

Understanding the phylogeographic patterns of European hedgehogs, *Erinaceus concolor* and *E. europaeus* using the MHC

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The genome of the European hedgehog, *Erinaceus concolor* and *E. europaeus*, shows a strong signal of cycles of restriction to glacial refugia and postglacial expansion. Patterns of expansion, however, differ for mitochondrial DNA (mtDNA) and preliminary analysis of nuclear markers. In this study, we determine phylogeographic patterns in the hedgehog using two loci of the major histocompatibility complex (MHC), isolated for the first time in hedgehogs. These genes show long persistence times and high polymorphism in many species because of the actions of balancing selection. Among 84 individuals screened for variation, only two *DQA* alleles were identified in each

species, but 10 *DQB* alleles were found in *E. concolor* and six in *E. europaeus*. A strong effect of demography on patterns of *DQB* variability is observed, with only weak evidence of balancing selection. While data from mtDNA clearly subdivide both species into monophyletic subgroups, the MHC data delineate only *E. concolor* into distinct subgroups, supporting the preliminary findings of other nuclear markers. Together with differences in variability, this suggests that the refugia history and/or expansion patterns of *E. concolor* and *E. europaeus* differ.

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Introduction

It has been shown that the genomes of many European species of plants and animals have been affected by the dramatic climate changes associated with cyclical glacial and interglacial periods (Hewitt, 1996, 1999; Comes and Kadereit, 1998; Taberlet *et al.*, 1998). During colder periods, temperate species have gone extinct, dispersed to new locations or survived in refugia, expanding out in postglacial periods (Hewitt, 2000). Mitochondrial DNA (mtDNA) studies show that the genomes of European hedgehogs bear the marks of glacially induced patterns of repeated expansion and contraction (Santucci *et al.*, 1998; Seddon *et al.*, 2001). It is likely that the two parapatric species of European hedgehogs, the white-breasted *Erinaceus concolor* in eastern Europe and the brown-breasted *E. europaeus* in western Europe (Reeve, 1994), found refuge in Iberia, Italy and the Balkans, expanding northwards in interglacial periods (Santucci *et al.*, 1998; Seddon *et al.*, 2001). The deep split between *E. concolor* and *E. europaeus* and the further subdivisions within the species (Santucci *et al.*, 1998; Seddon *et al.*, 2001) show that there have been strong effects of climate on the distribution of genetic variation of the hedgehogs. However, nuclear intron sequence data show a different

picture of divergence between postulated refugial groups in *E. concolor* but not *E. europaeus*. The addition of further loci is required to determine if this pattern is consistent across nuclear markers.

The class II major histocompatibility complex (MHC) genes include loci that display unusually high levels of polymorphism (reviewed in, eg, Potts and Wakeland, 1990; Meyer and Thomson, 2001). This high level of variation is consequent on the MHC molecules intrinsic role in the normal functioning of the immune system, with polymorphism concentrated in the peptide binding region (PBR) sites involved in pathogen recognition (Hughes and Nei, 1988, 1989). Polymorphism is thought to be maintained by balancing selection primarily driven by pathogen-based selection, through heterozygote advantage (Doherty and Zinkernagel, 1975; Hughes and Nei, 1988) or frequency-dependent selection (Bodmer, 1972; Takahata and Nei, 1990). The action of balancing selection also results in the long persistence times of MHC alleles. Transspecies polymorphism, in which alleles show relatedness extending beyond speciation events, indicates balancing selection in and from the distant past (Klein, 1987; Figueroa *et al.*, 1988; Garrigan and Hedrick, 2003). Hence, MHC genes are likely to provide a highly polymorphic system of alleles, many of which will predate the glacially induced bottlenecks of the European hedgehog. The aim of this study, therefore, is to assess the level of MHC variation in European hedgehogs to determine if the pattern of variation provides consistent differences in nuclear loci from the mtDNA patterns, furthering our understanding of the history of these species.

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Materials and methods

Samples

A total of 84 samples from European hedgehogs, 22 *E. concolor* and 62 *E. europaeus*, were screened for variation in this study. The samples represent a subset of those used in previous mitochondrial studies (Seddon *et al*, 2001), and they were selected to represent high mtDNA divergence and widespread locations. The locations of the samples by country are indicated in Table 1a and b, with more specific information available in online supplementary information.

