Genetic rescue in a severely inbred wolf population

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Abstract

Natural populations are becoming increasingly fragmented which is expected to affect their viability due to inbreeding depression, reduced genetic diversity and increased sensitivity to demographic and environmental stochasticity. In small and highly inbred populations, the introduction of only a few immigrants may increase vital rates significantly. However, very few studies have quantified the long-term success of immigrants and inbred individuals in natural populations. Following an episode of natural immigration to the isolated, severely inbred Scandinavian wolf (*Canis lupus*) population, we demonstrate significantly higher pairing and breeding success for offspring to immigrants compared to offspring from native, inbred pairs. We argue that inbreeding depression is the underlying mechanism for the profound difference in breeding success. Highly inbred wolves may have lower survival during natal dispersal as well as competitive disadvantage to find a partner. Our study is one of the first to quantify and compare the reproductive success of first-generation offspring from migrants vs. native, inbred individuals in a natural population. Indeed, our data demonstrate the profound impact single immigrants can have in small, inbred populations, and represent one of very few documented cases of genetic rescue in a population of large carnivores.

Keywords: *Canis lupus*, genetic rescue, heterozygosity, immigration, inbreeding, inbreeding depression

Received 18 March 2016; revision received 14 July 2016; accepted 29 July 2016

Introduction

In the past decades, several species of large mammals have recolonized parts of their historic distribution, either by translocation or by natural immigration (Hoffmann *et al.* 2011; Chapron *et al.* 2014). In particular, this is the case for large carnivores that have shown dramatic recovery in recent years, also in human-dominated landscapes (Chapron *et al.* 2014). However, due to competition with growing human populations and increasing fragmentation of natural habitats, many of these populations are likely to remain small and semi-isolated, which is expected to negatively affect their

long-term viability (Keller & Waller 2002; Kenney *et al.* 2014).

Small isolated populations experience reduced genetic diversity and increasing levels of inbreeding, leading to inbreeding depression (Wright 1931; Nei *et al.* 1975), increased genetic load and reduced evolutionary potential (England *et al.* 2003; Mattila *et al.* 2012). In situations where natural population sizes remain small through extended periods of time, careful monitoring is crucial. The mitigation of inbreeding depression and genetic load relies either on purging of harmful alleles or gene flow from neighbouring populations. While it seems that purging in most cases is not efficient in small populations (Hedrick 1994; Wang 2000; Boakes *et al.* 2007), outcrossing by just a few individuals into small inbred populations may lead to increased individual and/or

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population fitness (Madsen *et al.* 1999; Hogg *et al.* 2006; Johnson *et al.* 2010; Heber *et al.* 2013; Frankham 2016). Such genetic rescue (Ingvarsson 2001; Tallmon *et al.* 2004; Whiteley *et al.* 2015) may result both from natural immigration and from human-assisted outcrossing attempts. Still, the use of genetic recue as a conservation strategy is limited, not least due to the associated economical costs, political jurisdictional barriers, the risk of spreading disease and parasites, concerns about population/lineage 'purity' and outbreeding depression (Frankham 2015).

The return of the grey wolf (*Canis lupus*) to the Scandinavian Peninsula by a few founders with episodic immigration events over a 30-year period has in combination with the reconstruction of a near-complete pedigree (Liberg *et al.* 2005; Fig. 1) provided a unique possibility to study inbreeding depression and genetic rescue in a large carnivore. Two decades after the species was declared functionally extinct in Scandinavia, the peninsula was recolonized by two immigrant wolves (Vila *et al.* 2003) and first-time breeding was documented in 1983 (Wabakken *et al.* 2001). During the 1980s, the whole population comprised just this pair and its descendants within one single territory (Wabakken *et al.* 2001; Vila *et al.* 2003). The founding pair

reproduced in 1983–85, followed by no reproduction in 1986 and for the next five years four successive sibling or parent–offspring pairs reproduced within the same territory. Annual numbers of wolves never exceeded 10 individuals until 1991, when a new immigrant male wolf arrived and mated with a female from the founder family (Wabakken *et al.* 2001; Vila *et al.* 2003; Liberg *et al.* 2005). This immigration event initiated a rapid population increase and expansion, and a fast drop in the average inbreeding coefficient $\bar{f}$ (Wabakken *et al.* 2001; Vila *et al.* 2003; Liberg *et al.* 2005). However, after a few years the average inbreeding started to increase again and by 2006, it had reached 0.30 (Bensch *et al.* 2006). Inbreeding depression was documented from reduced litter size of highly inbred individuals (Liberg *et al.* 2005). The population has also shown an increasing incidence of various congenital defects (Räikkönen *et al.* 2013).

