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SHORT COMMUNICATION

The occurrence of mountain hare mitochondrial DNA in wild brown hares

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Abstract

If interspecific hybrids are fertile and backcross to either parental species, transmission of mitochondrial DNA over the species barrier can occur. To investigate if such transmission has occurred between the brown hare *Lepus europeus* Pall and the mountain hare *L. timidus* L. in Scandinavia, an analysis of genetic variation in mitochondrial DNA from 36 hares, collected from 15 localities, was performed. Sequence divergence of mtDNA between species was estimated at 8 ± 1% (SD). Intraspecific mtDNA sequence divergence varied between 0.09 and 0.38% in brown hares and 0.10 and 1.44% in mountain hares. In six out of 18 brown hares examined, two different haplotypes of mountain hare origin were detected, demonstrating a transmission of mtDNA haplotypes from mountain hares to brown hares. The results indicate that interspecific hybridization between the two species occurs in wild populations.

Keywords: *Lepus*, Lagomorpha, hybridization, introgression, mitochondrial DNA, genetic variation

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Introduction

The brown hare *Lepus europeus*, Pallas 1778 was introduced to Sweden from northern Europe as a game animal during the nineteenth century (Lönnberg 1905). The first successful introduction was made on the island of Ven in Öresund 1857–58 (Sjögren 1971), followed by several introductions on the mainland. Today, the brown hare inhabits southern and central Sweden, preferring agricultural areas (Frylestam 1990). The mountain hare *Lepus timidus*, Linnaeus 1758 has inhabited Scandinavia since the end of the most recent glacial period (Liljegren & Lagerås 1993) and occurs today south to 56°N, including the islands of Öland and Gotland (Angerbjörn & Flux 1995).

The morphological differences in fur and skeleton between the brown and the mountain hare are well defined (Angerbjörn & Flux 1995). However, since the first introductions of the brown hare to Sweden, morphological intermediates between the brown hare and the mountain hare have been reported to occur in the wild (Lönnberg 1905). These individuals, showing intermediate characters, have been considered hybrids between the two species (Lönnberg 1905). In captivity, mountain hare females spontaneously mate with brown hare males and produce viable offspring, but the reverse crosses has to be performed by insemination (Gustavsson & Sundt 1965). The F_1 hybrids are morphological intermediates between the species (Notini 1941) and are often considered fertile (Lönnberg 1905; Gustavsson 1971; Schröder *et al*. 1987). However, an attempt to demonstrate hybridization among wild hares in Finland with species-specific immunoglobulin markers was unsuccessful (Schröder *et al*. 1987).

Mitochondrial DNA (mtDNA) has a rapid substitution rate (Brown *et al*. 1979), and is therefore a useful marker when studying genetic divergence between and within closely related species or subspecies. We hypothesized, that female hybrids are fertile and backcross to either parental species. This backcross could result in a transmission of mitochondrial DNA across the species border. Individuals of one species carrying mtDNA from the other species could be detected, as earlier described for Scandinavian *Mus* (Ferris *et al*. 1983) and *Clethrionomys* species (Tegelström 1987).

We have analysed restriction fragment length polymorphisms (RFLP) in mtDNA from Scandinavian brown and mountain hares. Our estimates of genetic distance in

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mtDNA between and within the two species demonstrate the occurrence of different mtDNA haplotypes from mountain hares among wild brown hares.

Materials and methods

Kidneys were collected from 18 brown hares *Lepus europeus* and 18 mountain hares *Lepus timidus* shot by hunters in eight and nine localities, respectively, representing a total of 15 localities (Fig. 1). This sample of hares was collected from sympatric (localities 2–11) and allopatric populations (locality 1 for brown hares and 11–15 for mountain hares) of the two species during the hunting season in 1988–94. The kidneys were frozen at –20 °C and later stored at –80 °C. Mitochondria were isolated from 0.5–2.0 g of kidney by differential centrifugation (Lansman *et al*. 1981; Jones *et al*. 1988). MtDNA was purified by phenol/chloroform extraction as described by Jaarola & Tegelström (1995) with some modifications (Bilton & Jaarola 1996).

