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Differentiation and levels of genetic variation in northern European lynx (*Lynx lynx*) populations revealed by microsatellites and mitochondrial DNA analysis

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Abstract

The European lynx (*Lynx lynx*) has experienced significant decline in population numbers over large parts of its former distribution area in central and northern Europe. In Scandinavia (Sweden and Norway), the species has been subject to intense hunting and in the early 20th century the population size may have been as low as about 100 animals. During the rest of the century there have been alternating periods of restricted hunting and total protection. Future management of the Scandinavian lynx population will require insight into what effects demographic bottlenecks may have had on genetic variability and structure. For this purpose, 276 lynxes from Sweden, Norway, Finland, Estonia and Latvia were analysed for polymorphism at 11 feline microsatellite loci and at the mitochondrial DNA (mtDNA) control region. Scandinavian lynxes were found to be fixed for a single mtDNA haplotype, while this and three additional haplotypes were seen in Finland and the Baltic States (Estonia and Latvia); the haplotypes were all very similar, only differing at 1–4 sites within a 700 bp region sequenced. Microsatellite variability was moderate (H_e = 0.51–0.62) with lower heterozygosity and fewer alleles in Scandinavia than in Finland and the Baltic States together, though significant so only for the latter. Heterozygosity data in Scandinavia were consistent with a recent population bottleneck. Various analyses (e.g. F_{st}, individual-based tree, assignment test) revealed distinct genetic differentiation between Scandinavian lynxes and animals from Finland and the Baltic States. Some structure was evident within Scandinavia as well, suggesting an isolation by distance. The observed partition of genetic variability between Scandinavia and the eastern countries thereof indicates that lynx populations from the two regions may need to be separately managed. We discuss what factors can have contributed to the population genetic structure seen in northern European lynx populations of today.

Introduction

The Eurasian lynx (*Lynx lynx*) has been present in Northern Europe since the last Ice Age but the colonisation history and current population structure are not well known. In Sweden and Norway, the species has been aggressively pursued (e.g. for its coveted pelt) since the 16th century (Liberg 1997), which has led to a strong population decline during the past two centuries. Hunting statistics from Sweden shows a

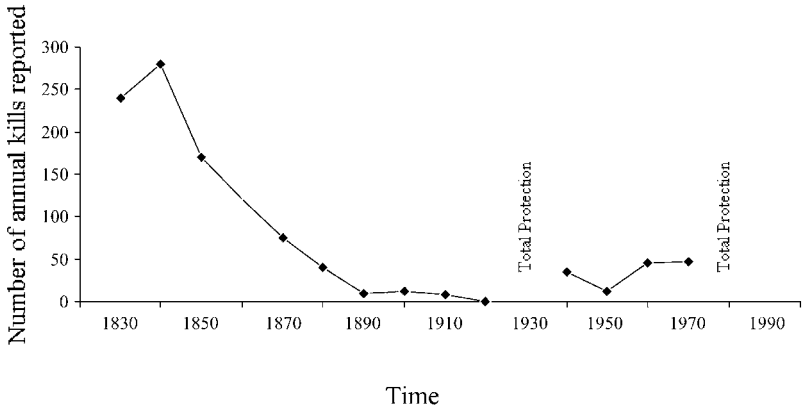

Figure 1. Hunting statistics of lynx in Sweden during 1830–1990. Data from Liberg (1997).

decrease in the numbers of lynx killed, from 250 per year in 1835 to 0 in 1927 (when laws in Sweden gave protection to the lynx; Figure 1). The total number of animals in Sweden at the end of the 1920's was estimated at less than 100 individuals, and could have been as few as 30 (Lönnberg 1930). There are no estimates of the population size in Norway at this time, however, Liberg (1997) suggests that the lynxes occasionally found in Norway at this time were primarily immigrants from the Swedish population. Moreover hunting statistics show a similar trend to that observed in Sweden. During the rest of the 20th century there have been alternating periods of restricted hunting and total protection, resulting in a rapid increase and expansion of the population to the south and north, with locally fluctuating lynx numbers (Heggberget and Myrberget 1980; Ahlén and Tjernberg 1996). In 1998, the Swedish lynx population size was estimated to be about 1000–1500 and a 1996 estimate for Norwegian lynxes was 500–600 (R. Franzén pers. comm.). These numbers are considerably higher than what has been the case in Scandinavia during most of the 20th century.

In Finland too, the lynx population was almost eradicated in the middle of the 20th century (Pulliainen 1968). A recent Finnish population increase is characterised by an expansion to the north and southwest from the more densely occupied areas in the east and south-east of the country (Breitenmoser and Breitenmoser-Würsten 1990). However, in the northernmost part, where reindeer husbandry is common place, lynx densities still remain very low (Liberg 1997). The lynx population size in Finland is currently estimated at 700–800 individuals (R. Franzén pers. comm.). The lynx population sizes in Estonia and Latvia are estimated to be 500–1300 and 400–675, respectively (IV Baltic Theriological Conference). The lynx populations in Finland and the Baltic States are presumably contiguous to the larger Russian population.

Lynx populations in southern and western Europe experienced strong decrease in overall population size during the 19th century. By the turn of the century this resulted in extirpation from central and southern Europe, except in the Carpathians and Balkans (Breitrenmoser and Breitenmoser-Würsten 1990). No information is available about the situation in European Russia, although a similar population decrease might be expected. A natural recovery, however, coupled with several reintroduction programs, has resulted in many countries in continental Europe now supporting lynx populations (Breitenmoser 1983; Cop and Frkovich 1998; Huber and Kaczensky 1998).

Genetic variation is important for the viability of populations in both long- and short-term perspectives (Frankel and Soulé 1981; Lande 1988). Loss of variation due to genetic drift in connection with population bottlenecks has been shown to cause decreased fitness in natural populations, or even extinction (Saccheri et al. 1998; Westemier et al. 1998). Particularly in stressful environments, an increase in homozygosity in an isolated population can greatly enhance the risk of extinction (Bijlsma et al. 1997). A captive Swedish lynx population has been shown to experience inbreeding depression (Laikre 1999). A proper management of Scandinavian lynx populations may therefore require detailed knowledge on the effects of either historic or more recent (e.g. associated with overhunting in the 19th and 20th centuries) demo-

graphic bottlenecks might have had on genetic variability and structure.

Using microsatellite genotyping and analysis of mitochondrial DNA (mtDNA) control region sequences, we addressed conservation genetics aspects of northern European lynx populations. Specifically, we sought to elucidate what evolutionary forces have acted as to maintain or reduce current levels of genetic variability.

