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Short Communication

Evaluating ex situ conservation projects: Genetic structure of the captive population of the Arabian sand cat

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ABSTRACT

Ex situ conservation plays an increasingly important role in the conservation of endangered species. Molecular genetic markers can be helpful to assess the status of captive breeding programmes. We present the first molecular genetic analysis of the captive population of the Arabian sand cat (*Felis margarita harrisoni*) using microsatellites. Our data indicates that the captive population of *F. m. harrisoni* comprises three genetic clusters, which are based on different founder lineages. Genetic diversity was relatively high, the effective population size even exceeded the number of founders. This was presumably caused by subsequently integrating unrelated, genetically diverse founders into the captive population and a careful management based on minimizing kinship. However, we detected an error in the studbook records, which might have led to incestuous matings and underlines the usefulness of molecular evaluations in captive breeding programmes for endangered species.

Ex situ conservation and reintroduction projects play an increasingly important role in the conservation of threatened species (Fischer and Lindenmayer 2000; Frankham et al. 2010). However, the success of such breeding programmes is strongly determined by genetic processes, such as inbreeding depression, loss of genetic diversity (reviewed in Witzenberger and Hochkirch 2011), outbreeding depression (e.g. Marshall and Spalton 2000) and adaptation to captivity (e.g. Williams and Hoffman 2009). In order to avoid such negative effects, studbooks have been established for many endangered species. Data on the genetic diversity and the relationships within a captive population can provide valuable information for the improvement of ex situ conservation programmes for endangered species (O'Brien 2006).

The sand cat (*Felis margarita*) is a small felid species with a disjunct distribution in northern Africa and western Asia, where it occurs at low densities. It is classified as near threatened on the IUCN red list (Mallon et al. 2008). Four subspecies have been described, only two of which (*F. m. harrisoni* and *F. m. scheffeli*) have ever been represented in captivity (Akers 2009). The first individuals bred in captivity mostly belonged to the subspecies *F. m. scheffeli*, a subspecies which is endemic to Pakistan. Nowadays, the captive population consists almost exclusively of the Arabian sand cat (*F. m. harrisoni*), which occurs on the Arabian Peninsula and in Jordan (Akers 2009). Based upon the international studbook, coordinated ex situ breeding of this subspecies started in the 1970s and the current population can be traced back to 18 founders of *F. m. harrisoni* (Akers 2009). Further 10 potential founders have been integrated but have yet to provide offspring. Since 1988 the captive population of the Arabian sand cat has been coordinated globally by an international breeding programme, which is however split into two more or less independent regional subsections: The North American Species Survival Plan (SSP) which provided the basis for this breeding population and the European endangered species programme (EEP),which was established in 1998 (Magiera 2011). Until the end of 2009 the complete captive population has reached 200 individuals (Akers 2009), 102 of which are registered in the EEP (Magiera 2011).

Here, we present the first genetic analysis of the captive population of the Arabian sand cat using nine microsatellite loci. Our aim was to analyze whether the relatively small breeding stock is affected by inbreeding and the loss of genetic diversity and if the pedigree information is correct.

The European studbook for *F. m. harrisoni* currently records 23 holders (Magiera 2011). All holders registered in the EEP studbook were contacted and asked to contribute samples to this analysis. We obtained 44 samples from nine zoos. DNA was extracted from tissue and blood using the DNeasy blood and tissue kit (Qiagen) following the manufacturer's instructions (with a special protocol for blood). For hair samples we extracted the DNA with a modified Chelex 100 protocol, using 400 μl of a 10% Chelex solution with addition of 7 μl Proteinase K (18 mg/ml) per sample and an overnight lysis step (Estoup et al. 1996; Walsh et al. 1991).

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All samples were genotyped at nine di-nucleotide repeat microsatellite loci (FCA08, FCA23, FCA45, FCA58, FCA77, FCA90, FCA126, FCA132 and FCA149). These primers were originally characterized in the domestic cat (Menotti-Raymond et al. 1999; Menotti-Raymond and O'Brien 1995). Amplification was performed in a Mastercycler (Eppendorf) using the 2.5× 5PRIME HotMasterMix (5PRIME). For each PCR we used 5 μl reaction mix containing: 1.2 μl genomic DNA, 2.2 μl HotMasterMix, 2.2 μl water and 0.1 μl of the forward and reverse primers. The PCR conditions were as recommended by the manufacturer, with an annealing temperature of 55 °C for most of the primers (exceptions: FCA45: 62 °C, FCA77: 58 °C, FCA90: 52 °C). The 5′-end of each forward primer set was labelled with a fluorescent marker (5-FAM, TMR or JOE). The products were genotyped on a MegaBACE 1000 automated DNA sequencer (GE Healthcare). Fragment lengths were determined using MegaBACE Fragment Profiler (Amersham Biosciences). To minimize genotyping errors such as allelic dropout due to low DNA concentrations we applied a multiple tube approach, as recommended in Taberlet et al. (1996). Samples were only included into further analyses if they yielded unambiguous results in three independent replications.