By 2007, two male immigrant wolves reached the breeding range of the Scandinavian wolf population, established territories with local females and reproduced successfully for three consecutive years (2008–2010). During these three years, the yearly number of packs groups was 28–31 and the estimated population size increased from about 240 to 340. Here, we compare
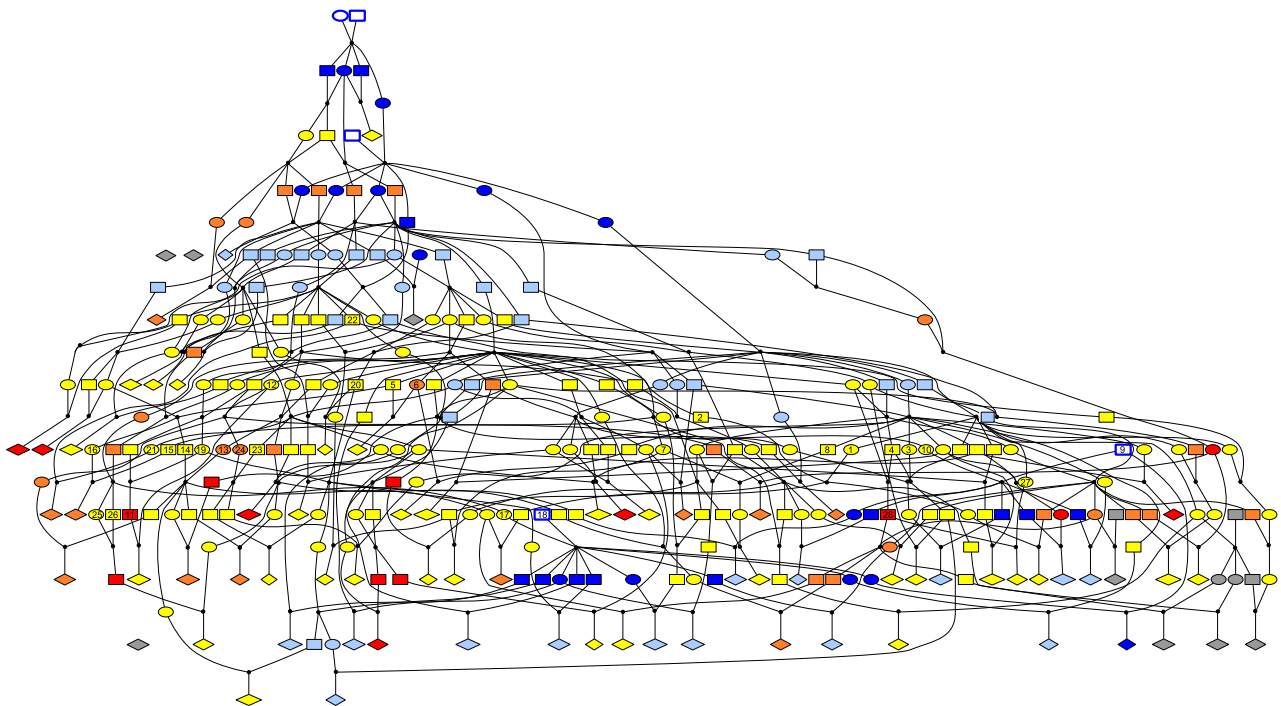
Fig. 1 Pedigree of breeding Scandinavian wolves from 1983 to 2012. Ellipses are females, rectangles are males, and diamonds represent litters where no offspring has yet entered the breeding population. The colours represent the inbreeding coefficient *f* of the individuals and litters (dark blue: $0 \leq f < 0.1$, light blue: $0.1 \leq f < 0.2$, yellow: $0.2 \leq f < 0.3$, orange: $0.3 \leq f < 0.4$, red: $0.4 \leq f < 0.5$, grey: unknown *f*). Founders, assumed to be nonrelated and thus their offspring $f = 0$, are nonfilled symbols with a blue frame. The pedigree also contains the identity (numbers) of the 28 wolves (Table S3, Supporting information) included in the 14 target pairs.

the fitness of these two immigrant males and their first-generation offspring with that of 12 native, inbred pairs and their offspring, produced during the same three years. To account for possible environmental variation, all 12 pairs selected started to breed the same year as the two migrants.

Materials and methods

Study design, sampling and DNA analysis

The Scandinavian wolf population has been monitored continuously since 1978; first based on snow tracking and since 1998 also by radiotelemetry and DNA analysis (Wabakken et al. 2001; Liberg et al. 2012a).

The primary goal of the joint Norwegian-Swedish monitoring programme has been to identify all resident wolves and wolf pairs that scent-mark a territory and to determine the annual number of successful reproductions. In short, the monitoring of wolves in Scandinavia was based on the active search for snow tracks during winter by trained personnel. Snow tracks consisting of groups with two or more wolves were followed for at least 3 km. During tracking, number of animals in the group tracked was determined, territorial scent markings and oestrous bleedings were recorded, and scats/urine were collected for DNA analysis. The confirmation of a scent-marking pair or family group (i.e. group with at least three wolves) in an area was made from at least three tracking events each comprising 3 km of continuous tracking. Tracks observed <100 km from each other and within an area of <2000 km^2 were considered the same territory unless proven otherwise from, for example, DNA analysis. Biological material (e.g. faeces, oestrous blood and hair) from wolves that was found during tracking was collected and used for DNA analysis. From these samples, scent-marking individuals and possible pups in the respective territories were identified and separated from other scent-marking individuals and pups in neighbouring territories. The monitoring was performed in accordance with Swedish criteria for monitoring of large carnivores (NFS 2007:10, Swedish environmental protection agency), also described in Wabakken et al. (2001). In addition, scats from all scent-marking pairs were sampled every winter for DNA analysis.

For identification and parentage analysis, we used between 19 and 36 microsatellite markers specified in Table S1 (Supporting information). The markers used depended on the year of analysis where more markers were used from 2010 and onwards in order to increase information content and to increase the comparability between analysis made by Swedish and Norwegian laboratories (Grimsö Wildlife Station, SLU and NINA, Trondheim, respectively). The comparability of microsatellite scores between the two laboratories was assured when both laboratory-genotyped samples taken at the same time from individuals that was caught for radiocollaring ($n = 19$) or found dead ($n = 72$).

Samples for microsatellite analyses were derived both from dead and live-captured radiocollared wolves (Sand et al. 2006), and noninvasively, primarily from scats, and less frequently from hair and blood in snow. Genomic DNA from tissue and blood was isolated using standard phenol/chloroform–isoamylalcohol extraction, manual extraction with the QIAamp tissue kit (Qiagen, GmbH, Hilden, Germany), or automated extraction using either a Genemole DNA Extraction robot (Mole Genetics, Lysaker, Norway) or a Maxwell instrument (Promega, Fitchburg, Wisconsin) following the protocols provided by the manufacturers. DNA from scats was prepared using either the QIAamp DNA Stool Kit (Qiagen), the ISOLATE Faecal DNA Kit (Bioline, London, UK), or the PowerMaxTM Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA) in accordance with the manufacturers' instructions. DNA extraction from hair follicles and small samples of blood involved 4-h incubation at 55 °C in 200 μL lysis buffer (0.1 M Tris, 0.005 M EDTA, 0.2% SDS, 0.2 M NaCl) and 60 mg proteinase K followed by precipitation and separation in 20 μL sodium acetate (3M) and 440 μL ethanol (95%). The precipitate was solved in 20–100 μL distilled water.