Materials and methods

Samples

Eighty-nine samples from Sweden and 107 samples from Norway were collected from legally harvested lynxes during the 1996–1998 and 1993–1997 hunting seasons, respectively. Similarly, 48 lynx samples were collected in Finland in 1997–1998, 24 in Estonia in 1998–1999 and eight in Latvia in 1997–1998. Samples consisted of muscle or tissue from internal organs and were stored in ethanol or frozen at –20 °C.

Populations were defined as Scandinavia (Sweden and Norway), Finland and the Baltic States (Estonia and Latvia). The separation between Scandinavia and Finland was justified by the fact that the density of lynxes in Northern Finland is low. The Baltic States and Finland are partly separated from each other by the Baltic Sea. Scandinavian animals were also tentatively assigned to sub-populations (Figure 2), to test for a possible contact zone between southern and north-eastern postglacial immigrants, as has been seen for other mammals (e.g. Jaarola et al. 1999). Sweden was thus divided into three sub-populations: southern Sweden (SS, n = 32), central Sweden (CS, n = 33) and northern Sweden (NS, n = 24), with the central sub-population approximately corresponding to the area where a contact zone has been seen in other species (European shrew *Sorex araneus*, Fredga and Nawrin 1977; bank vole *Clethrionomys glareolus*, Tegelström 1987; field vole *Microtus agretis*, Jaarola and Tegelström 1995; brown bear *Ursus arctos*, Taberlet et al. 1995). The mountain range along the border between central and northern Norway and Sweden (which corresponds to the political border between the two countries in this region) was kept as a boundary between Swedish and Norwegian sub-populations in order to test for a possible west-east differentiation. Finally, Norway was divided into five sub-populations (SSN, n = 25; SN, n = 25; CN, n = 26; NN, n = 16; NNN, n = 15), where SN, CN and NN correspond to SS, CS and NS in latitude.

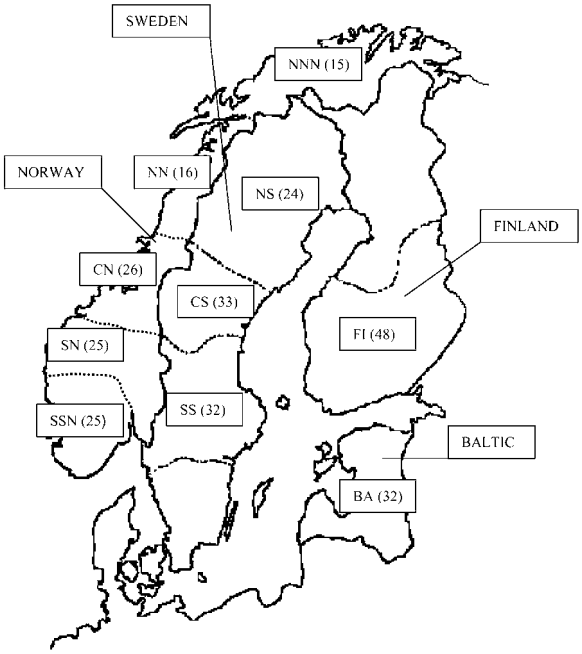

Figure 2. Map depicting the populations and sub-populations defined in this study. BA = Baltic States (Estonia and Latvia), FI = Finland, NNN = north northern Norway, NN = northern Norway, CN = central Norway, SN = southern Norway, SSN = south southern Norway, NS = northern Sweden, CS =central Sweden and SS = southern Sweden. Sample size is indicated in parentheses.

Tissue samples (20–50 μg) were minced and placed in 500 μl of extraction buffer (0.1 M Tris, 0.005M EDTA, 0.2 M NaCl, 0.007 M SDS, adjusted to pH 8.5) with 15 μl of 20 mg/ml proteinase K. The samples were incubated at 37 °C overnight and were then extracted once with phenol/chloroform (1:1) and once with chloroform. DNA was recovered by precipitation in 95% ethanol and the resulting pellet was washed with 70% ethanol, dried and resuspended in ddH_2O.

Mitochondrial DNA analysis

We amplified a 700 bp segment of the mtDNA control region using the primers L15997 (Ward et al. 1991) and H16498 (Meyer et al. 1990). The internal primers *Ll*1(5′-GATTTGCGGGCCAGTGGTT-3′, amplifying a 230 bp fragment in combination with L15997), and *Ll*2(5′-TAGTGCTTAATCGTGCATTATA-3′, amplifying a 195 bp fragment in combination with H16498; see Figure 3), were later designed to optimise fragment lengths for single-stranded conformation poly-

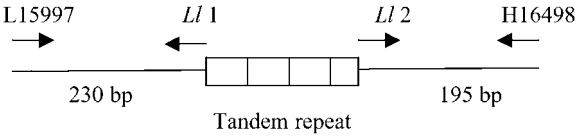

Figure 3. Schematic representation of the part of the lynx mitochondrial DNA control region analysed in this study. External and internal primers used in PCR amplifications are indicated. The location and structure of a 80 bp tandem repetitive element, in this case repeated 3.5 times, is also shown.

morphism (SSCP) analysis (Hayashi 1991). The 195 and 230 bp fragments flank a 80 bp tandem repeat located in the control region (Hoelzel et al. 1994), which was found to be repeated 3.5 times (3 full copies followed by 46 bp from a fourth copy) in all of our samples (Figure 3). The tandem repeat was initially excluded from the SSCP analyses because its high slippage rate often induces heteroplasmy (multiple different sequences in a single individual) in carnivores (Hoelzel et al. 1994).

Twenty-five ng DNA were used in 20 μl PCR reactions containing 1xPCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $mgCl_2$, 0.01% w/v gelatin), 5 μM of each primer, 16 mM dNTP and 0.5 U AmpliTaq polymerase (Perkin Elmer). The PCR profile included one cycle of 94 °C for 3 min, 55 °C for 30 s and 72 °C for 1 min, followed by 32 cycles of 94 °C for 30 s 50 °C for 30 s and 72 °C for 1 min, and a final extension step of 72 °C for 10 min.

The two smaller fragments (195 bp and 230 bp) were separated on native 12% polyacrylamide gels. The different SSCP morphs were visualised with silver staining according to Bassam et al. (1991). For subsequent sequencing, representative samples of the different morphs identified with SSCP were amplified with the primers L15997 and H16498, using the same PCR conditions as above. Products were purified with Qia-quick PCR purification kit (Qiagen) and sequenced using the BigDye^{TM} Terminator Cycle Sequencing chemistry (Perkin Elmer), following manufacturer's protocols. The sequences were recorded with an ABI377 semi-automated sequencing instrument (Perkin-Elmer). Some products were sequenced manually according to Hultman et al. (1989). The different haplotypes sequenced have been submitted to GenBank (accession numbers: AY034813-AY034816).

