Conserv Genet (2008) 9:389–399
DOI 10.1007/s10592-007-9351-x

RESEARCH ARTICLE

# Genotypic and phenotypic consequences of reintroduction history in the black-footed ferret (*Mustela nigripes*)

**Samantha M. Wisely · Rachel M. Santymire · Travis M. Livieri · Sara A. Mueting · JoGayle Howard**

Received: 14 October 2006 / Accepted: 21 May 2007 / Published online: 13 June 2007


**Abstract** Population augmentation with translocated individuals has been shown to alleviate the effects of bottlenecks and drift. The first step to determine whether restoration for genetic considerations is warranted is to genetically monitor reintroduced populations and compare results to those from the source. To assess the need for genetic restoration, we evaluated genetic diversity and structure of reintroduced ($n = 3$) and captive populations of the endangered black-footed ferret (*Mustela nigripes*). We measured genotypic changes among populations using seven microsatellite markers and compared phenotypic changes with eight morphometric characters. Results indicated that for the population which rapidly grew post-reintroduction, genetic diversity was equivalent to the captive, source population. When growth languished, only the population that was augmented yearly maintained diversity. Without augmentation, allelic diversity declined precipitously and phenotypic changes were apparent. Ferrets from the genetically depaupertate population had smaller limbs and smaller overall body size than ferrets from the two populations with greater diversity. Population divergence ($F_{ST} = 0.10 \pm 0.01$) was surprisingly high given the common source of populations. Thus, it appears that 5–10 years of isolation resulted in both genotypic divergence and phenotypic changes to populations. We recommend translocation of 30–40 captive individuals per annum to reintroduction sites which have not become established quickly. This approach will maximize the retention of genetic diversity, yet maintain the beneficial effects of local adaptation without being swamped by immigration.

**Keywords** Genetic monitoring · Genetic restoration · Population bottleneck · Reintroduction · Translocation

S. M. Wisely (✉) · S. A. Mueting
Division of Biology, Kansas State University, 116 Ackert Hall, Manhattan, KS 66506, USA
e-mail: wisely@ksu.edu

R. M. Santymire
Davee Center for Epidemiology and Endocrinology, Lincoln Park Zoo, 2001 N. Clark Street, Chicago, IL 60614, USA

R. M. Santymire
Department of Reproductive Science, Conservation & Research Center, Smithsonian's National Zoological Park, 1500 Remount Road, Front Royal, VA 22630, USA

T. M. Livieri
Prairie Wildlife Research, P.O. Box 308, Wellington, CO 80549, USA

J. Howard
Department of Reproductive Sciences, Smithsonian's National Zoological Park, 3001 Connecticut Avenue NW, Washington, DC 20008, USA

## Introduction

The restoration of an endangered species back to the wild holds both the promise of the perpetuation of a species (May 1991), and the perils of biological, technical or organizational failure (Reading and Clark 1996). Reintroductions can be a tool to reestablish species which are locally scarce or critically endangered (e.g., Kennamer et al. 1992; Kierulff and DeOliveira 1996; Ostermann et al. 2001; Ralls and Ballou 2004). Although restoration of species has become increasingly common globally (World Conservation Union 1995), reintroduction attempts often fail for reasons that are not well understood (Griffith et al. 1989). Many biological challenges face reintroduced populations as a result of naivety to the local environment

which can lead to increased competition, disease exposure, or predation, all of which decrease survival and reproduction rates (Sarrazin and Legendre 2000; Bar-David et al. 2005). Thus, the number of animals that survive to reproduce may be substantially lower than the number released leading to a population bottleneck and a high risk of extinction (Snyder et al. 1996).

In addition to the demographic vulnerability to extirpation imposed by small population size, bottlenecks also rapidly decrease genetic variation (Nei et al. 1975; Frankham 1996). Founder effects and genetic drift can increase the probability of extinction of a reintroduced population (Reed et al. 2002; Saccheri et al. 1998). In the short term, the loss of genetic variation can lead to the expression of deleterious alleles which may cause inbreeding depression (Charlesworth and Charlesworth 1987, e.g., Westermeier et al. 1998; Madsen et al. 1996; Reed and Frankham 2003). Deleterious alleles can become fixed in small populations when the force of genetic drift exceeds that of selection, which in turn decreases fitness and increases the probability of extinction (Lande 1994, e.g., Florida panther, *Puma concolor coryi*, Roelke et al. 1993). In the long term, reduced genetic variation can decrease the evolutionary potential of a species, increasing its risk of extinction in the face of a changing environment (Franklin 1980; Lande 1988; Hedrick 1994, e.g., Saccheri et al. 1998; Hoffman et al. 2003; but see Ellegren et al. 1993).

Evidence is mounting, however, that small, isolated populations suffering from inbreeding depression may be amenable to genetic restoration (Ingvarsson 2001; Tallmon et al. 2004; Hedrick 2005). Increased fitness in natural populations of adders (*Vipera berus*, Madsen et al. 1996), and prairie chickens (*Tympanuchus cupido*, Westermeier et al. 1998) was reported after immigrants bred with local populations. In these cases only low levels of immigration were necessary to derive a benefit; indeed, genetic restoration of a wolf population (*Canis lupus*, Vilà et al. 2003) occurred as a result of one immigrant. To lessen the risk of extinction associated with isolated, reintroduced populations, conservation practitioners may therefore choose to genetically augment populations with in situ translocations or ex situ captively bred animals to initiate genetic restoration.

Genetic monitoring is a first step in establishing the need for genetic restoration and is increasingly being incorporated for at risk species (Miller et al. 1999, e.g., Tallmon et al. 2004; Swanson et al. 2006). The level of isolation and genetic drift among reintroduced populations can be estimated by comparing levels of genetic variation in source and reintroduced populations. Once informed of the genetic landscape of a reserve network, practitioners may choose to augment restored populations based on levels of divergence from the source population. A much more difficult phenomenon to monitor is inbreeding depression. When is a loss of genetic variability sufficiently great to put a population at risk? Although deleterious effects of inbreeding have been documented in wild populations, the level of inbreeding which is deleterious is species specific and depends on the evolutionary history of a species (Keller and Waller 2002). Some species such as the Florida panther appear extremely vulnerable to the adverse effects of inbreeding (e.g., Roelke et al. 1993), while other populations with low levels of neutral genetic variation appear to prosper (e.g., Ellegren et al. 1993).

