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Species assignment and hybrid identification among Scandinavian hares *Lepus europaeus* and *L. timidus*

Carl-Gustaf Thulin, John Stone, Håkan Tegelström & Christopher W. Walker

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In Scandinavia, suspected hybrids between the native mountain hare *Lepus timidus* and the introduced brown hare *L. europaeus* have been observed by hunters since the first introductions of brown hares in the late 19th century. Several attempts to verify the status of these suspected hybrids have been unsuccessful. Recently, however, the transmission of mitochondrial DNA (mtDNA) from mountain hares to brown hares was documented and interpreted as a consequence of hybridisation and subsequent introgression. However, mtDNA markers alone will not allow identification of first-generation hybrids because of the strictly maternal inheritance of mitochondria. Here, we combine mtDNA data with analyses of variation in seven microsatellite loci among brown hares, mountain hares and putative hybrids. Our purpose was to determine species differentiation in nuclear DNA markers, elucidate the extent of interspecific gene flow, identify true hybrids within our sample and evaluate the ability of hunters to identify hybrids. The estimated genetic difference between species was low (F_{ST} = 0.18 - 0.24, Rho_{ST} = 0.09 - 0.16). We believe these low estimates result from a reticulated mode of evolution among hares, with repeated gene flow over the species barrier. Population assignment tests and randomly assembled, artificial, hybrid genotypes were used to classify individuals independently from the morphologically assessed species identity. More than half (57%) of the putative hybrid specimens were assigned unambiguously to either species and first-generation hybrids seem to be rare. Morphological plasticity and backcrossing, which confound species identification within the genus *Lepus*, might explain invalid classifications by hunters.

Key words: assignment, hares, hybridisation, introgression, Lepus, microsatellites, mtDNA

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This publication is dedicated to the memory of Håkan Tegelström

The introduction of species by humans disrupts natural biogeographic barriers and has important effects on the native fauna and flora (Ebenhard 1988). Hybridisation with introduced species and subsequent introgression of foreign genetic material may threaten locally adapted native species (Rhymer & Simberloff 1996, Simberloff 1996). The European brown hare *Lepus europaeus* was introduced to southern Sweden during the late 19th century and has expanded gradually northwards as a result of semi-natural dispersal and continued introductions. Since the initial introduction, hunters have reported hybrids between brown hares and native mountain hares *L. timidus*, a species that colonised Scandinavia naturally after the most recent glacial period. The specific status of suspected hybrids has been difficult to assess because of morphological plasticity within both species (Lönnberg 1905, Gureev 1964, Flux & Angerman 1990). However, genetic investigations have revealed that hybridisation can cause transmission of mitochondrial DNA (mtDNA) across the species barrier (Thulin et al. 1997, Thulin & Tegelström 2002). Brown hare males mate with mountain hare females, and at least the F_1 hybrid females are fertile and backcross with brown hare males. Subsequent backcrosses by female offspring to brown hare males will, in a few generations, result in phenotypic brown hares that carry mountain hare mtDNA. Approximately 15% of all Swedish brown hares sympatric with mountain hares carry mtDNA of mountain hare origin, whereas the reverse transmission has not been detected (Thulin & Tegelström 2002). Because the maternal inheritance of mtDNA limits the detection of genetic introgression, nuclear genetic markers are needed to evaluate the degree of interspecific gene flow between the species and to identify first-generation hybrids.

The lagomorph genus *Lepus* lacks species differentiation in genetic characters such as chromosome number (Robinson et al. 1983) and karyotype banding and structure (Gustavsson 1971) and is poorly differentiated in allozyme markers (Hartl et al. 1993, Suchentrunk et al. 1999, 2000). This poor differentiation may be a result of the recent history of the genus, with the earliest fossils dated about 500,000 years BP (Kurtén 1968), in combination with a reticulated mode of evolution in which *Lepus* species in secondary contact interbreed and repeatedly exchange genes and genomes (Thulin et al. 1997, Alves et al. 2003). During the last decade, microsatellite markers have proven useful in genetic studies of populations or species with low levels of allozyme polymorphism (Hughes & Queller 1993, Takezaki & Nei 1996, Estoup et al. 1998). Large numbers of alleles segregating at hypervariable microsatellite loci can reveal differentiation between populations and individuals, especially if several loci are combined. In population studies, analyses involving individual genotypes provide better resolution than those involving allele frequency differentiation (Paetkau et al. 1995). Such assignment methods entail calculations of genotype probabilities to determine the population origin for individuals and to assess genetic exchange between populations. Thus, assignment analysis using hypervariable microsatellite data could potentially be used to evaluate reproductive success for introduced species that hybridise with native species.

