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Biased geographical distribution of mitochondrial DNA that passed the species barrier from mountain hares to brown hares (genus *Lepus*): an effect of genetic incompatibility and mating behaviour?

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Abstract

Through interspecific hybridization and subsequent backcrossing, genes and genomes may be transferred over the species barrier. In Sweden, the introduced brown hare *Lepus europaeus* hybridizes with the native mountain hare *L. timidus*. To investigate the direction and the extent of transfer of mitochondrial DNA (mtDNA) between the species, the mtDNA haplotypes were screened in 522 brown hares and 149 mountain hares from areas of sympatry and allopatry. A total of 51 brown hares with mountain hare mtDNA, but no mountain hares with brown hare mtDNA were detected. Thus, mtDNA transfer over the species barrier is directed from mountain hares to brown hares. We argue that frequency-dependent hybridization and/or interspecific male competition mediates this directionality. Further, the percentage of brown hares with transmitted mountain hare mtDNA was lower in areas of former species sympatry (0.6%) compared to areas of current sympatry (15%). Thus, the transferred mtDNA may disappear from brown hare populations if there is no continuous input through hybridization. We suggest that specimens with an alien mtDNA experience a fitness reduction as a result of a functional incompatibility between the cytoplasmic mitochondrial genomes and the cell nucleus.

Key words: *Lepus*, hares, hybridization, introgression, mtDNA, selection

INTRODUCTION

Interspecific hybridization and subsequent backcrossing may generate the transfer of species-specific genes and genomes across the species barrier (introgression). If the hybridization and introgression are a result of human interference, such as introduction of alien species or habitat changes, the transfer of genes may constitute a threat to the unique evolutionary lineages within species through the mixing of gene pools (Ebenhard, 1988; Lehman *et al*., 1991; Rhymer, Williams & Braun, 1994; Rhymer & Simberloff, 1996; Simberloff, 1996). Hybridization between the native *Lepus timidus* (mountain hare) and the introduced *L. europaeus* (brown hare) in Sweden has been reported in wild sympatric populations of the two species since the latter were introduced during the 19th century (Lönnberg, 1905). Hybrids are easily acquired in captivity (Gustavsson & Sundt, 1965) and the F_1 hybrids are morphological intermediates between the species (Notini, 1941). The hybrids are often considered fertile (Lönnberg, 1905; Gustavsson, 1971; Schröder *et al*., 1987), but an attempt to demonstrate hybridization among wild hares in Finland with species-specific immunoglobulin markers was unsuccessful (Schröder *et al.*, 1987). Recently, however, mitochondrial DNA (mtDNA) lineages were detected in Swedish *L. europaeus* specimens that were identical, or resembled, mtDNA of *L. timidus* specimens sampled at the same locality (Thulin, Jaarola & Tegelström, 1997). Presumably, these lineages were transferred over the species barrier through interspecific hybridization between *L. europaeus* males and *L. timidus* females, after which hybrid females backcross to *L. europaeus* males and thereby mediate the mtDNA introgression. However, the investigated *L. europaeus* specimens were sampled only in a restricted region in central Sweden and may in principle be the result of two single hybridization events because only two transferred mtDNA lineages were detected (Thulin, Jaarola *et al.*, 1997). Further, because only six *L. timidus* specimens from areas of species sympatry were investigated, the possibility of bi-directional mtDNA introgression could not be assessed.

According to the principal expectations described by Hubbs (1955), animal hybridization usually occurs

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when one species is rare and the other is common. This phenomenon, referred to as 'Hubbs principle', has recently been extended by Wirtz (1999) to form a 'sexual selection hypothesis' of hybridization, where females of the rare species hybridize with males of the common species. After the introduction of *L. europaeus* to southern Sweden, the native *L. timidus* in this area started to disappear and is today absent (Thulin, 2000). Thus, in areas in Sweden where *L. europaeus* occurs, *L. timidus* is either absent, declining and/or rare. Therefore, hybridization and introgression between these species may follow the principles of Hubbs (1955) and Wirtz (1999). Further, hybridization between these species could be in accordance with the suggestions of Grant & Grant (1997), stating that unidirectional hybridization between females of the smaller species (*L. timidus*) and males of the larger species (*L. europaeus*) is the expected direction of mating when two species with different body sizes hybridize. Interestingly, in captivity, the *L. timidus* female spontaneously mate with the *L. europaeus* males while the reciprocal crossing has to be performed with artificial insemination (Gustavsson & Sundt, 1965). The latter incompatibility indicates that there is a unidirectional, behavioural barrier to reproduction, perhaps related to the reproductive behaviour of hares.

