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Genetic variation and structure in Scandinavian red deer (*Cervus elaphus*): influence of ancestry, past hunting, and restoration management

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In the 19th century, the red deer (*Cervus elaphus*) population in Sweden experienced a rapid decline in numbers and distribution. A small population was, however, remnant in the southernmost province (Skåne) of the country, presumably corresponding to the nominate form of red deer (*Cervus elaphus elaphus* Linnaeus, 1758). After management, reintroductions, and supplementary release during the 20th century the Swedish *C. elaphus* population recovered. The recovery was partially uncontrolled, and included introductions of *C. elaphus* of continental origin. In northern central Sweden (Jämtland) the current *C. elaphus* population may stem from natural colonization from Norway and/or from specimens of Swedish origin that have escaped from enclosures. To evaluate the status of the current, partially separated populations, we investigated variation at microsatellite markers in 157 *C. elaphus* specimens from ten locations in Sweden and Norway. Analyses suggest that the highest-likelihood phylogenetic structure among the individuals sampled is described four distinct genetic clusters: (1) animals from the province of Västergötland in south-western Sweden; (2) deer from the southernmost province of Skåne; (3) deer from the provinces Jämtland, Blekinge, and Västmanland; and (4) Norwegian deer. *Cervus elaphus* from a captive herd at the Skåne Zoo cluster with deer from Skåne or deer from Västergötland, depending on the method of analysis. A number of populations in Sweden may genetically match the nominate form of red deer (*C. e. elaphus*). The recently established *C. elaphus* population in Jämtland seems to stem mainly from escapees from enclosures, with a mixed ancestry from the wild remnant population in Skåne and continental deer, whereas the influx from Norway is minor, if any. Our results show the need for a detailed assessment of genetic differentiation, and emphasize the value of local management plans when planning and managing introductions.

ADDITIONAL KEYWORDS: colonization – management – microsatellites – migration – mtDNA – structure – translocation.

INTRODUCTION

The possibility that there is a drastic genetic restructuring when a population becomes small and isolated was first raised by Huxley (1938) and Mayr (1942). During events of population decline, small population size leads to an increased importance of random drift, which may cause loss of genetic variation and thereby induce genotypic and phenotypic differentiation from ancestral populations (e.g. Larsson *et al*., 2008). Great

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uncertainty remains, however, about how quickly isolated populations diverge from their parental stock, and which changes in the genome accompany this divergence (Grant, 2001). These considerations have recently become accentuated by the increased levels of human-induced habitat loss, and by the fragmentation of the ranges of natural populations (Höglund, 2009).

Extensive reintroductions and translocations will also affect the genetic structure of populations. Supplemental release will result in the admixture of contingents with different gene pools (e.g. Hindar, Ryman & Utter, 1991; Williamson & May, 2005). During introductions with a limited number of founders, the genetic signature of the populations may differentiate considerably from the ancestral composition (Baker & Moeed, 1987, Hedrick, 2001).

Intense hunting pressure has often limited the distribution of species; however, in recent years, favoured game species have also been protected and managed to resume their lost distribution, and even expand into novel habitats. Research on managed populations can increase our understanding of the speed of evolutionary divergence, local adaptation, and the forces driving these processes (Sakai *et al*., 2001). A problem with investigations of natural populations with unknown history is the need to infer the time and circumstances of the founding event, and the subsequent ecological history from molecular, biogeographical, or palaeontological data (St Louis and Barlow 1988).

Subfossil findings of red deer (*Cervus elaphus*) in Sweden date back to the end of the preboreal and early boreal periods, at the end of the most recent glacial period: 9500–8000 BP (Ahlén, 1965; Lepiksaar, 1986). Potentially, *C. elaphus* colonized the Scandinavian Peninsula from the south, i.e. from continental Europe (Ekman, 1922). The Swedish and Norwegian populations may have colonized Scandinavia once or separately, and under either scenario have been separated since prehistoric times (Haanes *et al*., 2010b). Allozyme studies of genetic differentiation concluded that genetic variation among the current Scandinavian *C. elaphus* populations still contained a signal from the ancestral state (Gyllensten *et al*. 1983). During approximately the last 50 years, however, several undocumented reintroductions and supplementary releases were carried out in Sweden, Norway, and Denmark (Nielsen *et al*., 2008; Haanes *et al*., 2010a). Thus, the genetic signal of the ancestral subspecies may have been obscured.