In this paper, we determine the degree of microsatellite differentiation between brown hares and mountain hares. We also evaluate the accuracy of hunter identification of hybrids between the species. Therefore, to enable molecular identification of hybrids, we assign all specimens on the basis of their individual multilocus genotypes to either species (brown hares or mountain hares) or to a category of artificial hybrids constructed by randomised allocation of alleles from the separate species.

Material and methods

Samples

In this study, we used a total of 199 hares from a variety of Scandinavian locations. Tissue from the hares was collected by hunters and kept frozen. The sample included brown hares and mountain hares with allopatric and sympatric distributions, and suspected hybrids as defined by their hunters. We included as many localities as possible to assess the genetic diversity present within each species (Fig. 1). Suspected hybrids comprised individuals whose phenotype deviated from what is considered 'normal' within each species. In Sweden, mountain hares turn light grey or white during the winter, whereas brown hares remain brown or grey-brown year-round. In addi-

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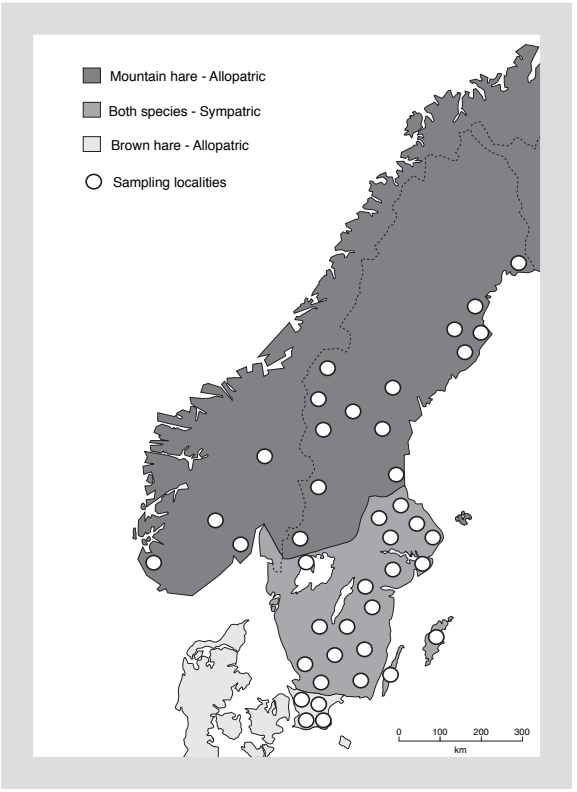


Figure 1. Distributions of brown hare (□) and mountain hare (■), and their overlapping occurrence (■), in Scandinavia, with indication of sampling localities (○).

tion, ear length is greater for brown hares than for mountain hares (Angerbjörn & Flux 1995). Thus, a specimen that appeared to be a brown hare, but with unusually short ears and white or light grey patches was considered a hybrid (i.e. suspected hybrid). Although this approach was subjective, it proved to be the only feasible way to obtain a substantial sample size of suspected natural hybrids. It also provided means to evaluate the ability of hunters to identify hybrids and the reliability of historical accounts of hybridisation (e.g. Lönnberg 1905).

The maternal mtDNA origin (i.e. mountain hare type or brown hare type) for each suspected hybrid specimen was determined in the manner described by Thulin & Tegelström (2002). Brown hares that carried introgressed mountain hare mtDNA were assigned to a separate group, and suspected hybrids were sorted according to which species mtDNA they carried. Consequently, the sample was divided into the following seven categories according to species characteristics (morphology), mtDNA and geographic location:

1. LeA - Allopatric brown hares (with species-specific morphological characteristics and mtDNA) from the southernmost parts in Scandinavia (Skåne), where the mountain hare has disappeared (N = 30 individuals).
2. LeS - Brown hares (as above) that were sympatric with mountain hares, from localities in central Sweden (N = 30 individuals).
3. LtA - Allopatric mountain hares (as above) from northern Scandinavia (N = 30 individuals).
4. LtS - Mountain hares (as above) that were sympatric with brown hares, from localities in central Sweden (N = 30 individuals).
5. Le* - Brown hares containing mountain hare mtDNA and, thus, from hybrid ancestry (N = 51 individuals).
6. HLt - Suspected hybrids containing mountain hare mtDNA (N = 19 individuals).
7. HLe - Suspected hybrids containing brown hare mtDNA (N = 9 individuals).

Laboratory techniques

DNA was isolated from approximately 1- mm^3 tissue (kidney or muscle) with the Chelex-100 method (Walsh et al. 1991). DNA concentrations were measured using a Hoefer fluorometer. We used the five microsatellite primer pairs Sat2, Sat5, Sat8, Sat12 and Sat13, which were developed originally for the European wild rabbit *Oryctolagus cuniculus* by Mougel et al. (1997). Monique Monnerot (CNRS, Paris, France) kindly provided primer aliquots. Two additional microsatellite primers for loci Sol8 and Sol30, also developed for rabbits, were synthesised in accordance with sequences presented by Rico et al. (1994). The microsatellite markers we used have previously been shown to be hyper-variable and also reveal differences between brown hares and mountain hares (Surridge et al. 1997, Andersson et al. 1999). Optimal amplification conditions were achieved as described by Andersson et al. (1999).