Laboratory procedures

Genomic DNA was extracted by standard phenol/chloroform procedures (Sambrook *et al*, 1989). For *DQA*, PCR amplification employed a published forward primer (Slade *et al*, 1993) and a new reverse primer within the intron close to exon 2 (5'-CTCTGTCAGT AAGAAGTATGGA-3'). For *DQB*, two primers were designed within exon 2 in regions conserved across species: 5'-GATTCCTGTACCAGTTAAAGG-3' and 5'-TTGTRTCTGCASACCGTGTC-3'. A lack of conserved regions in DRB exon 2 precluded its use in this study.

The PCR reactions contained 0.2 μ M of each primer, 0.1 mM dNTPs, 2.0 mM MgCl₂, 0.05 U/ μ l AmpliTaq Gold (Applied Biosystems) *Taq* polymerase in 1 \times AmpliTaq buffer and approximately 1 ng DNA/ μ l reaction mix. The PCR programme had an initial denaturation of 95°C for 7 min, 35 cycles of 95°C for 40 s, 50°C for 1 min and 72°C for 1 min and 30 s, and a final extension of 72°C for 10 min.

All samples were screened for polymorphism at exon 2 of *DQA* and *DQB* using single-strand conformation polymorphism (SSCP) (Orita *et al*, 1989). Standard 12% polyacrylamide gels were run at 4°C (*DQA* and *DQB*) or 12°C (*DQB*), with 2% glycerol added for *DQA*. For *DQB*, a subset of homozygous samples, representing each configuration pattern on the SSCP gels, as well as all heterozygous samples were selected for sequencing. For the *DQA* locus, which appeared monomorphic on SSCP, all samples were sequenced after more than one allele was revealed by preliminary sequencing. PCR products were purified by EXOsap-IT (USB), and sequenced using a MegaBACE 1000 instrument (Amersham Biosciences).

Heterozygous samples in which one or both *DQB* alleles were absent in homozygous form were cloned, unless only one site differed between the two alleles. PCR products were purified using the QIAquick protocol (Qiagen), ligated using the pGEM-T Vector System (Promega) and transformed with JM109 *Escherichia coli*-competent cells (Promega). For each sample, a total of 10–12 colonies were sequenced. Alleles seen only in heterozygous form were confirmed when at least three cloned sequences showed concordance or, in one case, with two cloned sequences of each allele, differing only at sites previously identified as heterozygous. Sequences have been deposited in GenBank (Accession numbers: AY929164–AY929183).

Data analyses

DQA and *DQB* hedgehog sequences were aligned with related sequences in other mammalian species. For *DQA*, the hedgehog sequences formed a monophyletic clade in

a neighbour-joining tree (Jukes–Cantor distances, 1000 bootstrap iterations), more closely related to *DQA* sequences of other mammals than to other MHC alpha genes. A similar pattern was seen with hedgehog *DQB* sequences most closely related to mammalian *DQB* sequences, confirming that the expected genes have been amplified.

After translation, the absence of stop codons within the amino-acid sequences of *DQA* and *DQB* suggested that the sequences encoded functional proteins. No more than two alleles were found in any individual, indicating that single-copy loci had been amplified. PBR sites were identified for *DQA* and *DQB* according to the predictions in Brown *et al* (1988, 1993), who modelled the PBR sites in human class II MHC molecules.

The number of nonsynonymous substitutions per nonsynonymous site (d_N) and the number of synonymous substitutions per synonymous site (d_S) were calculated in MEGA v 2.1 (Kumar *et al*, 2001) using the Nei–Gojobori method with a Jukes–Cantor correction and the standard error estimated by bootstrapping (500 iterations). The mean pairwise number of nucleotide differences was calculated in MEGA v 2.1 (Kumar *et al*, 2001) and the nucleotide diversity was calculated in DnaSP v 4.0 (Rozas *et al*, 2003). Neighbour-joining trees were constructed for all hedgehog *DQA* and *DQB* alleles using Jukes–Cantor distance measures with 1000 bootstrap replicates in MEGA v 2.1 (Kumar *et al*, 2001).