The transfer of genes over a species barrier provides novel genetic material for natural selection to work upon and, thus, may actually increase the evolutionary potential of a species (Anderson & Stebbins, 1954; Arnold, 1997). Assuming an evolutionary potential for transferred genes, they should be conserved within the new species, and ultimately become fixed. If the novel genes are evolutionary neutral, they should instead be affected by random processes, and therefore most probably disappear if the introgression is stochastic and restricted (cf. Avise, Neigel & Arnold, 1984). Finally, if there is a functional mismatch between the transferred and the species-specific genes, individuals with this genotype should be selected against and, thus, the transferred genes and genomes would rapidly disappear. As *L. timidus* in southern Sweden started to vanish a few decades after *L. europaeus* was established, the transfer of genes between the two species through hybridization was interrupted. If this newly allopatric *L. europaeus* population in southern Sweden was not exposed to a severe bottle-neck, which could randomly eliminate transferred genes, or if the transferred genes were selectively neutral or favoured, any genes transferred in previous sympatry with *L. timidus* would still be present.

To test if transfer of mtDNA is unidirectional from *L. timidus* females to *L. europaeus*, mtDNA origin was examined in hares of both species with sufficient geographic representation of their respective distribution in Sweden. The results are evaluated in relation to general principles of courting behaviour and the reproductive biology of hares. The persistence of transferred mtDNA in areas of former sympatry between the species was also investigated. The evolutionary importance of the mtDNA introgression observed among these hare species is discussed, along with possible implications of hybridization for their coexistence.

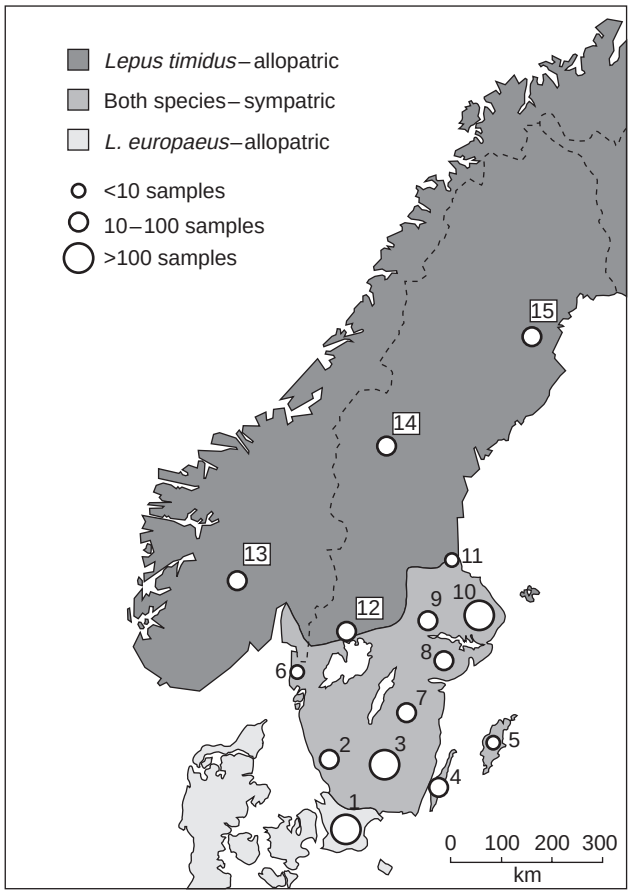

Fig. 1. The distribution of *L. europaeus* and *L. timidus* in Scandinavia. The circles with numbers indicate samples from this specific region, with the exact number of individuals given in Table 1.

