# Captive breeding and reintroduction of the lesser kestrel Falco naumanni: a genetic analysis using microsatellites

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Abstract We used microsatellites to assess ongoing captive breeding and reintroduction programs of the lesser kestrel. The extent of genetic variation within the captive populations analysed did not differ significantly from that reported in wild populations. Thus, the application of widely recommended management practices, such as the registration of crosses between individuals in proper stud books and the introduction of new individuals into the genetic pools, has proven satisfactory to maintain high levels of genetic variation. The high rates of hatching failure occasionally documented in captivity can therefore not be attributed to depressed genetic variation. Even though genetic diversity in reintroduced populations did not differ significantly when compared to wild populations either, average observed heterozygosities and inbreeding coefficients were significantly lower and higher, respectively, when compared to the captive demes where released birds came. Monitoring of reproductive parameters during single-pairing breeding and paternity inference within colonial facilities revealed large variations in breeding success between reproductive adults. The relative number of breeding pairs that contributed to a large part of captive-born birds (50–56%) was similar in both cases (28.6 and 26.9%, respectively). Thus, the chances for polygyny (rarely in this study), extra-pair paternity (not found in this study) and/or social stimulation of breeding parameters do not seem to greatly affect the genetically effective population size. Independently of breeding strategies, the release of unrelated fledglings into the same area and the promotion of immigration should be mandatory to counteract founder effects and avoid inbreeding in reintroduced populations of lesser kestrels.

Keywords Genetic diversity · Conservation genetics · Effective population size · Founder effect · Mixed reproductive strategies

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## Introduction

Captive breeding of endangered species has become a widespread practice to provide individuals for reintroduction or supplementation programs for extinct or declining populations. Although traditional approaches have tried to identify ecological and behavioural constraints affecting the short-term success of these initiatives (e.g. Hirzel et al. 2004; Martínez-Meyer et al. 2006), most monitoring programs do not take full advantage of the potential afforded by molecular markers. Monitoring population genetic metrics can provide insights into relevant processes that are difficult or impossible to study via traditional approaches (e.g. Schwartz et al. 2006). For example, captive breeding and reintroduction programs could potentially be counterproductive if the genetic consequences of the various

management options are not fully considered (Woodworth et al. 2002; Gilligan and Frankham 2003). In this respect, loss of genetic variation linked to founder effects and inbreeding may have serious fitness consequences and can jeopardize the evolutionary and adaptive potential of populations and species (Frankham et al. 2002).

The lesser kestrel Falco naumanni was one of the most abundant raptors in Europe before a sharp population decline which began in the late 1960 s (Bijleveld 1974). As a result, this small migratory and facultatively colonial falcon totally or partially disappeared from several locations of its former breeding range (Biber 1990), and is now patchily distributed from Portugal to China (Cramp and Simmons 1980). To date, numerous captive breeding programs have successfully contributed to the reinforcement and re-establishment of decimated or extinct populations in Western Europe (e.g. Pomarol 1993) by using the method of hacking (Sherrod et al. 1981).

In this study, we have performed the first genetic assessment of ongoing captive breeding and reintroduction programs of the globally vulnerable lesser kestrel (BirdLife International 2004). Firstly, we investigated levels of genetic diversity in captive populations. Hatching failure, one of the most cited fitness consequences of inbreeding in birds (e.g. Keller 1998; Morrow et al. 2002), has been occasionally high in captivity in lesser kestrels (<50% of fertile eggs; Colás et al. 2002), contrasting with the normal values of this parameter in the wild (<10% of fertile eggs, e.g. Serrano et al. 2005). In fact, hatching success in captivity is the only parameter which has not exceeded the performance of the species in the wild (Pomarol et al. 2004a).

Secondly, we compared single-pairing (one male and one female) versus colonial captive breeding (multiple males and females) strategies. We focused on variations in breeding success as primary determinants of genetically effective population size (e.g. Nunney and Elam 1994; Hedrick 2005). To this aim, we calculated the minimum number of breeding pairs that contributed to a high proportion of fledglings at two captive centres working on single-pairing into individual pens. Paternity of fledglings within colonial enclosures can only be confirmed through genetic inference, and therefore, we employed polymorphic microsatellites to infer kinship. Two hypotheses can be made in this respect. The first hypothesis would predict an increase of the variance in male breeding success because of mixed reproductive strategies such as those observed, although at low rates, in wild colonies [see exceptional polygynous mating systems in Tella et al. (1996) and low extra-pair paternity rates <7.5% in Alcaide et al. (2005)]. Alternatively, the simulation of colonial environments may stimulate the breeding behaviour of individuals which could otherwise remain sexually inactive (see for instance Waas et al. 2005), with the subsequent increase in overall productivity compared to single-breeding pairs.