The number of MHC alleles was compared with the number of mitochondrial types (Seddon *et al*, 2001). To control for biases in the level of variation due to the selection of samples, 10 000 data sets of 22 *E. concolor* and 62 *E. europaeus* individuals were created by resampling with replacement. The MHC/mtDNA ratio was calculated for each data set and the mean values compared using two-tailed *t*-tests.

Results

DQA sequences

Low levels of diversity were found at *DQA* in the hedgehog. In *E. concolor*, only two alleles were identified: *ErcoDQA*01*, which was found in all samples north of the Bosphorus, and *ErcoDQA*02*, which was identified in all samples found south of the Bosphorus (Table 1a). One nonsynonymous substitution, located within a PBR site, distinguishes the two alleles. In *E. europaeus* two alleles were found. *EreuDQA*01*, which was identical to *ErcoDQA*01*, was identified in many samples across a wide area, while *EreuDQA*02* was found only twice in homozygous form and once in heterozygous form among individuals from Estonia (Table 1b). One nonsynonymous substitution, located within a non-PBR site, distinguishes the two alleles. The phylogenetic relationship among the alleles is shown in online supplementary information.

DQB sequences

The *DQB* locus displayed higher diversity than *DQA* in the European hedgehogs. In *E. concolor*, 10 alleles were found, identified as *ErcoDQB*01* to *ErcoDQB*10* (Table 1a). These alleles are distinguished by 14 substitutions at a total of 13 polymorphic sites with eight amino-acid changes at seven polymorphic sites.

Table 1 Distribution of *DQA* and *DQB* alleles by locality for (a) *E. concolor* and (b) *E. europaeus*

Country	N	DQA		DQB		mtDNA
		H_{obs}	Alleles	H_{obs}	Alleles	
<i>(a) E. concolor</i>						
Estonia	1	—	*01	—	*01	C1
Poland	1	—	*01	1.0	*01, *05	C1
Austria/Hungary	5	—	*01	0.60	*01, *05, *06, *07	C1
Italy	4	—	*01	0.50	*01, *04, *06, *08	C1
Croatia/Serbia	2	—	*01	0.50	*01, *03, *04	C1
Greece/Turkey (Thrace)	4	—	*01	0.50	*03, *06, *09	C1
Israel/Turkey (Anatolia)	5	—	*02	0.20	*02, *10	C2
<i>(b) E. europaeus</i>						
UK	6	—	*01	—	*01	E2
Denmark/Norway/Sweden	5	—	*01	0.40	*01, *02	E1
Estonia/Russia	7	0.14	*01, *03	0	*01, *02	E1
Netherlands	7	—	*01	0.43	*01, *03	E1, E2
Germany	11	—	*01	0.09	*01, *04	E1
Switzerland	6	—	*01	0.50	*01, *03	E1, E2
France	6	—	*01	0	*01, *03	E2
Spain/Portugal	6	—	*01	0.17	*01, *05	E2
Italy/Corsica	8	—	*01	0	*01, *02, *06	E1, E3

N = number of samples; H_{obs} = observed proportion of heterozygotes. mtDNA haplotype groups are given according to Seddon *et al* (2001).

Although rare alleles were found across most of the distribution area of *E. concolor*, the highest number of alleles was found in central Europe, in the region of the Alps (Figure 1), although increased sampling through the postulated refugial region of the Balkans, where greater variation may exist, might change this. As for *DQA*, there is no overlap in allele distribution across the Bosphorous. In *E. europaeus*, six alleles were identified (*EreuDQB*01–EreuDQB*06*; Table 1b), defined by nine substitutions at nine polymorphic sites and six amino-acid changes at six polymorphic sites. As for *DQA*, one allele is shared between the two species (*ErcoDQB*01* and *EreuDQB*01*). Allele *EreuDQB*01* seems fixed in many regions, and importantly, allele *EreuDQB*03* is found across the boundary of two mitochondrial clades (Figure 1).

At the *DQB* locus, *E. concolor* shows greater variation than *E. europaeus* in the number of alleles and nucleotide diversity, which is significantly higher in *E. concolor* (*E. concolor* 0.025 ± 0.0013 , *E. europaeus* 0.0077 ± 0.0012 , $t = 9.8$, $P < 0.005$). Higher heterozygosity levels are also noted in *E. concolor* (*E. concolor* 45.5%, *E. europaeus* 16.1%). However, the lower values in *E. europaeus* are probably strongly influenced by allele fixation in north and far western Europe. Although more alleles are found in *E. concolor*, the alleles are not more divergent, as shown by the lack of a significant difference between the two species in mean pairwise number of nucleotide differences (*E. concolor* 5.62 ± 1.51 , *E. europaeus* 4.20 ± 1.38 , $t = 0.69$, $P > 0.05$).