MATERIALS AND METHODS

Samples from hares were collected with the help of hunters from 15 regions at a total of 160 localities across Scandinavia (Fig. 1). In total, 671 samples were included in this study, 522 from typical *L. europaeus* specimens and 149 from typical *L. timidus* specimens (Table 1). The external morphological differences between the two species are well defined (Hewson, 1993), especially during the hunting season, when *L. timidus* in Sweden has a white or light grey winter pelage, while *L. europaeus* remains brown throughout the year. External characters, that differ between the species throughout the year, include the iris colour (brown in *L. timidus*; light yellow in *L. europaeus)*, ear length (shorter than the nose when bent forward in *L. timidus*; longer in *L. europaeus*), tail colour (white in *L. timidus*; white with black triangle in *L. europaeus*) and appearance (tight and 'rabbit-like' in *L. timidus*; lanky in *L. euro-*

Table 1. The numbers of investigated *L. europaeus* (N_{Le}), *L. timidus* (N_{Lt}) and total number of hares (N_{Total}) from each region. The number of detected *L. europaeus* with *L. timidus* mtDNA are given for each region (N_{Le*}). The geographic location of regions is shown in Fig. 1.

Region (number)	N_{Le}	N_{Lt}	N_{Total}	N_{Le*}	% Le*
1. Skåne	180	–	180	1	0.6
2. Halland	29	–	29	3	10
3. Småland	93	20	113	23	25
4. Öland	21	–	21	3	14
5. Gotland	5	3	8	1	20
6. Bohuslän	–	2	2	–	–
7. Östergötland	18	1	19	–	0
8. Södermanland	29	4	33	2	7
9. Västmanland	26	1	27	1	4
10. Uppland	120	16	136	17	14
11. Gästrikland	–	1	1	–	–
12. Värmland	1	18	19	–	–
13. Norway	–	28	28	–	–
14. Jämtland	–	25	25	–	–
15. Norrbotten	–	30	30	–	–
Total	522	149	671	51	10

paeus). Nine *L. europaeus* with short ears and/or white spots in the pelage and 19 *L. timidus* with long brown ears and brown patches in the white (or light grey) winter pelage were excluded from the present study because of suspected hybrid ancestry. Tissue from various sources and of different quality was used (skin, bowel, muscle, etc.).

Sequencing

A 307 base pair stretch of the mtDNA cytochrome *b* gene was amplified, using the polymerase chain reaction (PCR) technique (Saiki *et al.*, 1985), from species-specific mtDNA from 1 L. europaeus and 1 *L. timidus* using the universal primers (target sequences) L14181 and H15149 (Kocher *et al.*, 1989). Purified mtDNA was used as the template for the PCR to certify amplification of the correct region and minimize the possibility of amplification of any homologous DNA sequence in the cell nucleus (cf. Zhang & Hewitt, 1996). Approximately 80 ng of the purified PCR product was ligated into the pCR™ 2.1 vector (Invitrogen) following the manufacturers instructions. INVaF competent bacteria were transformed with the ligated pCR™ 2.1 vector using the Original TA Cloning Kit (Invitrogen). DNA was extracted from positive transformants (bacteria that successfully incorporated the ligated vector) and then used as template for DNA sequencing with the dideoxy chain termination method (Sanger, Nicklen & Coulson, 1977), using the T7Sequencing™Kit (Pharmacia Biotech). The standard M13 reversed and the M13 (-20) forward primers were used in the sequencing reaction. Radioactive labelling of DNA sequences was performed by incorporation of α-^{33}P labelled dATP in the sequencing reaction. The sequencing reaction products were separated electrophoretically on 6% polyacrylamide gels that were subsequently dried and then exposed to autoradiographic film, from which the DNA sequence was interpreted. The obtained 307 base pair sequences are accessible through the EMBL Nucleotide Sequence Database under the accession numbers AJ250143 (*L. europaeus*) and AJ250144 (*L. timidus*).

