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Rescue of a severely bottlenecked wolf (*Canis lupus*) population by a single immigrant

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The fragmentation of populations is an increasingly important problem in the conservation of endangered species. Under these conditions, rare migration events may have important effects for the rescue of small and inbred populations. However, the relevance of such migration events to genetically depauperate natural populations is not supported by empirical data. We show here that the genetic diversity of the severely bottlenecked and geographically isolated Scandinavian population of grey wolves (*Canis lupus*), founded by only two individuals, was recovered by the arrival of a single immigrant. Before the arrival of this immigrant, for several generations the population comprised only a single breeding pack, necessarily involving matings between close relatives and resulting in a subsequent decline in individual heterozygosity. With the arrival of just a single immigrant, there is evidence of increased heterozygosity, significant outbreeding (inbreeding avoidance), a rapid spread of new alleles and exponential population growth. Our results imply that even rare interpopulation migration can lead to the rescue and recovery of isolated and endangered natural populations.

Keywords: wolf; genetic rescue; bottleneck; founding effect; migration; Scandinavia

1. INTRODUCTION

Human pressure is leading increasingly to the isolation of natural populations (Ceballos & Ehrlich 2002), with a consequent loss of genetic variability (Frankham 1995) and an elevated risk of extinction (Saccheri *et al.* 1998; Lande 1999; Bijlsma *et al.* 2000; Higgins & Lynch 2001). However, laboratory and translocation experiments have indicated that small and inbred populations may be rescued by the contribution of only a small number of immigrants (Westemeier *et al.* 1998; Madsen *et al.* 1999), preventing inbreeding depression (Spielman & Frankham 1992; Ebert *et al.* 2002) and inducing profound changes in genetic structures (Ball *et al.* 2000; Saccheri & Brakefield 2002). Nevertheless, the impact of such rare migration on the survival of natural populations is unclear. To address such issues in a natural population would require the close monitoring of a population, at an individual level, at and after the time of immigration.

Wolves have been competing with humans for millennia. Such conflict has led to intense persecution with active efforts towards their extermination, especially efficient through poisoning and firearm hunting from the nineteenth century (Mech 1970). As a result of extermination campaigns, wolf (*Canis lupus*) populations have been decimated and fragmented worldwide. On the Scan-

dinavian peninsula, hundreds of wolves were killed every year as late as the mid nineteenth century. After a dramatic decline, the population was considered extinct by the 1960s (Wabakken *et al.* 2001a). However, in 1983, a breeding wolf pack was unexpectedly discovered in southern Scandinavia, more than 900 km from the nearest known occurrence in Finland and Russia, sparking controversy as to its origin. The newly established population comprised only one breeding pack per year and consisted of fewer than 10 individuals during the 1980s. Preliminary genetic analysis showed a loss of genetic variability due to inbreeding in this newly founded population (Ellegren *et al.* 1996). However, without changes in habitat, prey availability, legal protection or the level of illegal hunting, the population suddenly started to grow exponentially after 1991 (mean annual growth rate of 29% (Wabakken *et al.* 2001a)). Despite both legal and illegal hunting, there are now 10–11 breeding packs and an estimated total population size of 90–100 individuals (figure 1a).

The rigorous monitoring and continuous sampling of this wolf population since its foundation provides an ideal opportunity to follow the changes in genetic diversity in a small and isolated population. Using a variety of molecular tools, including maternally (mitochondrial DNA (mtDNA)), paternally (Y chromosome) and bi-parentally (autosomal and X-chromosome microsatellites) inherited markers, we have been able to track the population's origin, to follow the changes in genetic diversity over time and to assess the impact of rare immigrants on this natural population.