Most markers were amplified in a multiplex PCR set-up using Qiagen Multiplex PCR Kit (Qiagen) and 1 μL eluted DNA. In some cases, multiplex optimizations were unsuccessful, and PCR amplifications were performed by single primer pair reactions. We used 1 μL eluted DNA together with 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 1.5–2.25 mM MgCl$_2$, 0.2 mM dNTP, 0.5 μg of bovine serum albumine (BSA), 1 μM of each primer, 0.1 U of AmpliTaq or 0.3 U. Qiagen HotStar DNA polymerase, adding up to 10 μL with distilled water. Fragments were separated and visualized by capillary electrophoresis using an ABI3730XL DNA Analyzer (Applied Biosystems) at SciLifelab (Uppsala University, Uppsala, Sweden), or the DNA laboratory at NINA (Trondheim, Norway). Genotyping was performed using the software package GENEMAPPER 3.5 (Applied Biosystems).

Noninvasive samples were replicated four times to account for the occurrence of allelic dropout and false alleles (Taberlet et al. 1996). We constructed consensus genotypes from the replicated PCR runs using the threshold rule that alleles had to appear at least twice for a heterozygous genotype and three times for a homozygous genotype. Samples that showed signs of

DNA contamination were omitted from further analysis.

In total, 2276 samples collected between 1 May 2008 and 2 December 2012 were analysed. These consisted of 2052 noninvasive samples and 224 invasive samples from dead ($n = 166$) and radiocollared ($n = 58$) wolves. Genotypes consisting of at least 10 genetic markers could be produced for 1518 samples and qualified to be analysed further.

Population assignment

The power to detect an immigrant increases with the extent of differentiation between the populations compared (Rannala & Mountain 1997). The presence of genetic differentiation between wolves in Scandinavia and Finland was investigated using Nei's G_{ST} (Nei 1987) and the harmonic mean of locus specific Jost's D (Jost 2008), see the detailed discussion of these measures by Meirmans and Hedrick (2011). Calculations were based on 92 wolves born in Scandinavia (invasively sampled between 2003 and 2007) and 64 Finnish wolves (invasively sampled between 1977 and 2002) provided by Jouni Aspi, University of Oulu, Finland. This analysis suggested a clear differentiation between the two populations ($G_{ST} = 0.101$, $P < 0.001$; $D = 0.393$, $P < 0.001$). Estimates and probabilities of differentiation, using 9999 permutations, were made using GENALEX 6.5 (Peakall & Smouse 2012).

To detect migrant wolves, we used Bayesian individual assignment (Rannala & Mountain 1997) with $\alpha = 0.05$ in the program GENECLASS 2 (Piry *et al.* 2004). As references, we used the same 92 Scandinavian and 64 Finnish wolves, 34 domestic dogs (consisting of both purebred dogs and mixed breeds) and 19 red foxes. A first assignment run was made with the two wolf populations pooled. In 57 cases of 1518 samples analysed, the genotypes assigned better with dogs ($n = 21$) or foxes ($n = 36$) and were omitted from further analysis. A second assignment run, with the two wolf populations used as separate references, was made with the remaining samples to detect first-generation migrants.

Individual identification

For samples that assigned with the Scandinavian wolf population, we identified individuals with unique genotypes using probability of identity of siblings (PID$_{sib}$) <0.05 as a threshold. PID$_{sib}$ was calculated in CERVUS 3.0.3 (Waits *et al.* 2001; Kalinowski *et al.* 2007). To account for the occurrence of allelic dropout, we allowed two mismatches (with the maximum rate of two of 20 markers mismatching) and manually checked

whether mismatching markers could be explained by allelic dropout. The average PID$_{sib}$ for all samples that had matching genotypes and was offspring in one of the 14 study pairs (see below) was on average 5×10^{-4} and ranged between 8×10^{-12} and 3×10^{-2}. Matching genotypes were combined to one consensus genotype used for further analysis. For more details, including consensus genotypes and PID$_{sib}$ values, see 'MEC_SupplementaryData.xlsx' on the Dryad data repository.

PID$_{sib}$ does not account for occurrence of inbreeding, which may cause elevated rates of genotype identity, especially among full-sibs (Liu & Weir 2005). We therefore calculated the probability of genotype identity among full-sibs given the allele composition in the parental genotypes and after excluding markers where deviations from Mendelian inheritance indicated the presence of allelic dropout. The probability of genotype identity, given parental genotypes, varied between 6×10^{-14} and 5×10^{-4} (median 9×10^{-10}).

For samples that assigned with the Finnish population, individual identity was confirmed with PID$_{sib}$ that was based on allele frequencies from 64 Finnish wolves invasively sampled between 1977 and 2002.

Parentage and pedigree

A combination of microsatellite genotypes (Table S1, Supporting information) and field observations, such as information on territorial, scent-marking pairs (Wabakken *et al.* 2001), was used to determine parents and reconstruct the pedigree (Liberg *et al.* 2005). Territorial pairs were confirmed from snow tracking conducted within the joint Swedish-Norwegian wolf monitoring programme (Wabakken *et al.* 2013). The parentage of individuals was determined by genetic exclusion (Table S2, Supporting information) both manually based on field observation data from genetically identified pairs and using CERVUS 3.0.3. Using CERVUS, we ran a simulation based on allele frequencies from the Scandinavian population with the purpose to estimate the critical values of the difference in log-likelihood (LOD) values (delta) between different pairs, that is putative parents. In the simulations, we assumed the parental sexes to be known, with 200 candidate mothers and 200 candidate fathers and 95% of the parents sampled. Moreover, we assumed 73.5% of the loci to be typed (which corresponds to the average success rate of the noninvasive samples in the data set), and 2% of the loci to be mistyped. As it is not possible to define specific pairs as potential parents in CERVUS, we included all known pair members as potential parents for all individuals and chose the output to include all parents with positive LOD scores. Matching parents were checked

manually for deviations from Mendelian inheritance, which could not be explained by allelic dropout.