In Europe, a drastic decline in both the numbers and the geographical distribution of *C. elaphus* resulted from intense hunting and land use during mainly the 18th and 19th centuries (Kuehn *et al*., 2003, 2004; Skog *et al*., 2009; Fickel *et al*., 2012). In Sweden, *C. elaphus* disappeared apart from a remnant population in Skåne (Lönnberg, 1906). In 1907 the entire Swedish population was estimated to consist of less than 50 individuals (Ahlén, 1965; Lavsund, 1975). The species was, however, saved from extermination and subsequently allowed to increase in Skåne during the 20th century. Today, this population is estimated to include approximately 2000–2500 deer before the harvest. In other areas in Sweden populations have been re-established by the supplemental release of *C. elaphus* with mixed Skåne and continental European heritage, but also partially by restocking or introductions of *C. elaphus* from continental Europe, mainly during the 1950s and 1960s (Lavsund, 1975). Thus, potentially the ancestral structure/composition of the Swedish nominate form of the red deer (*C. e. elaphus*) was severed. A similar population history, with drastic declines in the 19th century and with subsequent recovery, has been documented in Denmark (Nielsen *et al*., 2008) and in Norway, which is inhabited by the subspecies *Cervus elaphus atlanticus* (Haanes *et al*., 2010b).

The principal aims of this study were: (1) to resolve the potential substructuring of Swedish *C. elaphus* populations using highly polymorphic microsatellite markers and mitochondrial DNA (mtDNA) fragments, and compare Swedish and Norwegian *C. elaphus*; (2) to evaluate the levels of genetic variation within the populations studied; and (3) To provide background data that could be used as a guideline for the management of the Swedish *C. elaphus* population.

MATERIAL AND METHODS

GENOTYPING

In total, tissue samples from 157 legally hunted deer were collected from several localities in Sweden and from one locality in Norway from 1998 to 2009 (Fig. 1). The geographical origin of each individual was classified as belonging to the discrete population of origin. Samples were selected to represent areas where the two described subspecies occur (Kvam in Norway and the Vomb, Hjularöd, Övedskloster, and Christinehof estates in Skåne, Sweden), as well as areas where *C. elaphus* populations of various ancestries had been re-established (Blekinge, Västmanland, Västergötland, and Jämtland). In addition, samples from one zoological park population (the Skåne Zoo), presumed to belong to the southern subspecies *C. e. elaphus*, were collected.

Tissue (~0.5 cm^3) was obtained from each individual and was stored in 70% ethanol at room temperature (20°C) until DNA extraction. DNA was extracted from approximately 25 mg of tissue using a salt extraction