Ideally, fitness traits that relate directly to the intrinsic growth rate provide the best indicator of inbreeding depression, yet for small populations of highly endangered, or at risk species, demographic monitoring may not be feasible. For these populations, monitoring changes in morphometric traits may provide an indirect measure of the phenotypic effects of inbreeding, because increased genetic load and reduced overdominance can affect morphological features (DeRose and Roff 1999). A negative correlation between body size and inbreeding has been found in such species as Iberian lynx (*Lynx pardinus*, Pertoldi et al. 2006) and the endangered Mexican wolf (*C. lupus baileyi*, Fredrickson and Hedrick 2002).

The highly endangered black-footed ferret passed through a population bottleneck which at its nadir was <10 individuals. From 1987 to 1990 no individuals of this species existed in the wild. In the past 15 years, animals have been reintroduced to >11 reintroduction sites which are physically isolated from one another, and all in situ animals are presumed to have been propagated in captivity or descended from captively bred individuals. In addition to the population bottleneck and isolated nature of reintroduced populations, black-footed ferrets have a short generation length of 2.3 years in captivity, and is likely shorter in the wild (Wisely et al. 2003). Short generation length increases the risk of inbreeding and genetic drift in reintroduced populations by increasing the pace of evolution and reducing the effective population size (Lande 1995). Recognizing that this species may be susceptible to the deleterious effects of inbreeding, the Black-Footed Ferret Recovery Team provided provisions in the recovery plan for translocations among these reintroduced populations in an effort to retain genetic diversity within the entire network of reserves (U.S. Fish and Wildlife Service 1988; Brussard and Gilpin 1989). To assess the need for translocations to bolster genetic diversity and reduce genetic drift, we genetically evaluated three reintroduced populations and the captive population, the source of all reintroductions, to determine which populations may be experiencing inbreeding or genetic drift. To determine if phenotypic changes accompanied the loss of genetic diversity, we compared body size among reintroduced populations using eight morphometric characters.

## Methods

### Reintroduction locations

We analyzed populations of the black-footed ferret from three reintroduction sites in South Dakota, Wyoming, and Arizona. These populations were the first to be established in the recovery program, persisting for 9–14 years, thus these populations had the potential to be most affected by stochastic genetic processes. At the time of data collection, no migration or translocation had occurred among these reintroduction sites. In South Dakota, animals were introduced into the Conata Basin on Buffalo Gap National Grassland in 1996. A total of 144 individuals were released between 1996 and 1999. The population grew exponentially almost immediately (Schroeder et al. 2000), and at the time of our sampling, only wild born animals remained in the population. Sylvatic plague was not known at the site. In Wyoming, a total of 228 captively bred animals were reintroduced to the Shirley Basin from 1991 to 1994. Plague was endemic and an active outbreak occurred in 1991 that persisted throughout the release period. This population remained at low levels (only five animals were found between 1995 and 1997) until 2003 when 52 animals were detected (Grenier 2004). At the time of our sampling, only wild born animals were present. In Arizona, 188 individuals were released from 1996 to 2004. The population persisted at low levels ($n = 24$ in 2003), with a mixture of wild and captive born animals present at the time of our sampling (Van Pelt and Winstead 2003). Plague was endemic to the area, but no known outbreak occurred on the release site. These three reintroduced populations experienced substantially different environmental characteristics and population trajectories (Fig. 1).

All source animals used for reintroductions came from a captive population with a known pedigree. Average pedigree-based inbreeding in the captive population ranged between 0.09 and 0.11 during the periods of reintroduction, and gene diversity was 38% in 1999 (Wisely et al. 2003). Effective population size based on pedigree structure ($N_e$, Lacy 1995) of the captive, source population was 4.10 and the captive population was genetically managed to equalize founder representation among individuals (mean kinship strategy, Ballou and Lacy 1995; Garell et al. 1998).

### Animal handling

Ferrets are a socially polygamous carnivore. Juveniles born during the summer remain with the mother until early to mid September when they disperse. Ferrets were captured during fall or spring monitoring surveys at the three reintroduction sites from September 2002 to April 2005. In the fall, trapping occurred after juveniles dispersed which decreased the probability of sampling within family groups; in the spring all animals were assumed to be adults with established territories. We also excluded from our sample any captive animals that had been released within 5 months of our trapping effort in order to more accurately sample the population which was likely to breed. All animals were trapped at night using modified cage traps and returned to the same trap location following examination. Anesthesia was induced and maintained by inhalation of isoflurane gas (4 ml/min induction, 2–2.5 ml/min maintenance). Eight morphological characters were measured by one coauthor (RMS, Table 1) with digital calipers (±0.02 mm, Mitutoyo Corporation, Japan) for all adult, wild born animals captured during this study. Because we wanted to minimize the duration of animal handling, measurements were not repeated. We excluded measurements when morphological structures were missing or damaged. Blood was also collected from 78 captive animals from 2002 to 2004 during routine health examinations.

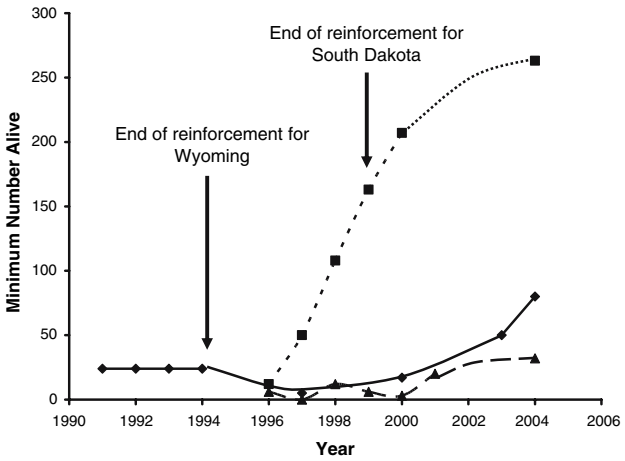


**Fig. 1** Estimates of the minimum number of black-footed ferrets alive at reintroduction sites in South Dakota, Arizona, and Wyoming. The *solid line* represents Wyoming, the *dashed line* is Arizona, and the *stippled line* is South Dakota. Estimates were based on spotlight surveys conducted from 1991 to 2005. Note that the number of surviving individuals was substantially less than the number of released individuals even for the most successful reintroduction site (South Dakota)

### DNA analysis

We placed 250 µl of whole blood drawn from the jugular vein of anesthetized animals into 1 ml of standard lysis buffer. The sample was stored at 4°C until it was extracted in the lab. DNA was extracted from 400 µl of the blood-lysis buffer solution using DNAeasy blood extraction kit (Qiagen, Inc.) following the manufacturer's protocol. We amplified seven microsatellite loci, six of which were