Indicators of evolution at *DQB*

The presence of alleles shared between the species suggests the long-term action of balancing selection and this is further supported by the absence of species-specific clades in a phylogenetic analysis of *DQB* alleles (Figure 2). In contrast, there is little support for balancing selection in the patterns of nonsynonymous substitutions. In the presence of balancing selection, values d_N and d_S for PBR and non-PBR sites (Table 2) are expected

to show a d_N/d_S ratio greater than one at the functionally important PBR sites and a d_N/d_S ratio less than one at non-PBR sites. This pattern is noted for *E. europaeus* but not for *E. concolor*. However, the low number of variable PBR sites results in high standard errors when calculating d_N and d_S values for PBR sites.

For both species, there is a strong bias towards PBR variability in the 5' region of the exon. All variable PBR sites in *E. concolor* and six out of seven variable PBR sites in *E. europaeus* occur in the 5' sequence region that encodes the β -floor of the peptide binding cleft, with no or very low variability at PBR sites in the α -helix. Furthermore, there is higher nucleotide divergence calculated over all sites in the β than the α regions, reaching significance in *E. europaeus* (Jukes–Cantor distance: *E. concolor* – β 0.041 ± 0.013 and α 0.014 ± 0.010 , $t = 1.6$, $P > 0.05$; *E. europaeus* – β 0.033 ± 0.012 and α 0.0051 ± 0.0051 , $t = 2.1$, $P < 0.05$).

Number of *DQB* alleles compared with mtDNA results

To obtain a measure of the relative level of variation at the *DQB* locus, the number of alleles was compared to the number of mtDNA types (Seddon *et al*, 2001) (Table 3). It is clear that the level of MHC variation in comparison to the level of mtDNA variation is lower in *E. europaeus* than in *E. concolor*. While this could be biased by the selection of samples for high mtDNA variation, resampling with replacement shows that the MHC/mtDNA ratio remains significantly lower in *E. europaeus* ($t = 583$, $P < 0.0001$). The level of mtDNA variation is similar between the two species and so the observed MHC/mtDNA difference is likely to reflect lower MHC variation in *E. europaeus*.

Discussion

Demographic effects on MHC variation

The overall distribution of MHC alleles in hedgehogs across Europe is consistent with patterns observed for mitochondrial markers and nuclear introns, suggesting