Radioactive Polymerase Chain Reaction (PCR) was performed in 10 μl volumes by incorporating α-^{33}P-labelled dATP (Amersham Pharmacia Biotech). The final PCR mixture contained 1 μl template DNA (5-150 ng), 1X buffer (Mg^{2+} free, distributed with the polymerase), 1.5 mM $MgCl_2$, 75 μM/nucleotide (dGTP/dCTP/dTTP), 400 nM primer, 0.5 units Taq polymerase (Promega) and 0.125 μCi α-^{33}P-labelled dATP. Amplifications were conducted in a PTM-100 cycler (MJ Research) with touchdown cycles as follows: 95°C/30 seconds, 94°C/30 seconds, T_a (-0.5°C/cycle)/30 seconds, 72°C/45 seconds (the last three cycles repeated 20 times), 94°C/30 seconds, T_a-10°C/30 seconds, 72°C/45 seconds (the last three cycles repeated 10 times) and a final 2-minute elongation at 72°C. The annealing temperatures (T_a) of the seven primer pairs vary from 50 to 68°C (Table 1).

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Table 1. Investigated microsatellite loci, touchdown PCR annealing temperatures (T_a), numbers of alleles detected (N_a), average F_{ST} between species categories (Av. F_{ST}) and literature references.

Locus	T_a (°C)	N_a	Av. F_{ST}	Reference
Sol 8	65-55	10	0.41	Rico et al. (1994)
Sol 30	68-58	35	0.20	Rico et al. (1994)
Sat 2	65-55	34	0.21	Mougel et al. (1996)
Sat 5	65-55	11	0.25	Mougel et al. (1996)
Sat 8	68-58	6	0.22	Mougel et al. (1996)
Sat 12	65-55	11	0.04	Mougel et al. (1996)
Sat 13	60-50	7	0.14	Mougel et al. (1996)

The PCR products (2 µl) were applied to a 6% denaturing polyacrylamide gel and separated electrophoretically for 4,000-7,000 volt hours^{-1} in 1xTBE buffer (containing Tris, Boric acid and EDTA) on an IBM sequence apparatus. To visualise the PCR products, dried gels were exposed to autoradiographic film for a period ranging from 10 to 24 hours. A known plasmid DNA sequence was applied to a separate lane on each gel to accurately measure different allele sizes.

Data analysis

Alleles were scored manually, and complete genotypes over six of the seven loci were obtained for the 199 specimens included in the study. Despite repeated template extractions and PCR amplifications, we failed to obtain genotypes for 10 specimens at locus Sat5. These specimens all belonged to the brown hare categories LeA (4) and LeS (2), and brown hares with mountain hare mtDNA, category Le* (4). As incomplete genotypes still may be useful in our search for differences between the two hare species, we decided to include locus Sat5 in all further analyses. Exact tests for deviations from expected Hardy-Weinberg genotype distributions and for genotypic disequilibrium were performed using the computer program package Genepop 3.1 (Raymond & Rousset 1995). Expected and observed heterozygosities were calculated using the computer program Genetix 4.02 (Belkhir et al. 2000). Probability values were corrected for multiple comparisons according to the sequential Bonferroni procedure for multiple tests (Rice 1989). To estimate genetic differentiation between categories, F_{ST} (Weir & Cockerham 1984) and Rho_{ST} (Michalakis & Excoffier 1996) were calculated using Genepop 3.1. To address the potential mutation bias for highly variable loci when investigating hybrid zones with microsatellite markers (e.g. Balloux et al. 2000), average pair wise F_{ST} between species categories (i.e. LeA, LeS, LtA & LtS) was compared over loci. Allele frequencies over the seven loci were also used to calculate Nei's genetic distance (Nei 1972) between the categories with the computer program Gendist, available in the computer program package Phylip 3.5c (Felsenstein 1993). To visualise relationships between categories, the genetic distances were used to construct an unrooted neighbour-joining tree with the computer program Neighbour, also available in Phylip 3.5c.