Finally, we evaluated the extent of genetic variation that has been successfully transmitted from captive stocks to reintroduced populations to help optimize the main genetic goal of a reintroduction program. In this respect, it is widely assumed that high levels of genetic diversity maximize the possibilities of re-establishing a self-sustaining population in the long term (e.g. Ballou and Lacy 1995; Frankham et al. 2002).

## Materials and methods

### Captive, reintroduced and wild populations

In Spain, three captive populations kept by non-government organizations for Native fauna and its Habitat Rehabilitation "GREFA" (www.grefa.org), Defence and Study of Natural Environment "DEMA" (www.demaprimilla.org) and the wildlife recovery center of Torreferrusa attached to the Catalonian government "TORREF" (http://mediambient.gencat.cat/cat/el_medi/fauna/fauna_auctoctona/centres/torreferrussa.jsp) were investigated (see Fig. 1). Founder individuals of captive demes, usually injured birds which could not be rehabilitated and returned into the wild, were derived from different locations belonging to the main Spanish population or translocated from other captive populations. Management actions of breeders encompass the registration of crosses between individuals in proper stud books and the introduction of new individuals into

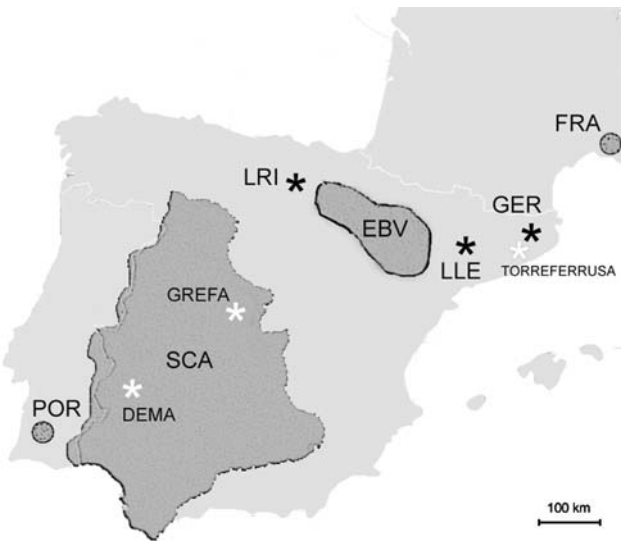


Fig. 1 Breeding distribution of the lesser kestrel in Western Europe. Reintroduced (black asterisks) and captive (white asterisks) populations investigated in this study are indicated. See Table 1 for codes

Table 1 Polymorphism statistics of wild (W), captive (C) and reintroduced (R) populations of lesser kestrels across 8 microsatellites

| Population size | Code | N | Number of alleles per locus | | | | | | | | He–Ho | Rs | $F_{IS}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Fp5 | Fp13 | Fp31 | Fp46 | Fp79 | Fp89 | Cl347 | Cl58 | | | |
| Southern France (W) ↘100 BP | FRA | 26 | 5 | 3 | 6 | 6 | 17 | 3 | 6 | 3 | 0.60–0.60 | 4.59 | 0.04 |
| Ebro Valley (W) ↘1,000 BP | EBV | 174 | 6 | 4 | 7 | 10 | 33 | 4 | 10 | 5 | 0.65–0.64 | 4.92 | 0.026 |
| Spanish core area (W) 12,000–19,000 BP | SCA | 207 | 6 | 4 | 7 | 9 | 38 | 4 | 11 | 5 | 0.65–0.65 | 5.12 | 0.014 |
| Portugal (W) ↘300 BP | POR | 25 | 6 | 3 | 6 | 7 | 19 | 3 | 8 | 3 | 0.66–0.65 | 5.06 | 0.016 |
| GREFA (C) ↘100 BP | | 32 | 6 | 3 | 7 | 9 | 25 | 4 | 9 | 3 | 0.68–0.67 | 5.33 | 0.028 |
| DEMA (C) ↘100 BP | | 59 | 6 | 4 | 7 | 7 | 28 | 4 | 8 | 4 | 0.67–0.68 | 5.04 | −0.007 |
| Gerona (R) ↘50 BP | GER | 14 | 5 | 4 | 6 | 4 | 16 | 3 | 5 | 3 | 0.64–0.62 | 4.93 | 0.078 |
| Lleida (R) ↘100 BP | LLE | 25 | 5 | 3 | 4 | 7 | 21 | 4 | 8 | 4 | 0.63–0.61 | 4.95 | 0.060 |
| La Rioja (R) ↘50 BP | LRI | 16 | 4 | 4 | 5 | 7 | 14 | 3 | 8 | 3 | 0.63–0.64 | 5.02 | 0.011 |