Analysis of mtDNA variation

For an initial screening of haplotypes, mtDNA from all 36 samples was digested with the tetranucleotide restriction enzyme *Mbo*I. Restriction fragments were separated by electrophoresis in 5% polyacrylamide gels (Tegelström 1986, 1992). Lambda DNA digested with *Bgl*I was used as a size marker. The fragments were visualized by silver staining (Guillemette & Lewis 1983; Tegelström 1986, 1992). Four brown hares and seven mountain hares with species-specific mtDNA were not further analysed in order to keep the investigation limited. Extended investigations of intraspecific variation and diversity of these species are in progress and will be presented elsewhere. The remaining 14 brown hares and 11 mountain hares (referred to as Le and Lt, respectively) nevertheless represent a wide range of Scandinavian localities (Fig. 1). MtDNA from six of these brown hares (referred to as Le*) showed *Mbo*I-restriction patterns identical or similar to the patterns of mountain hare mtDNA. The mtDNA from the

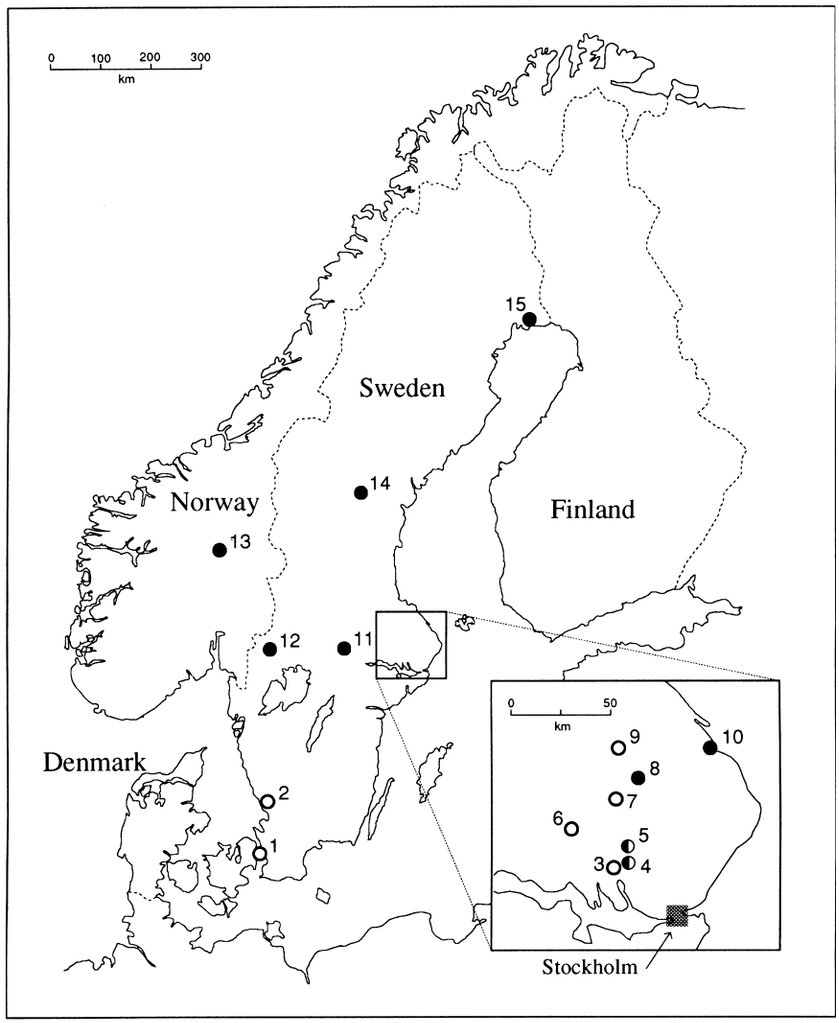

Fig. 1 Map of collection sites. Empty circles refer to localities where only brown hares were collected, filled circles to localities where only mountain hares were collected and half-filled to localities where samples from both species were collected. The region of Uppland, where brown hares carrying mountain hare mtDNA were found, is shown in the enlarged inset.

other eight brown hares and the 11 mountain hares showed variable, but species-specific, *Mbo*I-restriction patterns.