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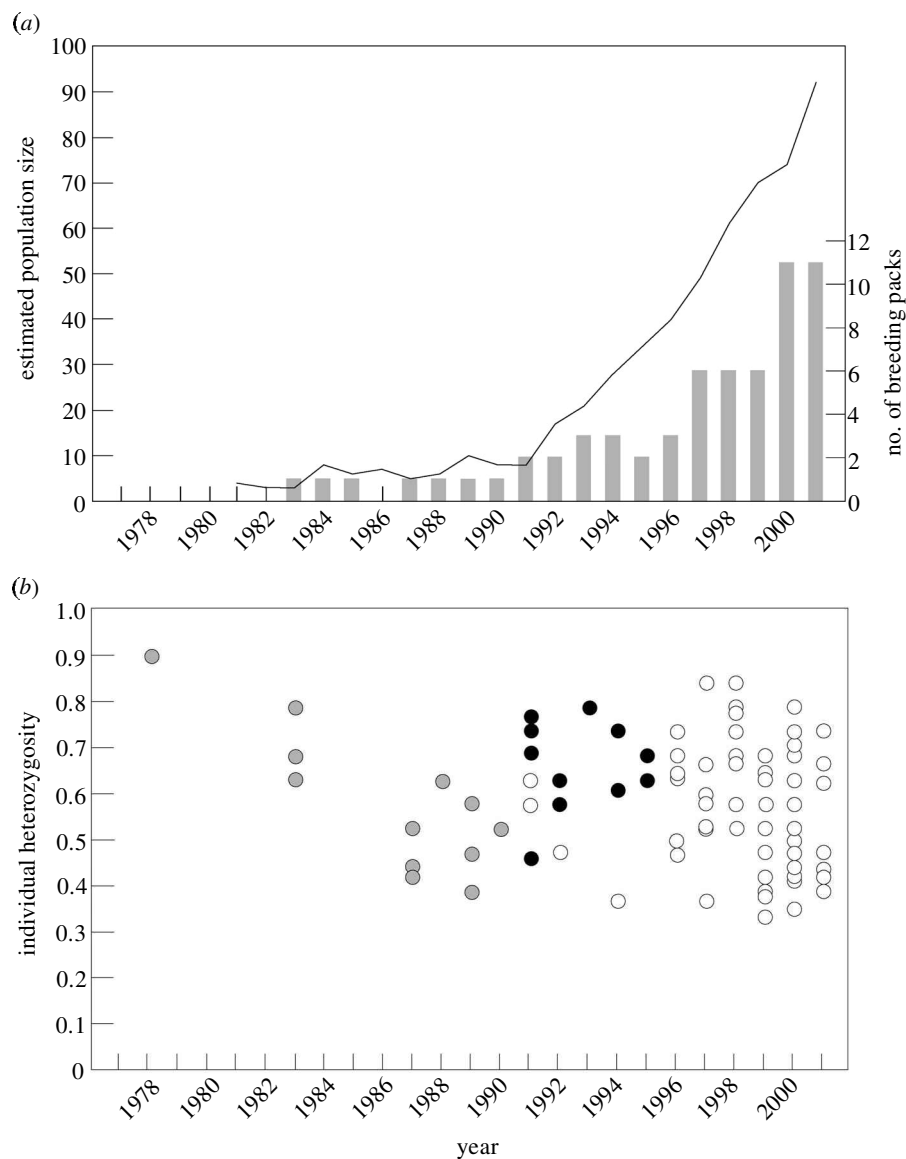


Figure 1. (a) Estimated total number of individuals (line, left-hand scale) and packs where breeding was confirmed or likely to have occurred (columns, right-hand scale) in the Scandinavian wolf population since the first recorded breeding in 1983 (based on Wabakken *et al.* 2001a). (b) Individual microsatellite heterozygosity (at 19 autosomal microsatellite loci) in Scandinavian wolves by year of birth. Each of the circles represents one individual. The leftmost grey circle, with an estimated date of birth of 1978, represents the founding female. Samples with birth dates prior to 1991, when the population included only one pack, are shown in grey. Black circles indicate wolves born between 1991 and 1995 that carry a number of new alleles and express the increase in heterozygosity resulting from the reproduction of one immigrant.

2. MATERIAL AND METHODS

(a) Samples

We analysed tissue or blood samples from 94 Scandinavian wolves collected between 1984 and 2001, 93 wolves from Finland and northwest Russia (Tvier and Smoliensk regions) that are referred to as the eastern population and 66 pure and mixed-breed dogs. One Scandinavian wolf, sampled in 1984 in southern Norway, had a genetic composition indicating that it was a migrant from the eastern wolf population and that it did not contribute to the extant Scandinavian wolf population (e.g. it has mtDNA and Y-chromosome haplotypes that differ from any other animal in the Scandinavian population). This individual was excluded from all subsequent analyses. We also analysed the historical Scandinavian wolf population, represented by teeth samples of 30 museum specimens obtained between 1829 and 1965, i.e. pre-dating the extinction of the 1960s (Flagstad

et al. 2003). Information on the demographic changes in the Scandinavian population has been published (Wabakken *et al.* 2001a) and is available in the yearly status reports of the Swedish Nature Conservation Agency (2002; see also Aronson *et al.* 1999, 2000, 2001) and Norwegian Nature Conservation Agency (2002; see also Aronson *et al.* 2002, Wabakken *et al.* 1999, 2001b, 2002).