Based on the reconstructed pedigree, we calculated the inbreeding coefficient using CFC v1.0 (Sargolzaei *et al.* 2005). For more detailed description of the reconstruction of the pedigree, see Appendix S1 (Supporting information).

Pairing and breeding success

Successful pairing was defined in accordance with national criteria (NFS 2007:10, Naturvårdsverket), including the identification and tracking of two wolves moving together and scent-marking their territory regularly. Successful breeding was confirmed when offspring was identified and parentage confirmed. Pairing and breeding success and failure were defined as paired or nonpaired and breeding or nonbreeding, respectively.

Several offspring to the 14 target pairs were killed legally before they managed to pair ($n = 28$) or breed after being paired ($n = 7$). In addition, two wolves died accidently shortly after being released from capture and anaesthesia (hereafter referred to as legally killed). These 37 offspring were omitted from further analysis, to reduce the anthropogenic effect on pairing and breeding success. We repeated the analyses by including the legally killed wolves and found that these did not affect the final conclusions of the study (see Appendix S1, Supporting information).

Very few pups were genetically identified before their first winter, as sampling was mainly conducted during snow tracking. To avoid results being biased by early pup mortality, for which we have too little data in our study material, all offspring included in the study was observed some time during autumn, winter and early spring (i.e. between September and April).

The yearly genetic contribution of immigrants (founders) to the breeding population was defined as the expected proportion of genes from the founder carried by all newborn litters, calculated as the average pairwise genetic relatedness between the founder and all newborn litters (see Lacy 1989).

Statistical analysis

We identified a total of 135 offspring to the 14 pairs which started to breed in 2008. One more pair started to breed 2008, but was excluded from the study as no parents could be assigned to one of the pair members (M-09-04 in Full; Table S2, Supporting information) and consequently no inbreeding coefficient could be assigned to the offspring. In two of the pairs, the male wolf had an immigrant origin (Table S3, Supporting information). Removing legally killed wolves left a data set of 105 and 98 wolves for pairing and breeding success, respectively. In the final data set, the distribution between offspring from the 12 native, inbred families and immigrant offspring were 78 vs. 27 for the analyses of pairing success and 72 vs. 26 for the analyses of breeding success, respectively. The frequency distributions of successful and nonsuccessful pairing and breeding for the two groups were tested against an independent frequency distribution using 2×2 chi-square tests (Sokal & Rohlf 1995).

Several studies, including one on the Scandinavian wolf population (Bensch *et al.* 2006), show that marker-based estimators of heterozygosity or homozygosity may be stronger correlated with fitness than pedigree-based inbreeding (e.g. Hansson *et al.* 2004; Bensch *et al.* 2006; Forstmeier *et al.* 2012). Moreover, after recent founder events or strong bottlenecks pedigree-based f may show weaker correlation with the proportion of the genome that is identical by decent (IBD_G) than marker-based estimates (Kardos *et al.* 2014). We therefore complemented the study by also using standardized multilocus heterozygosity (stMLH)ʹ, that is the proportion of heterozygous loci among loci typed divided by population mean multilocus heterozygosity (MLH) of the typed loci (Slate *et al.* 2004).

To test whether the inbreeding coefficient f and stMLH predicted the probability of pairing success and breeding success, respectively, we used a generalized linear mixed models (GLMMs) approach in R (v 2.15.2), with the lmer function in lme4 assuming a binomial distribution, logit-link function and Laplace maximum likelihood. Natal observation (i.e. whether or not an individual was genetically identified at least once within the territory of birth) was included in the analyses as a fixed factor. The reason for this is that animals, sampled only after natal dispersal, already have passed several critical periods in life causing a bias in comparison with animals observed earlier in life due to (i) the mortality during natal dispersal and (ii) a methodological bias towards sampling of territorial pairs and packs over solitary individuals in the Scandinavian wolf monitoring programme (Liberg *et al.* 2012a). To account for unequal offspring sample size between parental pairs, we used parental pair as a random factor. The main conclusions of the study did not change when models were run without parental pair as a random factor and the effect of inbreeding changed only slightly (see Appendix S1, Supporting information). We used the Akaike's information criterion, corrected for small sample sizes (AICc) to compare all models (Table S4, Supporting information). After ranking model i in relation to ΔAICc (AICc$_i$ – minimum AICc), models with ΔAICc ≤2 were chosen as the most parsimonious models and

were reported together with the number of parameters (*K*). To better interpret the relative likelihood of the reported models, we also calculated the Akaike's weights w_i (Burnham & Anderson 2002). We used w_i to scale the parameter estimates from the most parsimonious models (Buckland *et al.* 1997). High correlation between stMLH and *f* ($r = -0.76$, $n = 133$, $P < 0.001$), only slightly weaker than the correlation ($r_e = -0.83$) predicted by Slate *et al.* (2004), prohibited the inclusion of these two predictors in the same models. All models with *f* and stMLH as predictors were therefore run separately. To find out whether the general conclusions from our study were explained largely by individuals in the larger spectra of *f* and smaller spectra of stMLH, models were run without individuals with *f* > 0.4, consisting of offspring to a pair (Sång1) that were full-sibs or individuals with 5% of the lowest stMLH values, that is stMLH < 0.661. The omission of these individuals did not change the conclusions drawn from the study (Table S5, Supporting information).