The number of alleles detected at each marker in each population is indicated in its corresponding column. The number of individuals sampled at each population (N), expected heterozygosities (He), average observed heterozygosities (Ho) and allelic richness (Rs) estimates are showed. Allelic richness estimates were based on a minimum number of 14 individuals. Estimated population sizes in breeding pairs (BP) when the samples were taken are also given. See Fig. 1 for geographical locations

the genetic pools to avoid inbreeding. The proportion of birds which annually die (about 5%) is easily replaced given that this option is not constrained by the number of lesser kestrels available in the study area (see Table 1; Pomarol et al. 2004a for more details). To date, different captive stocks have contributed to several reintroduction and reinforcement programs in Spain and France (e.g. Pomarol et al. 2004a, http://crecerellette.lpo.fr/life/life.html).

Three reintroduced populations of lesser kestrels (Lleida and Gerona in Catalonia plus La Rioja, Fig. 1) were also investigated. The lesser kestrel disappeared from Catalonia (North Eastern Spain) as a breeding species in 1986. A reintroduction program beginning in 1989 has led to a population distributed in two main nuclei (Gerona and Lleida) which was estimated at 94 breeding pairs in 2003 (see Pomarol et al. 2004b). The lesser kestrel also disappeared from La Rioja (Central Northern Spain) around the second half of the XX century. After an evaluation of habitat suitability for the reintroduction of the species, the first colony was founded in 1997 by the release by hacking and subsequent return after migration of captive-born birds (Lopo et al. 2004 for more details). The population size of this colony was estimated at 13 breeding pairs by 2003. Finally, four geographically distinct natural populations (Southern France, Ebro Valley, Spanish core area and Portugal) were analysed to provide comparative data (see Table 1; Fig. 1).

## Sampling and DNA extraction

Biological samples for genetic analyses were obtained from wild and reintroduced populations during the 2002 and 2003 breeding seasons. Only one nestling per brood was analysed to minimize the sampling of related individuals. In 2004, we sampled the breeding stocks of DEMA and GREFA (see Table 1) as well as the captive-born progeny produced at the two largest colonial pens of DEMA (N = 96 nestlings).

The DNA extraction protocol we used follows that described by Gemmell and Akiyama (1996). Blood and feathers tips were digested by incubating with proteinase K in 300 µl of a buffered solution for at least 3 h. Proteins were selectively discarded by adding 1 volume of a 5 M LiCl solution and two volumes of chloroform–isoamylic alcohol (24:1). After centrifugation at maximum speed, DNA was precipitated using two volumes of absolute ethanol. Pellets thus obtained were dried and washed twice with 70% ethanol, and later stored at −20°C in 0.1 ml of TE buffer.

## Microsatellite genotyping

We amplified nine microsatellite markers originally isolated from the peregrine falcon Falco peregrinus (Fp5, Fp13, Fp31, Fp46-1, Fp79-4, Fp89, Fp107, see Nesje et al. 2000; Cl347 and Cl58, see Alcaide et al. 2008a). For each locus, the polymerase chain reaction (PCR) was carried out in a PTC-100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) using the following PCR profile: 35 cycles of 40 s at 94°C, 40 s at 55°C, 40 s at 72°C and finally, 4 min at 72°C. Each 11 µl reaction contained 0.2 units of Taq polymerase (Bioline, London, UK), 1× manufacturer-supplied buffer, 1.5 mM $MgCl_2$, 0.02% gelatine, 0.12 mM of each dNTP, 5 pmol of each primer and, approximately, 10 ng of genomic DNA. Forward primers were 5′-end labelled with HEX, NED or 6-FAM fluorocroms. Amplified fragments were resolved on an ABI Prism 3100 Genetic Analyser and later scored

using the GenMapper software version 3.5 (Applied Biosystems, Foster City, CA, USA).

