ELECTRONIC APPENDIX

This is the Electronic Appendix to the article

Severe inbreeding depression in a wild wolf *Canis lupus* population

by

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Supplementary Material

Laboratory procedures

DNA-analyses were based on different types of material. Muscle or blood drawn directly from the animal is here called "tissue". Some genotypes are based on faeces, collected during tracking or other field activities, and for a few individuals we also have analysed oestrus blood found on snow as a complement to the analyses of faeces. Genotypes based on tissue are more reliable than those based on faeces and oestrus blood which, because of low concentration or poor DNA quality, more frequently are affected by allelic dropouts and false alleles.

We scored the tissue and blood samples for allelic variation at 32 autosomal microsatellite loci. The oestrus and faecal samples were typed on a subset (16) of these loci (and denoted with an ***)**. The loci were: u20*, u109*, u123, u172, u173, u204, u213, u225, u250* (Ostrander *et al*. 1993), c2001*, c2010*, c2054*, c2088*, c2137*, c2140*, c2159*, c2168, c2201* (Fransisco *et al*. 1996), AHT002*, AHT004, AHT101*, AHT106 (Holmes *et al.* 1993), AHT124, AHT125, AHT126 (Holmes *et al.* 1994), AHT103, AHT119*, AHT121*, AHT133, AHT136, AHT138 (Holmes *et al.* 1995) and vwf *(Shibuya *et al*. 1994). One marker on the Y-chromosome MS 41B (Sundqvist *et al*. 2001) was also scored for all types of samples.

We used PCR conditions similar to the original reports (exact PCR conditions and profiles are available upon request). The amplified microsatellite alleles were separated by electrophoresis in 6% acrylamid gels. To visualise the fluorescein - labelled primers and PCR products the gels were scanned in a FluorImager (Molecular Dynamics, Sunnyvale, California, USA). In every gel and for every locus, alleles from four reference individuals were run as size standards.

1 Determination of parentage

The pedigree was determined by a manual parentage analysis, where each individual was checked against every possible pair. In this procedure we used material from 163 wolf individuals, of which 113 were based on muscle from dead wolves or blood from anaesthetized wolves, and the rest from faeces and/or oestrus blood. In strongly inbred populations, such as the one studied here, it is problematic to use standard programs to assign kinship and paternity because genetically similar individuals are more frequent than inferred from a background distribution of alleles (Waits et al. 2001). The present data is also complicated because we did not have genotypes from certain key individuals. To obtain a pedigree the genotypes of these key individuals had to be reconstructed. We started from the earliest known reproducing pair and worked up the pedigree using the following assumptions and procedures. For each year, we assumed that we knew the presence of all packs of reproducing wolfs in Scandinavia (see Wabakken et al 2001). In years when there were reproducing pairs with unknown genotypes (one or both pair mates were not sampled for DNA) we took all wolves estimated to have coincident birth years and manually worked out all possible full-sib clusters. Sib-clusters or individual wolves that completely matched the genotypes of known pairs were accepted as positively identified offspring of those pairs. We then used the non-matched sib-cluster(s) to reconstruct the missing parental genotypes. Reconstructed genotypes were based on 3-6

putative offspring but were always partial. We compared all identified kinship relationships with our field data (time of year, locations, wolf pack-sizes) to validate the parental assignments.

Missing genotype of one parent was reconstructed from genotypes of the known parent and pups of that pair. Of the 48 breeding wolves in the pedigree used in the analysis, genotypes of 16 were reconstructed, 25 were based on tissue (muscle or blood drawn directly from the animal) and 7 were based on faeces/oestrus blood

To check the validity of our manual assigning procedure we scored the number of mismatches between each individual and all breeding pairs in the population. In our dataset of 32 microsatellites, we had 106 different alleles, i.e. 3.3 per locus, and in the limited dataset of 16 microsatellites used for faeces analysis, we had 60 different alleles, i.e. 3.8 per locus. There was not a single case where the fit was ambiguous, i.e. where two different breeding pairs could have been the parents of a certain tested individual. In 90 % of the cases with a mismatch, there were at least 6 non-matching alleles for cases including 32 scored loci, and at least 4 non-matching alleles for cases with 16 scored loci (faeces and oestrus blood). We allowed up to 2 mismatches in accepted parent-offspring ties, but only if these mismatches could be caused by allelic dropouts (i.e we did not allow any mismatch in cases where both parents and the tested offspring were heterozygous). The full details of the parental reconstructions for each breeding pair are given below.