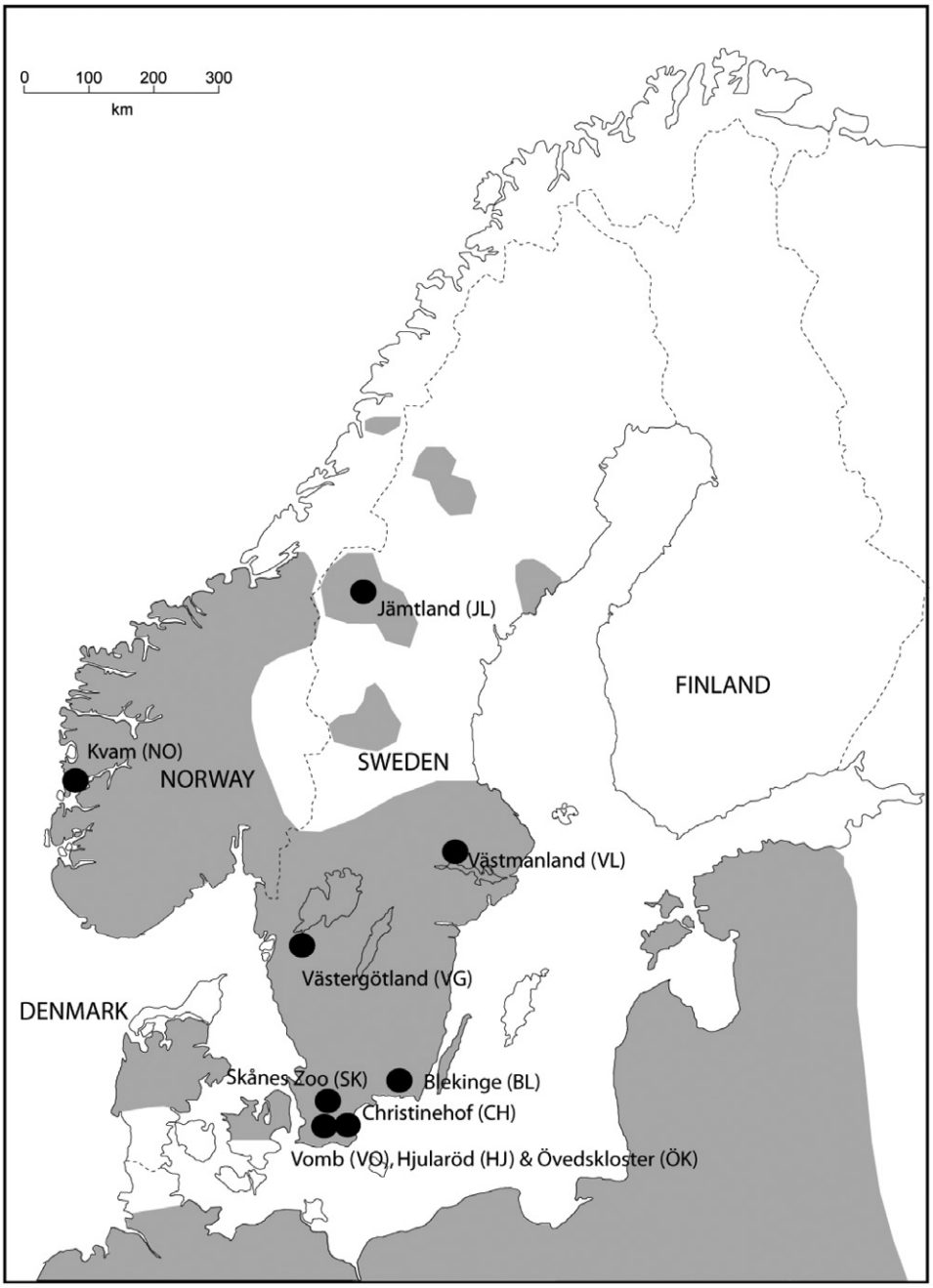

Figure 1. The approximate current distribution of red deer (*Cervus elaphus*) in Northern Europe (in grey), and the locations of sampling sites. Population abbreviations are given after the name of each population.

protocol (Paxton *et al*., 1996). Nine bovine microsatellite markers previously shown to be polymorphic in *C. elaphus* and other cervids (ranging between four and nine alleles; Talbot, Haigh & Plante, 1996; Slate *et al*., 1998; Thevenon *et al*., 2004) were amplified: INRA121, IDVGA55, BMC1009, VH110, BM757, BL42, BM848, TGLA53, and BM203. Following Bonnet *et al*. (2002), the amplification of polymerase chain reaction (PCR) products at individual loci was performed using a multiplex protocol, whereby groups of three loci were amplified in single PCRs.

The microsatellite markers were labelled with fluorescent dyes (HEX, NED, or FAM), and were divided into three multiplexes according to size. multiplex PCR reactions were run in 10-μL volumes containing 1 μL of each multiplex mix, 1 μL of diluted DNA, 5 μL

of PCR mastermix, and 3 μL of RNase-free water. PCR conditions were an initial denaturation cycle at 95 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, annealing at 62 °C for 90 s and 72 °C for 90 s. The PCRs then had a final extension at 72 °C for 10 min. PCR products were scored on a MegaBACE 1000 (Amersham Biosciences). To do this, 96-well plates were loaded. Each well was composed of 2 μL of ten-fold diluted PCR products, 7.8 μL of H_2O, and 0.2 μL of MegaBACE size standard. Genotypes for each sample were scored using the software FRAGMENT PROFILER 1.2 (Amersham Biosciences, 2003). Peak scoring was performed manually.

An 826-bp fragment of the control region of the mitochondrial control region (CR) was amplified with the primers and protocol described in Zachos *et al*. (2003) and Nielsen *et al*. (2008) for 21 of the samples. We sequenced the mtDNA fragments with the forward PCR primer and resolved 325 unambiguous base pairs for 11 individuals.

Statistical analyses

To check for null alleles, stuttering, and large allelic dropout, the data set was analysed with MICRO-CHECKER 2.2.3 (Van Oosterhout *et al*. 2004). Putative linkages among loci were checked with GENEPOP on the web (Raymond & Rousset, 1995, Rousset 2008). Calculations of global and pairwise F_{ST} levels (Weir & Cockerham, 1984) were performed using GENETIX 4.05.2 (Belkhir *et al*., 2000). We obtained confidence limits for all estimates using 1000 bootstrap replications. Allelic richness (AR), the rarefied number of alleles in a population (El Mousadik & Petit, 1996), normalized to the smallest complete sample number (here eight), across loci, was obtained using the R patch STANDARICH 1.0 (Alberto *et al*., 2006). The expected and observed frequencies of heterozygotes (H_E and H_O, respectively) for all loci were obtained using GENETIX. F_{IS}, the standardized deviation among average observed and expected heterozygosities, was calculated according to Weir & Cockerham (1984). We used the R package ADEGENET (Jombart, 2008) to create principal components analysis (PCA) plots to illustrate the multidimensional relationships between each individual genotype in a two-dimensional plot.

We used the model-based approach in STRUCTURE (Pritchard, Stephens & Donnelly, 2000) to find the number of genetic clusters (K) in Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD), and assign individuals to these clusters using the admixture model. We followed the approach suggested by Evanno, Regnaut & Goudet (2005) to infer the most likely number of K, adjusting for an increase in variance as K increases, and looking at the modal value of ΔK with the aid of STRUCTURE HARVESTER (Earl & von Holdt, 2012).