We calculated the annual finite rate of population growth (λ) from 1990 through 2012 based on annual estimates of population size adjusted for the number of wolves legally harvested each year. This adjustment is important because both the number of legally harvested wolves (0–65) and the proportion of the total population size (0.00–0.18) varied between years. The adjusted population growth rate, assuming that legal harvest is additive to other sources of mortality, is therefore a measure of the annual potential finite rate of population growth (λ_p), without legal harvest, calculated as:

$$\lambda_p = (N_{t+1} + H_{t \to t+1})/N_t,$$

where N_t is the total population size at year t and $H_{t \to t+1}$ is the number of wolves legally harvested between year t and $t+1$. The average annual population increase was described by the geometric mean, and we statistically tested for a difference in growth rate before (2002–2007) and after (2007–2012) the immigration event, using a one-tailed *t*-test (two-sample, equal variance) based on logarithm-transformed λ_p-values (Alf & Grossberg 1979).

Results

During 2008–2010, we identified a total of 135 offspring produced by the 14 target pairs (Table 1). Among these, 34 were offspring of the two immigrants and consequently fully outbred (*f* = 0), assuming that the immigrants were unrelated. The remaining 101 were offspring of the 12 native, severely inbred pairs ($0.26 \leq f \leq 0.49$). Pairing and breeding success for offspring to immigrants was considerably higher compared to offspring from inbred, native pairs, leading to a rapid spread of the immigrant genomic ancestry in the population (Fig. 2, dark blue and light blue). By winter 2012/2013, in a population of 380 wolves (Wabakken *et al.* 2013), at least one wolf in 23 of 64 territorial pairs (36%) were descendants of the two

Table 1 Breeding success and pedigree-based inbreeding coefficient (*f*) for the offspring of 14 Scandinavian wolf pairs that reproduced first time in 2008. Parameters of breeding success are reported as number of litters, number of identified offspring number of legally killed offspring before reaching pairing or breeding status, number and proportion of offspring with successful pairing and breeding later in life

Territory (pair ID)	*f*	Litters	No. of offspring	Legally killed		Successful pairing	Successful breeding	Pairing success*	Breeding success*
				Unpaired	Unbred				
Galven (Gal)	0.00	3	14	6	6	6	6	0.75	0.75
Kynna (Kyn2)	0.00	3	20	1	2	13	11	0.68	0.61
Dals Ed-Halden (DE5)	0.26	3	15	0	0	2	2	0.13	0.13
Aamäck (Amä1)	0.27	3	17	6	8	5	2	0.45	0.22
Äppelbo (Äpp)	0.27	3	11	2	2	4	2	0.44	0.22
Lövsjön (Löv2)	0.28	2	12	2	2	4	4	0.40	0.40
Osdalen (Osd2)	0.28	2	8	1	3	4	2	0.57	0.40
Sandsjön (Snd1)	0.28	1	2	0	0	0	0	0.00	0.00
Bredfjäll (Bre1)	0.29	1	1	0	0	0	0	0.00	0.00
Julussa (Julu5)	0.29	1	3	0	0	0	0	0.00	0.00
Kloten (Klot)	0.30	3	14	4	4	8	7	0.80	0.70
Kroppefjäll (Krp3)	0.33	2	5	2	3	1	0	0.33	0.00
Ockelbo (Ock2)	0.35	1	4	0	1	1	0	0.25	0.00
Sången (Sång1)	0.49	3	9	6	6	1	1	0.33	0.33

*Proportion of the total number of identified offspring (without legally shot animals before reaching pairing/breeding status) that successfully built a pair and reproduced, respectively.
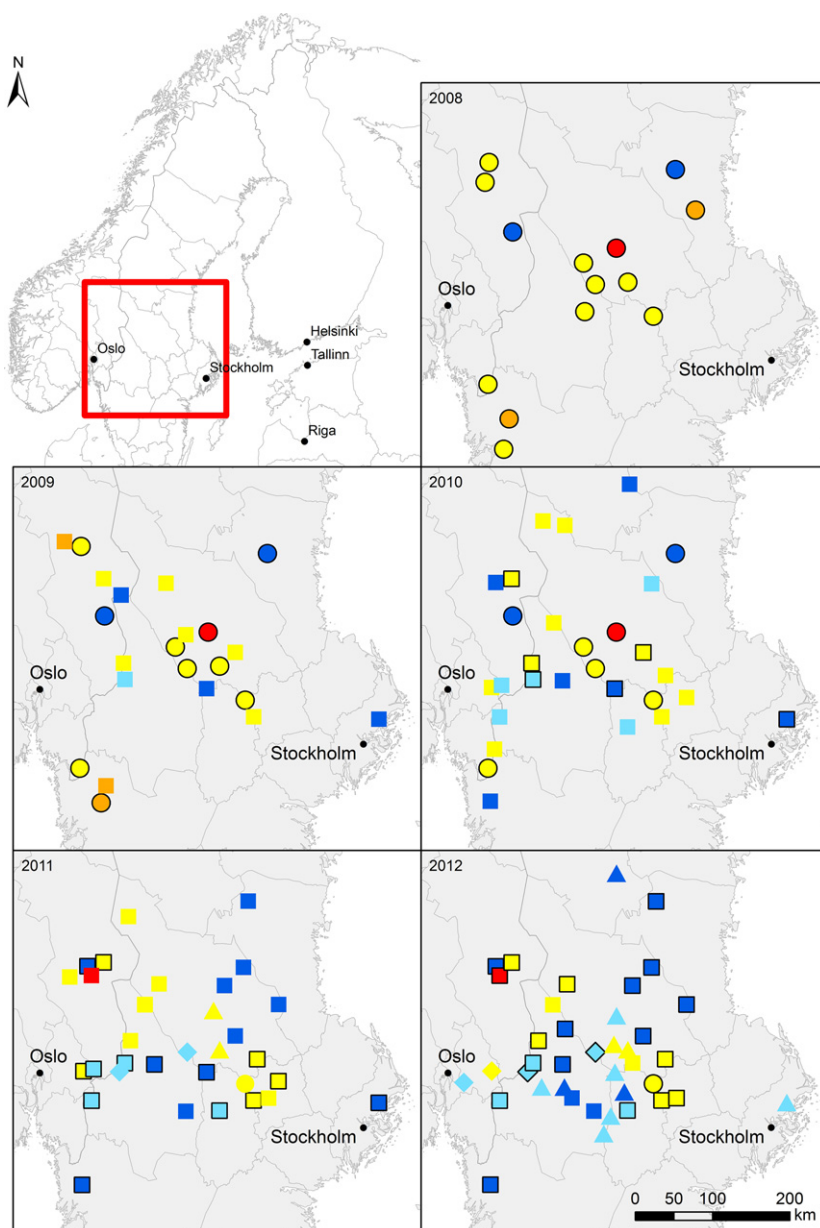
Fig. 2 Geographic distribution of the original wolf pairs and their descendants. The location of wolf territories in Scandinavia 2008–2012, illustrating wolf pairs that can be traced back to the 14 original pairs (circles) that started to reproduce in 2008. Squares and triangles represent pairs with first- (F1) and second-generation (F2) descendants, respectively, whereas diamonds symbolize pairs with both one F1 and one F2 descendant. Edge-lined symbols represent breeding pairs. Dark blue represents the original pairs with an immigrant and pairs composed of immigrant descendants with partners descending from inbred pairs other than the 12 in the study. Light blue symbolizes pairs composed by an immigrant descendant and a descendant from one of the 12 inbred families included in the study (i.e. born 2008). Yellow, orange and red, respectively, represent the inbred native pairs with medium ($f = 0.2$–0.3), high ($f = 0.3$–0.4) and very high ($f > 0.40$) inbreeding levels and their descendants.