(b) Genetic methods

Genomic DNA was extracted using conventional protocols (Sambrook *et al.* 1989). DNA from historical specimens was extracted using the Isoquick DNA extraction kit (Orca Research Inc., Bothell, WA, USA) and analysed in a separate laboratory dedicated to low-copy DNA research to minimize the risks of contamination. The authenticity of the results was verified by replicates carried out by different researchers (Hofreiter *et al.*

2001). Partial mitochondrial control region sequences were obtained for the wolf samples as described in Vilà *et al.* (1999). Four Y-chromosome-linked microsatellites (Sundqvist *et al.* 2001) were typed and combined into haplotypes. Five X-chromosome-linked microsatellites (*REN296K08*, *FH2548*, *FH2584*, *FH2985* and *FH3027*; obtained from the Fred Hutchinson Cancer Research Center (FHCRC) Dog Genome Project (http://www.fhcrc.org/science/dog_genome/dog.html) and 19 autosomal microsatellite markers (*c2001*, *c2006*, *c2010*, *c2017*, *c2054*, *c2079*, *c2088* and *c2096* (Francisco *et al.* 1996), *vWF* (Shibuya *et al.* 1994), *u109*, *u173*, *u225*, *u250* and *u253* (Ostrander *et al.* 1993) and *PEZ01*, *PEZ03*, *PEZ05*, *PEZ06* and *PEZ12* and *PEZ20* (Perkin-Elmer, Zoogen; see the FHCRC Dog Genome Project)) were used to genotype the modern wolf samples. A subset of 12 autosomal microsatellite markers (*c2001*, *c2054*, *c2088*, *c2096*, *vWF*, *u109*, *u173*, *u225*, *u253*, *PEZ01*, *PEZ03* and *PEZ05*), chosen because of their reliable amplification even in samples of low quality, was successfully typed in the historical samples. In dogs (*Canis familiaris*), 15 autosomal microsatellites were typed (*c2001*, *c2010*, *c2017*, *c2054*, *c2079*, *c2088*, *c2096*, *vWF*, *u250*, *u253*, *PEZ01*, *PEZ03*, *PEZ05*, *PEZ06* and *PEZ12*). The X-linked and autosomal microsatellites were amplified by polymerase chain reaction (PCR) in 10 µl reactions containing 20 ng of DNA template, 2.0 mM MgCl₂, 0.2 mM dNTPs, 3.2 pmol of each primer and 0.5 units of DNA polymerase (Amplitaq Gold (Applied Biosystems, Foster City, CA, USA) for recent samples and HotStar DNA Polymerase (QIAGEN, GmbH, Hilden, Germany) for historical samples). The PCR profile was identical across all markers and included an initial denaturation step of 95 °C for 10 min (15 min for HotStar amplifications), 11 touch-down cycles with 94 °C for 30 s, 58 °C for 30 s decreasing by 0.5 °C in each cycle and 72 °C for 1 min, 28 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min and a final extension of 72 °C for 10 min. All microsatellite amplifications and sequencing reactions were run on an ABI377 (Applied Biosystems) sequencing instrument.

(c) Data analysis

Factorial correspondence analysis (Benzécri 1973), which displays the genetic similarity among samples in a two-dimensional graphical space, and an assessment of the genetic variability in each population were undertaken using the software GENETIX v. 4.02 (Belkhir *et al.* 2001). The degree of differentiation between populations was measured by the statistic θ , an estimator of F_{ST} (Weir & Cockerham 1984), and by Nei's distance (1978), and its significance was assessed against 1000 permutations in GENETIX. Individual pairwise relationships were assessed in KINSHIP v. 1.0 (Goodnight & Queller 1999) by determining the likelihood ratio of a pair of autosomal microsatellite genotypes for a specified primary and null relationship and attributing significance by simulation.