We used runs without a location prior for 50 replicates each at $K = 1$–10 with a burn-in of 50 000 and 100 000 iterations (Pritchard *et al*., 2000). To account for 'label switching' and to take an average over all runs (50), the output files were aligned in CLUMPP (Jakobsson & Rosenberg, 2007). The averaged STRUCTURE outputs were then visualized using the software DISTRUCT (Rosenberg, 2004).

We aligned the mtDNA sequences with the ClustalW algorithm in CODON-CODE ALIGNER 2.0.6 (Codon Code Corporation) with the aid of 41 European *C. elaphus* CR sequences downloaded from GenBank. We constructed a minimum spanning network of all the sequences using TCS 1.21 (Clement, Posada & Crandall, 2000), and calculated Φ_{ST} for the Scandinavian sequences against the European sequences, with and without the sequences from Norway, with the software ARLEQUIN 3.0 (Excoffier, Laval & Schneider, 2005).

RESULTS

At each microsatellite locus we found between 15 and 34 alleles. The total number of alleles observed within populations ranged from 24 in the Vomb population to 49 in the Jämtland population, and the average mean number of alleles per locus per population was 5.68. With MICRO-CHECKER we could find no evidence for stuttering, nor large allele dropout for any locus; however, we did find an excess of homozygotes, except for TGLA53. Given that some populations may have been inbred this is not surprising. None of the loci appeared linked. The Västergötland population had the highest number of private alleles (13), thereafter Jämtland (12), and Norway (9). The Skåne Zoo and Västergötland populations had the highest values of AR (Table 1). For these data, the two individuals from Hjularöds gods (see below) were merged with Vomb. It is apparent that observed heterozygosity tended to exceed the expected heterozygosity in the population from Skåne Zoo and the estate populations in Skåne (Vomb, Övedskloster, and Christinehof), whereas the reverse was observed among the populations in south (Blekinge), central (Västergötland, Västmanland), and northern (Jämtland) Sweden and Norway (Kvam). Thus, F_{IS} tended to be negative in the zoo and estate populations, but was neutral or positive in other populations.

We observed a clear population structure among predefined populations, with a global F_{ST} of 0.223 (95% confidence limits: 0.151–0.290). Pairwise F_{ST} values were all significant, except for comparisons involving the populations on the estates in Skåne and among the populations in Blekinge, Västmanland,

Table 1. Populations, sample size (N), expected and observed heterozygosity (H_E and H_O), and their respective standard deviations. Also given are F_{IS} and allelic richness (AR ± 1SD). The first population listed is a captive population, whereas the latter populations are free living. All values for F_{IS} are significantly different from 0 (at table-wide Bonferroni corrected $P < 0.008$), except for the estimates for Skåne Zoo, Norway, Västmanland, and Blekinge

Population	N	H_E	H_E (SD)	H_O	H_O (SD)	F_{IS}	AR
Skåne Zoo	18	0.760	0.056	0.784	0.050	*–0.006*	5.30 ± 0.71
Vomb	6 (+2)	0.664	0.065	0.833	0.062	–0.288	5.00
Övedskloster	9	0.684	0.070	0.815	0.053	–0.204	4.90 ± 0.15
Christinehof	26	0.623	0.090	0.797	0.034	–0.288	4.56 ± 0.35
Kvam, Norway	29	0.594	0.107	0.565	0.041	*0.051*	4.53 ± 0.14
Jämtland, Sweden	10	0.689	0.030	0.474	0.065	0.312	4.47 ± 0.36
Västmanland, Sweden	10	0.610	0.080	0.550	0.064	*0.098*	4.30 ± 0.22
Blekinge, Sweden	27	0.643	0.056	0.602	0.039	*0.064*	4.87 ± 0.69
Västergötland, Sweden	20	0.693	0.044	0.429	0.050	0.310	5.60 ± 0.69

Table 2. Pairwise F_{ST} values among the red deer (*Cervus elaphus*) populations sampled in Sweden and Norway

	Vomb	Öveds kl	C-hof	Kvam	Jämtland	Västmanland	Blekinge	Västergötland
Skåne Zoo	0.083	0.087	0.109	0.248	0.134	0.221	0.202	0.150
Vomb		*–0.002*	*0.012*	0.334	0.112	0.213	0.177	0.222
Övedskloster			*0.001*	0.317	0.100	0.193	0.155	0.208
Christinehof				0.334	0.141	0.233	0.194	0.243
Kvam					0.286	0.313	0.303	0.334
Jämtland						*0.019*	*0.005*	0.161
Västmanland							*–0.006*	0.189
Blekinge								0.199

Non-significant values are set in *italics*. All other estimates are significant at table-wide Bonferroni corrected $P < 0.0012$.

and Jämtland (Table 2). A two-dimensional PCA identified four main non-overlapping clusters: (1) Norwegian deer; (2) deer from the estates in Skåne and Skåne Zoo; (3) the Västergötland population; and (4) deer from Jämtland in northern Sweden, Västmanland (central Sweden), and Blekinge (southern Sweden), respectively (Fig. 2).