migrants. In sharp contrast, offspring to the three native families with the highest inbreeding coefficients ($f > 0.3$) had very poor breeding success.

In quantitative terms, pairing success was almost two times higher for offspring to immigrants compared with offspring from the 12 inbred families (Fig. 3A); 70% vs. 38% ($\chi^2 = 8.20$, $P = 0.004$). The difference in breeding success was even clearer (Fig. 3B). Whereas 65% of the offspring to immigrants reproduced, only 26% of the offspring to native, inbred wolves did so ($\chi^2 = 11.5$, $P = 0.001$). A GLMMs approach showed that the inbreeding coefficient was an important explanatory variable for the significant difference in pairing and

breeding success (Table S4, Supporting information, Fig. 3C,D). By including parental pair as a random factor and natal observation as fixed factor, we addressed whether the difference in pairing success between the immigrant and native offspring was an effect of unequal sample size of the different parental pairs or biased sampling of individuals that had already reached pairing status. The weighted probability of pairing ($\beta = -5.10$, 95% CI: -9.62; -0.58) and breeding ($\beta = -5.64$, 95% CI: -9.86, -1.44) decreased significantly with increasing f. Similarly, when using molecular data (Table S4, Supporting information; Fig. 3E,F), individuals with higher stMLH showed higher pairing
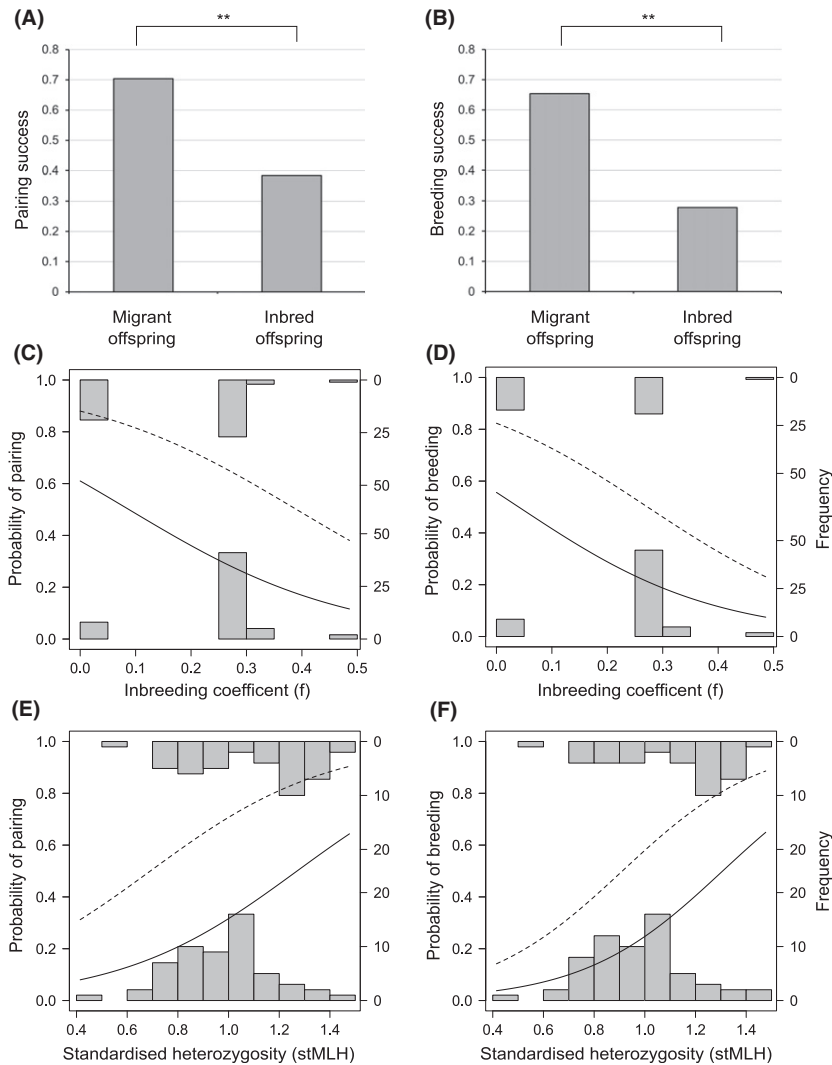
Fig. 3 Pairing and breeding success of offspring to immigrant and inbred pairs. Observed pairing and breeding success of outbred offspring to immigrant wolves (i.e. all have $f = 0$) and inbred offspring ($0.26 < f < 0.49$) where both parents are native wolves (A, B; ** ⟹ $P < 0.01$), and their probability to establish with a partner and breed in relation to the inbreeding coefficient f (C, D) and standardized heterozygosity stMLH (E, F). In panels C–F, the histograms represent the frequency distribution of f values (C, D) and stMLH values (E, F) among individuals that were successful (upper histogram presented upside down) and unsuccessful (lower histogram) in pairing or breeding. Solid curves represent the success of individuals first identified within the natal territory, and dashed curves represent the success of individuals identified first time outside the birth site, that is postdispersal.