**Table 1** Mean ± SE in mm for eight morphometric measurements from 77 adult black-footed ferrets (*Mustela nigripes*)

| | UCC | LCC | IW | NP | TA | UCW | RL | FA |
|---|---|---|---|---|---|---|---|---|
| Females | | | | | | | | |
| South Dakota, $n = 20$ | 10.06 ± 0.08 | 7.15 ± 0.12 | 6.82 ± 0.05 | 10.43 ± 0.11 | 129.40 ± 1.40 | 3.74 ± 0.05 | 60.99 ± 0.20 | 52.33 ± 0.38 |
| Wyoming, $n = 9$ | 9.85 ± 0.11 | 6.80 ± 0.08 | 6.55 ± 0.07 | 10.64 ± 0.08 | 127.67 ± 2.19 | 3.71 ± 0.06 | 59.95 ± 0.32 | 51.01 ± 0.36 |
| Arizona, $n = 11$ | 9.97 ± 0.10 | 6.60 ± 0.17 | 6.44 ± 0.11 | 10.49 ± 0.07 | 132.17 ± 2.96 | 3.59 ± 0.06 | 59.36 ± 0.68 | 50.37 ± 0.85 |
| Males | | | | | | | | |
| South Dakota, $n = 21$ | 10.92 ± 0.10 | 7.52 ± 0.12 | 7.29 ± 0.05 | 11.86 ± 0.12 | 134.24 ± 1.71 | 4.21 ± 0.07 | 69.35 ± 0.39 | 58.97 ± 0.40 |
| Wyoming, $n = 6$ | 10.80 ± 0.16 | 7.71 ± 0.17 | 7.08 ± 0.06 | 11.98 ± 0.21 | 132.60 ± 2.25 | 4.12 ± 0.09 | 67.97 ± 0.48 | 56.78 ± 0.24 |
| Arizona, $n = 10$ | 10.89 ± 0.08 | 7.41 ± 0.09 | 7.04 ± 0.07 | 12.06 ± 0.15 | 142.50 ± 2.52 | 4.11 ± 0.07 | 69.19 ± 0.29 | 58.98 ± 0.31 |

*UCC* distance from medial edges of maxillary canines, *LCC* distance from medial edges of mandibular canines, *IW* distance from lateral edges of $I^3$'s, *NP* greatest distance between lateral edges of the nose pad, *P24* distance from anterior edge of $P_2$ to posterior edge of $P_4$, *TA* distance from the base of the tail to the end of the last vertebrae, *UCW* distance from the anterior to the posterior edge of the maxillary canine, *RL* tibia length, *FA* ulna length

designed from other mustelids: Mvis002, Mvis022, Mvis072, Mer095 (Fleming et al. 1999); Mvis9700, Mer049 (Wisely et al. 2003), and one black-footed ferret specific primer, Mnig31 (Forward primer: 5′-gcactacgaggacccgacta-3′, Reverse primer: 5′-gtaagcaaaaggggataggg-3′) which was designed using the protocol of Hamilton et al. (1999). Two microsatellites, Mnig31 and Mer095 were amplified using a three primer system following the protocol of Schuelke (2000). Specifically, the universal sequence, M-13 was added to the 5′ end of the forward primer of each microsatellite primer pair. Fluorescently labeled M-13 was added to the polymerase chain reaction (PCR) of these primers. The other five primers were directly dye labeled with the fluorescent molecules, FAM-6, HEX or TAMRA (Applied Biosystems, Inc.). We amplified DNA using 20 µl reactions which included 2 µl of DNA, 2.7 mM $MgCl_2$, 1× buffer without $MgCl_2$, 0.2 mM dNTPs, 0.1 µg/µl bovine serum albumin, 0.8 M betaine, and one unit of *Taq* polymerase. For three primer reactions, we used 1 µM each of the dye labeled M-13 primer, and reverse primer, and 0.3 µM of the forward primer. For two primer reactions, we used 1 µM each of the forward and reverse primers. All reactions were amplified in an Eppendorf Mastercycler (Eppendorf, Inc.). Three primer reactions used the following protocol: 95°C for 5 min then 30 cycles of 95°C for 30 s, 45 s of optimum annealing temperature (55°C for Mer095 and 61°C for Mnig31), followed by 45 s of extension at 72°C. These 30 cycles were followed by 30 cycles of 95°C for 30 s, 45 s at 53°C, and 45 s at 72°C followed by a 72°C extension for 10 min. For two primer reactions, we used the following protocol: 94°C for 2 min, then for 30 cycles: 15 s at 94°C, 15 s at 54°C, and 30 s at 72°C. The program finished at 72°C for 10 min. All reactions were performed with a negative control to confirm that we had no contamination in the reaction. Genotypes were visualized on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Inc.) and interpreted with either Genemarker, v. 1.2 or Genemapper, v. 3.5. We reamplified homozygous individuals 2–4 times to assess the rate of allelic dropout.

## Data analysis

Genotypic data from seven microsatellite loci were assessed for Hardy–Weinberg equilibrium for each population using an exact probability test generated by a Markov chain analysis in GENEPOP 3.3 software (Raymond and Rousset 1995). Linkage disequilibrium was tested by the genotypic equilibrium test in GENEPOP 3.3. All tests were adjusted for multiple comparisons using a sequential Bonferroni correction (Rice 1989). We tested for differences in the average number of alleles per locus and expected heterozygosity using Kruskal–Wallis rank tests. A posteriori we used pair-wise tests to determine significant relationships among pairs of populations.

We used the program, BOTTLENECK, to determine if the genetic signal of a bottleneck could be detected for each of the populations (Cornuet and Luikart 1996) using a two-phased model of mutation. This model modified the single step, stepwise mutation model such that 10% of the mutations added more than a single step per mutation event (Luikart et al. 1998). We used the Wilcoxon signed rank test to determine if allelic diversity was reduced more quickly than heterozygosity as expected soon after a bottleneck; monomorphic loci were removed from this analysis. We also examined the distribution of allele frequencies to compare the occurrence of low frequency alleles among populations. Low frequency alleles occur at a higher proportion in populations which are in drift-mutation equilibrium than in bottlenecked populations (Luikart et al. 1998).