Simulations in STRUCTURE also gave strong support for four genetic clusters in our data (Fig. 3): one cluster mostly composed of animals from Skåne Zoo and Västergötland; a second cluster of deer from the estates in Skåne, likely to represent the nominate subspecies (*C. e. elaphus*); a third cluster of deer from Norway (representing *C. e. atlanticus*); and, finally, a fourth cluster of deer from Jämtland, Västmanland, and Blekinge. Individuals with genotypes distinct from their respective geographic location were found in all areas, except from the estates in Skåne. In the Skåne Zoo, four individuals looked like they had estate ancestry. In Norway, one individual had a genotype resembling the Västergötland–Zoo cluster in Sweden. In the Jämtland–Västmanland–Blekinge cluster, three individuals resembled estate deer and two resembled the Västergötland–Zoo animals. Finally, in Västergötland one individual showed evidence of Norwegian ancestry.

Both Norwegian and Swedish deer nested with *C. elaphus* from Western Europe when the mtDNA sequences where analysed in a minimum spanning network (Fig. 4). Five of the sequences from Swedish deer were nested in a unique subclade. Also, the sequences from deer from northern Sweden (SC1–SC3) were identical to those from a deer from the estates in south Sweden (SC7), and differed from the Norwegian deer (SC5 and SC6). Moreover, one sequence from a deer from Skåne Zoo (SC4) only differed by one nucleotide substitution from these sequences. Two sequences from deer from the estates in Southern Sweden were the same as a previously published sequence from GenBank. The Φ_{ST} for Scandinavian sequences versus continental European was 0.136 ($P < 0.01$), and excluding Norway Φ_{ST} took the value 0.100, $P < 0.003$.

DISCUSSION

We found that both Norwegian and Swedish deer belonged to the previously described mitochondrial

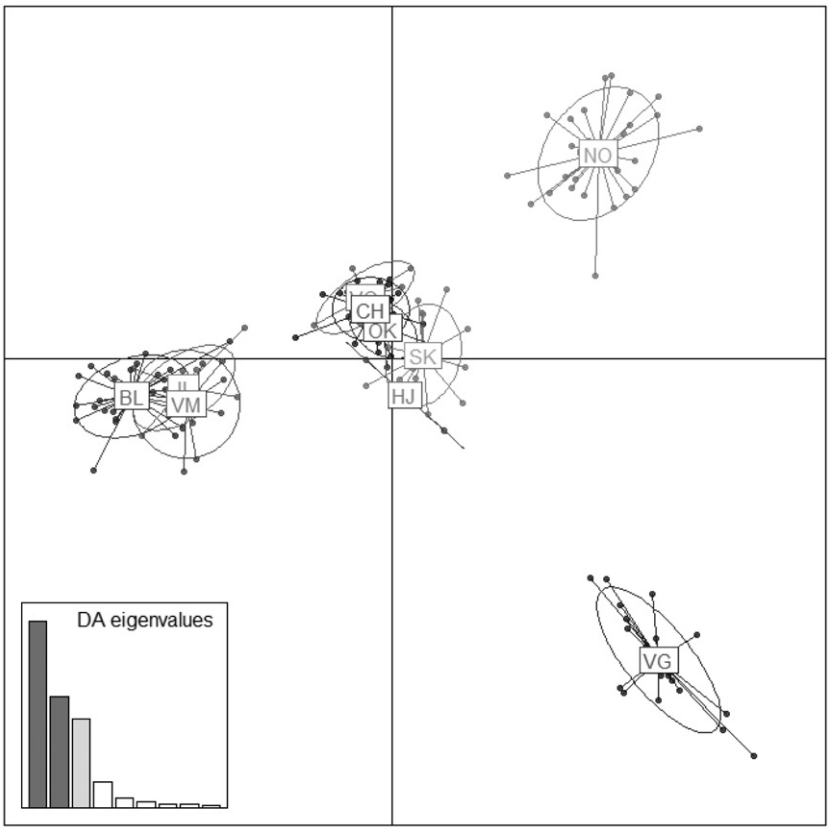

Figure 2. Two-dimensional principal components analysis (PCA) plot of the ten populations genotyped. Eigenvalues corresponding to the represented components are filled in black. Points represent individual genotypes; populations are labelled inside their 95% inertia ellipses (with abbreviations as in Figure 1).

haplogroup A from Western Europe, and our results thus confirm the results from previous studies (Skog *et al*., 2009; Niedzialkowska *et al*., 2012; Zachos & Hartl, 2012). We also found that at least some Swedish deer were nested in a unique subclade. Also, deer from northern Sweden were similar to deer from southern Sweden, and differed from Norwegian deer with respect to mtDNA. This supports the notion that the deer in Norway and Sweden have separate population histories.