MtDNA screening

To find species-diagnostic restriction endonuclease cutting sites (DNA sections recognized by a specific DNA cutting enzyme) in our obtained DNA sequences, the search function in Word 6.0 (Microsoft) was used. The 2 endonucleases *Dde*I (recognition sequence CTNAG) and *Hinf*I (recognition sequence GANTC) were chosen for the analysis. For *Hinf*I, 2 recognition sites were detected in the *L. europaeus* sequence (resulting fragments are 72, 100 and 135 base pairs long) and 1 in the *L. timidus* sequence (resulting fragments are 100 and 207 base pairs long). *Dde*I cut the *L. europaeus* sequence in to 2 fragments (97 and 210 base pairs long, respectively) while no recognition site was present in the *L. timidus* sequence.

Screening of mtDNA haplotypes was performed with PCR, using total DNA isolated with Chelex-100 (Walsh, Metzger & Higuchi, 1991) as a template. Proteinase treatment was performed overnight to get a satisfactory disruption of cellular membranes. The composition of the PCR was: 1 µl template (5–150 ng total DNA), 1X buffer (Mg^{2+} free, distributed with the polymerase), 1.2 mM $MgCl_2$, 60 µM /nucleotide dNTP, 400 nM of each primer and 0.5 units *Taq* polymerase (Promega). The PCR reactions were carried out in a PTM-100 cycler (MJ Research) with cycles as follows: 94°/30 min, 92°/30 min, 53°/30 min, 72°/45 min, the last 3 steps repeated 35 times, with a final elongation at 72° for 2 min. The obtained PCR product was digested with the restriction endonucleases *Hinf*I and *Dde*I, the obtained fragments were separated in 2% agarose gels and visualized by ethidiumbromide staining. The visualized fragments were interpreted as being of *L. europaeus* or *L. timidus* mtDNA type depending on their length.

Sequence analysis

Kimura 2-parameter genetic distance (Kimura, 1980) was calculated in the computer package Phylip (Felsenstein, 1993), with the ratio transition/transversion weighted 2:1 (standard settings). Standard deviations of percentage sequence divergence were calculated as described by Upholt (1977). Genetic distances for synonymous and non-synonymous DNA nucleotide substitutions respectively (e.g. a 'synonymous substitution' will not alter the amino-acid coded for) were calculated in the computer package Synonym, constructed and kindly provided by Dr Pekka Pamilo (University of Oulo). Fishers' exact test of differentiation of mtDNA haplotype distribution between and

within the species were calculated in the computer package Genepop 3.1 (Raymond & Rousset, 1995). Corrections for multiple comparisons were performed as described by Rice (1989).

To identify non-synonymous nucleotide substitutions in the mtDNA, the amino acid sequence coded for by a 702 base pair cytochrome *b* motif from 1 *L. europaeus* and 1 *L. timidus* were obtained from the NCBI GenBank (AF010162 and AF010155; Halanych *et al.*, 1999). The *L. europaeus* specimen is the same individual that was sequenced in the present study for 307 base pairs of the cytochrome *b* gene. The *L. timidus* specimen was from the Chukotka Peninsula, far east Russia (Siberia).

RESULTS

The 307 base pair stretch of the mitochondrial DNA cytochrome *b* gene showed 29 synonymous substitutions that differed between the species: 28 transitions and one transversion.Twenty-five transitions were at twofold degenerate sites and the remaining three at fourfold degenerate sites. The Kimura 2-parameter genetic distance between the two sequences was estimated at 0.10 and the percentage sequence divergence at 9.4% (± 1.7%). Similarly, mtDNA from the two species show a 8% (± 1%) sequence divergence using the RFLP technique (Thulin, Jaarola *et al.*, 1997) and direct sequencing of a 410 base pair long fragment of the control region shows a 12–14% sequence divergence (Thulin, Isaksson & Tegelström, 1997). The genetic distance at synonymous sites was estimated at 0.36 (± 0.06), i.e. more than one-third of the synonymous sites have been altered. This observation is in agreement with the previously described saturation of synonymous DNA sequence substitutions between mtDNA from different *Lepus* taxa (Halanych *et al.*, 1999).