We used two different approaches to identify individual hybrids; both involved varieties of population assignment tests. First, we calculated probabilities that each individual genotype was drawn from each of the *a priori*-defined categories (Paetkau et al. 1995, Waser & Strobeck 1998). This test entailed three assumptions: conditions for Hardy-Weinberg equilibrium and linkage equilibrium were met and alleles were present in all categories. To address violations of the last assumption, alleles that were not represented in a category were included with frequency 1/2N, where N individuals were screened in that specific category. These calculations were performed with the computer program DOH, kindly provided over the Internet by John Brzustowski (http://biodb.biology.ualberta.ca/jbrzusto). In addition, the method described by Rannala & Mountain (1997) was used, wherein the probabilities for specific allele frequencies over all populations were derived using a Bayesian approach. Thus, the difficulty raised by absence of alleles in the first method was avoided, and the only explicit assumption required was the one involving linkage equilibrium. To assess individual assignment reliability, a computer simulation procedure was implemented on the data set, wherein 100,000 multilocus genotypes were synthesised by pseudo-randomly drawing alleles according to their proportions in *a priori*-defined categories. The latter calculations were performed with the computer program GeneClass, which was kindly provided by Jean-Marie Cournet (www.montpellier.inra.fr/URLB/index.html).

In the second approach, we obtained 'true hybrid' genotypes and constructed an artificial F_1 hybrid population of 30 individuals by using a pseudo-random number generator to select genotypes which were combined virtually. The allele association was achieved on the basis of allele proportions within allopatric categories of brown hares (LeA) and mountain hares (LtA; see also Thulin 2000, Vilà et al. 2003). This 'artificial hybrid' category (AHyb) was then included in the assignment tests. To assess the confidence of assignments, we constructed 100 artificial F_1 hybrid populations of 30 specimens each, ran a frequency-based assignment test 100 times and sorted the assignment likelihoods for all individuals. We did not include artificial backcross categories because our microsatellite markers did not provide sufficient resolution to enable extraction of backcrosses

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from our sample. Inclusion of artificial backcross categories would therefore confound the analyses.

Results

In total, 114 alleles were detected among the seven microsatellite loci, ranging from six to 35 per locus (see Table 1), with a mean of 16.3 alleles/locus. No linkage disequilibrium was detected, but there was significant homozygote excess in five hare categories (LeA, LtA, Le*, HLt, HLe) at three loci (Sol30, Sat2 and Sat5). Only the category Le* revealed homozygote excess at all three loci. Because all our categories consist of individuals from several geographic areas, the basic criteria for Hardy-Weinberg distribution were unfulfilled. Thus, a Wahlund effect (Wahlund 1928) may explain some of the homozygous excess we observe. At locus Sat5, among the categories LeA and Le*, the homozygous excess may have been caused by a null allele because a total of eight specimens among these categories (four from each) repeatedly failed to produce any product at locus Sat5. The observed and expected heterozygosities of the respective categories and microsatellite loci are presented in Table 2.

The categories of each of the pure species (i.e. LeA & LeS for brown hares and LtA & LtS for mountain hares) reveal species-specific relationships (i.e. brown hare categories are more related to each other than to mountain hares and vice versa). In casual comparison, there are no congruent differences between F_{ST} estimates from microsatellite loci with high and low number of alleles, respectively (see Table 1). The estimates of genetic differentiation (Table 3) between these species categories range within 0.18-0.24 (F_{ST}) and 0.09-0.16 (Rho_{ST}). The category of brown hares with mountain hare mtDNA (Le*) does not differ from the pure brown hare categories (F_{ST} = 0-0.02, Rho_{ST} = 0) and differs least from brown hares sympatric to mountain hares (LeS).

Table 3. Estimates of F_{ST}, Rho_{ST} and Nei's genetic distance, following Weir & Cockerham 1984, Michalakis & Excoffier 1996 and Nei 1972, respectively. Calculations were performed by the computer programs Genepop 3.1 (Raymond & Rousset 1995) and Phylip 3.5c (Felsenstein 1993). The categories are Scandinavian brown hares and mountain hares in allopatry and sympatry, respectively (LeA, LeS & LtA, LtS), brown hares with introgressed mountain hare mtDNA (Le*) and presumed hybrids with brown hare and mountain hare mtDNA, respectively (HLe & HLt).

Categories	F_{ST}	Rho_{ST}	Nei's D
LeA & LeS	0.0275	-0.0033	0.1169
LeA & LtA	0.2142	0.1111	0.6321
LeA & LtS	0.2421	0.1552	0.6806
LeA & Le*	0.0227	-0.0080	0.0946
LeA & HLt	0.1465	0.0497	0.5144
LeA & HLe	0.0162	0.0400	0.1384
LeS & LtA	0.1813	0.0885	0.5549
LeS & LtS	0.1945	0.1144	0.5332
LeS & Le*	-0.0052	0.0043	0.0291
LeS & HLt	0.0962	0.0242	0.3620
LeS & HLe	0.0186	0.0034	0.1668
LtA & LtS	0.0268	-0.0060	0.0540
LtA & Le*	0.1771	0.1404	0.5529
LtA & HLt	0.0475	0.0034	0.1129
LtA & HLe	0.2063	0.0489	0.6475
LtS & Le*	0.1910	0.1726	0.5443
LtS & HLt	0.0362	0.0052	0.0733
LtS & HLe	0.2261	0.0561	0.6257
Le* & HLt	0.0978	0.0608	0.3652
Le* & HLe	0.0177	0.0191	0.1543
HLt & HLe	0.1143	-0.0011	0.4708