Reconstruction of ancestry of different pairs

Much of the field data referred to is published in Wabakken et al. 2001.

The Aa-pair, 1983-1985.

The first founding pair was detected in winter 1982/83 (single wolves had been tracked during several winters before this in the same region). According to field data this pair produced 3 litters between 1983 and 1985. No other wolf reproduction was known in Scandinavia in this period. In July 1985, the female was shot, but the male managed to raise at least some of the pups born in that year. We have tissue from the female, and from 3 pups retrieved as dead and aged, born in 1983-85. The genotype of the father was reconstructed from that material. On 14 of the 32 autosomal loci both alleles could be reconstructed, but only one of the alleles at the remaining loci. A total of 88 different alleles were recorded for this pair on the 32 autosomal loci scored, i.e. on average 2.75 alleles per locus.

The Ab, Ac, Ad and B pairs in the period 1987-1994.

Towards the end of winter 1985/86, the field personnel noted that the characteristic foot prints of the old male of the Aa-pair was not seen any more, and was not reappearing the following winters. Other wolves, presumably offspring of the Aa pair, however remained in the original territory of the Aa-pair, hereafter called " the A territory". In 1986 there was no reproduction in this territory, but in1987 a new litter was recorded, and reproduction was thereafter registered each year until at least 1992, and possibly until 1995 (see fig. 2 and 3 in Wabakken et al. 2001). All these reproductions occurred within the A territory, but seemingly from several different pairs in succession. Before we

can discuss how the kin relationships of these pairs were reconstructed, we however first have to look at the formation of the B pair.

In winter 1990/91 a wolf pair appeared in a new area 250 km north of the A territory, closer to the source population in Finland. Tracks from a lone wolf, producing oestrus blood almost every winter, had been recorded in that area since 1985. This new pair, called the B-pair, reproduced for three seasons, 1991-1993, after which first the male disappeared, and a few years later also the female. We have samples from 14 wolves retrieved as dead, aged, and determined born during the period 1987-1992. Four of these wolves, all born in 1991 or 1992, carried alleles, not seen in any wolf born before 1991. Also, a new Y-chromosome allele appeared in these four wolves (they were all males). None of these four wolves were retrieved from the area of the Aa territory, nor from the area of the new pair (B), but from various places scattered all over southern Scandinavia. We concluded that these four wolves were offspring of the B pair, and later had dispersed. On basis of their genotypes we further concluded that the B pair consisted of a female offspring of the Aa pair and an immigrating male from the source population in Finland/Russia (see Wabakken et al. 2001 and Vila et al. 2003). The genotypes of the male and female in this pair were reconstructed from their four presumed offspring born in 1991 and 1992 and from another 3 wolves born in 1993, and later judged to have come from this pair (see below). The correct allocation of the offspring alleles to mother and father respectively was facilitated by the 18 "new" alleles on 14 of the 32 loci scored, all judged to originate from the father. Both field data (occurrence every winter in this area of a female already from 1985 and up to the pair formation in 1990/91) and the reconstructed genotype support that the female of this pair was a daughter of the original Aa pair, and not of any of the succeeding incestuous pairs discussed below.