### Genetic analyses

We excluded locus Fp107 from our analyses since previous paternity and population genetic studies conducted for lesser kestrels have shown the occurrence of null alleles and significant heterozygosity deficits at this locus (see Alcaide et al. 2005, 2008a, b, 2009). No mismatches in the segregation of alleles from parents to offspring, significant deviations from Hardy–Weinberg expectations or evidence of linkage disequilibrium between any pair of loci have been detected in previous studies after using the same molecular methods. We therefore employed the permutation test (N = 10,000) implemented in the program FSTAT ver 2.9.3 (Goudet 2001) to test for significant differences in genetic diversity among captive, wild and reintroduced populations. In order to avoid putative biases caused by uneven sampling, the software FSTAT calculates a standardised estimate of allelic richness ($R_S$) independent of sample size. Average observed heterozygosity (Ho) and the inbreeding coefficient $F_{IS}$ were also calculated and compared using FSTAT. The extent of population differentiation was calculated according to the traditional $F_{ST}$ estimate using the software GENETIX 4.04 (Belkhir et al. 1996). The significance of pairwise $F_{ST}$ estimates was given by a P-value calculated using 10,000 random permutations tests that were further adjusted according to sequential Bonferroni corrections for multiple tests (Rice 1989).

### Paternity inference within colonial enclosures

We inferred paternity at the two largest colonial breeding pens that were kept at DEMA facilities during the 2004 breeding season. Such colonial enclosures contained 36 and 16 adult kestrels, respectively, supplied with ad libitum feeding. All individuals were identifiable through PVC rings. Colonial enclosures consisted of several labelled nest-boxes which could be manipulated from the exterior of the building. Thus, eggs could be easily removed without disturbing the whole colony. All eggs were labelled according to where the nests they were laid to control for the origin of the artificially reared nestlings. Nests boxes were also provided with devices to observe the inside of the nest. Incubating females could therefore be identified. This fact, jointly with the registration of copulation events between marked birds, allowed us to elucidate what breeding pairs were attending each particular nest.

All adult birds and nestlings were genotyped at six out of the nine microsatellite markers mentioned above (Fp5, Fp31, Fp46-1, Fp79-4, Fp89 and Cl347). Locus Fp107 was excluded because of mismatches, probably due to the amplification of null alleles, in the segregation of alleles from parents to offspring (see Alcaide et al. 2005 for details). There was no special reason for excluding Loci Fp13 and Cl58 except for their comparably low polymorphism and because of the aim of accelerating the data collection process without compromising the resolution power of the molecular approach. Parentage exclusion for first and second parents, as well as the probability of two individuals sharing the same genotype was calculated with CERVUS 2.0 (Marshall et al. 1998) and IDENTITY 1.0 (Wagner and Sefc 1999), respectively. Mendelian inheritance was checked at every locus in each particular case. Those nestlings sharing alleles from their putative parents at all loci were considered actual offspring of the couple. The genotypes of the remaining males in the colony were also checked to assure unequivocal paternity assignments. Those cases in which nestlings would fail to match any of the two alleles of the putative father at two or more loci were considered as the result of extra-pair paternity.

### Calculation of variances in breeding success of captive kestrels during single-pairing breeding strategies

From 1996 to 2007, the number of fledglings produced by 35 reproductive lesser kestrels kept in TORREF was registered. The number of fledglings produced by 70 reproductive adults kept in GREFA was available from 2005 to 2007 breeding seasons. In both cases, we focused exclusively on those kestrels that raised offspring, so these numbers did not include non-breeding birds. We calculated the minimum number of breeding pairs that contributed to a high proportion of fledglings during the period of time investigated in each particular case.

## Results

### Genetic diversity in captive populations

The permutation test performed in FSTAT did not report statistically significant differences in allelic richness (5.04 vs. 5.18), average observed heterozygosities (0.64 vs. 0.68) or the inbreeding coefficient $F_{IS}$ (0.021 vs. 0.006) between wild and captive populations after analysing eight polymorphic microsatellite markers (all two-tailed P-values $\geq$ 0.05, see Table 1).