Nucleotide diversity (π, Nei and Li 1979) and its standard deviation (SD) in different populations were calculated using DnaSP (Rozas and Rozas 1997). A minimum spanning network (Excoffier et al. 1992) was constructed for the different mtDNA types found.

Microsatellite genotyping

Eleven polymorphic microsatellite loci were scored (Appendix 1) using primers developed for the domestic cat (Menotti-Raymond and O'Brien 1995; Menotti-Raymond et al. 1999). Eight loci contained dinucleotide repeats and three loci contained tetranucleotide repeats. According to genetic map information from domestic cat, only two of the markers analysed herein are located on the same chromosome, viz. *Fca45* and *Fca90* (Menotti-Raymond t al. 1999). However, there was no evidence for linkage disequilibrium between these two markers, as tested by the program Genetix (Belkhir 2000) (data not shown). PCR reactions for each of these loci were essentially performed as for mtDNA amplifications except that total reaction volumes were 10 μl. Three different scoring methods were applied to identify the alleles at each locus: use of fluorescently labelled primers with PCR products analysed on an ABI377 instrument, use of radioactive labelled primers with products visualised by autoradiography (Sambrook et al. 1989) or unlabelled primers with products visualised by silver staining (Bassam et al. 1991). The results from the different methods were checked for consistency by analysing several of the samples with all methods and comparing the scores.

To derive specific information from lynx about the sequence and repeat structure of the microsatellites analysed, at least two lynx alleles (amplified from homozygote individuals) were sequenced for each locus. Sequencing was performed as described above for mtDNA. From this information we indirectly inferred the number of repeat units contained within alleles of different sizes.

Microsatellite data analysis

Observed (H_o) and expected (H_e) heterozygosity and mean number of alleles per population were calculated using the program BIOSYS-2 (Swofford et al. 1981). To take the varying sample sizes into account in population comparisons, 100 data sets, each consisting of 80 randomly chosen individuals, were simulated from the larger Scandinavian sample to match the sample size of Finland + Baltic. The program GENEPOP (Raymond and Rousset 1995) was used to test if alleles deviated from Hardy-Weinberg equilibrium applying a Markov chain exact test (Guo

Table 1. Frequency (%) of mtDNA control region variants, nucleotide diversity (±SD) and number of variable sites found in different north European lynx populations

	Type 1	Type 2	Type 3	Type 4	Nucleotide diversity	Number of variable sites
Scandinavia	100	—	—	—	0	0
Finland	89.6	8.3	2.1	—	0.00066 (0.00027)	4
Baltic	65.6	15.6	12.5	6.3	0.00215 (0.00039)	5

and Thompson 1992) and Bonferroni correction for multiple tests. GENEPOP was also applied to calculate allele frequencies at different loci and to estimate the genetic exchange between the sub-populations in the form of effective number of migrants per generation (N_em) (Slatkin 1985). Differentiation between populations was characterised using F-statistics (Weir and Cockerham 1984), based on the infinite allele model (IAM). R-statistics (Slatkin 1995; Michalakis and Excoffier 1996) based on the stepwise mutation model (SMM) would have been an obvious alternative approach. However, it has been suggested that SMM-based statistics are not appropriate for use when populations under examination have a recent common origin (Paetkau et al. 1997), as can be expected in this case. Moreover, it has been indicated that R-statistics have poorer performance than F-statistics when fewer than 20 microsatellite loci are studied (Gaggiotti et al. 1999). The program Genetix (Belkhir 2000) was used to calculate levels of population subdivision, F_{ST}.

To test for significant differentiation we used a permutation test (1000 permutations) implemented in Genetix. Mantel's test (Mantel 1967) was used to assess the correlation between pairwise F_{ST} and geographic distance using the program MANTEL v. 2.0 (A. Liedloff, Queensland University, Brisbane, Australia). The geographic distance in kilometres between sub-populations, via the nearest land route, was roughly estimated from points in the centre of each sub-population. To test for possible effects from a recent bottleneck we used a test based on the difference between expected and observed heterozygosities implemented in the program BOTTLENECK (Cornuet and Luikart 1996). An assignment test (Paetkau et al. 1995) was used to calculate the likelihood of each individual multi-locus genotype having originated in each of the defined sub-populations. The test was performed by the assignment calculator provided by Dr J. Brzustowski (available at http://gause.biology.ualberta.ca/jbrzusto/Doh.html). The frequency 1/2N was used for alleles missing from one population, where N is sample size for that population. Individuals were assigned to the population with the highest likelihood. The evolutionary relationship between the sub-populations was calculated according to Nei's D mean distance using MICROSAT (Minch et al. 1995). This distance measure was chosen based on the results of Takezaki and Nei (1996), showing it to be superior in recovering the correct topology than other similar distance measures. A neighbour-joining tree of populations was constructed using this distance, and the support for the branches was estimated using 1000 bootstrap replicates. Genetic distances between all pairs of individuals (D_{xy}) were calculated as $1 - P_{xy}$, where P_{xy} is the proportion of shared alleles (Bowcock et al. 1994). The individual pairwise distances were calculated in MICROSAT. These distances were used to construct an unrooted neighbour-joining dendrogram in PAUP* 4.0 (Swofford 1998).

Results

Mitochondrial DNA

We analysed mtDNA control region variability in 276 Scandinavian, Finnish and Baltic lynxes by SSCP analysis of 195 bp and 230 bp fragments flanking both sides of a tandem repetitive portion of the control region (Figure 3). The overall level of genetic variability was very low with only one haplotype found in Scandinavia. This variant (type 1) was also the most common in Finland and the Baltic States. In addition, two other variants were found in Finland and the Baltic States, and a fourth type was unique to the Baltic population (Table 1). To complete the search for different haplotypes we also sequenced a 700 bp fragment, including the middle tandem repeat, in a large proportion of the samples. This extended search did not provide additional haplotypes to the ones identified by SSCP.