The suspected hybrids with mountain hare mtDNA (category HLt) and brown hare mtDNA (category HLe) follow their maternal heritage. Thus, the HLt category is related to the mountain hares and the HLe category to the brown hares. The relationships between the categories are visualised in the neighbour-joining tree constructed from Nei's genetic distance (Fig. 2), wherein the categories form two separate groups.

The different assignment tests (Tables 4 and 5) revealed that: 1) pure-species individuals from the brown hare

Table 2. Observed (H_{obs}) and expected (H_{exp}) heterozygosities for the different hare categories, including randomly synthesised artificial F_1 hybrids (AHyb).

	LeA		LeS		LtA		LtS		Le*		Hlt		Hle		AHyb	
Locus	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}	H_{obs}	H_{exp}
Sol 8	0.47	0.49	0.73	0.72	0.53	0.48	0.27	0.24	0.65	0.67	0.68	0.59	0.56	0.53	0.93	0.68
Sol 30	0.47	0.52	0.67	0.76	0.83	0.93	0.87	0.92	0.63	0.73	0.74	0.89	0.56	0.72	1	0.89
Sat 2	0.80	0.87	0.77	0.86	0.50	0.50	0.63	0.72	0.74	0.86	0.89	0.86	0.89	0.89	1	0.91
Sat 5	0.42	0.83	0.57	0.74	0.40	0.42	0.13	0.18	0.32	0.78	0.58	0.65	0.44	0.72	0.63	0.58
Sat 8	0.27	0.42	0.37	0.47	0.13	0.13	0.10	0.10	0.45	0.50	0.16	0.15	0.22	0.57	0.30	0.34
Sat 12	0.90	0.84	0.70	0.82	0.63	0.78	0.87	0.83	0.90	0.85	0.68	0.80	0.56	0.77	0.87	0.83
Sat 13	0.73	0.75	0.70	0.74	0.57	0.68	0.77	0.64	0.65	0.76	0.74	0.68	0.67	0.69	0.77	0.77
Total	0.58	0.67	0.64	0.73	0.51	0.59	0.52	0.52	0.62	0.73	0.64	0.66	0.56	0.69	0.79	0.71

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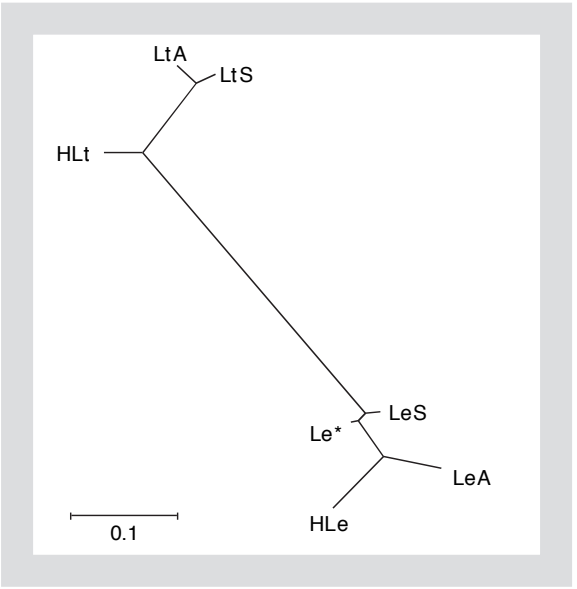


Figure 2. Relationships between the seven hare categories illustrated by a neighbour-joining tree constructed using Nei's genetic distance (Nei 1972).

categories LeA & LeS and the mountain hare categories LtA & LtS were never assigned to the other species; 2) artificial hybrids (AHyb) were assigned primarily to their own category (among the 3,000 artificial hybrids, 94.1% assigned to their own artificial hybrid category); 3) brown hares with mountain hare mtDNA, category Le*, were assigned to other brown hare categories (i.e. LeA, LeS, Le* or HLe); 4) suspected hybrids with mountain hare mtDNA, category HLt, were assigned to all categories. The results from the computer simulation indi-

Table 4. Assignments resulting from: 1) the allele frequency method described by Paetkau et al. (1995); 2) a Bayesian approach, after Rannala & Mountain (1997); 3) a simulation based on Bayesian probability, using 100,000 simulated individuals and an assignment threshold of 0.05. The *a priori* categories are presented in the left column followed by the respective assignments and the total individuals assigned to the right. In the simulation (3), each individual could have been assigned to several or none of the categories. Thus, total assignments may exceed actual specimen numbers in the specific categories.