We tested our data for the occurrence of null alleles with Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) and for linkage disequilibrium with Fstat 2.9.3.2 (Goudet 1995). The genetic structure of our sample was analyzed using structure 2.3.3 (Pritchard et al. 2000). For this we assumed admixture to be possible and chose the correlated allele frequency model with a burn-in of 100,000 simulations followed by one million Markov chain Monte Carlo simulations. Tests were run for $K = 1–10$ with ten iterations per K. As the method for inferring the optimal K value suggested by Evanno et al. (2005) tends to result in low K values (Campana et al. 2010; Hausdorf and Hennig 2010) and generally works better for scenarios with strong genetic differentiation (Waples and Gaggiotti 2006), we used both the method described by Pritchard et al. (2000) and the method suggested by Evanno et al. (2005) to infer K. In our case, both methods revealed an identical value for K. The individuals were assigned to genetic clusters using the highest assignment probability. A F_{ST} based AMOVA with 9999 iterations was performed in GenAlEx 6.4 (updated from Peakall and Smouse 2006) with the three structure based genetic clusters as populations.

We used Fstat to calculate the number of alleles (n_a) and the inbreeding coefficient (F_{IS}). Expected and observed heterozygosities (H_e and H_o) for each locus and population were determined in GenAlEx. The accuracy of linkage disequilibrium methods to assess the effective population size has been criticized by several authors (e.g., Beebee 2009; England et al. 2006), while the Bayesian approach was rated to be more accurate (Beebee 2009). Therefore, the effective population size (N_e) of the captive populations was determined with ONeSAMP (Tallmon et al. 2008), which uses a Bayesian approach. The upper and lower bounds of the prior distribution for N_e were 2 and 500, respectively. Additionally we performed a Wilcoxon signed rank statistic implemented in the software package Bottleneck 1.2.02 (Cornuet and Luikart 1996) to test for genetic signatures of population bottlenecks. The two-phase mutational model (TPM) is the most likely mutation model for microsatellites (Piry et al. 1999). We therefore used the TPM and set the proportion of stepwise mutations to 0.3 (setting the proportion to 0.7 did not change the results).

The mean relatedness between individuals was calculated using Coancestry (Wang 2010). This programme calculates seven different relatedness estimators. We tested all of them and assessed their performance by comparing the results with known relationships. Based upon these comparisons, we chose the estimator based on Queller and Goodnight (1989), as it showed the smallest deviation from our known relationships and the smallest variance. Additionally, we tested the relatedness between the breeding pairs and the four sampled founders with the same estimator. To measure individual F, four estimators are available in Coancestry. We used the TrioML estimator based on Wang (2007) as it fitted our data best based on the relatedness data for the whole captive population (see above).

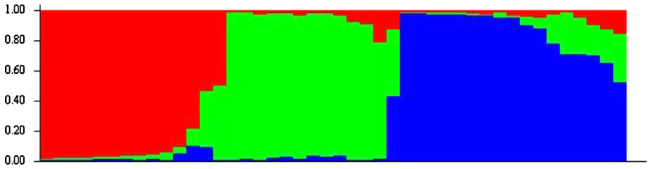

Fig. 1. Genetic clusters obtained from the Structure analysis ($K = 3$) for all samples ($n = 44$). For definition of clusters see text. Each individual is represented by a single vertical line, divided into K colours. The coloured segment shows the individual's estimated proportion of membership to that genetic cluster.

There was no evidence for null alleles, large allele drop-out or scoring errors and all pairwise tests for linkage disequilibrium were not significant ($P > 0.05$). However, two juveniles did not match their parents and siblings at six of the nine analyzed loci but perfectly matched a second breeding pair of the concerning zoo.