Both captive populations analysed (DEMA and GREFA) were genetically differentiated from the Ebro Valley and the French populations, but pair-wise $F_{ST}$ estimates did not significantly differ from 0 when compared to wild populations from southwestern Iberia (SCA and POR, Table 2).

Table 2 $F_{ST}$-pairwise values (above diagonal) between four geographically distinct natural populations of lesser kestrels (W), captive (C) and reintroduced populations (R)

| | EBV (W) | SCA (W) | POR (W) | FRA (W) | GER (R) | LLE (R) | LRI (R) | GREFA (C) | DEMA (C) |
|---|---|---|---|---|---|---|---|---|---|
| EBV (W) | | 0.003* | 0.005 | 0.012* | 0.008 | 0.006 | 0.013 | 0.010* | 0.008* |
| SCA (W) | | | 0.004 | 0.016* | 0.010 | 0.006 | 0.009 | 0.006 | 0.006 |
| POR (W) | | | | 0.027* | 0.007 | 0 | 0.010 | 0.003 | 0.008 |
| FRA (W) | | | | | 0.019* | 0.028* | 0.032* | 0.033* | 0.025* |
| GER (R) | | | | | | 0.001 | 0.030* | 0.017 | 0.013 |
| LLE (R) | | | | | | | 0.012 | 0.010 | 0.010 |
| LRI (R) | | | | | | | | 0.013 | 0.014 |
| GREFA (C) | | | | | | | | | 0.005 |

Significant values after Bonferroni corrections for multiple tests are indicated by asterisks. See Fig. 1 for geographic locations

## Single pairing versus colonial breeding strategies

The analysis of the breeding performance data set from the captive stocks of GREFA and TORREF revealed that, in both cases, only a low proportion of breeding pairs (28.6%) contributed to at least one half of the total number of fledglings produced (50 and 56%, respectively). Paternity inference within the colonial enclosures kept at DEMA facilities revealed similar results, with only seven breeding pairs (26.9% of the reproductive birds) contributing to 56% of the fledglings produced during the 2004 breeding season. Concerning mixed-reproductive strategies, we detected two cases of sequential polygyny, i.e. males raising two broods with successive females, in the largest colonial pen in DEMA. On the contrary, no genetic evidence of extra-pair paternity was found. All paternity assignments were assigned unequivocally, especially due to the highly polymorphic locus Fp79-4 (Table 1). The combined probability of exclusion for the microsatellite marker set that we used was estimated at 0.95. The likelihood of two individuals carrying an identical genotype was estimated at $6.21 \times 10^{-6}$.

## Genetic diversity in reintroduced populations

We did not find statistically significant differences in allelic richness (5.04 vs. 4.97), average observed heterozygosities (0.64 vs. 0.62) or the inbreeding coefficient $F_{IS}$ (0.021 vs. 0.049) between wild and reintroduced populations (all two-tailed P-values $> 0.05$). However, reintroduced populations showed statistically significant lower average heterozygosities (0.62 vs. 0.68) and higher inbreeding coefficients $F_{IS}$ (0.049 vs. 0.006) in relation to the captive demes from which released birds came (two-tailed P-values = 0.012 and 0.031, respectively).

Reintroduced populations only showed statistically significant evidence of genetic differentiation when compared to the geographically isolated population from Southern France (Fig. 1; Table 2). Genetic divergence in relation to the French population is comparably high in spite of the geographic proximity of reintroduced populations. Thus, reintroduced populations somewhat depart from the isolation-by-distance patterns documented for natural populations of lesser kestrels in Eurasia (see Alcaide et al. 2008a, b, 2009 for details).

## Discussion

This study supports the utility of several management recommendations, such as the registration of crosses between individuals in proper stud books and the frequent introduction of new individuals into the genetic pools, to maintain high levels of genetic diversity in captive populations of lesser kestrels without previous genetic monitoring. Polymorphisms statistics at 8 microsatellite markers in lesser kestrels argue against low genetic variation as a primary cause of the comparably low and occasionally very low hatching rates documented in captivity (see Colás et al. 2002; Pomarol et al. 2004a). Rather, high rates of hatching failure could be linked to other factors such as the feeding conditions of the breeding stock and/or the management of the eggs (e.g. Pomarol et al. 2004a). $F_{ST}$-pairwise estimates also revealed that both captive demes analysed did not differ significantly from their natural source population, a fact that reinforces the absence of strong fluctuations in the distribution of allele frequencies.