To estimate the variation between species-specific mtDNA haplotypes, the selected 25 samples were analysed with a total of six tetranucleotide restriction enzymes (*Hae*III, *Hpa*II, *Hin*fI, *Mbo*I, *Sau*96I and *Rsa*I). Each obtained restriction fragment pattern was given a specific letter: capital letters if Le and small letters if Lt or Le*. Thus, each haplotype was given a six letter composite code and every unique code was given a number (Table 1). By estimating gain and/or loss of restriction sites between restriction morphs for each enzyme, a minimum path network of site differences among fragment profiles was constructed (Avise *et al*. 1979; Lansman *et al*. 1983; Avise 1989). Five small fragments were assumed in order to explain all mutations, since fragments smaller than 100 base pairs were usually undetectable. Intraspecific sequence divergence (d), haplotype (h) and nucleotide diversity (π) was estimated from site data with equations given by Nei & Li (1979), Nei & Tajima (1981) and Nei (1987), using the computer package REAP version 4.0 (McElroy *et al*. 1992). The minimum path networks for different enzymes were summarized to construct a parsimonious network of phylogenetic relationship between haplotypes (Fig. 2).

To estimate interspecific genetic distance, mtDNA from one individual of each species carrying species-specific mtDNA haplotypes (number 1 and III in Fig. 2) was digested with a total of eight tetranucleotide restriction enzymes (*Hae*III, *Hpa*II, *Hin*fI, *Mbo*I, *Sau*96I, *Rsa*I, *Dde*I and *Taq*I). Fragments were separated as described by Tegelström & Jaarola (1989), and sequence divergence between the two species was estimated from fragment data as described by Nei & Li (1979) and Nei (1987). Standard deviations where calculated as suggested by Upholt (1977).

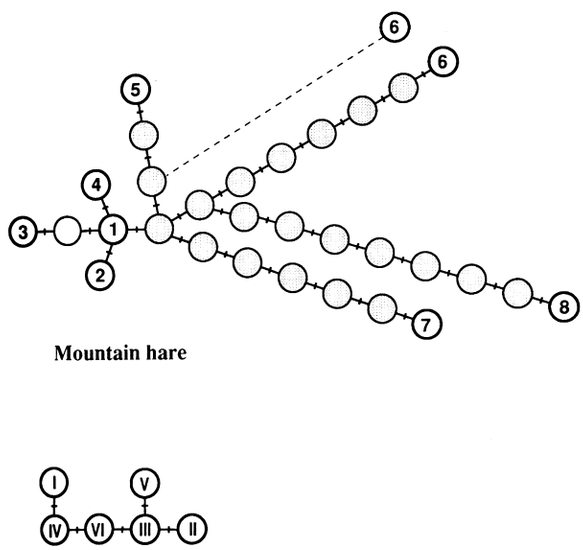

Fig. 2 Hand-drawn parsimonious phylogenetic network of site differences between eight mountain hare and six brown hare mtDNA haplotypes. Hypothetical haplotypes, not observed in the sample, are marked grey. Restriction site changes are depicted along branches linking haplotypes. An alternative clustering of mountain hare haplotype 6 is depicted with a dashed line.

Results and discussion

The total number of restriction fragments per individual varied between 122 and 126 in the mountain hare and 129 and 132 in the brown hare, corresponding to about 500 bp, or 3% of the mitochondrial genome. The mtDNA sequence divergence between the mountain hare and the brown hare was estimated at 8 ± 1% (SD). This is a high but relatively normal divergence between mammalian species of the same genus (Tegelström *et al*. 1988; Martin & Palumbi 1993). Assuming a divergence rate of 2–4%/ Myr (Brown *et al*. 1979; Martin & Palumbi 1993) the estimated time since divergence of the mountain hare and the brown hare maternal lineages will be about 2–4 Myr.

Altogether, eight mountain hare and six brown hare mtDNA haplotypes were identified (Table 1). The sequence divergence between mountain hare haplotypes (including Le*) varied between 0.10 and 1.44%. The parsimony network of site differences (Fig. 2) shows that mountain hare haplotypes 1–4 cluster tightly together, with haplotype 5 added more apart. Haplotypes 6–8 constitute more distantly related branches. The Swedish mountain hares are divided in two morphological subspecies, *Lepus timidus timidus* (Linnaeus 1758) and *L. t. sylvaticus* (Nilsson 1831) (Angerbjörn & Flux 1995). Bergengren (1969) proposed different postglacial colonization routes to Scandinavia for these subspecies: a northern route for *L. t. timidus* and a southern route for *L. t. sylvaticus*. In our investigation, *L. t. sylvaticus* is represented by two individuals from localities 8 and 12, carrying haplotypes 1 and 3, respectively (Table 1 and Fig. 2). Although our estimates of sequence divergence indicate a relatively large intraspecific mtDNA variation within mountain hares (0.10–1.33%, Le* excluded), no dichotomous subdivision of mtDNA haplotypes can be observed as expected from a two-directional colonization route. All mountain hares examined carried species-specific mtDNA.