Although the first breeding female was sampled, the genotype of the first breeding male was inferred indirectly. His reconstructed genotype was based on the genotypes of three wolves available from the first litter (born in 1983), which were confirmed to be full siblings by both KINSHIP analysis and an examination of the compatibility of all loci, and that of their presumed mother. The genotype of the unsampled father was reconstructed manually for each locus by deducing the inheritance of alleles from each parent to the offspring. Similarly, the genotype of a presumed immigrant, breeding for the first time in 1991, was inferred by attributing to it the alleles present in its offspring

but not previously seen in the Scandinavian population, together with alleles at homozygous loci in the offspring. At some loci in both reconstructed genotypes, we could infer only one of the two alleles. In such cases, we assumed that the individual was homozygous at these loci to facilitate the estimation of its probable origin. The origin of the founders (including the reconstructed genotypes) was determined with a Bayesian approach as implemented in the program STRUCTURE (Pritchard *et al.* 2000). Individual polymorphism was estimated as the proportion of heterozygous autosomal microsatellite loci (Ellegren *et al.* 1996). The age of most sampled wolves from the extant Scandinavian population has been estimated (Wabakken *et al.* 2001a) but if unknown, we assumed the collection year minus one for the analysis of the changes in heterozygosity through time.

3. RESULTS AND DISCUSSION

(a) Founding of the Scandinavian wolf population

Samples for DNA analysis are available for a significant part of the Scandinavian wolf population from 1984 to 2001 ($n = 93$), i.e. from one year following the re-establishment of the population until the present. Eleven animals with an estimated year of birth between 1983 and 1990 show no more than four alleles at any of 19 autosomal microsatellite loci and no more than three alleles at any of five X-linked microsatellite loci. Among these animals, there is a maximum of two alleles per locus that are not carried by the first breeding female, which died in 1985 and is sampled. The markers used for genotyping are highly variable in outbred wolf populations (table 2) and so these results indicate that there were only two founders. The reconstructed paternal genotype can, together with the genotype of the first breeding female, explain the presence of all alleles seen in the population among animals born prior to 1991. Finally, all animals born before 1991 carry the same mtDNA haplotype and the six males sampled from this time-period are fixed for the same Y-chromosome haplotype, as revealed by four highly variable Y-chromosome-linked microsatellites (Sundqvist *et al.* 2001). From this, we conclude that only two individuals, one male and one female, founded the population.

The origin of the founders was traced using a Bayesian approach (Pritchard *et al.* 2000) to estimate the probability of their genotypes corresponding to individuals originating from (i) the historical Scandinavian wolf population; (ii) the neighbouring eastern wolf population; or (iii) dogs.

For this analysis, no information about the origin of the samples was specified in the program STRUCTURE, and the individual genotypes (using the nine autosomal microsatellite loci typed in the three datasets) were divided into three groups, each one of them as close as possible to Hardy-Weinberg and linkage equilibrium. The resulting groups corresponded very closely to the three populations. The founding female has a probability of 0.95 of originating from the eastern wolf population, but less than 0.01 of originating from the historical population. This female also possesses six alleles found in the eastern wolf population but not seen in the historical Scandinavian wolf population. Similarly, the inferred founding male has a high probability (0.98) of being an immigrant from the neighbouring eastern population. The probabilities of a dog ancestry are lower than 0.05 for both founders, and

Table 1. Differentiation between populations at autosomal microsatellite loci.

(F_{ST} estimated by θ (Weir & Cockerham 1984), above the diagonal, and the distance of Nei (1978), below the diagonal). The number of microsatellites considered varies between the comparisons (see § 2).

	wolves			
	Scandinavia	eastern	historical	dogs
Scandinavia	—	0.136**	0.265**	0.213**
eastern	0.310**	—	0.089**	0.103**
historical	0.791**	0.333**	—	0.184**
dogs	0.603**	0.399**	0.704**	—