Among the 522 *L. europaeus* specimens included in the study, 471 carried species-specific mtDNA and 51 (10%) carried transmitted *L. timidus* mtDNA (Table 1). All 149 *L. timidus* had species-specific mtDNA and, thus, introgression of mtDNA is directed from *L. timidus* to *L. europaeus* ($P > 0.001$, Fishers' exact test). In the only area where *L. europaeus* currently is allopatric (see Fig. 1), only one out of 180 investigated individuals had transmitted mtDNA. The additional 50 individuals were detected in areas where the two species are sympatric (Fig. 1, Table 1). Thus, the frequencies of transmitted mtDNA differed between *L. europaeus* from areas of current and former sympatry with *L. timidus* ($P > 0.001$, Fishers' exact test). MtDNA transferred from *L. timidus* to *L. europaeus* was detected in all regions except for one (No. 7, Östergötland) (Table 1). When the frequency of transmitted mtDNA among *L. europaeus* was tested for differentiation between specimens from the different regions, the allopatric *L. europaeus* from the region Skåne (No. 1) remain significantly different from the *L. europaeus* in Småland (No. 3) and Uppland (No. 10), ($P > 0.001$, Fishers' exact test). The regional samples of *L. europaeus* in sympatry with *L. timidus* did not show any significant differences in the frequency of transmitted mtDNA.

Two non-synonymous substitutions were located in the 234 amino acids coded for by the 702 base pair cytochrome *b* sequence of the respective species (Halanych *et al.*, 1999). An alanine in the *L. europaeus* mtDNA has been replaced with a leucine in the *L. timidus* sequence, and a methionine has been replaced with valine (amino acid nomenclature following Stryer, 1988). Under the assumption that the changes in cytochrome *b* are representative for all coding parts of the whole mtDNA genome (*c.* 11 000 base pairs) and extrapolating this frequency of amino acid substitutions ($2/234 \approx 0.00855$), there would be *c.* 31 ($0.00855 \times 11\,000/3 \approx 31$) amino acid substitutions that differ between the species' respective mitochondrial genomes.

DISCUSSION

Unidirectional introgression

There is a significant bias in the direction of mtDNA transmission over the reproductive barrier from the native to the introduced species. Approximately 10% of the Swedish *L. europaeus* examined have *L. timidus* mtDNA but, conversely, we did not detect any *L. timidus* with *L. europaeus* mtDNA. Our results suggest that the *L. timidus* females mate with *L. europaeus* males, and at least some of their female offspring back-cross to *L. europaeus* males, thereby generating the observed transmission (see also Thulin, Jaarola *et al.*, 1997). However, wild *L. europaeus* females rarely, or never, mate with *L. timidus* males and, thus, their mtDNA is not transferred over the species barrier. This theory of unidirectional introgression is supported by the results from captive breeding, where *L. timidus* females spontaneously mate with *L. europaeus* males, while *L. europaeus* females do not mate with *L. timidus* males (Gustavsson & Sundt, 1965) and, likewise, verify the expectations from Hubbs (1955) and Wirtz (1999), that females of the rare species (*L. timidus*) hybridize with males of the common species (*L. europaeus*). In addition, the suggestion of Grant & Grant (1997) that unidirectional hybridization usually occurs between females of the smaller species (*L. timidus*) and males of the larger species (*L. europaeus*) is also in accordance with our observations.

The failure of *L. timidus* females to discriminate against *L. europaeus* males may be related to the courtship behaviour of the species. During courtship, several males usually follow the females before, and up to, oestrus (Flux, 1970; Holley & Greenwood, 1984). Among *L. europaeus*, dominant males commonly mate-guard a female that is close to oestrus, and as a consequence they gain more matings than their subordinates (Holley, 1986). Such mate-guarding also occurs among *L. timidus* males, but seems rare: Flux (1970) recorded one male driving off four competitors,

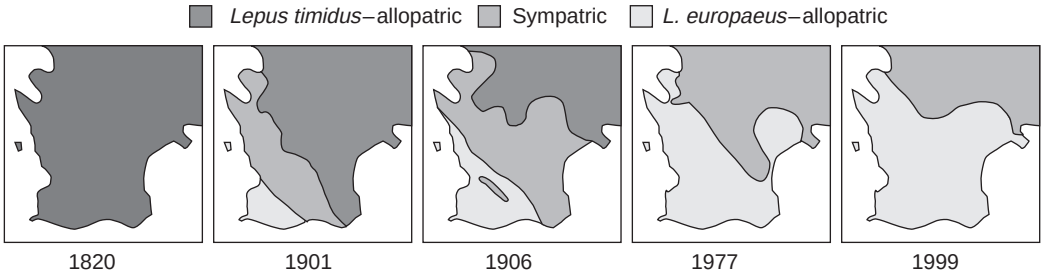

Fig. 2. Distribution of *L. europaeus* and *L. timidus* in south Sweden (Skåne), before and after the first introductions of *L. europaeus*, as depicted from Nilsson (1820), Lönnberg (1908) and Gerell (1977). The 1999 distribution is based on a telephone survey with local hunters (Thulin, 2000).