($\beta = 2.85$, 95% CI: 0.26, 5.45) and breeding probability ($\beta = 3.62$, 95% CI: 1.09, 6.16).

The rapid spread of the immigrant genomes in the population was accompanied by a marked reduction in the average inbreeding coefficient; from 0.30 in 2007 to 0.24 in 2012 (Fig. S1A, Supporting information). The genetic contribution to the breeding population from each of the immigrants increased from 3.8% in 2008 to 5.3% and 7.1%, respectively, in 2012, implying that one-eighth (12.4%) of the founder contribution could be attributed to these two immigrants only three years after their first offspring became sexually mature (Fig. S1B, Supporting information). Notably, the new genomic ancestry was already distributed across more than one-third of all territorial pairs in 2012 (see Fig. 2).

The immigrants are also likely to have had a positive effect on the population growth rate. During the 1990s, the average annual population increase, corrected for legal hunt, was estimated to 23.4%. Between 2002 and 2007, the corresponding figure had dropped to 12.7%, with negative or negligible population growth in three out of six year-to-year intervals (Fig. 4). After the two immigrants started to reproduce in 2008, the population growth rate again seemed to stabilize at a higher level. We recorded an average annual growth of 26.7% between 2007 and 2012, which is similar to the period of population increase during the 1990s and markedly higher than in the early 2000s, although not statistically significant ($P = 0.08$; one-tailed *t*-test).

Discussion

Wolves are socially monogamous, normally maintaining their pair bonds throughout life (Mech & Boitani 2003). Finding and accepting a mate is therefore a critical part of wolf life history. Little is known about the mechanisms behind this process, but it is likely that both physical and behavioural aspects are involved. Several
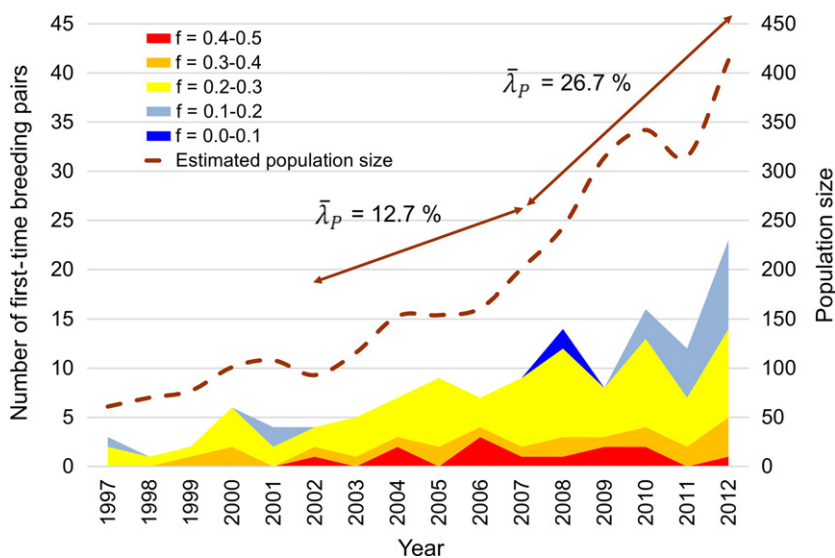
Fig. 4 Annual population size and inbreeding levels of first-time breeding pairs in the Scandinavian wolf population from 1997 to 2012. Population size is the mean census size using yearly direct count estimates from the combination of snow tracking and DNA analysis carried out within the framework of the Scandinavian wolf monitoring programme. Inbreeding levels refer to the inbreeding coefficients of the offspring in litters to first-time breeding pairs. The angles of the arrows represent the average annual population growth rate ($\bar{\lambda}_p$) before and after 2008, when the two immigrants started to breed. The population growth rate is corrected each year for the number of legally shot wolves (see Materials and methods).

studies have indicated that canids are capable of discriminating and avoiding mating with close relatives and pack members (Smith *et al.* 1997; Geffen *et al.* 2011). Also in other species, active mate choice for unrelated individuals has been demonstrated (Hoffman *et al.* 2007). Such inbreeding avoidance may have contributed to the higher pairing success of the migrant offspring in this study. Yet, there is little support in the literature for canids being able to discriminate between individuals that are not pack members (e.g. full-sibs from earlier litters), and whether they are closer or more distantly related (Geffen *et al.* 2011). A more likely underlying mechanism is inbreeding per se, which may negatively affect the probability of survival until reaching pairing and breeding status and the competitive ability to find a partner and establish a pair bond. It has been documented in wolves that inbreeding depression affects the earlier stages in life, before and during the very first months after birth (Liberg *et al.* 2005; Fredrickson *et al.* 2007). However, the dispersal phase is also a critical period with high mortality (Mech & Boitani 2003). Indeed, there are studies on wolves suggesting that body weight, a common indicator of body condition, is negatively affected by inbreeding (Fredrickson & Hedrick 2002). The high incidence of congenital defects among Scandinavian wolves indicates that their physical status might also be affected by inbreeding (Räikkönen *et al.* 2013).