Genetic diversity in reintroduced populations did not differ significantly from natural populations in the absence of previous genetic monitoring either. From the perspective of population structuring, the departure of reintroduced populations from naturally occurring isolation-by-distance patterns (see Alcaide et al. 2008a, b) can be attributed to the lack of migration-drift equilibrium in recently founded populations (see for instance Leberg and Ellsworth 1999; DeYoung et al. 2003). However, our results suggest that

uneven contributions of reproductive birds to the captive-born progenies may be responsible for a non-optimal transmission of genetic diversity from captive stocks to reintroduced populations. This fact, which has been already documented in the literature for other captive flocks (e.g. McLean et al. 2008), is particularly important in lesser kestrels since many of the most prolific breeding pairs are forced to produce a second and even a third clutch during the same breeding season (Pomarol et al. 2004a; J. L. Antolín et al., personal communication). As this study shows, large variations in reproductive success of individuals are similarly occurring for both single-pairing and colonial breeding facilities, with only about one-fourth of the reproductive birds producing 50–56% of fledglings. Hence, the occurrence of polygynous behaviours at low rates does not seem to significantly decrease the effective population size. The lack of extra-pair fertilizations, on the other hand, suggests that an increase in mate guarding might have overridden the effects of large breeding densities or female promiscuity in colonial breeding systems with ad libitum feeding. Our results do not seem to support smaller variances in individual breeding success linked to social stimulation of breeding and a broader availability of potential mates either.

Founder effects during both captive breeding and settlement stages can be counteracted by minimizing the release of related birds into the same location. A recent study by Lenz et al. (2007) also suggests the utility of manipulating sex-ratios to increase the effective population size during captive breeding of this species. Immigration is particularly important to diminish average genetic similarity and increase overall heterozygosity, as it has been already demonstrated by Ortego et al. (2007) in natural population of lesser kestrels. The effect of conspecific attraction in this respect is particularly well documented (Serrano and Tella 2003; Serrano et al. 2004, but see Calabuig et al. 2008), and thus, birds kept in pens or even decoys can be regularly used in newly established colonies to promote both settlement of released individuals and recruitment of wild birds. As Pomarol et al. (2004b) have previously indicated, immigration from the close Ebro Valley population may have decisively contributed to population growth in the reintroduced populations in Catalonia (GER and LLE). Such gene flow events may also explain the lack of significant patterns of genetic differentiation between natural and reintroduced populations (Table 2). Although immigration may involve individuals dispersing long distances, as exemplified by one bird from the Ebro Valley (North Eastern Spain) recruited as a breeder 300 km away in the reintroduced population of Villena (Middle Eastern Spain, M Alberdi, personal communication), dispersal probabilities between populations sharply decrease with geographic distance in this species (Serrano and Tella 2003; Alcaide et al. 2008a, 2009). Given that reintroduction programs may be necessary in highly isolated areas where natural colonization and immigration are highly improbable, reintroducing genetically diverse birds may be of importance to guarantee population persistence.

In conclusion, this study revealed high levels of genetic variation for ongoing but non-genetically monitored captive breeding and reintroduction programs of the lesser kestrel. However, we found a significant loss of genetic variation from captive flocks to reintroduced populations because of large variances in breeding performance of individuals. Although the lesser kestrel program does not seem to be seriously compromised by this finding, this information could be crucial for highly endangered species in which the number of founders remains below the recommended minimum (20–30 individuals), and where the incorporation rates of new birds to refresh the genetic pools and natural gene flow is comparably low. Undoubtedly, genetic monitoring is a desirable practice to maximize reproductive success and genetic variation in captive-born individuals which will be subsequently released into the wild or used to supplement the captive stocks (Frankham et al. 2002; see examples in Gautschi et al. 2003; Ralls and Ballou 2004; Hedrick and Fredrickson 2008). Genetic monitoring can however become costly and time-consuming, especially if molecular markers for the target species are not available. Since some conservation initiatives cannot simply afford it, the experiences summed from other captive breeding and reintroduction programs can become of high assistance.

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