but stated 'Males do not normally fight one another, even when chasing the same female'; and Wolfe (1995) saw only two male–male fights on 32 occasions when females were approached by several males. When males of both species court a *L. timidus* female, the *L. europaeus* males may have a size advantage and chase away the *L. timidus* males simply because they are smaller and weigh less (*c*. 0.5 kg less; after Hewson, 1993). Thus, the *L. timidus* female will be constrained in her mate-choice to a *L. europaeus* male as she approaches oestrus. Even if she has an opportunity to reject him and search for a conspecific male, it may be more costly to do so, and risk the reproduction, than to accept him as a mate. The importance of these priorities, which depend on time of oestrus and male availability, has been shown to affect choice among species with single-sex discrimination (Real, 1990); and among hybridizing flycatcher species *Ficedula hypoleuca* and *F. albicollis*, females tend to hybridize more frequently at the end of the mating season (Veen *et al*., 2001).

Further, it seems plausible that hare females choose males indirectly (after Wiley & Poston, 1996) and, thus, the female will automatically choose the dominant mate-guarding male when she reaches oestrus. Conversely, following this line of reasoning, the *L. europaeus* females that live in sympatry with *L. timidus* males will rarely, or never, have the opportunity to mate with *L. timidus* males, as they are outcompeted by *L. europaeus* males. In the fragmented landscape in south- and central Sweden where the two species are sympatric (Fig. 1), *L. europaeus* is common while *L. timidus* is relatively rare and restricted to particular habitats, such as deep forests and isolated islands, and only one out of four hares killed by hunters is a *L. timidus* (Swedish Association for Hunting and Wildlife Management, Wildlife Monitoring). Thus, by chance alone, a *L. timidus* female will more likely encounter a *L. europaeus* male than a conspecific, and may occasionally accept hybridization as the only option for reproduction. Among other species, it has been shown that female choice varies with age and timing of oestrus (Johnstone, Reynolds & Deutsch, 1996; Veen *et al*., 2001).

Interspecific competition between *L. europaeus* and *L. timidus* is believed to restrict the geographic range of *L. timidus* (Lönnberg, 1905; Sjögren, 1971; Thulin, in press). The species shows tendencies of spatial competitive exclusion in favour of *L. europaeus* where sympatric (Lind, 1963; Hewson, 1976), but the characteristics of the interactions between the species is unknown. The two species are sympatric in parts of their natural range, but in general there is a subdivision of habitat choice where they occur in sympatry: *L. europaeus* occupies open agricultural areas and *L. timidus* is restricted to deep forests, high mountains or other barren biotopes. The unidirectional hybridization and introgression observed in the present study may be of importance for the possible coexistence of these species. For every interspecific mating, the local *L. timidus* population will also lose a species-specific litter, so the observed loss of habitat and the resulting decrease in population density of *L. timidus* may be further extended as a direct consequence of unidirectional hybridization (see also Thulin, in press). This phenomenon, 'extinction by hybridization', was previously described by Rhymer & Simberloff (1996) and is a possible effect of hybridization between native and introduced species (Ebenhard, 1988; Simberloff, 1996).

Geographic distribution of transmitted mtDNA

There is no significant difference between the frequency of transmitted *L. timidus* mtDNA among *L. europaeus* from regions where the two species are sympatric (Table 1), implying a geographic homogeneity in the degree of hybridization and introgression in areas of sympatry. Interestingly, however, the frequency is much lower among *L. europaeus* from Skåne (region No. 1, in southernmost Sweden), where *L. timidus* occurred < 100 years ago (Fig. 2), compared to the regions of current sympatry ($P > 0.001$).