We tested for genetic structure among the three reintroduced populations using both classical population genetic approaches and coalescent based methods. Two

measures of population differentiation, $F_{ST}$ and $\rho_{ST}$, were calculated using GENEPOP 3.3. We reported only $F_{ST}$ values, because $F_{ST}$ and $\rho_{ST}$ estimates were equivalent in all pairwise population comparisons. We also used a coalescent model to estimate $N_m$ (Beerli and Felsenstein 2001) using a Markov Chain Monte Carlo simulation with the software program MIGRATE (V. 1.6.9, Beerli 1997–2003). Microsatellite mutation processes were estimated using a Brownian motion model. In an initial run of the program, we used estimates of $F_{ST}$ to begin the iterative process of calculating $N_m$ Resulting values from this initial simulation were then used as starting values for three subsequent simulations. We used default search parameters for every simulation; all simulations (including the initial one) gave similar results. For maximum likelihood estimation, we selected a full migration model to estimate $N_m$.

To visualize genetic similarities among the three reintroduced populations and the source population we used the Bayesian clustering method of the program structure (Version 2.0, Pritchard et al. 2000). This program assigns individuals from the three reintroduced populations and the source population to new clusters of individuals based on the correspondence of individual allele frequencies and newly created cluster allele frequencies. The program estimated the probability of a set of allelic frequencies given $K$ clusters [Pr($X/K$)]. We estimated Pr($X/K$) at $K = 1$–5; we assumed the model with the largest Pr($X/K$) to most accurately predict the number of clusters in our sample. Because the three reintroduced populations were recently established from the source population, we created a model which allowed admixture of individuals among clusters and allowed clusters to have correlated allele frequencies. The program started with a random configuration of parameter values; therefore, we allowed the simulation of population assignments to run 100,000 times before collecting data (burn-in period), and another 100,000 times to estimate the parameters. This burn-in period appeared to be sufficiently long; all parameter estimations converged before burn-in ended.

To test the hypothesis that animals from different reintroduction sites were of different body sizes, we reduced the morphological variables to one principal component. Many morphological studies (e.g., Fleischer and Murphy 1992; Komers and Komers 1992), including two for this species (Wisely et al. 2002, 2005), inferred the first principal component (PC1) to represent overall body size. We analyzed factor components from the PC1 with an ANOVA with sex and location as fixed factors and $\alpha = 0.05$. We tested a posteriori for pairwise differences among populations in body size with Tukey's HSD test. Because black-footed ferrets are known to be sexually dimorphic (Anderson et al. 1986; Wisely et al. 2005) we did not report results for differences among sexes.

## Results

### Genetic data

We extracted DNA from 185 black-footed ferrets across the three locations and the captive population. We reamplified 40% ($n = 517$) of the genotypes and found no evidence for allelic dropout. The populations were in Hardy–Weinberg equilibrium except for locus Mvis022 at the Arizona population ($P < 0.02$, Table 2). One pair of loci (Mer049 and Mer095) at one population (Arizona) was in linkage disequilibrium, however this pair was in equilibrium at the other three populations. We found a significant difference in allelic diversity among populations ($H = 12.5$, $df = 3$, $P = 0.006$). All a posteriori pair-wise tests involving Wyoming were significant at $\alpha < 0.05$; no other pair-wise comparisons of allelic diversity among populations were significant (Table 2). This reduction in allelic diversity in Wyoming was further apparent in comparisons of polymorphic loci; the seven polymorphic loci in the source population remained polymorphic in the South Dakota and Arizona populations, however four loci were monomorphic in the Wyoming population. At the Arizona location, we documented the presence of an allele at locus Mvis002 not present in the other reintroduced populations, nor in the captive population. We found no significant difference in expected heterozygosity among populations ($H = 1.6$, $df = 3$, $P = 0.67$).

**Table 2** Descriptive genetic parameters for three reintroduced populations of black-footed ferrets

| Population | $n$ | $H_e \pm$ SE | $H_o \pm$ SE | $A$ | $P$-value (%) |
|---|---|---|---|---|---|
| South Dakota | 44 | 0.41 ± 0.01 | 0.40 ± 0.01 | 2.00 | 100 |
| Wyoming | 32 | 0.21 ± 0.04 | 0.21 ± 0.01 | 1.43 | 43 |
| Arizona | 31 | 0.34 ± 0.02 | 0.28 ± 0.01 | 2.14 | 100 |
| Captive | 78 | 0.37 ± 0.01 | 0.38 ± 0.01 | 2.00 | 100 |

We estimated expected heterozygosity ($H_e$) and observed heterozygosity ($H_o$) averaged across loci ± SE, the average number of alleles per locus ($A$), and the percentage of polymorphic loci ($P$)

Among reintroduced populations we found evidence of a population bottleneck in the South Dakota population. South Dakota had significant heterozygote excess ($P = 0.004$) based on the Wilcoxon signed rank test, Wyoming had marginally significant heterozygote excess ($P = 0.06$), and Arizona did not exhibit heterozygote excess ($P = 0.15$). The Wyoming population, however, had four monomorphic loci which reduced the number of loci available to test to three which reduced the power of the Wilcoxon signed rank test. All three populations had modes shifted away from a high proportion of low frequency alleles expected under equilibrium (Fig. 2); Arizona had the highest frequency of rare alleles (alleles with a frequency <0.01).

Both classical and coalescent based methods of measuring genetic structure revealed divergence among the three reintroduced populations. Across all populations $F_{ST} = 0.10 \pm 0.01$. Arizona, with the most recent translocations from the captive population, and Wyoming, the longest established population, were the most differentiated ($F_{ST} = 0.28$). The two populations which were the least temporally separated, South Dakota and Arizona, from their common source population were the least genetically differentiated of the reintroduced populations ($F_{ST} = 0.05$). South Dakota and Wyoming were moderately differentiated ($F_{ST} = 0.16$). South Dakota was substantially less differentiated from the source population ($F_{ST} = 0.005$) than either Wyoming ($F_{ST} = 0.12$) or Arizona ($F_{ST} = 0.10$).

Results of the Bayesian cluster analysis demonstrated that the distribution of allele frequencies was best explained by partitioning reintroduced and source individuals into three clusters (Fig. 3). Individuals from Wyoming most frequently were assigned to Cluster 2, South Dakota and Arizona individuals were assigned most often to Clusters 3 and 1, and individuals from the source population were evenly assigned to all three clusters. Coalescent based estimates of $N_m$ were similar among program runs which suggested that an appropriate number of Markov chain simulations were used per run. $N_m$ estimates indicated that migration was least among Wyoming and Arizona, but substantial among other pairs of populations (Table 3).