The most likely number of genetic clusters (K) revealed by structure was three (Fig. 1), independent of the method used to determine the optimal K. According to studbook data, these three clusters can be explained by their ancestry. Our sample includes genetic information from twelve of the original 18 founders (four via direct sampling and further eight by indirect sampling of their descendants). Individuals assigned to cluster 1 (red in Fig. 1) show a direct descent from three founders captured at Ad Dawhah, Qatar (studbook # 403, 409 and 504). Nearly all individuals from this cluster are currently kept in the Al-Wabra Wildlife Preservation (Qatar). Individuals assigned to cluster 2 (green) can be traced back to eight founders. These include individuals which are descendants from four founders from Al-Wabra (studbook # 403, 409, 504, 510), two from Saudi Arabia (studbook # 231 and 340) and two from Riyadh Zoo (Saudi Arabia, studbook # 261 and 228). Cluster 3 (blue) includes individuals with a complex ancestry, which can be traced back to eleven founders. It includes all already mentioned founder lineages (except for studbook # 510), with the addition of one Saudi Arabian founder from Riyadh Zoo (studbook # 235) and another Saudi Arabian breeding line which originated in the Society of Scientific Care Inc (USA, studbook # 143, 149 and 150). The individuals assigned to cluster 2 and 3 are now living in several zoos without any obvious geographic correlation to the genetic clusters. Individuals from Al-Wabra Wildlife Preservation are found in all clusters except cluster 3, which comprises only sand cats from European zoos. Cluster 2 is a mixture of sand cats from Al-Wabra Wildlife Preservation and Ebeltoft Zoo. The AMOVA revealed, that most of the genetic variation occurred within individuals (85%), the difference among individuals was not significant (0%), but a significant portion was explained by the difference between the three genetic clusters (15%).

H_e and H_o were rather high in the ex situ population of the Arabian sand cat and mean F_{IS} was not significant (Tables 1 and 2). The estimated N_e was 22.16 (median, 95% CI: 18.7–37.6). We detected a significant sign for a recent genetic bottleneck (Table 1). The mean relatedness between dyads was $r = -0.024 \pm 0.009$. The mean

Table 1
Genetic parameters of the captive populations of *Felis m. harrisoni* (with n = number of samples, n_a = mean number of alleles per locus, H_0 and H_e = observed and expected heterozygosity) and two-tailed P-values of the Wilcoxon test for heterozygosity excess or deficiency under the two-phase mutational model (TPM).

n	n_a	H_0		H_e		TPM
44	4.67	0.649	95% CI: 0.58–0.72	0.655	95% CI: 0.60–0.71	0.00391*

* Significant values ($P < 0.05$).

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Table 2
Comparison between studbook and genetic data for the captive population of *F. margarita harrisoni* based on the 2009 data with $N_c = 200$; f = inbreeding coefficient derived from studbook data, F_{IS} = inbreeding coefficient derived from molecular genetic data.

Studbook		Genetic data		
f	0.095	F_{IS}	0.020	95% CI: −0.06–0.10
n effective founders	18	N_e	23	95% CI: 18.7–37.6
Ratio N_e/N	0.12	Ratio N_e/N	0.15	

individual inbreeding coefficient (F) was 0.100 ± 0.016. Within the breeding pairs of *F. m. harrisoni* we found a mean r of 0.025 ± 0.08. Most sand cat breeding pairs (56%) had a low relatedness $r \leq 0.05$. However, there were two breeding pairs which showed very high relatedness values of 0.45 and 0.32. In both cases studbook data documented, that these were pairings of parents with their offspring. The mean relatedness between the 4 sampled sand cat founders was -0.089 ± 0.07. All dyads in this comparison showed negative relatedness values except for studbook # 403 and 504, which had a comparably high relationship of $r = 0.171$. The relationship of this last dyad was much higher than the mean relatedness in the whole captive population (see above).

There was a difference in the inbreeding coefficients derived from genetic and studbook data (Table 2), with a higher value for the studbook inbreeding coefficient f than for the molecular genetic inbreeding coefficient F_{IS}. Based on the studbook records, there were 18 founders for the sand cat population (with further ten potential founders). Our genetic data suggests an effective population size (N_e) of 23. The results concerning the ratio of N_e/N were more or less similar for genetic and studbook data.

Our sample of the captive population of the Arabian sand cat can be divided into 3 genetic clusters (Fig. 1). This division is presumably influenced by the geographical origin of the founders. Unfortunately, the studbook information on the founder origin is rather poor (e.g. "Saudi Arabia"). However, the population subdivision might also be influenced by skewed breeding success in the zoos. Zoos within the natural range of the species keep the majority of founders, larger breeding groups and have a high breeding success.

Our data shows that the genetic diversity and N_e of *F. m. harrisoni* was relatively high (Table 1). F_{IS} was low, suggesting that inbreeding does not play a substantial role. The captive population showed significant traces of a recent genetic bottleneck (Table 1), which might be a result of a founder effect caused by the transfer of individuals to the captive population. Alternatively, it might have occurred due to the population decline in the wild population. Taking all genetic measures into account, the choice of the founders and coordinated breeding apparently made it possible to (at least partly) compensate for the small population size and to retain a relatively high degree of genetic diversity. As the genetic diversity of ex situ populations is mainly determined by the gene pool of the founders and their reproductive success, it seems likely that the founders of the Arabian sand cat population were not closely related. This is also suggested by the fact that they were caught and integrated into the captive population over several years (captures occurred between 1977 and 2008 at different sites, Akers 2009). The high genetic diversity of the captive population also resulted in a high N_e, which even exceeds the actual number of founders. Although N_e is usually smaller than the census population size N_c (Frankham et al. 2010), Kalinowski and Waples (2002) state, that N_e can exceed N_c in intensively managed populations. The integration of the additional 10 potential founders (which have not yet reproduced) will probably even further improve the genetic diversity in the captive population.