** Significantly different from 0 at $p < 0.01$.

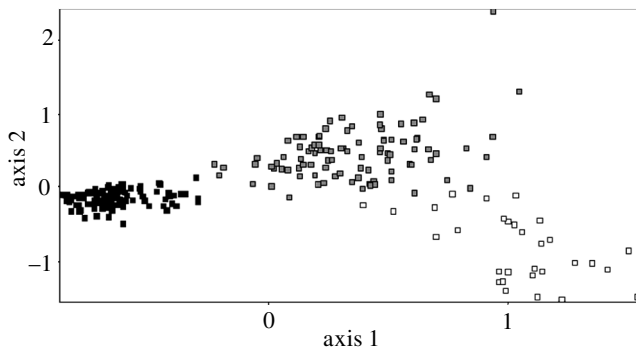


Figure 2. Factorial correspondence analysis of wolves based on the 12 microsatellite loci described in table 2. The contemporary Scandinavian wolf population (black squares) shows the lowest genetic variability and appears to be differentiated from both the historical Scandinavian (1829–1965; white squares) and neighbouring eastern (grey squares) wolf populations. The axes explain 11% of the total variance.

the probability of being F_1 hybrids is similarly reduced (data not shown). As genetic variability is partitioned within populations and, even more dramatically, among dog breeds, it is difficult to fully characterize the diversity of the possible source populations for the migrants and this must be considered in the interpretation of the analysis. However, these results answer a long-term debate on the origin of the population, countering earlier suggestions of a small group of wolves surviving undetected in Scandinavian forests, unauthorized releases from captive colonies in Scandinavian zoos (Ellegren *et al.* 1996) and hybridization between dogs and wolves.

(b) Loss of genetic diversity

Even though eastern immigrants founded the population, a factorial correspondence analysis (figure 2) indicates that contemporary Scandinavian wolves are highly differentiated both from the neighbouring eastern population and from historical Scandinavian wolves. All wolf populations are significantly differentiated, as well as wolves and dogs (table 1; permutation test of θ using 1000 re-samplings, $p < 0.01$ in all comparisons (Belkhir *et al.* 2001)). Moreover, the genetic variability is significantly lower in the contemporary Scandinavian wolf population than in the eastern or historical wolves (table 2; Wilcoxon's signed rank test for the expected heterozygosity and number of alleles, $p < 0.05$ in both cases). Low

genetic diversity and strong differentiation are consistent with a very small number of individuals founding the Scandinavian population and stress the influence of genetic drift (Barton & Charlesworth 1984; Merilä *et al.* 1996; Tarr *et al.* 1998). The founding effect may have formed, in just 15 years, a population that is highly differentiated from the source population.

The founding female was killed in 1985. As the genotypes of some offspring born in 1985–1990 are incompatible with them being fathered by the inferred founding male, and with no evidence of new alleles arriving during this time period, this necessarily implies mating between siblings. Consistent with close inbreeding, a sharp decline in individual heterozygosity (figure 1b) is observed during the 1980s. The expected loss of heterozygosity per generation corresponds to $(1 - 1/(2N_e))$, where N_e is the effective population size (Hartl & Clark 1997). Thus, in a population with just two reproducing individuals, we should expect a 25% loss in heterozygosity per generation, although the strict application of such a formula may not be appropriate in extremely small populations. Our observations roughly correspond to this expectation, as the average heterozygosity decreases from 0.70 in the individuals born in 1983 to 0.49 in the individuals born between 1985 (after the replacement of both parents) and 1990 (a decrease in heterozygosity of 30%; figure 1b). Moreover, relatedness values between pairs of individuals from this time (inferred from microsatellite genotypes) are similar to those observed after sibling mating in the captive Scandinavian zoo population (Ellegren 1999), for which multiple deleterious effects associated with inbreeding depression have been described (Laikre & Ryman 1991; Laikre *et al.* 1993).

(c) Migration from neighbouring populations

By contrast to the homogenous allele composition of wolves from the 1980s, ten new alleles over 19 loci suddenly appeared in a group of six wolves that were born in 1991 and 1992 and identified as siblings by KINSHIP analysis (figure 1b). These siblings, all males, also carry a new Y-chromosome haplotype, showing that a male new to the population fathered them. Reconstruction of the genotype of this new male and its comparison with eastern wolves and dogs at 15 autosomal microsatellite loci assigns it with a probability of 0.99 to the eastern wolf population, confirming a new immigrant.

The arrival of the male immigrant led to an increase in individual heterozygosity (figure 1b), from a mean of 0.49

Table 2. Variability at autosomal microsatellite loci for the Scandinavian, the eastern and the historical Scandinavian wolf populations, and for dogs.