1) Frequency	LeA	LeS	LtA	LtS	Le*	HLt	HLe	AHyb	Total
LeA	20	4	0	0	5	0	1	0	30
LeS	5	6	0	0	18	0	0	1	30
LtA	0	0	22	7	0	0	0	1	30
LtS	0	0	8	20	0	1	0	1	30
Le*	4	24	0	0	17	1	4	1	51
HLt	0	0	2	4	3	7	1	2	19
HLe	1	0	0	0	4	1	3	0	9
AHyb	0	1	0	1	0	0	1	27	30
2) Bayesian									
LeA	26	1	0	0	3	0	0	0	30
LeS	1	26	0	0	3	0	0	0	30
LtA	0	0	27	3	0	0	0	0	30
LtS	0	0	1	29	0	0	0	0	30
Le*	3	7	0	0	39	0	1	1	51
HLt	0	0	0	3	2	14	0	0	19
HLe	0	0	0	0	0	0	9	0	9
AHyb	0	0	0	1	0	0	0	29	30
3) Simulation									
LeA	26	17	0	0	21	2	3	2	71
LeS	9	28	0	0	27	1	1	2	68
LtA	0	0	28	13	1	17	0	11	70
LtS	0	0	17	28	1	20	0	22	88
Le*	15	36	0	0	46	3	4	2	106
HLt	1	2	7	6	2	16	0	6	40
HLe	2	3	0	0	4	1	9	0	19
AHyb	0	0	0	1	0	1	0	29	31

Table 5. Assignments of the 28 suspected hybrids to categories of allopatric brown hares (LeA), allopatric mountain hares (LtA) or artificial F_1 hybrids (AHyb). Identification numbers are given in the top rows (HLt-27, -76.... for suspected hybrids with mountain hare mtDNA and HLe-142, -272.... for suspected hybrids with brown hare mtDNA). The assignment test, as described by Paetkau et al. (1995), was repeated 100 times, once for each of the reconstructed AHyb populations of 30 individuals. In addition, the designated assignments of each specimen from the simulation presented in Table 4 are given, along with a consensus from both tests, where Lt = mountain hare, Le = brown hare, Le* = brown hare with mountain hare mtDNA, H = putative hybrid. Specimens marked '?', fail to be assigned to any class in the simulation, or assigns differently in the two different approaches undertaken. These specimens could be F_1 hybrids, but also recent backcrosses. HLe284 is marked Lt* because it seems to be a mountain hare with brown hare mtDNA. Criteria for the designation are presented in detail in 'Material and methods'.

	HLt-27	-76	-77	-78	-79	-80	-81	-192	-194	-200	-201	-229	-230	-263	-492	-493	-641	-701	-715
LeA	0	0	0	0	0	0	0	0	95	0	0	0	0	0	100	0	55	0	8
LtA	100	100	100	100	30	100	100	100	0	100	100	100	100	100	0	98	0	32	0
AHyb	0	0	0	0	70	0	0	0	5	0	0	0	0	0	0	2	45	68	92
Simulation	Lt	Lt	H	Lt	Lt	H	H	Lt	Le*	H	Lt	Lt	Lt	Lt	Le*	H	?	H	H
Consensus	Lt	Lt	?	Lt	?	?	?	Lt	Le*	?	Lt	Lt	Lt	Lt	Le*	?	?	H	H
	HLe-142	-272	-273	-284	-441	-480	-484	-638	-729										
LeA	100	100	100	0	100	100	48	22	100										
LtA	0	0	0	99	0	0	0	0	0										
AHyb	0	0	0	1	0	0	52	78	0										
Simulation	Le	Le	Le	?	Le	Le	Le	Le	Le										
Consensus	Le	Le	Le	Lt*	Le	Le	?	?	Le										

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cate that mountain hares vary more in their assignments than do brown hares in theirs and are more prone to be assigned to the artificial hybrid category. Similarly, artificial hybrids are more prone to be assigned to their mountain hare parental category (4.3%) than to brown hares (1.6%).