The captive population of the Arabian sand cat is managed through the most widely accepted and applied breeding strategy of "minimizing kinship" (Falconer and Mackay 1996). This strategy aims at conserving a maximum of genetic diversity by reducing the mean relatedness within the population (Ballou and Lacy 1995; Saura et al. 2008). However, detailed studbook information is needed to assemble optimal breeding pairs with this approach (Ballou and Lacy 1995; Caballero and Toro 2000). In the case of the Arabian sand cat these detailed studbook records are available. Both the studbook and the genetic data show, that the captive population has a very low mean relatedness.

With 18 founders the captive population of Arabian sand cats reaches the critical founder size which is needed to conserve a high amount of genetic diversity and avoid inbreeding (Witzenberger and Hochkirch 2011). These founders have been integrated into the captive population over a time span of 20 years, which is conform with recommendations to minimize genetic differentiation between wild and captive populations and avoid adaptation to captivity (Frankham 2008; Gilligan and Frankham 2003; Robert 2009; Williams and Hoffman 2009). The fact that the zoos which keep the highest number of individuals and house several successful breeding pairs are situated within the natural range of the species will help to impede an undesirable genetic adaptation to captivity. The current size of the captive population (~200) is probably also sufficiently high to retain this good status for a longer period (reviewed in Witzenberger and Hochkirch 2011), as long as it is carefully managed and the captive population size is not significantly reduced. Up to now, the genetic diversity in the captive population ($H_e = 0.655$) exceeds the genetic diversity found in captive populations of other endangered species, which have a mean H_e of 0.57 (Witzenberger and Hochkirch 2011).

The benefit of genetic monitoring is exemplified by the fact that our data revealed a mistake in the studbook records. We assume, that the 2 mismatching juveniles had accidently been assigned to the wrong parents. Erroneous data like this can have a strong influence on the statistical values that are calculated from studbook data (f, N_e/N,...). Mistaken identities or other erroneous information in studbooks have already been discovered in several breeding programmes (e. g., Bowling et al. 2003; Marshall et al. 1999; Signer et al. 1994). The detection of such wrongly assigned parentages by genetic analyses can prevent that individuals supposed to be unrelated are accidently paired with close relatives. Studbook errors can unintentionally accelerate the loss of genetic diversity and thus lead to severe inbreeding problems. Hence, genetic monitoring programmes become increasingly important for the management of ex situ conservation projects (Frankham 2009; O'Brien 2006; Saura et al. 2008).

The genetic inbreeding coefficient F_{IS} was lower than the value calculated from the studbook. This is rather surprising, as close relationship between founders as well as homoplasy and null alleles may increase F_{IS} values considerably. Therefore, one would expect F_{IS} values to exceed the studbook inbreeding coefficient f. We found a rather similar N_e/N ratio in the molecular genetic and studbook data (Table 2). This ratio usually lies between 0.15 and 0.4 (with a mean of 0.3) for captive populations (Ballou et al. 2010). In the ex situ population of *F. m. harrisoni* the N_e/N ratio was 0.15 for the genetic and 0.12 for the studbook data. Therefore, the genetic contribution of the founders seems to have been rather evenly distributed. However, it needs to be noticed that the number of available microsatellite loci for this study was rather low.

Although our data suggest that the captive population of the Arabian sand cat is in a good condition, there are still several

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factors which can act as impediments for successful captive breeding programmes. In the Arabian sand cat 57 wild individuals were captured over the years, but only 18 of them provided enough surviving offspring to contribute to the genetic diversity of the ex situ stock. Such a low breeding success is problematic in species with a higher extinction risk than in the Arabian sand cat. Stress during transportation, infections, injuries and also incompatibilities with exhibition mates can always lead to a loss of valuable individuals, which needs to be avoided. Hence, new individuals from the wild should soon be integrated into the captive stock. The genetic value of each new individual could be assessed, if gene banks for ex situ conservation programmes were established.

Other factors which might thwart captive breeding programmes are the high costs and the legal regulations for the international exchange of individuals. This problem can lead to more or less isolated breeding lines on the different continents. Regional studbooks can also be misleading, as only a fraction of the captive population is considered. This might increase the probability to disregard holders. Furthermore, the regulations and high fees of zoo associations (e. g. AZA, EAZA) can complicate the cooperation with (sometimes very successful) breeders, who are not members of these associations (Clegg 2011).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2004.08.011.

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