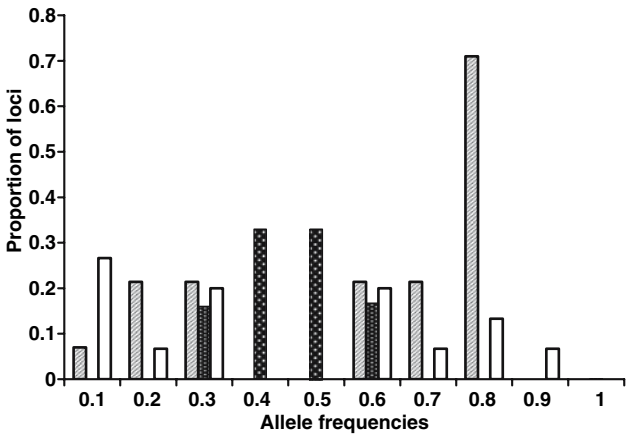


**Fig. 2** The allele frequency distribution of seven microsatellite loci for three populations of reintroduced black-footed ferrets. For populations in equilibrium, a unimodal distribution of alleles at low frequencies is expected; for populations that have recently experienced a bottleneck, allele frequencies will be multimodal and allele frequencies will be high (adapted from Luikart et al. 1998). *Striped line* indicates South Dakota, *stippled line* indicates Wyoming, *white line* indicates Arizona

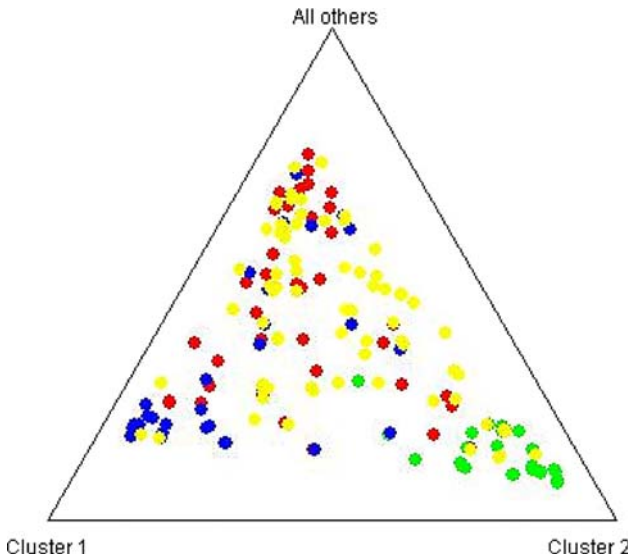


**Fig. 3** Results of the Bayesian cluster analysis of the three reintroduced populations (South Dakota, Wyoming, and Arizona) and the source population grouped into three clusters ($K = 3$). Each *interior angle* of the *triangle* represents one of three clusters; the distance from a *point* to *each angle* represents the proportion of ancestry comprised by each cluster. The *color of the point* represents the population (South Dakota *red*, Wyoming *green*, Arizona *blue*, source *yellow*). Fifty three percent of South Dakota individuals were classified as Cluster 3, 57% of Arizona individuals were classified as Cluster 1, and 88% of Wyoming individuals classified as Cluster 2. Source animals were distributed among the three clusters

**Table 3** Estimates of the effective number of migrants per generation ($N_m$) for three reintroduced populations of the black-footed ferret using microsatellite data in the program MIGRATE

| Population | $N_m$ ($x$ = receiving population) | | |
|---|---|---|---|
| | 1, $x$ | 2, $x$ | 3, $x$ |
| 1. South Dakota | – | 3.7 | 4.9 |
| 2. Wyoming | 6.8 | – | 0.4 |
| 3. Arizona | 5.2 | 0.8 | – |

### Morphological data

We collected morphological measurements from 77 adults across the three populations (Table 1). The PC1 of the eight morphological variables had an eigenvalue of 5.2 which captured 65% of the variance in those eight variables. All principal component scores in the eigenvector of PC1 were large and positive as expected if PC1 represented overall body size. The minimum score was for tail length (0.52) and the largest value was for tibia length (0.95). The effect of location on PC1 was highly significant in our ANOVA model ($F_{2,56} = 6.3$, $P = 0.003$), but we found no significant interaction between gender and location. A posteriori tests revealed that Wyoming animals were significantly smaller than either South Dakota ($P \ll 0.001$) or Arizona ($P \ll 0.001$) animals, but that animals from South Dakota and Arizona ($P = 0.80$) were not significantly different from one another.

## Discussion

### Reintroduction history and genetic diversity

Compared to the source population, neither South Dakota nor Arizona appears to have lost measurable amounts of genetic diversity. South Dakota retained all alleles present in the source population (average number of alleles was 2 for both the source and South Dakota), and measures of gene diversity were similar through time for South Dakota. Diversity measured in 1999 ($H_e = 0.38 \pm 0.07$, $A = 2$, Wisely et al. 2003), the year population augmentation with captive animals ended, remained similar to measures of gene diversity in this population 4 years later ($H_e = 0.41 \pm 0.01$, $A = 2$) suggesting that no diversity was lost once the demographic reinforcement ended. The maintenance of diversity is not surprising given the exponential growth that this population experienced during those years. Populations that experience growth or are maintained at a large size are less likely to lose genetic diversity than small populations which are subject to the effects of drift, inbreeding, and bottlenecks (Gilpin and Soulé 1986).

Gene diversity was also similar between the source population ($H_e = 0.37 \pm 0.01$) and Arizona ($0.34 \pm 0.02$). Allelic diversity actually increased for the reintroduced Arizona population due to the presence of a new allele at Mvis002. This allele did not appear to be a PCR artifact; it was independently amplified on each of four occasions. This allele was present in very low frequency in Arizona; only one copy in one wild born individual was detected. This allele was not present in either the extant captive population or the founders of the captive population (Wisely et al. 2003), suggesting that this allele was a new mutation. We attribute the maintenance of genetic diversity, despite persistently low population size in Arizona, to reinforcement with translocated individuals from captivity. The addition of animals through time also appeared to obscure the signature of a population bottleneck in the Arizona population. In South Dakota, despite the maintenance of genetic diversity, we found evidence for a genetic signal of a population bottleneck, as predicted when founding animals are released over a short time frame (Robert and Couvet 2004).

The paucity of allelic diversity and fixation of alleles at 4 of 7 loci support the observation that the Wyoming population passed through multiple generations of a bottleneck. A comparison of allelic diversity between the source and Wyoming populations revealed that Wyoming lost 28% (4 out of 14 alleles) of its allelic diversity. Persistently low population size during the mid 1990s reinforced the loss of diversity and likely contributed to genetic drift in this population. It appears that the persistent low population size characteristic of a prolonged bottleneck led to diminished genetic diversity, increased inbreeding, and increased drift in Wyoming.