The remaining 10 wolves, born in 1987-1992, only contained alleles from the original Aa pair. Four of them also had been retrieved as dead in the original territory of the Aa pair, further supporting the presumption that they were born in that territory. By combining the birth dates of these 10 wolves with their genotypes, we could conclude that there in succession had been formed at least three different incestuous mating pairs involving descendants of the original Aa pair. The first pair bred only in 1987, the second in 1988 and 1989, and the third in 1990-1992, possibly even up to 1995. We can not determine the exact relations of these mating pairs, other than that the first one must have been two full sibs, born by the Aa pair. The two extreme possibilities are either 1) that all three pairs (Ab, Ac, Ad) consisted of different constellations of offspring of the Aa pair, or 2) that the first incestuous pair (Ab) was between full sibs as in the first alternative, but that the second mating (Ac) was between offspring of the former pair (Ab), and that the third pair (Ad) was offspring of the second. The first alternative gives the lowest inbreeding levels, and the second the highest. We have chosen an intermediate level, namely that after the same female paired with two different brothers in succession (Ab and Ac), she finally paired with one of her sons (Ad). The rationale for this scenario is that mammalian females generally are much more restrictive in their mate choice than males, and that this also goes for a reluctance to mate with close relatives. We therefore find it less probable that in this small group of wolves there would occur more than one non-discriminating female. The reason that we assumed that the female finally paired (Ad) not with a third brother, but with a son, is that this last pair formed in 1990, five years after the last of her brothers were born, and it is unlikely that a brother would have stayed that long in the territory.

However, we have tested several possible alternatives, including the two extremes, and none is changing our results more than marginally. Most inbreeding coefficients in the pedigree changed with <0.01 unit, and none >0.03 units (e.g. a change of *F* from 0.22 to 0.25). The only exceptions are for offspring of Ac and Ad where the changes are larger. The relative relations between inbreeding levels in the different pairs were almost constant and the demonstrated effect of inbreeding on litter size is unaffected. In our chosen alternative, we found $R^2 = 0.39$ in the regression between litter size and *F* in pups. In the high and low inbreeding scenarios R^2 changed to 0.42 and 0.32. The probability that R^2 does not deviate from zero is below 0.001 in all cases.

The C, D and E pairs, 1993-1996.

In 1993 a new pair (C) reproduced in an area 60 km east of the original A-territory, and in 1994 another pair (D) bred close to the C-territory. We have no genetic material from these two pairs, but from 9 presumed pups of those two pairs, born in the period 1993-96. Based on genotypes of these pups, they could be split up on two sib groups, and based on birth year these two groups could be allocated to either of these two pairs (C reproduced in 1993 and 1994, D in 1994-1996). All these pups contained "new" alleles originating from the immigrating male in pair B. However, none of the five males among them carried the "new" Y chromosome allele, but the "old" from the A territory. We thus concluded that in both these pairs, the females were offspring of the B pair, while the males were born in the A territory. Again the "new alleles" facilitated the reconstruction of the genotypes of these two pairs, as we now knew that only the females could carry these alleles.

The E pair utilized approximately the same territory as the C pair before it. The E pair started breeding in 1995, and both partners were anaesthetized and radio marked in 1998. This was the first pair from which we had direct information of the genotypes in both male and female. We here found the same situation as in the C and D pairs, a female with "new alleles" obviously born by the B pair, and a male with "old alleles", obviously originating from one of the pairs in the A territory. We have identified 9 pups as being the offspring of this pair, born in the period 1995-1998. Both partners in this pair died during 1999.

From which of the incestuous pairs in the A territory did the successive founders of packs come?

As seen from the pedigree, 6 breeding wolves outside of the A territory originated in this territory. How could we determine from which of the four (Aa, Ab, Ac Ad), very closely related pairs in this territory, they came?

First, none could descend from the Ab pair as that pair only bred 2 wolves, and both have been retrieved dead too early to be considered here. The Ac pair bred 4 or five pups, 3 of which have been retrieved dead at times or places that will exclude them here. Remain one or two pups of this pair not accounted for. The Ad pair bred for several years and field data indicate that a minimum of 8 pups and possibly as many as 14 pups may have been produced by this pair. Only 4 of these were retrieved as dead in times and places that can exclude them as candidates.

The female of the B pair has already been treated in this respect (see above). The reconstructed male of the D pair, as well as the fully scored male of the E pair and female of the J pair, and the female of the I pair (genotype based on feces) all fitted into the sib group of the offspring of the Ad pair and

none into the sib groups of Aa, Ab and Ac. The reconstructed male of the C territory was ambiguous in this respect, and could fit into either of the sib groups bred by Ab, Ac or Ad (but not Aa). We chose to assign him together with the Ad sib group because this group contained more wolves likely to be alive at the given time, but there is a possibility that he might have descended from the Ac pair. That alternative, however, would again have very little effect on the inbreeding coefficients in the rest of the pedigree.