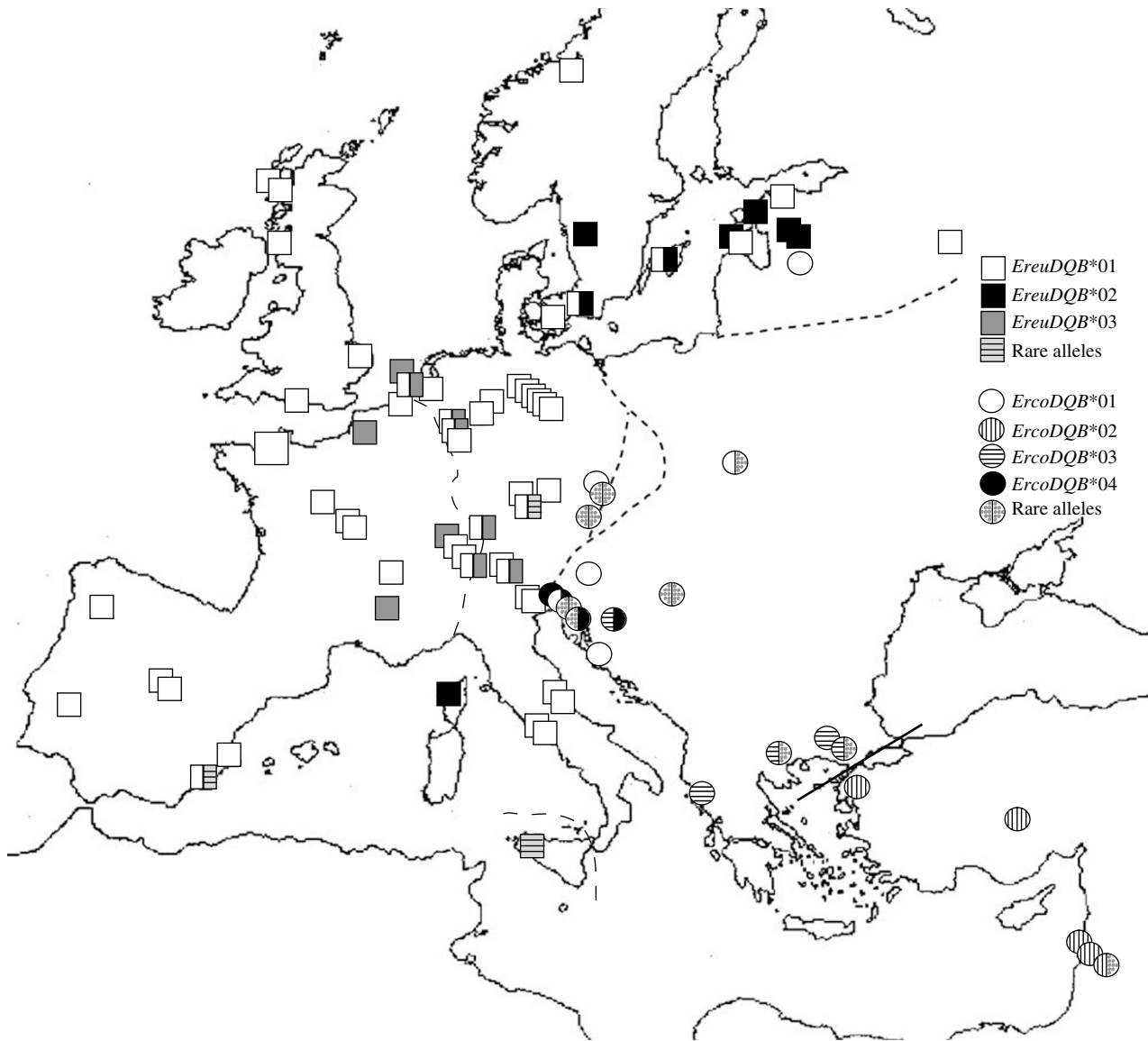


Figure 1 Distribution of *DQB* alleles among European hedgehogs, *E. concolor* and *E. europaeus*. Each figure (square, *E. europaeus*; circle, *E. concolor*) represents the genotype of one sample. Heterozygous samples are divided into two. Alleles with an intraspecific frequency of less than 10% are grouped as 'rare alleles'. The approximate separation of mitochondrial clades (Seddon *et al*, 2001) is shown by dashed lines in *E. europaeus* and by an unbroken line shows in *E. concolor*. The dotted lines indicate the boundaries between the two species.

that demography and range changes associated with cycles of restriction to glacial refugia and postglacial expansion have been the predominant force in shaping the patterns of MHC variation.

There are obvious similarities between the results from this study and previous analyses of mtDNA (Seddon *et al*, 2001), in terms of the geographic distribution of alleles. In particular, a deep subdivision in *E. concolor* is seen for both markers, coinciding with the Bosphorus. As for the mtDNA, there are no shared MHC alleles across this barrier, suggesting that there has been a lack of gene flow and effective isolation of these regions for a considerable period of time. One may broadly discern a similar western, central, eastern and Balkan genome pattern across Europe in the *DQB* allele data (Figure 1) as in the mtDNA (Seddon *et al*, 2001), but it is less distinct than the mtDNA. Other similarities are seen, with *EreuDQB*02* found in Corsica as well as Scandinavia

and Estonia, mimicking the long-distance colonisation in hedgehogs seen with mtDNA, and, in a manner similar to the divergent mtDNA haplotype identified only in Sicily, a unique allele (*EreuDQB*06*) was restricted to Sicily in this study (Seddon *et al*, 2001). Despite these apparent similarities, there are two important differences between the patterns of MHC and mtDNA variation.