Among the brown hares, polymorphism was detected with two restriction enzymes, *Hin*fI and *Rsa*I. The divergence between haplotypes was low and varied between 0.09 and 0.38%. The parsimony network of brown hare haplotypes is shown in Fig. 2. Because the brown hares introduced to Scandinavia were brought from various

Table 1 Assigned haplotype and composite designations of mtDNA from brown and mountain hares, respectively, along with the number of individuals carrying a specific haplotype. The six letter code designate, from left to right, restriction profiles produced by restriction enzymes *Hae*III, *Hpa*II, *Hin*fI, *Mbo*I, *Sau*96I and *Rsa*I. Locality number refers to the locality were the individual was shot (Fig. 1)

Haplotype number	mtDNA haplotype	Number of individuals	Locality number
Brown hare			
I	A A A A A A	1	1
I I	A A B A A B	1	1
I I I	A A C A A B	1	2
I V	A A D A A A	3	4, 5, 9
V	A A E A A B	1	7
V I	A A D A A B	1	6
1	c a a a a a	2	4, 5
8	d d e d e e	4	3, 7
Mountain hare			
1	c a a a a a	3	4, 5, 8
2	c a c a a a	1	14
3	c a a c a a	2	12
4	c a a a a d	1	11
5	c a f b c a	1	15
6	d c d a c c	1	13
7	b b b a b b	2	8, 10

parts of central and northern Europe, we suggest an overall low level of mtDNA variation in this species. This conclusion is strengthened by an investigation by Hartl *et al*. (1993), demonstrating low differentiation in mtDNA as well as in allozymes between brown hares from Austria.

A total of six brown hares (Le*) out of the 18 examined carried mtDNA similar or identical to mountain hare mtDNA. Haplotype 1 was found in two morphologically distinct brown hares and three mountain hares from localities 4, 5 and 8 in the region of Uppland (Fig. 1). We suggest that this haplotype has been transmitted from mountain hares to brown hares via hybridization in the wild, after the introduction of brown hares to Sweden. Although we can not dismiss the possibility of captive hybridization being the cause of mtDNA transmission, we believe that it is highly unlikely because haplotype 1 was detected in wild individuals of both species from the same localities. The one-way transmission of mtDNA, from mountain hares to brown hares, is inferred by the asymmetric patterns of mating observed in captivity (Gustavsson & Sundt 1965). Mountain hare haplotype 8 was found in four brown hares from two localities (3 and 7) in Uppland, but not in any mountain hare. This haplotype is separated from other mountain hare haplotypes by a sequence divergence of 0.83–1.44%. Although haplotype 8 might be carried by Scandinavian mountain hares, there is also a possibility that it has been introduced to Sweden along with some brown hares. If so, it would indicate that transmission of mtDNA over the species barrier via hybridization occurs on the European continent as well. The observed separation of haplotype 8 from the other investigated mountain hare haplotypes could reflect the genetic distance between the Alpine subspecies *L. t. varronius* (Miller 1901) and the Scandinavian subspecies described above. Continuous asymmetric transmission of mtDNA over time would create a mixture of mountain hare and brown hare haplotypes in populations of brown hares. Pérez-Suárez *et al*. (1994) describe an unusually high degree of intraspecific mtDNA variation in Iberian brown hares that, along with the present study, might be an indication of the existence of such a mixture. Also, continuous gene flow between the brown hare and the mountain hare, resulting in a diffusion of nuclear alleles, could explain the shorter time since divergence (0.5%/Myr) when estimated from allozyme data (Grillitsch *et al*. 1992) compared with the 2–4%/Myr estimated from mtDNA in the present investigation.

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