(Variability at the 12 loci shared among the wolf populations, a limitation imposed by the analysis of historical specimens, is shown in italics. H_E , unbiased expected heterozygosity. H_O , observed heterozygosity.)

population	<i>N</i>	loci	H_E (s.d.)	H_O (s.d.)	average no. alleles (s.d.)
Scandinavia	93	19	0.55 (0.03)	0.59 (0.01)	3.74 (1.24)
		<i>12</i>	<i>0.54 (0.04)</i>	<i>0.59 (0.01)</i>	<i>3.83 (1.34)</i>
eastern	93	19	0.77 (0.02)	0.69 (0.01)	8.00 (3.56)
		<i>12</i>	<i>0.77 (0.02)</i>	<i>0.71 (0.01)</i>	<i>8.00 (3.36)</i>
historical	30	12	0.71 (0.02)	0.58 (0.03)	5.75 (1.71)
dogs	66	15	0.70 (0.04)	0.58 (0.02)	8.13 (3.20)

(± 0.03) in eight animals born in 1985–1990 to 0.62 (± 0.03) in 16 animals born in 1991–1995 (Mann–Whitney *U*-test, $p = 0.014$). The increase in heterozygosity can be directly linked to the immigrant as all wolves but four that were born after 1993 ($n = 72$) carry at least one of his alleles.

The arrival of this immigrant coincides with the establishment of a second breeding pack in 1991 and the start of rapid and exponential population growth (figure 1). Moreover, a permutation test on all animals born after 1996 ($n = 56$) shows an excess of heterozygotes (the inbreeding coefficient, $F_{IS} = -0.08$; $p < 0.01$), implying an avoidance of mating with close relatives (outbreeding). An initial excess of heterozygotes could be explained by the admixture of two populations. However, by restricting the analysis to individuals born after 1996, i.e. after several generations from the arrival of the migrant male, it is improbable that any effect of admixture would remain and therefore we conclude that outbreeding occurred following the arrival of this immigrant.

(d) Implications for conservation

The selection of dissimilar mates can be advantageous since it may increase reproductive success (Amos *et al.* 2001) and thereby fitness (Ball *et al.* 2000; Hedrick & Kalinowski 2000; Keller & Waller 2002). As members of the same population are more likely to share deleterious mutations, the arrival of a migrant can induce hybrid vigour (Westemeier *et al.* 1998; Madsen *et al.* 1999; Ebert *et al.* 2002), which may lead to immigrant alleles being present in higher frequencies than predicted from neutral expectations (Whitlock *et al.* 2000; Ingvarsson & Whitlock 2000; Ebert *et al.* 2002; Saccheri & Brakefield 2002). The arrival of the immigrant male wolf is thus likely to have provided the possibility for inbreeding avoidance, decreased the risk of inbreeding depression and triggered population growth. Only immigrants that successfully reproduce within the population can contribute to the rescue of the population. In this sense, we have evidence of another immigrant wolf in Scandinavia in the early 1980s that failed to reproduce and did not contribute to the genetic makeup of the current population (see § 2a).

Many species have behavioural mechanisms to avoid inbreeding and the deleterious effects commonly associated with it (Keller *et al.* 1994; Pusey & Wolf 1996; Smith *et al.* 1997). Such mechanisms are likely to have contributed to the low rate of population growth in Scandinavian wolves prior to 1991. It is possible that the observed excess of heterozygotes results from a higher survival rate

of animals with ancestry from the new migrant, as has been observed in laboratory experiments (Spielman & Frankham 1992; Ball *et al.* 2000; Ebert *et al.* 2002; Saccheri & Brakefield 2002). However, to our knowledge, no field data are available that could indicate these differences in survival. Instead, it has been suggested that inbreeding avoidance may have limited the expansion of the wolf population at Isle Royale in Lake Superior following a population decline (Wayne *et al.* 1991). Hence, avoidance of the deleterious effects of incestuous mating in a small population may enhance the extinction risk from environmental or demographic stochasticity. However, as evidenced here, migration, even at a low rate, can be highly beneficial for a natural population through genetic rescue and by allowing non-incestuous mating, permitting increased fitness (Westemeier *et al.* 1998; Ingvarsson & Whitlock 2000) and rapid population growth.

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