The Skåne Zoo population, founded in the 1950s, was found to be genetically the most diverse. It is quite surprising that a small captive population shows such a high level of genetic variation, and this is most likely to be a result of introducing specimens of diverse, unknown origin into the captive herd. When analysed by STRUCTURE at $K = 4$, five of the zoo animals showed evidence of admixed ancestry. This captive population did not deviate from Hardy–Weinberg expectations. The populations in Norway, Blekinge, and Västmanland did not deviate significantly from Hardy–Weinberg expectations, whereas Västergötland and Jämtland did. The differences among populations in F_{IS} may reflect a larger extent of admixture in reared or intensively managed populations (the Skåne Zoo and the estate populations in Skåne) than in recently established or translocated wild populations (Blekinge, Västergötland, Västmanland, and Jämtland). We observed heterozygote deficiency (positive F_{IS}) in Jämtland (north Sweden) and Västergötland (western Sweden). Possibly this arose from these populations being founded by a few individuals released into relatively isolated places (Lavsund, 1975). Thus, subsequent gene flow has been very limited for a few decades. It is believed that the Jämtland population was founded by a few animals escaping from captive herds (Sennstam, 2005), with a mixed ancestry from the wild remnant population in Skåne and continental deer (Lavsund, 1975). The population in Västergötland was also founded during the 1950s by a few released animals (two males, five females, and an unknown number of calves), stemming from wild individuals caught in Skåne (Lavsund, 1975). Variation in F_{IS} levels among populations has also been observed in *C. elaphus* populations in Spain (Perez-Gonzalez *et al*. 2012). In