A very limited number of studies have been able to document and quantify the breeding success of

offspring to migrants that have established in highly inbred wild populations (Whiteley *et al.* 2015). In the highly inbred Florida panther, the offspring of introduced reproducing females were very successful, but no direct comparison between them and offspring to native pairs was performed (Hedrick 2010; Johnson *et al.* 2010). In two severely bottlenecked and isolated populations of South Island robins (*Petroica australis*) in New Zealand (Heber *et al.* 2013), the offspring to immigrants showed considerably higher juvenile survival as well as a much higher breeding success than native offspring. Still, the reproductive success was only 1.6 times larger in migrant offspring vs. inbred offspring, compared with the ratio of 2.5 found in our study. On Isle Royale, the breeding success of a single migrant wolf's offspring greatly exceeded that of the native wolves (Adams *et al.* 2011; Hedrick *et al.* 2014). Indeed, gene flow from just one or two individuals has the potential for rapid and dramatic improvement for both very small populations, like that on Isle Royale, as well as in larger populations, such as the Scandinavian wolf population.

Our results demonstrate a substantial effect of the immigrants on individual fitness that also appears to have stimulated the growth of the population. Although not statistically significant ($P = 0.08$), the average annual population growth rate, corrected for legal hunting, more than doubled after the two immigrants arrived. It is even possible that population growth during the last period was limited by additional anthropogenic effects that we have not accounted for. The

culling of several adult paired and reproductive wolves during the rather extensive quota harvests in Sweden 2010 and 2011 may have resulted in a more permanent reduction of the potential for population growth beyond the immediate numeric reduction. Protection of domestic sheep and reindeer in western and northern Scandinavia by increased lethal control has also limited the geographical expansion of the wolf population. Wolves are not allowed to reproduce in these areas, further limiting the growth potential of the population.

Without the recent immigration event, inbreeding levels in the Scandinavian wolf population would have continued to increase, with a higher proportion of highly inbred pairs, most likely with low reproductive success, as indicated by all available data (Liberg *et al.* 2005). It is possible that purging of deleterious alleles could have reduced inbreeding depression, but empirical studies suggest that purging is inefficient in small populations (Hedrick 1994; Wang 2000; Boakes *et al.* 2007), and that detrimental alleles may instead be randomly fixed by genetic drift, increasing the genetic load (Wang 2000). In any case, the nature of genetic rescue is highly ephemeral (Hedrick 2010; Hedrick *et al.* 2014) and more immigrants are needed.

Besides poaching, the high level of inbreeding and its negative consequences is the most critical conservation issue for the Scandinavian wolves (Liberg *et al.* 2012b). A recent review on the effects of outcrossing in inbred animal and plant populations recommended a wider use of outcrossing within species to genetically rescue inbred and fragmented populations to avoid population extinction from genetic factors (Frankham 2016). To increase the probability for success of such management actions, there is a demand for scientifically based guidelines on how it should be conducted, not least concerning the number and frequency of genetic translocation events needed to reach inbreeding levels with little or no effect on population fitness. Such guidelines require better knowledge on the expected genetic impact of outcrossing in the wild, not least in large carnivores where active translocations can be both expensive and controversial (Miller *et al.* 1999; Jule *et al.* 2008). Here, we show a substantial selective advantage for immigrants, demonstrated by a 2.5 times higher breeding success among immigrant offspring compared to inbred offspring. Even though the effect size may vary between species and situations, our study raises the importance of monitoring of inbred populations where the impact of multiple immigration events can be quantified. Our study is one of very few to quantify the relative fitness advantage of immigrants in a highly inbred, natural animal population, providing important knowledge to future genetic management.

Acknowledgements

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O.L. conceived and designed the set-up of the study, made the first analyses and took initiative to the study. P.W. organized and participated in fieldwork. M.Å. and Ø.F. were involved in the laboratory work. M.Å. performed the final analyses of genetic identity, parentage and reproductive success. H.S. and Ø.F. performed the analysis of population growth rate. M.Å. and Ø.F. wrote the first draft of the study. All authors discussed the results and contributed to the final manuscript. All authors gave their final approval for publication.

Data accessibility

Individual data and genotypes of the offspring in the study are available through Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.sr437. Other supporting data can be accessed in the Appendix S1 (Supporting information), including supporting tables.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Supplementary methods and results.

Fig. S1 Annual average inbreeding and genetic contribution of founders among cohorts.

Table S1 Microsatellite loci, their chromosomal position (Chr), number of alleles, expected heterozygosity (H_E) and observed homozygosity (H_O) among breeding individuals 1983–2012 and the percentage of these individuals that was successfully typed for each locus, respectively.

Table S2 Parental wolf pairs breeding in Scandinavia between 1983 and 2012, with Pair ID, Male and Female ID (invasively sampled individuals are in bold), their pedigree-based coancestry, i.e. the inbreeding coefficient f of their progeny (Falconer & Mackay 1996), first and last known year of breeding and whether successful breeding was confirmed from offspring DNA (bold letters indicate that at least one offspring has been invasively sampled).

Table S3 Population assignment of 30 identified wolves (numeric ID within parentheses) included in the 15 pairs that started breeding in 2008 in Scandinavia.

Table S4 Factors affecting pairing success and breeding success of Scandinavian wolves.

Table S5 The effect of pedigree-based inbreeding coefficients f (a-b) or marker-based standardized multi-locus heterozygosity stMLH (c-d) on pairing success and breeding success of Scandinavian wolves after excluding individuals with $f > 0.4$ and individuals with 5% of the lowest stMLH-values.