgehog (*Erinaceus europaeus*) in Britain. *Journal of Zoology of London*, **206**, 276–278.

Sokolov V-Ye, Aniskin VM, Lukyanova IV (1991) Karyological differentiation of two hedgehog species in genus *Erinaceus* (Insectivora, Erinaceidae) in the USSR. *Zoologichesky Zhurnal*, **70**, 111–120.

Swofford DL (1993) PAUP: *Phylogenetic Analysis Under Parsimony*, Version 3.1.1. Smithson Institution, USA.

Szymura JM (1993) Analysis of hybrid zones with *Bombina*. In: *Hybrid Zones and the Evolutionary Process*. (ed. Harrison RG), pp. 261–289. Oxford University Press, Oxford, New York.

Taberlet P, Bouvet J (1994) Mitochondrial DNA polymorphism, phylogeography and conservation genetics of the brown bear (*Ursus arctos*) in Europe. *Proceedings of the Royal Society of London, B*, **255**, 195–200.

Taberlet P, Fumagalli L, Hausser J (1994) Chromosomal versus mitochondrial DNA evolution: tracking the evolutionary history of the southwestern European populations of the *Sorex araneus* group (Mammalia, Insectivora). *Evolution*, **48**, 623–636.

Taberlet P, Fumagalli L, Wust-Saucy A, Cosson J (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7**, 453–464.

Talbot SL, Shields GF (1996a) Phylogeography of brown bears (*Ursus arctos*) of Alaska and paraphyly within the Ursidae. *Molecular Phylogenetics and Evolution*, **5**, 477–494.

Talbot SL, Shields GF (1996b) A phylogeny of the bears (Ursidae) inferred from complete sequences of three mitochondrial genes. *Molecular Phylogenetics and Evolution*, **5**, 567–575.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of the mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

Thorpe RS (1984) Primary and secondary transition zones in speciation and population differentiation: a phylogenetic analysis of range expansion. *Evolution*, **38**, 233–243.

Wilson AC, Cann RL, Carr SM *et al*. (1985) Mitochondrial DNA and two perspectives on evolutionary genetics. *Biological Journal of the Linnean Society*, **26**, 375–400.

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