Firstly, while the amount of mtDNA variation is similar between the two species (Seddon *et al*, 2001), there is more MHC variation in *E. concolor* than in *E. europaeus*. There are a total of 10 *DQB* alleles found among the 22 samples of *E. concolor*, while there are only six alleles identified among the 62 samples of *E. europaeus*. A contingency χ^2 test shows these ratios to be significantly different ($\chi^2 = 8.15$, $P < 0.01$), despite the relatively small sample sizes. Furthermore, the relative level of MHC variation at the *DQB* locus in comparison to the mtDNA variation is more than three times higher

in *E. concolor*. This finding supports the previous analysis of two nuclear introns in 23 hedgehog samples, in which higher nuclear diversity was noted for *E. concolor*. This suggests that the differences in variation between the species relate to the nuclear genome overall and not specifically to the MHC.

Secondly, postulated refugial clades within *E. europaeus* are not concordant among nuclear and mtDNA markers. Data from mtDNA (Seddon *et al*, 2001) clearly subdivide both species into monophyletic subgroups, suggesting separate past refugia and postglacial coloni-

sation routes. However, the nuclear intron results from the same study and the results from this MHC study, for both *DQA* and *DQB*, although limited by sample size, delineates only *E. concolor* into distinct subgroups.

Given there is little evidence for sex-biased dispersal in hedgehogs (Reeve, 1994), one possible explanation for the inconsistent pattern in mtDNA and nuclear loci is that the sorting of alleles is a result of repeated refugial separation. The similar level of intraspecific mtDNA divergence within *E. concolor* and *E. europaeus* suggests that the more recent refugial separations occurred at the same time in both species, with recent bottlenecks associated with contractions to the refugia accounting for the subdivision of mtDNA. However, the results from this MHC study and the previous nuclear intron study (Seddon *et al*, 2001) suggest additional older separation in refugia in *E. concolor*, allowing sorting of nuclear alleles, the signal of which has remained in the nuclear genome of *E. concolor*, while being eroded by more recent cycles in *E. europaeus*. The suggestion proposed by Seddon *et al* (2001) of a more severe bottleneck in the founding of *E. concolor* compared to *E. europaeus* is rendered unlikely by the relatively high level of MHC variation in *E. concolor*, which suggests that a large population size has been maintained during glacial periods.

A second explanation is a difference in past expansion patterns between the species. A more rapid expansion from refugia for *E. europaeus* would have resulted in a stronger founder effect and thus more severe loss of genetic variation (Hewitt, 1996; Ibrahim *et al*, 1996). If such rapid expansion occurred repeatedly over several glacial cycles, then a reduction in both MHC and mtDNA may be expected. In contrast, slower expansion patterns for *E. concolor* in the past could have maintained MHC variation. An extreme loss of genetic variation, by refugial bottlenecks or founder events, will obliterate

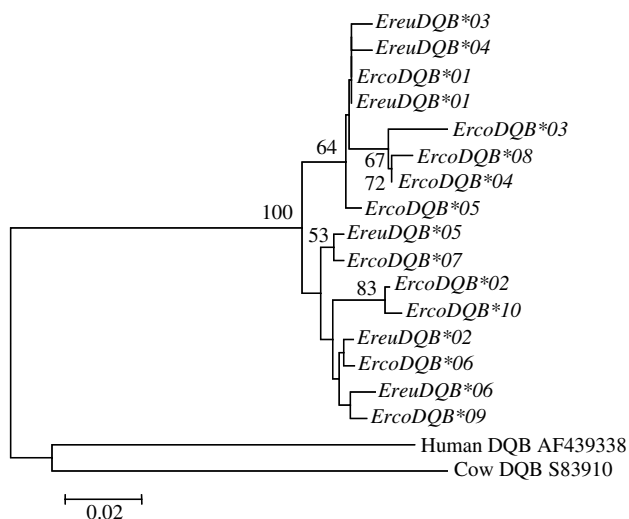


Figure 2 Neighbour-joining tree of the 15 *DQB* alleles identified in *E. concolor* and *E. europaeus*. The tree is based on Jukes–Cantor distances. Bootstrap values from 1000 iterations are shown where values exceed 50%. *DQB* sequences from human and cow are included as outgroups.