In the two different approaches to evaluate status of suspected hybrids, each specimen was designated as mountain hare, brown hare, brown hare with mountain hare mtDNA or potential hybrid. The designations are summarised in Table 5. In the simulation procedure implemented with the GeneClass software, a specimen that was assigned to one or more mountain hare categories was designated as being a pure mountain hare (thus, 'Lt' in Table 5). Similarly, if it was assigned to brown hare categories, it was a brown hare ('Le'), and, if the specimen carried mountain hare mtDNA but was assigned to brown hare categories, it was designated as being an 'Le*' specimen. Any specimen that was assigned to the artificial hybrid class was designated 'H' and those specimens that were not assigned to any category were marked '?' (see Table 5). In our alternative approach, wherein the putative hybrids were assigned repeatedly to the parental species or to an artificial hybrid population reconstructed 100 times, we applied an arbitrary 50% cut-off for assignments. If the assignment of a certain specimen was repeated in both approaches, this specific assignment was considered to be conclusive; otherwise it was represented by '?' (see Table 5). Thus, of 28 suspected hybrids (categories HLt and HLe) that were included in the investigation, eight specimens appeared to be pure mountain hares, six pure brown hares and two brown hares with mountain hare mtDNA (see Table 5). Thus, 12 suspected hybrids remained (HLt77, HLt79, HLt80, HLt81, HLt200, HLt493, HLt641, HLt701, HLt715, HLe284, HLe484 and HLe638). Among these, the assignments of nine specimens varied between the different tests undertaken (see Table 5). Potentially, all nine specimens could be F_1 hybrids, but they could also be recent backcrosses to either parental species. Two specimens (HLt701 & HLt 715) are consistently assigned to the artificial hybrid class (AHyb) and are most likely F_1 hybrids. Finally, the specimen HLe284 deserves extra attention, as it seems to be a brown hare with mountain hare mtDNA, something that has never been observed before (*cf.* Thulin & Tegelström 2002). In conclusion, more than half ($16/28 \approx 0.57$) of the individuals suspected by hunters to be hybrids seem instead to be either pure brown hares or mountain hares, while a few seem to be backcrosses or actual F_1 hybrids.

Discussion

Microsatellite markers are generally thought to be more sensitive markers for population studies than allozymes, largely because of the high allelic diversity that characterises microsatellite loci (Hughes & Queller 1993, Takezaki & Nei 1996, Estoup et al. 1998). The rapid mutation rate that underlies the allelic diversity of microsatellites may occasionally result in an underestimated genetic distance between species or subspecies that are readily defined with karyotype markers or allozymes (Balloux et al. 2000). The explanation for such observations is that homoplasy (i.e. identical alleles with different evolutionary histories) obscures the differences. Alternatively, a microsatellite mutation rate that exceeds the inflow of alleles from migration may also cause an underestimation of genetic structure (*cf.* Balloux et al. 2000). In general, a comparison to genetic differentiation estimates resulted from more slowly evolving genetic markers, such as allozymes, circumvent discrepancies related to the high mutation rates of microsatellites. Our situation is, however, somewhat different. Here we detect microsatellite differentiation between two species with previously documented low allozyme divergence (e.g. Hartl et al. 1993, Suchentrunk et al. 1999, 2000) and identical karyotypes (Gustavsson 1971). The previous allozyme investigations mostly cover other hare populations than those from Scandinavia. Suchentrunk et al. (1999) include a sample of Scandinavian mountain hares, but conclude that the allelic similarities hamper differential diagnosis between mountain hares and brown hares. Nevertheless, it seems like mountain hares and brown hares maintain species-specific differences in microsatellite loci despite a low level of allozyme differentiation and despite evidence of interspecific gene flow and a reticulated mode of evolution within the genus *Lepus* (Thulin et al. 1997, Alves et al. 2003). The microsatellite markers used here enable us to separate mountain hares and brown hares into two distinct groups, much as Goodman et al. (1999) were able to do among hybridising red deer *Cervus elaphus* and sika deer *Cervus nippon* in Scotland. In contrast to the results of Goodman et al. (1999), however, the sorting of mtDNA among hares is not species specific because of the biased mtDNA introgression. Nevertheless, using the microsatellites, we would be able to determine species origin for an anonymous hare sample regardless of phenotype or mtDNA haplotype.

The microsatellite genotypes of brown hares with introgressed mountain hare mtDNA are very similar to those of brown hares with species-specific mtDNA from the same geographic regions. This is evident from the

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assignment tests presented in Table 4 as well as from the neighbour-joining tree (see Fig. 2). None of these Le* specimens were assigned to the mountain hare categories in either assignment test, even though specimens occasionally had mtDNA that was identical to that of mountain hares from the same locality (*cf.* Thulin et al. 1997, 2003). If these Le* specimens were recent backcrosses, we could detect linkage disequilibrium among introgressed alleles (*cf.* Goodman et al. 1999), but we did not. Presumably, most transmitted mountain hare mtDNA was incorporated into the brown hare populations during the rapid expansion of brown hare populations that followed introduction, which, in our study area in southern and central Sweden, occurred 50-100 years ago (*cf.* Thulin 2003). During the 50 hare generations (minimum) since introduction and initial hybridisation, transferred nuclear markers from mountain hares have disappeared or been allotted equally among brown hare populations, while the transferred mountain hare mtDNA has persisted unchanged. Although nuclear and mitochondrial DNA may still be transferred across the species barrier, only a few specimens in our sample seem to be F_1 hybrids. Thus, present day hybridisation and interspecific gene transfer seem to be sporadic and lack the penetrative power expected in expanding populations, where most specimens contribute to the future gene pool (Hewitt 1993).