Table 2. Characteristics and genetic variability of 11 feline microsatellite markers genotyped in North European lynx populations

Locus	Repeat sequence[a]	Mean length[b]	Inferred range[c]	Expected heterozygosity[d]			Number of alleles[d]		
				Scandinavia[e]	Finland	Baltic	Scandinavia[e]	Finland	Baltic
Fca001	$(GT)_{14,18,19}(GA)_{19,21,25}$	—	—	0.59 (0.59)	0.69	0.74	5 (4.8)	8	8
Fca008	$(CA)_{20,22,23}$	22.7	19–26	0.48 (0.47)	0.70	0.73	5 (4.5)	4	5
Fca031	$(CA)_{21,24}$	20.8	20–27	0.05 (0.05)	0.82	0.67	3 (2.4)	7	6
Fca043	$(CA)_{9,10}$	9.4	8–13	0.46 (0.46)	0.67	0.69	3 (2.3)	4	5
Fca045	$(CA)_{16,17}$	16.5	16–20	0.48 (0.47)	0.32	0.23	2 (2.0)	3	3
Fca090	$(CA)_{10,16}$	12.2	10–17	0.48 (0.48)	0.51	0.58	4 (3.0)	4	4
Fca149	$(CA)_{16}$	16.1	15–17	0.27 (0.27)	0.30	0.15	3 (3.0)	3	2
F115	Complex A- and G-rich repeats[f]	—	—	0.79 (0.79)	0.78	0.73	11 (9.3)	10	11
Fca391	$(TGGA)_7 \ldots (AGAT)_{8,9}$	—	—	0.65 (0.65)	0.57	0.64	4 (4.0)	3	3
Fca506	$(CA)_{15,18}$	17.1	8–20	0.66 (0.66)	0.71	0.73	6 (5.3)	6	7
Fca559	$(GAAA)_{9,11,12,13}$	10.7	9–14	0.75 (0.74)	0.71	0.72	6 (5.8)	5	4
All loci				0.51 (0.51)	0.62	0.60	4.7 (4.2)	5.3	5.3

[a]Repeat sequence obtained from at least two lynx alleles.
[b]Mean number of repeat units seen in the total lynx sample, assuming that size variation was only due to varying number of tandem repeat units and based on the sequence information obtained for lynx. This was only calculated for perfect repeats.
[c]Inferred range of the number of repeat units according to the same assumptions as in b.
[d]Based on genotyping of 176 Scandinavian, 48 Finnish and 32 Baltic lynxes.
[e]Values in parantheses are obtained after 100 simulations with sample size equal to Finland+Baltic.
[f]$(GAA)_9(GA)_3(GAAA)_7(GAGA)(GAAA)(GA)_{15}(GAAA)_6(GAA)(GAAA)_{11}(GAA)_9(GA)_3(GAAA)_7(GAGA)(GAAA)(GAGA)(GAAA)(GAGA)(GAAA)(GAGA)(GAAA)_9(GAA)(GAAA)_9$ and $(GAA)_9(GA)_3(GAAA)_7(GAGA)(GAAA)(GA)_{15}(GAAA)_6(GAA)(GAAA)_{11}(GAA)_9(GA)_3(GAAA)_7(GAGA)(GAAA)(GAGA)(GAAA)(GAGA)(GAAA)(GAGA)(GAAA)_9(GAA)(GAAA)_{10}$

There were four segregating sites in the flanking regions and one in the tandem repeat, resulting in a mean sequence divergence of 0.38% (range: 0.15–0.61) including the repetitive region and 0.52% (range: 0.26–0.78) excluding it. In the two populations where a single mtDNA variant was not fixed, nucleotide diversity was less than 0.0025 (Table 1). A minimum spanning network shows the close relationship between the different haplotypes with type 2 in the centre differing with 2 substitutions from type 1 and type 3 and with 1 substitution from type 4. All substitutions were transitions.

Genetic variability of microsatellite loci

Levels of genetic variability at 11 feline di- and tetranucleotide microsatellite loci genotyped in all 276 animals corresponded to what might be considered as moderate for microsatellites, i.e. mean heterozygosities between 0.51–0.62 (Table 2) for the three defined populations (Scandinavia N = 196, Finland N = 48 and Baltic N = 32). Mean heterozygosity and mean number of alleles were lower in all of 100 simulated data sets of Scandinavian individuals of equal sample size to Finland+Baltic. Mean heterozygosity in the 100 resamples was 0.51 ranging from 0.50 to 0.53 compared to 0.63 for Finland+Baltic. The mean number of alleles in the 100 Scandinavian resamples (4.2 with a range from 3.8 to 4.5) was significantly lower compared to Finland+Baltic (6.0; $p = 0.015$, Wilcoxon signed rank test). To test if the levels of genetic variability in Scandinavia were consistent with the expected genetic effects of a recent bottleneck, we applied Cornuet and Luikart's (1996) test of comparison of observed and expected heterozygosities. There is significant support for a recent bottleneck ($p = 0.006$) in our data.

All populations were in Hardy-Weinberg equilibrium except SS for which locus *Fca008* showed a large excess of homozygotes ($H_o = 0.28$, $H_e = 0.58$), which could result from the existence of null alleles. Twenty alleles that were observed in the Finnish and Baltic populations were not observed in the much larger Scandinavian sample, whereas the reverse was only true for seven alleles. A complete presentation of allele frequencies in all subpopulations is presented in Appendix 1.

Use of microsatellites designed for another species may inherently be associated with finding relatively low levels of genetic variability, given that repeat lengths are not always conserved (Primmer et al. 1996) and that repeat length significantly affects the varia-

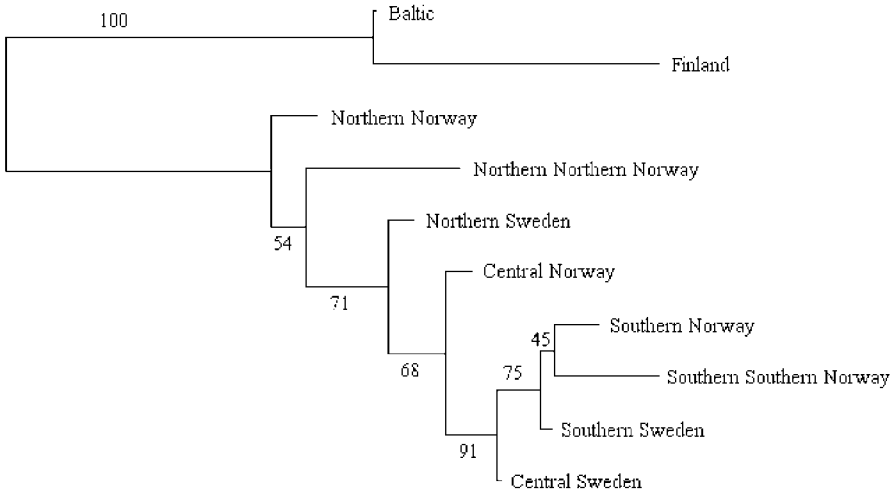

Figure 4. Neighbour-joining tree of lynx populations and sub-populations based on Nei's D distance with bootstrap support values based on 1000 replicates indicated at the branches.

bility of microsatellites (Weber 1990). However, by sequencing alleles of the feline markers used in this study, the number of repeat units found in lynxes generally proved to be in the order of what should be expected for polymorphic markers (Table 2). Thus, the moderate levels of microsatellite variability seen in lynxes, particularly in Scandinavia, seem not likely to be a consequence of the use of markers of inherent low variability for this species.