The situation after 1995

For the pairs formed after 1995, there is less ambiguity. From most of them we had tissue samples, and/or faeces samples, in some cases complemented with oestrus blood. Four pairs (Xa, Xb, Xc, Xd) could not be traced back to their ancestry. For three of them we had no samples at all, and for the fourth (Xc) we had tissue from both partners, but could not identify the origin of the female. She could come from either the Xa or the Xb pair, and we have ecological data pointing to the Xa pair, but as the exact ancestry of that pair is unknown, we did not include the Xc pair in the analysis. For details of sample sizes and types of samples, see table 1.

Table 1. Type of determination of genotypes of breeding animals and number of offspring determined for each pair. T = tissue, F = faeces, O = oestrus blood, R = reconstruction of genotype based on offspring profiles (in some cases combined with partner profile).
"Known offspring" includes individuals captured or retrieved within the territory of the presumed parents, or in some of the early cases also based on year of birth.

Pair ID	Male	Female	N DNA-typed known offspring	N pups derived (tissue)	N pups derived (faeces)
Aa	R	T	3	1	
Ab	R	R	2		
Ac	R	R	2	1	
Ad	R	R	6		2
B	R	R	4	1	2
C	R	R	4		
D	R	R	5	1	
E	T	T	3	5	1
F	T	T	3		
G	T	T	3	4	4
H	T	F	6	6	3
I	F	F, O	3	2	6
J	T	T	1		
K	T	T	2		1
L	R	T	3		1
M	T	T	7	2	2
N	R	T	2		2

O	T	R	2	1	2
P	T	T	2		2
Q	F	F, O	1	2	
R	F	F, O	4	2	1
S	T	T	1		
T	T	T	4	1	1
U	T	T			
Xc	T	T	8		
Wolves DNA typed on tissue but not included in pedigree				12	
Wolves DNA typed on faeces but not included in pedigree					8
Total			**81**	**41**	**38**

Demographic analysis

Effects on growth rate (λ) by falling winter litter sizes caused by inbreeding were tested in a Leslie matrix with 5 age classes: pups < 1 year, yearlings (< 2 years) and three adult classes, 2 year old, 3 year old and 4+ year. The purpose was not to make a thorough demographic analysis of the population, but to obtain a rough estimate of the demographic consequences of various levels of inbreeding. We therefore had to assume a "base line" demography, from which to deduce effects of increasing levels of inbreeding. The increase rate of our study population in the period 1990-1998 was $\lambda = 1.29$ (Wabakken et al. 2001). We have used data from the population during this period, when the inbreeding level still was low (see fig 1 in the paper) to model our base line population. Survival rates for age classes older than half year old pups, however have been taken from the whole study period. This was necessary to get reasonable sample sizes, and the rationale for this was that we have not been able to demonstrate any obvious effects of inbreeding on survival of these age classes.

We assumed equal sex ratio at birth, and lumped fecundity and early survival (0-0,5 year of age) into one demographic parameter, litter size during first winter ("winter litter size"), corresponding to the demographic parameter we used in the inbreeding analysis. Average winter litter size before 1998 in our study population was 4.1 (excluding the strongly inbred pair Ad). The data set was too small to allow separate analyses for the different age classes, so we arbitrarily set winter litter size to 3.5 pups in age classes 2 and 3 and 4.5 pups in age class 4. We have likewise arbitrarily set breeding frequencies among females to 43 % of 2 year old, 86 % of 3 year old, and 100 % of 4 years and older females.

We assumed no sex difference in survival, and annual rates were set to 0.7 (pups 05-1 year, i.e. those that had survived until their first winter), 0.5 (yearlings) and 0.86 (adults). These figures were based on data from 54 radio marked wolves, representing 85 "wolf-years". The method used was Kaplan-Meier with staggered entry design.

We adjusted our parameters to give a λ of 1.29 in our base line population, corresponding to the one we found in our 1990-1998 population. Our model population does not exactly picture our study population, but it is a realistic general wolf model.

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