Table 2 Synonymous (d_S) and nonsynonymous (d_N) changes at peptide binding region and nonpeptide binding region sites in hedgehog exon 2 *DQB* alleles

Species	N	Peptide binding region			Nonpeptide binding region				
		d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S		
<i>E. concolor</i>	10	0.037 ± 0.025	0.050 ± 0.053	0.75	ND	0.027 ± 0.013	0.029 ± 0.018	0.92	ND
<i>E. europaeus</i>	6	0.040 ± 0.023	0.000 ± 0.000	ND	*	0.019 ± 0.012	0.027 ± 0.021	0.71	ND

n = number of alleles; ND = not determined.

d_S and d_N values were calculated using the Nei–Gojobori method with a Jukes–Cantor correction. Standard errors were estimated by 500 bootstrap iterations. In all, 14 PBR codons and 48 non-PBR codons were analysed.

Statistical significance assessed by one-tailed *t*-test: *, $P < 0.05$.

Table 3 Comparison between MHC (*DQB*) and mitochondrial variation in hedgehogs

Species by mtDNA group	N	No. of <i>DQB</i> alleles	No. of mtDNA types	MHC/mtDNA	Unbiased MHC/mtDNA
<i>E. concolor</i>	22	10	22	0.455	0.608 ± 0.0008
C1	17	8	17	0.471	
C2	5	2	5	0.400	
<i>E. europaeus</i>	62	7	55	0.127	0.138 ± 0.0002
E1	39	4	38	0.105	
E2	23	3	17	0.176	

Unbiased MHC/mtDNA ratios were estimated by creating 10 000 data sets of 22 *E. concolor* and 62 *E. europaeus* individuals by resampling with replacement. MtDNA groupings (E1, E2, C1, C2) are based on Seddon *et al* (2001).

the signal of previous events. Hence, the loss of variability at mtDNA but not at nuclear DNA indicates that the recent bottleneck(s) were mild, affecting mtDNA, a marker sensitive to the effects of population size reductions because it has an effective population size one-quarter that of nuclear DNA, but not resulting in obvious reduction of nuclear variability.

MHC variation and effects of balancing selection

Although transspecies polymorphism in the hedgehog MHC suggests the action of balancing selection, a conflicting signal is given at the nucleotide level by an unexpectedly low d_N/d_S ratio in *E. concolor*. There are several explanations to explain an apparent reduction in selection. Firstly, there is a possibility that PBR sites may differ between different species and not reflect the predicted models of humans on which these calculations are based. This does not seem likely considering that the overall structure is often very conserved between species (Trowsdale, 1995). Secondly, environmental and behavioural factors that affect the strength of balancing selection have in some cases been proposed to account for low levels of MHC polymorphism (Slade, 1992; Murray *et al*, 1995; Ellegren *et al*, 1996). In hedgehogs, hibernation, in which body temperature and many physiological systems, including immune function, are greatly suppressed (Boyer and Barnes, 1999; Burton and Reichman, 1999; Nedergaard and Cannon, 1990) and may affect the strength of balancing selection. Thirdly, the inconsistent pattern between the β -floor and the α -helix encoding regions may indicate site-specific selection pressure as seen in the estuarine fish (*Fundulus heteroclitus*; Cohen, 2002). However, a higher d_S value in the β -floor region than for the α -helix region suggests different evolutionary histories. Similar patterns have been noted in DRB sequences (Gyllensten *et al*, 1991; Schwaiger *et al*, 1993) and a conserved recombination motif, present also in the hedgehog DQB sequences, has been identified (Wu *et al*, 1986).

Conclusions

The use of nuclear markers in addition to mtDNA has allowed us to draw more detailed conclusions about the demographic changes associated with the glacial refugia and postglacial expansion events. mtDNA is widely used in phylogeographic studies (reviewed by Taberlet *et al*, 1998; Hewitt, 1999), yet this marker is particularly sensitive to reductions in the effective population size and only provides information about the maternal line. In the European hedgehog, the use of mtDNA alone suggested distinct lineages in *E. europaeus* that were not supported by nuclear data. However, the clear separation of *E. concolor* into two clades, north and south of the Bosphorus, is noted with both MHC and mtDNA. Hence, these two species have different refugial histories, with inferred differences in population size or expansion patterns. This additional demographic information gained demonstrates that the use of more than one marker type is to be encouraged in phylogeographic studies.

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