Genetic relationships between populations

A neighbour-joining tree constructed from Nei's D distances showed differentiation between the Scandinavian sub-populations and, with strong bootstrap support, differentiation between the Scandinavian and the Finnish and Baltic populations (Figure 4). Scandinavian sub-populations appear in the tree according to their geographic location, with the northern sub-populations closer to the Finnish and Baltic populations, the central sub-populations in intermediate positions, and the southern sub-populations being the most divergent. Moreover, a neighbour-joining dendrogram based on the proportion of shared alleles placed all 196 Scandinavian and all 80 Finnish/Baltic animals on separate branches (Figure 5). Furthermore, an assignment test correctly assigned 274 of the 276 animals to either Scandinavia or Finland/Baltic (Table 3; the two cross-classified individuals cluster together with individuals from their respective populations in the individual tree (Figure 5), but they have both long branches indicative of uncommon genotypes combinations).

Table 3. Number of individuals from population *i* (row) assigned to population *j* (columns). Numbers in the box area refer to assignments within Scandinavian sub-populations

	BA	FI	NNN	NN	CN	SN	SSN	NS	CS	SS
BA	29	3	0	0	0	0	0	0	0	0
FI	6	42	0	0	0	0	0	0	0	0
NNN	0	0	8	4	1	0	0	2	0	0
NN	1	0	2	6	3	1	0	3	0	0
CN	0	0	2	3	4	8	2	0	5	2
SN	0	0	0	1	1	3	7	3	3	7
SSN	0	0	0	0	2	3	19	0	1	0
NS	0	0	1	4	3	2	0	8	5	1
CS	0	0	0	2	4	6	3	3	12	3
SS	1	0	0	1	2	13	0	1	2	12

Calculations of F_{ST} and N_em showed that the degree of genetic differentiation between Scandinavian sub-populations and the eastern populations was significant (Table 4). All F_{ST} values for these comparisons were in the range of 0.14–0.29. This high degree of differentiation may be explained by contrasting population histories (phylogeography, bottleneck effects) and/or by isolation by distance. If the latter would be the case, we should expect F_{ST} values between individual Scandinavian sub-populations and Finland/Baltic to increase with increasing geographic distance. However, no such

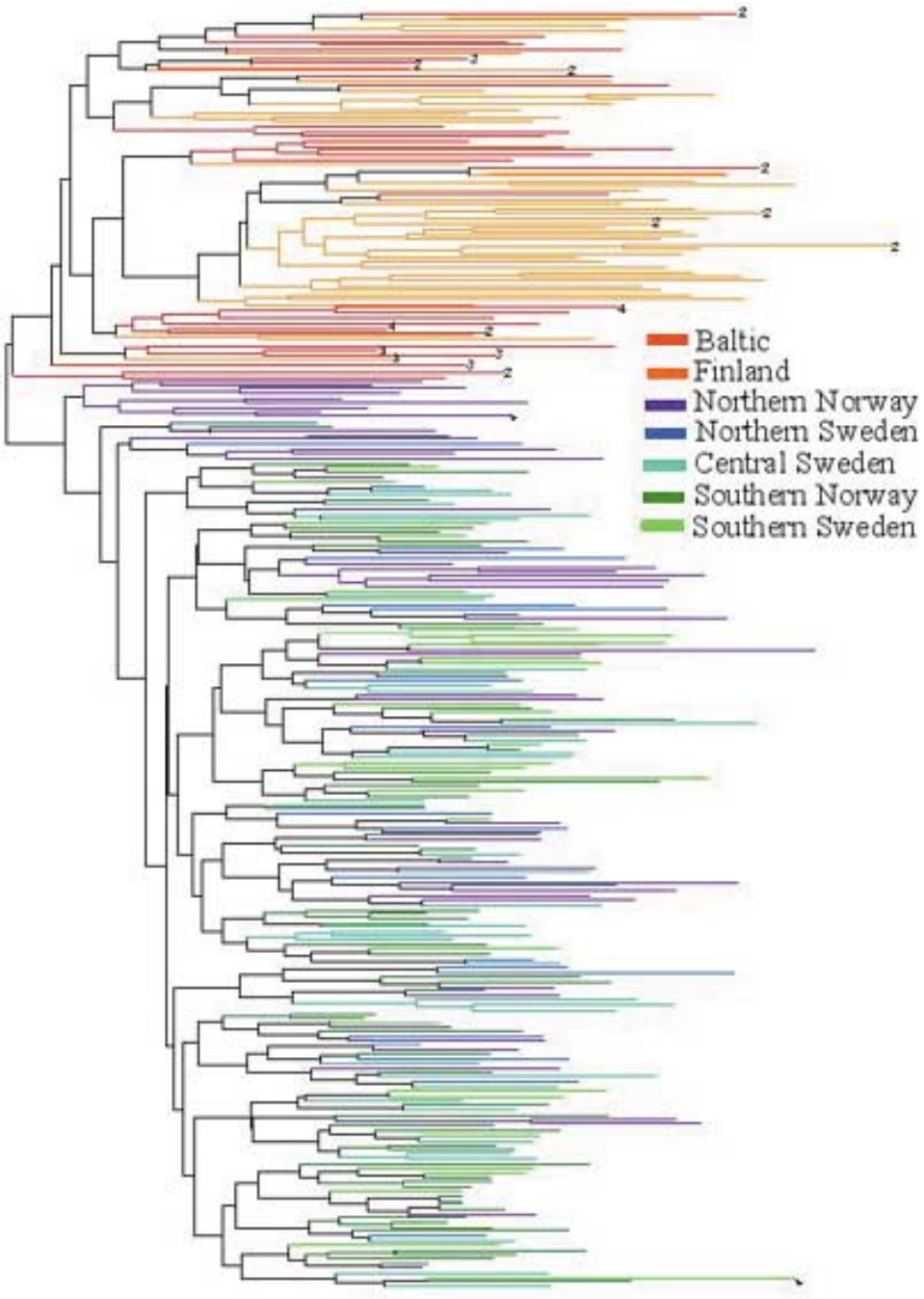

Figure 5. Dendrogram based on the distance 1 – [proportion of shared alleles] between all pairs of individuals. Each population is represented by a different colour (to simplify, Norway sub-populations are only represented by two colours). The mtDNA haplotype of each individual is indicated at the end of the branches when different from type 1 (see Table 1). Stars indicate Scandinavian samples assigned to the Baltic States in an assignment test (Table 3).