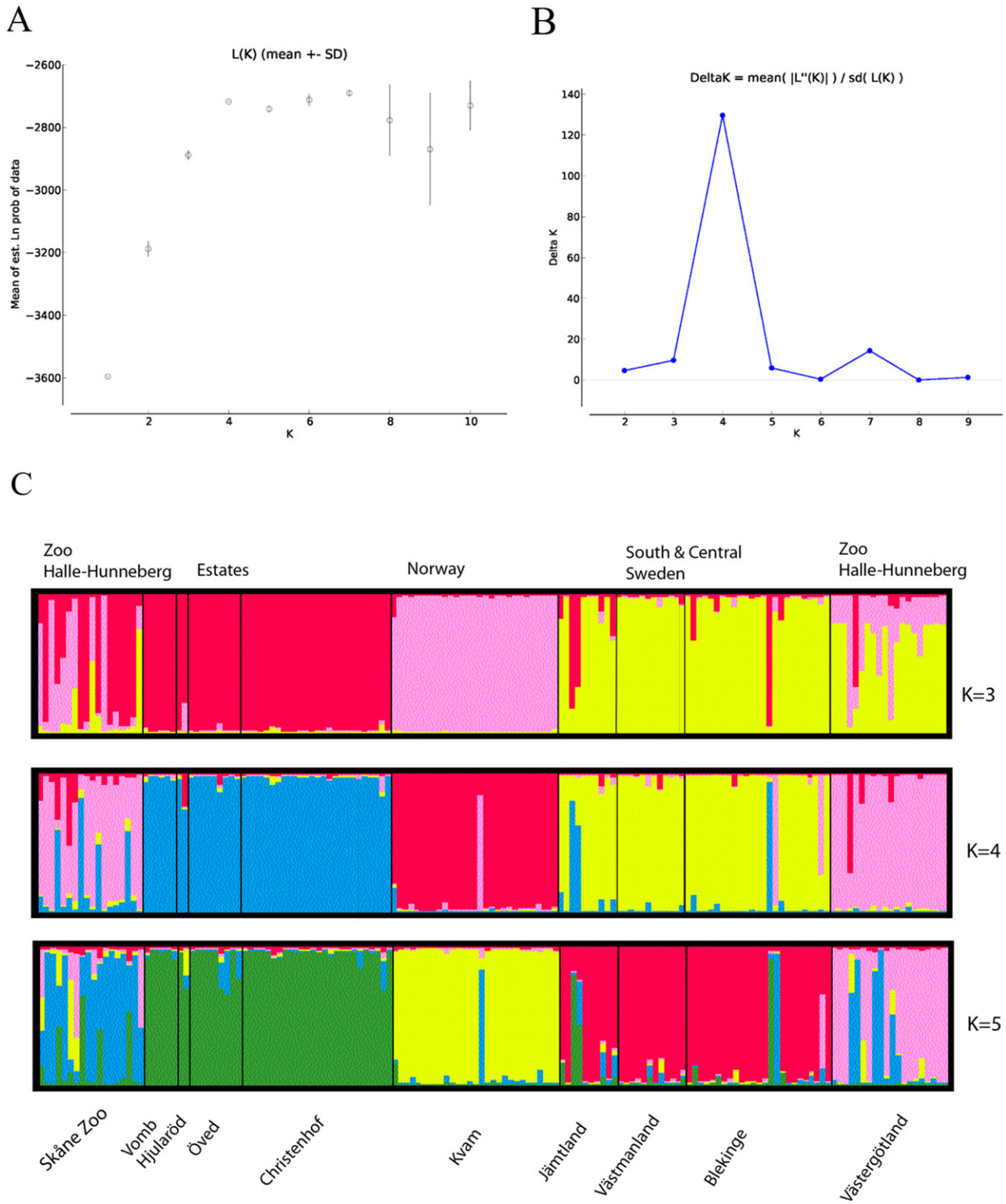

Figure 3. Results of STRUCTURE analyses. (a) Mean estimated log-normal (Ln) probability of the data in relation to the simulated number of clusters *K*. Vertical bars indicate the standard deviation among ten replicates. (b) Delta *K* in relation number of clusters *K*. (c) Average individual assignment probability (*y*-axis) of individuals for three values of *K*. Sampling sites (populations are given below the plot and STRUCTURE clusters for $K = 4$ are given above).

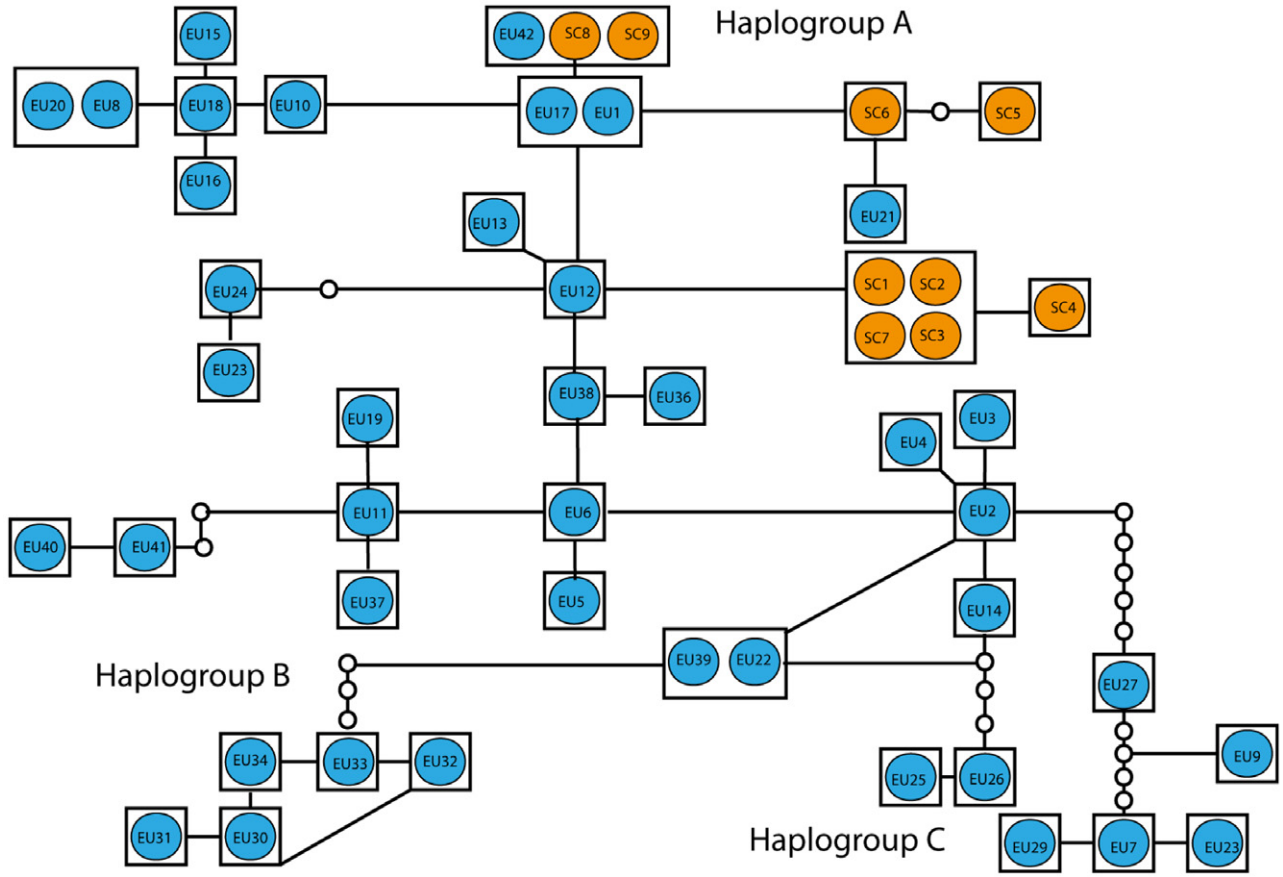

Figure 4. Minimum spanning network for red deer (*Cervus elaphus*) mitochondrial haplotypes. Scandinavian haplotypes found in this study are shown in orange; previously published haplotypes are shown in blue. Previously suggested haplogroups: A, Western Europe; B, North Africa and Sardinia; and C, South-East Europe.

closed populations local inbreeding will cause an increase in F_{IS} over time, and this effect is faster in small populations (Höglund, 2009).