During the late 19th century, the larger estates in Skåne imported *L. europaeus* from the European continent, as the native *L. timidus* gave few satisfactory hunting opportunities in the open agricultural landscape. The first introductions were made on the island of Ven in the middle of Öresund, during the 1858–59 hunting season. On the mainland, *L. europaeus* was first established in the south-west during the 1886–87

season and inhabited most of Skåne in the early 20th century (Fig. 2). Generally, after the introductions, the *L. europaeus* population densities increased rapidly for the first 10–30 years and then declined. The hunting bags across Sweden peaked in 1950–51 with 124 000 individuals reported (Swedish Association for Hunting and Wildlife Management, Wildlife Monitoring). It is conceivable that it was during this initial phase of population increase that most of the hybridization events took place, when the *L. timidus* females were encountered by the expanding *L. europaeus* males for the first time. The transmitted mtDNA lineages may have been incorporated into the *L. europaeus* populations partly because of the reduced rate of lineage extinction observed in expanding populations (Avise *et al.*, 1984). Such a reduction in lineage extinction, along with admixture of introduced specimens with different geographic origin, presumably accounts for the high overall mtDNA haplotype diversity ($h = 0.893 \pm 0.002$) observed among the Swedish *L. europaeus* (Thulin & Tegelström, 2001). Thus, the high variability in species-specific mtDNA has been retained, while there seems to be a loss of *L. timidus* mtDNA that has been transmitted to *L. europaeus*. Possible explanations for the significantly lower frequency of *L. timidus* mtDNA among *L. europaeus* in this area include: (1) that hybridization and introgression was less common in the region of Skåne when *L. europaeus* was introduced compared to the regions where the species presently occur in sympatry; (2) random genetic drift; (3) that released captive reared *L. timidus* are the cause for the high frequency of transmitted mtDNA among *L. europaeus* in areas of sympatry; (4) natural selection acting against *L. europaeus* specimens with *L. timidus* mtDNA.

Historical hybridization

Maybe hybridization and subsequent introgression was less common in areas of previous sympatry (Skåne) than it is today, i.e. the frequency of introgressed mtDNA was initially much lower. The only available documentation from the initial period when *L. europaeus* was introduced does, however, indicate that hybridization also occurred then (Lönnberg, 1905). Lönnberg describes a transition period, during which the species hybridized frequently on the larger estates, which had introduced *L. europaeus*. At first, the hunting bags included both the separate species and hybrids, but after some time the *L. timidus* fraction of the hunting bags declined. Soon thereafter, only a few hybrids were shot, until *L. timidus* disappeared completely (*cf.* Lönnberg, 1905). This pattern is very similar to that described by hunters today for areas where the species occur in sympatry. Thus, although circumstantial, we assume that hybridization and introgression was as frequent in Skåne as it is today where both species occur, and that the initial frequency of transmitted mtDNA was the same, i.e. *c.* 15%.

Random genetic drift

A possible explanation for the low frequency of transmitted mtDNA is that random genetic drift reduced the frequency after the mtDNA transfer over the species barrier. This is, however, improbable under the assumption that the initial frequency of transmitted mtDNA was *c.* 15%. Suppose that hybridization in Skåne gave rise to 250 females with introgressed mtDNA. There was a total of 1667 (250/0.15) females present, which equals the minimum number of females in the yearly hunting bags in Skåne from 1960 to 1999 (Swedish Association for Hunting and Wildlife Management, Wildlife Monitoring). Following the simulations of mtDNA lineage extinctions performed by Avise *et al.* (1984), the probability that all these 250 female lineages will be lost by random drift within 100 generations is 0.01. Further, in a population of stable size, initiated by 250 females, producing daughters according to a Poisson distribution, the probability that two or more of these founding mtDNA lineages will remain in the population after 100 generations is 0.95. In an expanding population, such as the *L. europaeus* population in Skåne, even 30–40 initial female lineages would account for two or more lineages remaining in the population ($P = 0.95$). In addition, even if random genetic drift caused mtDNA lineage extinction, we would still expect that *L. europaeus* individuals with *L. timidus* mtDNA would immigrate from the nearby regions Halland (No. 2) and Småland (No. 3) (Table 1, Fig. 1). As the percentage *L. europaeus* with transmitted mtDNA is 25% in Småland, every fourth migrant from there should carry alien mtDNA. Even low levels of genetic exchange, at a rate of one individual per generation, will maintain the same neutral alleles in two populations (Kimura & Ohta, 1971). Thus, given these circumstances, we believe that random drift has not caused the shift in frequency of introgressed mtDNA from 15% to 0.6% in < 100 generations.