Table 4. Pairwise N_em (above the diagonal) and F_{ST} estimates (below the diagonal) between lynx populations and sub-populations

	BA	FI	NNN	NN	CN	SN	SSN	NS	CS	SS
BA		4.2	1.3	1.6	1.4	1.0	0.8	1.2	1.1	0.9
FI	0,057***		1.0	1.2	1.0	0.8	0.6	0.9	0.9	0.8
NNN	0,162***	0,207***		5.3	3.5	2.0	1.2	5.0	2.2	1.6
NN	0,136***	0,168***	0,045***		11.4	3.7	1.9	22.7	4.2	2.5
CN	0,156***	0,205***	0,067***	0,021*		39.9	2.8	15.2	27.1	9.7
SN	0,207***	0,248***	0,111***	0,063***	0,006		8.8	11.0	49.7	999.0
SSN	0,246***	0,291***	0,172***	0,119***	0,081***	0,028***		2.6	3.3	4.8
NS	0,179***	0,211***	0,048***	0,011	0,016*	0,022*	0,089***		8.6	5.2
CS	0,184***	0,227***	0,102***	0,056***	0,009	0,005	0,071***	0,028***		16.3
SS	0,219***	0,251***	0,137***	0,090***	0,025***	–0,003	0,049***	0,046***	0,015*	

* $p < 0.05$; *** $p < 0.001$.

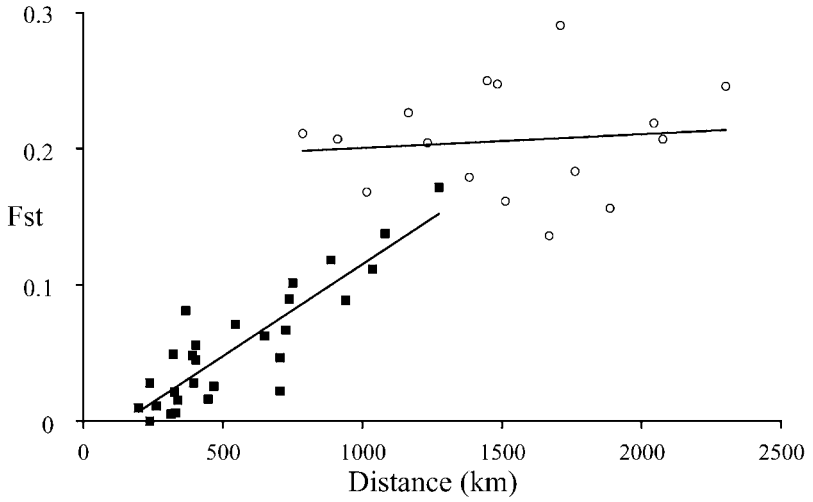

Figure 6. Relationship between genetic (F_{st}) and geographic (km) distance for lynx sub-populations. Comparisons between Scandinavian subpopulations and the Finnish and Baltic populations are indicated with open circles. Comparisons between Scandinavian sub-populations are indicated by filled squares. Regressions lines are for the two separate sets of comparisons.

trend was detectable (Figure 6; Mantel's test, $p = 0.17$). If the Finnish and Baltic populations were distinctly fractionated or the mtDNA haplotypes were only distributed among related individuals, animals with identical mtDNA should be more similar also at autosomal loci. However, mtDNA haplotypes do not appear grouped in the individual-based microsatellite tree (Figure 5), indicating that there is not obvious association between mtDNA haplotypes and microsatellite genotypes.

Genetic relationships between Scandinavian sub-populations

F_{ST} values between sub-populations within Scandinavia were generally lower than in the comparisons between Finnish and Baltic populations, yet statistically significant in most cases (Table 4). Within Scandinavia, differentiation was consistent with an isolation by distance, F_{ST} increasing with increasing genetic distance (Figure 6, Mantel's test $p < 0.01$). Genetic differentiation between northern and southern sub-populations was also evident from the frequent occurrence of private alleles. A total of four alleles at four loci in animals from the northern NS, NN and NNN sub-populations were absent in the south. Simi-

larly, six alleles seen in animals from the southern SS and CS sub-populations were not found in northern animals. Gene flow in Scandinavia seems thus most pronounced between neighbouring sub-populations. There was no evidence for a restriction in gene flow between neighbouring Swedish and Norwegian subpopulations.

Discussion

Population structure

This study provides strong evidence for population structuring among northern European lynx populations. Two important patterns were seen, distinct differentiation between Scandinavian and adjacent eastern populations, and structuring consistent with isolation by distance inducing a differentiation along a north-south gradient along Scandinavia. We can postulate at least two scenarios explaining the strong differentiation between Scandinavia and Finland/Baltic. First, similar to what has been documented for several other mammals (brown bear *Ursus arctos*, Taberlet et al. 1995; European shrew *Sorex araneus*, Fredga and Nawrin 1977; field vole *Microtus agretis*, Jaarola and Tegelström 1995; bank vole *Clethrionomys glareolus*, Tegelström 1987), the postglacial recolonization of Scandinavia by lynxes may have been through two different routes: one from the south through a land bridge from Denmark and another one from the east through Finland. The unique mtDNA haplotypes seen in Finland and the Baltic States could, in this perspective, be taken as indicators of a separate origin. However, the close similarity between haplotypes and the fact that the haplotype fixed in Scandinavia was also very common in Finland and Baltic argue against this idea. Moreover, in the case of contrasting evolutionary histories, we should reciprocally have expected a significant number of microsatellite alleles being unique to the different groups of animals. A large number of alleles found in Finland and the Baltic States were indeed not observed in Scandinavia, but only a few alleles observed in Scandinavia were lacking in the more eastern populations. Since the degree of variability differed between the populations, and since a differentiation with distance exists even inside Scandinavia, it is not apparent that a contrasting evolutionary history can be invoked. Firmly addressing phylogeographic patterns of lynxes in Europe will therefore require analysis of historical samples and/or samples from other geographical regions.

An alternative explanation to the differentiation between Scandinavia and Finland/Baltic is that, although populations are geographically very close, gene flow through the north of the Scandinavian peninsula is severely restricted. The vegetation in the far north of Finland and Scandinavia is dominated by tundra, whereas forest, the preferred habitat of the lynx, is scarce. The occurrence of carnivores in this area may also be in conflict with reindeer husbandry. Indeed, lynxes have been rare in the very north of Scandinavia and Finland until only recently (O. J. Sikku pers. comm.). Strong genetic drift during the documented bottlenecks in Scandinavia in recent centuries may have contributed to genetic differentiation. We consider this to be a likely explanation. In any case, our data suggest that the Scandinavian lynx population should probably be treated as a management unit separate from the Finnish and Baltic populations.