One purpose of our study was to evaluate the accuracy of hybrid classification by hunters. Thus, suspected hybrids might have been pure brown hares or mountain hares with characteristics that deviated from what is 'normal' within the two species or even within the specific area where they were shot. Because of overlap between the species in microsatellite allele distributions, we experienced similar difficulties in determining whether 'suspected hybrid' genotypes might have resulted from associations of rare alleles within either species rather than from alleles from both species. This overlap in allele distributions might explain why several mountain hares, especially those sympatric to brown hares, have a tendency to be assigned to the artificial hybrid category (see Table 4). An individual from one species may, by chance, have a genotype composed of alleles that occur in a higher frequency in the other species and, thus, appear to have a hybrid origin. However, assignment to the artificial hybrid category may also indicate past hybridisation and introgression. That artificial hybrids are assigned more often to mountain hares than to brown hares supports the hypothesis that interspecific gene flow is biased (Thulin et al. 1997, Thulin & Tegelström 2002). As mountain hare alleles have introgressed into brown hare genomes, the artificial hybrids are more likely to have a genotype similar to that of mountain hares. Recently, Alves et al. (2003) showed that brown hares and Iberian hares *L. granatensis* on the Iberian Peninsula carry mountain hare mtDNA despite the fact that the closest mountain hare populations occur in the Alps. The authors explain this phenomenon as resulting from ancient introgression and subsequent preservation of transferred mtDNA. Thus, a relevant question is whether there are any 'pure' brown hare populations anywhere, or whether brown hares repeatedly furnish themselves with genes and genomes from sympatric conspecifics.

Fewer than half of the putative hybrids included in our study may be F_1 hybrids or recent backcrosses, whereas most specimens are assigned unambiguously to either parental species. The tendency for hunters to assess apparently pure specimens as hybrids is likely explained by the morphological plasticity documented among hares (Gureev 1964, Flux & Angerman 1990). The winter pelage of mountain hares varies from brown throughout the year in Ireland, white with brownish ears in Scotland, blue/grey in southern Sweden and the Baltic countries to the characteristic pure white in northern Scandinavia and the Arctic (Angerbjörn & Flux 1995). In addition, local variation has been documented, often attributed to stress and hormonal disturbances caused by poor snow cover or fluctuating temperatures (Angerbjörn & Flux 1995). Among brown hare populations, pelage polymorphism is also common. Typical winter pelage for Swedish brown hares includes grey hind legs (C-G. Thulin, pers. obs.). In the Volga area in northern Russia, even completely white winter pelage occurs (Gureev 1964). This polymorphism might confound identification, and we believe it contributes considerably to misidentifying many hares as hybrids. In addition, we also expect that a few specimens within our sample are recent backcrosses. The inheritance of phenotypic characteristics of mountain hares and brown hares are largely unresolved, but the expected mixture of characters displayed by hybrids and backcrosses probably adds confusion to the identification of hybrids. To be able to sort out recent backcrosses from our sample, we need more markers than were included in this study, presumably markers fixed for different alleles among the respective species. Because of the seemingly continuous interspecific gene flow among hares, we believe that such diagnostic, genetic markers may be difficult to obtain.

Finally, one specimen (HLe284) in our sample warrants specific attention because it was described as a suspected hybrid, but was the only specimen with brown hare mtDNA that was assigned to mountain hares in the assignment tests. This specimen constitutes the only support to date for natural hybridisation between a brown

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hare female and a mountain hare male. Interestingly, this specimen was collected on the northernmost edge of the present brown hare distribution in Sweden (Gästrikland), where brown hare density is expected to be low in relation to local mountain hare populations. This fact indicates that hybridisation between brown hares and mountain hares might be frequency dependent (i.e. males from a population with high density hybridise with females from a population with low density; Wirtz 1999). However, introgression of brown hare mtDNA to mountain hares must be very rare, because no prior indications of this event were observed in a sample of 671 hare specimens of both species (Thulin & Tegelström 2002). Only by artificial insemination have captive breeders been able to produce viable hybrid offspring from brown hare females and mountain hare males (Gustavsson & Sundt 1965). Investigations that focus on populations in areas recently colonised by brown hares (e.g. during the last 5-10 years) are needed to verify potential bi-directional gene flow between brown hares and mountain hares.

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