Captive breeding

Wherever the local *L. timidus* population density is low, thousands of captive-reared specimens are released to supplement the wild stocks (Thulin, 2000). If supplementation is occurring in areas of sympatry, interspecific mating with the more common *L. europaeus* may be the only option for released *L. timidus* to reproduce. Captive-reared hares may also have disturbed mating preferences compared to wild individuals and thereby overcome any natural reluctance to interspecific matings. As a consequence, released *L. timidus* in areas of sympatry may account for some of the observed mtDNA introgression. However, the released captive-reared specimens have a very low monthly survival rate, on average only 11.2 days (Lemnell & Lindlöf, 1982). Further, observations of natural hybridization between these species (Lönnberg, 1905) predate large-scale breeding and transport of *L. timidus* by

c. 60 years (cf. Thulin, 2000). Thus, we believe that hybridization between released captive-reared *L. timidus* and wild *L. europaeus* may contribute to hybridization and introgression in areas of current sympatry, but would not alone cause the extremely high frequencies of transmitted mtDNA observed.

Selection against L. timidus mtDNA

As more than a third of the synonymous nucleotide sites have been altered between the *L. europaeus* and the *L. timidus* mtDNA cytochrome *b* sequences investigated, the synonymous substitution rate has reached saturation (cf. Halanych *et al.*, 1999). Also, in the corresponding amino acid sequence of a 702 base pair stretch of the mtDNA cytochrome *b* gene there are two amino acid substitutions. Presumably, there could be enough amino acid substitutions between the mtDNA genomes of the two hare species to alter specific functions and/or characteristics of certain gene-products that co-function with nucleic coded proteins. Most mitochondrial genes encode subunits, that in combination with other subunits coded in the nuclear genome, form functional enzymes, for example in the respiratory chain or in the mitochondrial ribosomes (cf. Moritz, Dowling & Brown, 1987; Avise, 1991).

Several studies have evaluated the specific contribution of selection on mtDNA variants among different strains of *Drosophila* (Clark & Lyckegaard, 1988; MacRea & Anderson, 1988; Fos *et al.*, 1990; Hutter & Rand, 1995; Kilpatrick & Rand, 1995) and among human hybrid cell lines (Dunbar *et al.*, 1995). MacRea & Anderson (1988) evaluated the relative impact of drift and selection on frequency changes of different mtDNA lineages in experimental populations of *Drosophila pseudoobscura*. They concluded that mtDNA haplotypes are not always neutral, although the causes of the dramatic changes they observed were not easily explained. Also, Hutter & Rand (1995) performed a reciprocal transfer of alien mtDNA to a novel nuclear background of *Drosophila* sibling species, and observed significant frequency increases of certain mtDNA lineages. Their results indicate a fitness association between the nuclear genetic background and specific mtDNA lineages. Likewise, Kilpatrick & Rand (1995) concluded that hitchhiking of mtDNA with specific nuclear genes causes shifts of mtDNA variant frequencies in hybrid strains. A mismatch between gene products encoded by *L. europaeus* nuclear DNA and *L. timidus* mtDNA may cause decreased fitness of an individual with such a genotype, perhaps because of a lowered metabolic ability, which in turn affects growth rate, adult body size and/or other fitness-related characters. *Lepus timidus* occurs in harsh arctic habitats and uses food resources of lower quality than the average *L. europaeus* encounters. We suggest that species-specific adaptations of *L. europaeus* and *L. timidus* causes a differentiation of the metabolism between the species and that mtDNA is an important component that contributes to this metabolic specificity. Fitness differences related to the mtDNA set-up would then be detectable between *L. europaeus* with species-specific and introgressed mtDNA.

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