Almost all Scandinavian sub-populations analysed in this study were moderately differentiated from each other, the pattern being consistent with isolation by distance. Significant gene flow is thus evident only for neighbouring sub-populations. One possible interpretation of our data is that, following severe bottlenecks during the last century leading to one or a few isolated groups of animals, lynxes are now spreading across Scandinavia in a process that initially has given rise to some structuring. The fact that we observed a number of private alleles in both southern and northern Scandinavia may suggest that lynxes survived in both the south (as suggested by Liberg 1997), and the north of Scandinavia during the most severe population contractions in the early 20th century. Since differentiation between neighbouring central and northern Swedish and Norwegian subpopulations (i.e. in a west-east direction) was not more pronounced than between neighbouring subpopulations in a north-south direction, the mountain range along the border between these parts of Norway and Sweden seems not to hinder gene flow between the countries. A common management of the Swedish and Norwegian populations therefore seems reasonable.

Low genetic variability in Scandinavian lynx populations

The overall level of genetic variability observed for northern European lynx populations was low

(mtDNA) to moderate (microsatellites). A single mtDNA control region haplotype was fixed in the Scandinavian population and this population also displayed a lower mean heterozygosity (0.51) at microsatellite loci than the Finnish and Baltic populations (0.60–0.62). In the latter, a limited number of mtDNA haplotypes were seen, but the nucleotide diversity was low (0.00066 and 0.00215, respectively). It might be argued that the moderate variability indicated from the set of 11 feline microsatellite markers we applied is at least partly a consequence of using heterologous markers (cf. Ellegren et al. 1995). However, eight out of these 11 markers have been used in cross-species amplification of leopard *Panthera pardus* (Spong et al. 2000) and lion *Panthera leo* (G. Spong pers. comm.) populations, and have revealed high genetic variability in these species (H_e = 0.80 and 0.76, respectively). We therefore speculate that northern European lynx populations have lower levels of genetic variability than what is seen for other felids.

It may be argued that the low variability seen in northern European lynx populations, in particular in Scandinavia, is a consequence of the heavy hunting pressure during the last two centuries, known to have translated into population decreases across the entire Europe (Breitrenmoser and Breitenmoser-Würsten 1990). Genetic drift, especially during bottlenecks, may thus have led to reduced levels of genetic variability. Although hunting is likely to have been intense also in Finland and the Baltic States, the contact with the larger Russian lynx population may have prevented loss of genetic variability to the same extent as hypothesised for Scandinavia. This scenario lends support from the bottleneck test. Alternatively, moderate variability may have been characteristic to all lynxes in Europe, resulting from their survival in single glacial refugium. The very low sequence divergence between mtDNA haplotypes observed in this study supports this idea. A similar interpretation, expansion from a single glacial refugium, has been used to explain the low mtDNA sequence diversity seen in otters (*Lutra lutra*) across Europe (Cassen et al. 2000). In theory, the low variability of Eurasian lynxes could be a species-specific trait due to either a recent origin or a slow mutation rate. However, the fossil record indicates that the lynx was present in Eurasia over one million years ago (Werdelin 1981), and substitution rates do not seem lower in lynxes than in other felids (Johnson and O'Brien 1997; Pecon and O'Brien 1998).

Four large carnivores inhabit the Scandinavian peninsula: the wolf *Canis lupus*, the wolverine *Gulo gulo*, the brown bear and the lynx. With this study, basic population genetic parameters of Scandinavian populations of all four species have now been addressed using molecular genetic approaches (Ellegren et al. 1996; Ellegren 1999; Waits et al. 2000; Walker et al. 2001). Interestingly, three of these species (wolf, wolverine and lynx) show very similar patterns, i.e. little or no mtDNA variability and low to moderate microsatellite variability. A common denominator for the three species is that due to persecution and over-hunting, they have all gone through severe bottlenecks in recent times. This might suggest that their reduced levels of genetic variability can commonly be explained by these bottlenecks. However, the situation is complicated by the fact that analyses of historical samples of wolverines (Walker et al. 2001) and wolves (Ø. Flagstad et al., in prep.) indicate that low variability is not necessarily a very recent phenomenon. Moreover, the population size of brown bears in Scandinavia has also been significantly reduced during the 20th century and, despite this, higher levels of genetic variability are seen in this species (Waits et al. 2000). This may relate to the documentation of two different immigration routes of brown bears in Scandinavia (Taberlet et al. 1995), which significantly can have contributed to increase the genetic heterogeneity of Scandinavian brown bears in a way that has not occurred in the other species.

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Appendix 1. Allele frequencies and expected heterozygosities for all loci and subpopulations. Allele sizes are given in bp