### Genetic differentiation among reintroduced populations

In small populations, genetic drift not only reduces genetic diversity, but also promotes divergence among populations via stochastic changes in allele frequencies. Both classical population genetic parameters ($F_{ST}$ and $\rho_{ST}$) and those based on coalescent approaches (implemented by the programs, structure and MIGRATE) suggested that the three reintroduced populations were differentiated from one another. Based on the divergent allele frequencies among populations, the Bayesian cluster analysis assigned reintroduced individuals into three clusters which suggests that some genetic differentiation occurred among these populations in spite of their common source population. Individuals from the source population did not assemble into any one cluster; rather, as would be expected for a source population, it was an admixture of the three clusters.

Estimates of migration ($N_m$) were 5–15 times lower for Wyoming than for South Dakota or Arizona (Table 3). $N_m$ was lowest and $F_{ST}$ was highest between Arizona and Wyoming suggesting that these two populations were the most dissimilar. Overall patterns of divergence were better explained by the time that had elapsed since reinforcement from the source population, rather than the number of years since reintroduction, suggesting that reinforcement partially, but not fully ameliorated genetic drift. That multilocus genotypes from all sampled black-footed ferrets clustered into three populations, which corresponded to the three reintroduced populations, suggests that these popu-

lations have diverged in spite of short amount of time that they were separated from the source population and in spite of reinforcement.

## Morphological divergence

Black-footed ferrets from Wyoming were smaller in overall body size than animals in South Dakota or Arizona. Differences in overall body size were exemplified by the differentiation of fore- and hind-limb length among populations (Fig. 4). Morphological measurements of museum specimens collected prior to extirpation from the wild indicated that animals from Wyoming and South Dakota were historically similar sized (Anderson et al. 1986). The similarly sized historical populations provide evidence that differences among contemporary populations were due to recent changes in the endogenous (genetic) or exogenous (environmental) influences on black-footed ferrets. The cause of these changes remains elusive however, and is potentially complicated by low power of the analysis due to small sample sizes. Nonetheless, rapid population turnover due to short generation time makes this species vulnerable to the processes of genetic drift which can increase the rate of adaptive evolution as well as increase the risk of inbreeding depression (Lande 1995).

Previous studies have found links between morphometry and inbreeding. Morphometric studies of in situ populations of the highly endangered Iberian lynx (Pertoldi et al. 2006) and Roesel's bush-crickets (*Metrioptera roeseli*, Berggren 2005) found divergent morphological traits among isolated populations and decreased character size in inbred populations. The authors attributed these findings to genetic drift among populations, and inbreeding depression within populations. For the Iberian lynx, the authors recommended that gene flow be initiated among established populations. Body size was also found to decrease as inbreeding increased in captive populations of the endangered Mexican wolf (Fredrickson and Hedrick 2002). Although morphometry may not be the most responsive attribute to inbreeding, it has shown utility as a correlate. For black-footed ferrets, although we cannot determine the cause of morphological differentiation, it appears that the Wyoming population is not only diverging genetically, but that phenotypic changes, the exact mechanism of which is not fully understood, have occurred.

## Implications for conservation and management

Genetic diversity was maintained for two of the three populations that were evaluated. Rapid establishment and population growth, which occurred in South Dakota, appears to have effectively maintained genetic diversity and reduced drift in this population (Green 1997; Gilpin and Soulé 1986).

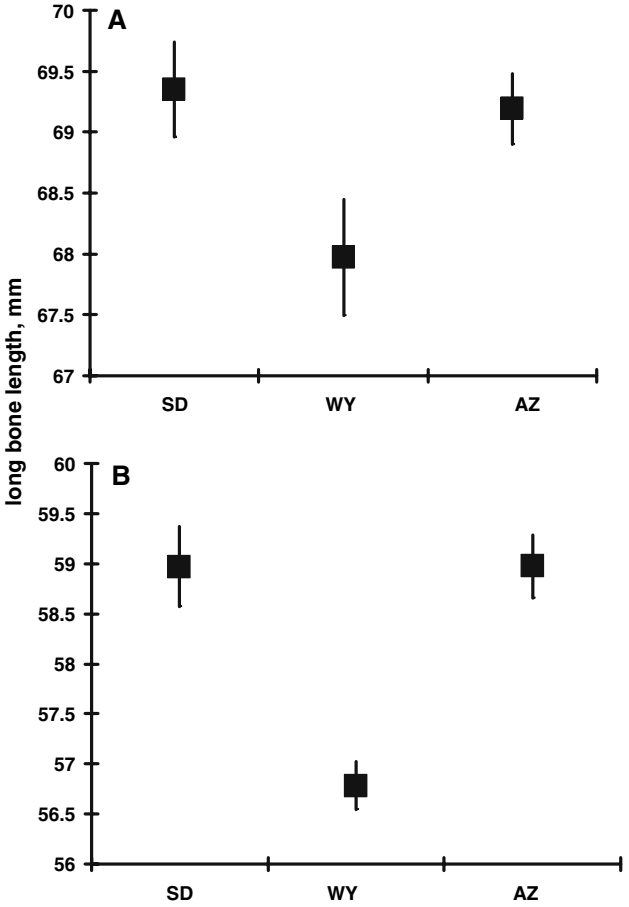


**Fig. 4** Mean and standard error of tibia length (*A*) and ulna length (*B*) of male black-footed ferrets reintroduced to Arizona, $n = 10$; South Dakota, $n = 21$; and Wyoming, $n = 6$. All animals were wild-born

Although rapid establishment did not occur in Arizona, supplementation with yearly augmentation of captive animals appears to have mimicked natural immigration which, even at low levels, maintains genetic diversity in small populations (Couvet 2002). In Wyoming, which experienced a population bottleneck and no reinforcement, substantial genetic diversity was lost. Based on our empirical observations, we recommend that reintroduced populations be monitored to evaluate establishment and population growth. If populations do not quickly become established, additional individuals should be released to dampen both demographic and genetic stochasticity. In addition, for black-footed ferrets, body size may respond to decreases in genetic diversity. Although further sampling is needed, morphometric evaluation may be a valuable tool to assess the phenotypic impacts on small, isolated populations.