We observed low differentiation among the estates in Skåne, and among the deer from Blekinge, Västmanland, and Jämtland. The other population differentiation estimates suggested significant genetic structure. This may be explained by a combination of factors. First, we expect the Norwegian population to be genetically differentiated from the southern Swedish populations, as the populations have been suggested to belong to different subspecies (Gyllensten *et al*., 1980, Haanes *et al*., 2010a). That all pair-wise F_{ST} values involving the Kvam population from western Norway indicate substantial differentiation may very well be a consequence of different subspecies ancestry. Second, the populations in the estates in Skåne may have become differentiated from other, recently founded wild populations in Sweden, and from the Skåne Zoo, because of the low number of founders involved in the establishment phase. Third, research in the particular area in Skåne where the estates are situated has shown that there are extensive movements of males between the estates, both during the rut (Jarnemo, 2011) and during seasonal migration between rutting areas and summer–winter areas (Jarnemo, 2008). Also, GPS-collared females commute up to 26 km between different estates (A. Jarnemo, unpubl. data). Fourth, the population showing the closest genetic affinity to the Norwegian population was the wild population in Västergötland, which is also closest geographically to the expanding Norwegian population (Haanes *et al*. 2010b). A few individuals from Västergötland and the Norwegian population were genetically similar, which may indicate limited gene flow between these units.

There are two alternative hypotheses regarding the geographic origin of the Jämtland population in northern Sweden. This population resides in an area where *C. elaphus* have been absent since prehistoric times. Thus, the Jämtland *C. elaphus* may have been established by escapees from deer imported to hunting enclosures (Sennstam, 2005). Such animals would ultimately be of the same stock as the

re-established populations in Blekinge and Västmanland. An alternative hypothesis is that a few individuals of Norwegian origin may have traversed the Scandinavian mountain range and established the Jämtland population during the recent, northward expansion of Norwegian *C. elaphus* along the west coast of Norway (Haanes *et al*., 2010b). Occasional *C. elaphus* were observed in Jämtland during the 1950s, long before the establishment of enclosures in the province (Lavsund, 1975). Immigration from Norway to Sweden has occurred in muskoxen (*Ovibos moschatus*), where a population in Sweden was established by spontaneous migration out of a reintroduced population in central Norway (Alendal, 1974). Both our nuclear and mitochondrial genetic data clearly favour the first hypothesis, and do not indicate any genetic exchange with Norwegian *C. elaphus*.

The STRUCTURE analyses suggested that the population in Västergötland was similar to the present population in Skåne Zoo. The Skåne Zoo population was established in 1951 using animals from the estates, and may thus at least partially originate from the populations used for other reintroductions. Furthermore, the PCA indicated an affinity between the estates in Skåne and Skåne Zoo. As the present Skåne Zoo population appeared to contain recently admixed deer (individuals from different genetic clusters), this raises the speculation that the Skåne Zoo population was once less admixed, and resembled the animals eventually reintroduced to Västergötland.

In general, our interpretation of the results from the present study is that the population at the estates in Skåne has been, and still is, a genetic entity, even after the 19th century decline. These populations are now somewhat different, however, from the captive deer at Skåne Zoo. During the re-establishment of wild populations further north in Sweden, such populations have become genetically diverged, mainly by the effect of the intensive force of genetic drift in small populations, with a local impact of *C. elaphus* strains introduced from continental Europe. The pattern is similar to what has been observed in Denmark, where enclosed populations were found to be divergent from free-living deer (Nielsen *et al*., 2008). The data presented here indicate that the genetic signature of the previously described subspecies of the Scandinavian *C. elaphus* (e.g. Gyllensten *et al*., 1980, Haanes *et al*., 2010a, b) is still detectable among current wild populations. The free-living populations in southern Skåne probably represent the genetically intact nominate subspecies, but to preserve its genetic distinctiveness, a local management plan is required. Similar conclusions have been drawn in Poland, where only one population was found to harbour autochthonous *C. elaphus* and all the rest contained introduced genotypes (Niedzialkowska *et al*., 2011, 2012), and in France, where two out of four populations studied were non-indigenous (Dellicour *et al*., 2011). In Skåne Zoo and in the Västergötland population, the impact of translocated specimens with unclear origin may be considerable, also stressing the need for careful management.

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