Locus	Allele	BA	FI	NNN	NN	CN	SN	SSN	NS	CS	SS
Fca001	176	—	0.021	0.100	0.344	0.380	0.380	0.320	0.333	0.554	0.469
	178	0.017	—	—	—	—	—	—	—	—	—
	180	0.150	0.188	0.733	0.531	0.400	0.520	0.640	0.625	0.351	0.406
	182	0.367	0.490	0.033	0.094	0.020	—	—	0.021	—	—
	184	0.083	0.031	—	—	—	—	—	—	—	—
	186	—	0.083	—	—	—	—	—	—	—	—
	188	0.017	—	—	—	—	—	—	—	—	—
	190	0.300	0.010	0.033	0.031	0.180	0.100	0.040	0.021	0.095	0.125
	192	—	—	0.100	—	0.020	—	—	—	—	—
	194	0.033	0.146	—	—	—	—	—	—	—	—
	196	0.033	0.031	—	—	—	—	—	—	—	—
H_e		0.743	0.694	0.440	0.589	0.662	0.575	0.486	0.497	0.561	0.60
Fca008	136	—	—	—	—	—	—	—	—	—	0.016
	138	0.113	0.240	0.233	0.031	0.192	0.260	0.140	0.182	0.324	0.203
	142	—	—	—	—	—	—	—	0.045	—	0.172
	144	0.290	0.104	0.700	0.688	0.750	0.740	0.840	0.682	0.581	0.594
	146	0.355	0.427	0.067	0.281	0.058	—	0.020	0.091	0.095	0.016
	148	0.226	0.229	—	—	—	—	—	—	—	—
	150	0.016	—	—	—	—	—	—	—	—	—
H_e		0.726	0.697	0.451	0.447	0.397	0.384	0.274	0.492	0.548	0.576
Fca031	228	0.417	0.202	0.917	1.000	1.000	1.000	1.000	0.896	0.986	0.969
	230	0.067	0.234	—	—	—	—	—	—	—	0.016
	232	0.383	0.053	—	—	—	—	—	—	—	—
	234	0.033	0.096	—	—	—	—	—	—	—	—
	236	—	0.106	—	—	—	—	—	—	—	—
	238	0.083	0.085	0.083	—	—	—	—	0.104	0.014	0.016
	242	0.017	0.223	—	—	—	—	—	—	—	—
H_e		0.667	0.824	0.152	0.00	0.00	0.00	0.00	0.187	0.027	0.061
Fca043	111	0.328	0.394	—	0.031	—	—	—	—	—	—
	113	0.328	0.191	0.600	0.406	0.692	0.708	0.620	0.604	0.622	0.750
	115	0.310	0.372	0.400	0.563	0.308	0.292	0.380	0396	0.378	0.250
	119	0.017	—	—	—	—	—	—	—	—	—
	121	0.017	0.043	—	—	—	—	—	—	—	—
H_e		0.688	0.668	0.480	0.517	0.426	0.413	0.471	0.478	0.470	0.375
Fca045	144	0.875	0.813	0.733	0.567	0.500	0.320	0.340	0.375	0.365	0.234
	146	0.094	0.146	0.267	0.433	0.500	0.680	0.660	0.625	0.635	0.766
	152	0.031	0.042	—	—	—	—	—	—	—	—
H_e		0.225	0.317	0.391	0.491	0.500	0.435	0.448	0.469	0.463	0.359
Fca090	105	0.565	0.667	0.321	0.60	0.596	0.708	0.750	0.563	0.568	0.717
	111	0.097	—	—	—	—	—	—	—	—	—
	115	0.032	0.188	—	—	—	—	—	—	0.014	—
	117	0.306	0.104	0.679	0.400	0.404	0.292	0.250	0.438	0.392	0.283
	119	—	0.042	—	—	—	—	—	—	0.027	—
H_e		0.577	0.508	0.436	0.480	0.481	0.413	0.375	0.492	0.523	0.406

Locus	Allele	BA	FI	NNN	NN	CN	SN	SSN	NS	CS	SS
Fca149	122	—	0.052	0.10	0.094	—	0.020	0.120	0.104	0.027	—
	124	0.917	0.823	0.60	0.844	0.885	0.900	0.860	0.813	0.878	0.875
	126	0.083	0.125	0.30	0.063	0.115	0.080	0.020	0.083	0.095	0.125
H_e		0.153	0.304	0.540	0.275	0.204	0.183	0.245	0.322	0.219	0.219
F115	231	0.017	0.011	—	—	—	—	—	—	0.028	—
	235	0.483	0.125	0.179	0.094	0.192	0.300	0.500	0.188	0.347	0.234
	237	0.083	—	0.036	—	—	—	—	—	—	—
	239	0.050	0.045	0.071	0.188	0.289	0.360	0.300	0.375	0.389	0.297
	241	0.033	0.057	0.036	0.313	0.269	0.180	0.020	0.250	0.083	0.109
	243	0.067	0.341	0.179	0.094	—	—	—	0.021	0.042	—
	245	0.050	—	—	—	—	—	—	—	—	—
	247	0.067	0.011	0.286	0.031	0.077	0.040	—	0.021	0.014	0.141
	251	0.117	0.284	0.143	0.219	0.115	0.020	—	0.063	0.042	0.141
	253	—	—	—	—	—	—	0.020	—	—	—
	255	0.017	0.068	0.071	0.063	0.039	0.10	0.020	0.083	0.056	0.141
	257	—	—	—	—	0.019	—	0.140	—	—	—
	259	0.017	0.023	—	—	—	—	—	—	—	—
	261	—	0.034	—	—	—	—	—	—	—	—
H_e		0.730	0.776	0.821	0.796	0.786	0.736	0.639	0.750	0.714	0.799
Fca391	224	—	—	0.143	0.167	0.208	0.435	0.780	0.229	0.264	0.516
	228	0.234	0.344	0.607	0.467	0.50	0.391	0.080	0.583	0.569	0.422
	232	0.453	0.552	0.071	0.233	0.167	0.065	0.120	0.083	0.056	0.031
	236	0.313	0.094	0.179	0.133	0.125	0.109	0.020	0.104	0.111	0.031
H_e		0.642	0.568	0.574	0.682	0.663	0.641	0.370	0.589	0.591	0.554
Fca506	179	0.016	—	0.167	—	—	—	—	0.105	—	—
	193	0.081	0.177	—	—	—	—	—	—	—	0.016
	195	0.323	0.344	0.200	0.467	0.375	0.313	0.348	0.368	0.210	0.290
	197	0.016	0.010	0.400	0.333	0.146	0.104	0.022	0.289	0.161	0.016
	199	0.097	0.042	0.233	0.167	0.438	0.583	0.630	0.211	0.548	0.597
	201	0.371	0.375	—	0.033	0.042	—	—	0.026	0.081	0.081
	203	0.097	0.052	—	—	—	—	—	—	—	—
H_e		0.733	0.705	0.717	0.642	0.645	0.551	0.481	0.724	0.623	0.553
Fca559	110	0.281	0.229	0.200	0.188	0.100	0.140	0.100	0.227	0.153	0.133
	114	0.375	0.177	0.267	0.281	0.360	0.260	0.320	0.114	0.333	0.267
	118	0.219	0.438	0.033	0.094	0.320	0.280	0.120	0.205	0.264	0.333
	122	0.125	0.042	0.333	0.344	0.200	0.320	0.460	0.432	0.250	0.267
	126	—	0.115	0.067	—	—	—	—	0.023	—	—
	130	—	—	0.100	0.094	0.020	—	—	—	—	—
H_e		0.717	0.710	0.762	0.750	0.717	0.732	0.661	0.707	0.733	0.729
$H_{E\ Tot}$		0.600	0.617	0.527	0.513	0.498	0.464	0.401	0.524	0.495	0.476
(S.E)		0.210	0.175	0.179	0.229	0.237	0.224	0.188	0.167	0.210	0.217
H_O		0.625	0.628	0.555	0.529	0.476	0.476	0.449	0.540	0.467	0.460
(S.E)		0.244	0.195	0.201	0.263	0.236	0.248	0.220	0.194	0.229	0.223
F_{IS}		–0.025	–0.007	–0.015	0.004	0.066	–0.004	–0.099	–0.008	0.072	0.049

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