For taxa with patchy distributions, such as the black-footed ferret, connectivity among reintroduction sites is currently impossible. The Black-Footed Ferret Recovery Plan calls for the exchange of one migrant per generation (U.S. Fish and Wildlife Service 1988), however theoretical

work since the publication of the recovery plan highlights that one migrant per generation is an idealized estimate without consideration to the behavior or social construct of the species, the genetic or spatial landscape of the populations, or the relationship of the effective population size ($N_e$) to the census population size (Mills and Allendorf 1996; Vucetich and Waite 2000). Although a formal estimate of the minimum number of migrants needed to maintain genetic diversity is beyond the scope of this paper, several attributes of the species suggest that the minimum number would be greater than one. Survival of ferrets released into occupied habitat is generally low (33% in Badlands National Park, Conservation Breeding Specialist Group 2003), and males are highly territorial suggesting that the census number of released animals would be substantially lower than the effective number of individuals. In addition, given the paucity of diversity in extant ferrets and the high relatedness of captive individuals, the influx of genetic material will more closely resemble that of the local population than it would in an idealized situation. Based on computer simulations by Allendorf and Phelps (1981), Mills and Allendorf (1996) suggested that ten effective migrants per generation would not swamp out adaptive alleles that have accumulated in reintroduced populations. For black-footed ferrets, this could mean a minimum translocation of 30–40 individuals, based on short term survival estimates of translocated animals. This estimate is similar to the average of 26 ± 6 individuals released per year for 8 years in Arizona (Van Pelt and Winstead 2003) where genetic diversity has been maintained.

To most rapidly enhance genetic diversity in reintroduced populations, we recommend that the source of reintroduced individuals be captively bred animals. Not only will this strategy minimize differences among reintroduced populations, but it will also alleviate concerns of disease transmission among in situ populations.

**Acknowledgments** All animal handling was authorized and coordinated by the U.S. Fish and Wildlife Service's Black-Footed Ferret Recovery Implementation Team. Animal handling protocols were reviewed and authorized by Kansas State University's Institutional Animal Care and Use Committee (protocol no. 2310). The authors thank M. Lockhart and P. Marinari of U.S. Fish and Wildlife Service; B. Van Pelt, C. King, S. Goodman, R. Lonsinger, J. Broescher, and A. Siniawski of Arizona Game and Fish; B. Oakleaf and M. Grenier of Wyoming Game and Fish Department; B. Perry and D. Sargent of USDA Forest Service, D. Albertson, G. Schroeder and B. Kenner of the National Park Service for accommodating us in the field. We thank the many additional people who volunteered their time to help survey for black-footed ferrets. We also thank D. Garelle of Cheyenne Mountain Zoo; E. Bronson of National Zoological Park, K. Orr of Phoenix Zoo, C. Eng, J. Bachtel, H. Mutlow, D. Gaspar, and J. Kreeger for veterinary services. Phoenix Zoo, Arizona Game and Fish Department, Wyoming Game and Fish Department, USDA Forest Service, National Park Service, National Fish and Wildlife Foundation, Division of Biology at Kansas State University, and Friends of the National Zoo provided financial support for this research. We thank M. Schwartz and anonymous reviewers for constructive comments on the manuscript.

## References

Allendorf FW, Phelps SR (1981) The use of allelic frequencies to describe population structure. Can J Fish Aquat Sci 38:1507–1514

Anderson E, Forrest SC, Clark TW, Richardson L (1986) Paleobiology, biogeography, and systematics of the black-footed ferret, *Mustela nigripes* (Audubon and Bachman), 1851. Great Basin Nat Mem 8:11–62

Ballou JD, Lacy RC (1995) Identifying genetically important individuals for management of genetic variation in pedigreed populations. In: Ballou JD, Gilpin M, Foose TJ (eds) Population management for survival and recovery: analytical methods and strategies in small population conservation. Columbia University Press, New York

Bar-David S, Saltz D, Dayan T, Perelberg A, Dolev A (2005) Demographic models and reality in reintroductions: Persian fallow deer in Israel. Conserv Biol 19:131–138

Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in *n* subpopulations by using a coalescent approach. Proc Natl Acad Sci USA 98:4563–4568

Berggren A (2005) Effect of propagule size and landscape structure on morphological differentiation and asymmetry in experimentally introduced Roesel's bush crickets. Conserv Biol 1095–1102

Brussard PF, Gilpin ME (1989) Demographic and genetic problems of small populations. In: Seal US, Thorne ET, Bogan MA, Anderson SH (eds) Conservation biology and the black-footed ferret. Yale University Press, New Haven

Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. Annu Rev Ecol Syst 18:237–268

Conservation Breeding Specialist Group (2003) Black-footed ferret population management workshop. World Conservation Union, Apple Valley

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014

Couvet JF (2002) Deleterious effects of restricted gene flow in fragmented populations. Conserv Biol 16:369–376

DeRose MA, Roff DA (1999) A comparison of inbreeding depression in life-history and morphological traits in animals. Evolution 53:1288–1292

Ellegren H, Hartman G, Johansson M, Andersson L (1993) Major histocompatibility complex monomorphism and low levels of DNA fingerprinting variability in a reintroduced and rapidly expanding population of beavers. Proc Natl Acad Sci USA 90:8150–8153

Fleischer RC, Murphy MT (1992) Relationships among allozyme heterozygosity, morphology and lipid levels in house sparrows during winter. J Zool Lond 226:409–419

Fleming MA, Ostrander EA, Cook JA (1999) Microsatellite markers for American mink (*Mustela vison*) and ermine (*Mustela erminea*). Mol Ecol 8:1351–1362

Frankham R (1996) Relationship of genetic variation to population size in wildlife. Conserv Biol 10:1500–1508

Franklin IR (1980) Evolutionary change in small populations. In: Soulé ME, Wilcox BA (eds) Conservation biology: an evolutionary–ecological perspective. Sinauer Associates, Sunderland

Fredrickson R, Hedrick P (2002) Body size in endangered Mexican wolves: effects of inbreeding and cross-lineage matings. Anim Conserv 5:39–43

Garell D, Vargas A, Marinari P (1998) Black-footed ferret species survival plan: annual report 1997. American Zoo and Aquarium Association (AZA), Bethesda

Gilpin ME, Soulé ME (1986) Minimum viable populations: processes of species extinction. In: Soulé ME (ed) Conservation biology: the science of scarcity and diversity. Sinauer Associates, Sunderland

Green RE (1997) The influence of the number of released on the outcome of attempts to introduce exotic birds to New Zealand. J Anim Ecol 66:25–35

Grenier M (2004) Finding ferrets. Wyoming Wildl 68:24–29

Griffith BJ, Scott JM, Carpenter JW, Reed C (1989) Translocation as a species conservation tool: status and strategy. Science 245:477–480

Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. Biotechniques 2:500–507

Hedrick PW (1994) Purging inbreeding depression and the probability of extinction: full-sib mating. Heredity 73:363–372

Hedrick PW (2005) Genetic restoration: a more comprehensive perspective than genetic rescue. Trends Ecol Evol 20:109

Hoffman AA, Hallas RJ, Dean JA, Schiffer M (2003) Low potential for climatic stress adaptation in a rainforest *Drosophila* species. Science 301:100–102

Ingvarsson PK (2001) Restoration of genetic variation lost—the genetic rescue hypothesis. Trends Ecol Evol 16:62–63

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. Trends Ecol Evol 17:230–241

Kennamer JE, Kennamer M, Brenneman R (1992) History. In: Dickson JG (ed) The wild turkey: biology and management. Stackpole Books, Harrisburg

Kierulff MCM, DeOliveira PP (1996) Re-assessing the status and conservation of the golden lion tamarin (*Leontopithecus rosalia*) in the wild. Dodo 32:98–115

Komers PE, Komers EJ (1992) Juvenile male magpies dominate adults irrespective of size differences. Can J Zool 70:815–819

Lacy RC (1995) Clarification of genetic terms and their use in the management of captive populations. Zoo Biol 14:565–577

Lande R (1988) Genetics and demography in biological conservation. Science 241:1455–1460

Lande R (1994) Risk of population extinction from fixation of new deleterious mutations. Evolution 48:1460–1469

Lande R (1995) Breeding plans for small populations based on the dynamics of quantitative genetic variance. In: Ballou JD, Gilpin M, Foose TJ (eds) Population management for survival and recovery: analytical methods and strategies in small population conservation. Columbia University Press, New York

Luikart G, Allendorf FW, Cornuet J-M, Sherwin WB (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. J Hered 8:238–247

Madsen T, Stille B, Shine R (1996) Inbreeding depression in an isolated population of adders *Vipera berus*. Biol Conserv 75:113–118

May R (1991) The role of ecological theory in planning reintroduction of endangered species. Symp Zool Soc Lond 62:145–163

Miller B, Ralls K, Reading RP, Scott JM, Estes J (1999) Biological and technical considerations of carnivore translocations: a review. Anim Conserv 2:59–68

Mills LS, Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. Conserv Biol 10:1509–1518

Nei M, Maruyama T, Chakraborty R (1975) Bottleneck effect and genetic variability in populations. Evolution 29:1–10

Ostermann SD, Deforge JR, Edge WD (2001) Captive breeding and reintroduction evaluation criteria: a case study of peninsular bighorn sheep. Conserv Biol 15:749–760

Pertoldi C, García-Perea R, Godoy JA, Delibes M, Loeschcke V (2006) Morphological consequences of range fragmentation and population decline on the endangered Iberian lynx (*Lynx pardinus*). J Zool 268:73–86

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Ralls K, Ballou JD (2004) Genetic status and management of California Condors. Condor 106:215–218

Raymond M, Rousset F (1995) GENEPOP (ver. 1.2): a population genetics software for exact test and ecumenicism. J Hered 86:248–249

Reading RP, Clark TW (1996) Carnivore reintroductions: an interdisciplinary examination. In: Gittleman JL (ed) Carnivore behavior, ecology, and evolution, vol 2. Cornell University Press, Ithaca

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. Conserv Biol 17:230–237

Reed DH, Briscoe DA, Frankham R (2002) Inbreeding and extinction: the effect of environmental stress and lineage. Conserv Genet 3:301–307

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225

Robert A, Couvet FSD (2004) Influence of the rate of introduction on the fitness of restored populations. Conserv Genet 5:673–682

Roelke ME, Martenson JS, O'Brien SJ (1993) The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Curr Biol 3:340–350

Saccheri I, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I (1998) Inbreeding and extinction in a butterfly metapopulation. Nature 392:491–494

Sarrazin F, Legendre S (2000) Demographic approach to releasing adults versus young in reintroductions. Conserv Biol 14:488–500

Schroeder GM, Albertson DD, Plumb GE (2000) Black-footed ferret reintroduction in Badlands National Park. Completion report 1993–1997. NPS Natural Resource Preservation Project 174. Badlands National Park, Interior

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234

Snyder NFR, Derrickson SR, Beissinger SR, Wiley JW, Smith TB, Toone WD, Miller B (1996) Limitations of captive breeding in endangered species recovery. Conserv Biol 10:338–348

Swanson BJ, Peters LR, Kyle CJ (2006) Demographic and genetic evaluation of an American marten reintroduction. J Mammal 87:272–280

Tallmon DA, Luikart G, Waples RS (2004) The alluring simplicity and complex reality of genetic rescue. Trends Ecol Evol 19:489–496

U.S. Fish and Wildlife Service (1988) Black-footed ferret recovery plan. U.S. Fish and Wildlife Service, Denver

Van Pelt WE, Winstead RA (2003) Review of black-footed ferret reintroduction in Arizona, 1996–2001. Nongame and Endangered Wildlife Program Technical Report 222. Arizona Game and Fish Department, Phoenix

Vilà C, Sundqvist AK, Flagstad O, Seddon J, Bjornerfeldt S, Kojola I, Casulli A, Sand H, Wabakken P, Ellegren H (2003) Rescue of a severely bottlenecked wolf (*Canis lupus*) population by a single immigrant. Proc R Soc Lond B 270:91–97

Vucetich JA, Waite TA (2000) Is one migrant per generation sufficient for the genetic management of fluctuating populations?. Anim Conserv 3:261–266

Westermeier RL, Brawn JD, Simpson SA, Esker TL, Jansen RW, Walk JW, Kershner EL, Bouzat JL, Paige KN (1998) Tracking

the long-term decline and recovery of an isolated population. Science 282:1695–1698

Wisely SM, Fleming MA, McDonald DB, Buskirk SW, Ostrander EA (2002) Genetic diversity and fitness in black-footed ferrets before and during a bottleneck. J Hered 93:231–237

Wisely SM, McDonald DB, Buskirk SW (2003) Evaluation of the species survival plan and captive breeding program for the black-footed ferret. Zoo Biol 22:287–298

Wisely SM, Santymire RM, Marinari PE, Kreeger JS, Livieri TM, Wildt DE, Howard JG (2005) Environment influences morphology and development of in situ and ex situ populations of the black-footed ferret. Anim Conserv 8:321–328

World Conservation Union (IUCN) (1995) IUCN species survival commission guidelines for reintroductions. IUCN, Gland