Analysisofrelatednessanddeterminationofthesourceoffoundersinthe captivebeardedvulture, *Gypaetusbarbatus* ,population

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Abstract

Genetic relatedness among founders is a vital parameter in the management of captive populations as kin structure can have a significant effect on subsequent population structure. Methods for inferring relatedness from microsatellite markers have all been developed for natural populations; their applicability to captive populations with unknown founder origins needs therefore testing. We used information derived from 14 microsatellites in 177 individuals and Queller and Goodnight's approach, to estimate relatedness in the captive bearded vulture population and to test the assumption of unrelated founders. Mean relatedness of known parent–offspring, full-sib and half-sib pairs within the captive population were in agreement with theoretical distributions. Pairwise relatedness values among the founders had a mean of -0.051 (SE $\pm$ 0.007) and their distribution did only differ marginally from the one found in the natural Pyrenean population. A maximum likelihood approach was used to determine the likelihood of founder pairs to be as closely related as full-sibs or parent–offspring. These results were combined with data from 268 bp mitochondrial DNA control region sequences and studbook information. We could exclude a close relationship among the majority of the 36 successfully reproducing founders. Our study therefore removes management concerns about hidden problems of inbreeding and inbreeding depression. It demonstrates the applicability of relatedness estimates based on microsatellite allele frequency data even in captive populations. Furthermore, we verified studbook information on the origin of two founders from the Pyrenees, and show the value of assignment tests based on microsatellites for deducing founder origins and their important role in future monitoring projects.

Introduction

The knowledge of kinship relationships and the degree of relatedness among individuals play central roles in several fields of biology, including quantitative genetics, social behaviour and conservation genetics (Lynch and Ritland 1999). Especially in captive breeding programmes, knowledge of relatedness is essential to minimise mating between close relatives and to reduce the risks of inbreeding depression and loss of genetic variation due to random genetic drift (Montgomery et al. 1997). The bearded vulture (*Gypaetusbarbatus* , Linnaeus 1758) captive-breeding programme was initiated in the late 1970s to establish a source population for reintroduction of bearded vultures in the Alps (Frey et al. 1995). The degree of relatedness among the 57 incorporated individuals (throughout this manuscript we will refer to them as founders, irrespectively of whether they have reproduced or not) is unknown and only vague information about their origin is available (Frey et al. 1995). Most of these founder birds seem to come

from several regions within the former Soviet Union including the Caucasus and Turkmenistan; three birds are recorded to come from the Pyrenees, two from Afghanistan, and one each from Crete and mainland Greece (Frey et al. 1995). In absence of detailed information about relatedness, all founder birds in the captive bearded vulture population were assumed to be unrelated. The determination of the relative degrees of relatedness among individuals in a captive population with founders that are derived directly from the wild or are descendants of individuals of unknown relationship can only be achieved through inferences using molecular markers (Avise 1995). To assess pairwise relatedness based on microsatellite data, two major classes of estimator, the maximum-likelihood and the methods-of-moments approaches have been used (Blouin et al. 1996; Nesje et al. 2000; Pouyaud et al. 1999; Ritland 2000). The first are best suited for discriminating among hypothesised relationships, while the latter are designed to estimate relatedness based on probability of identity-by-descent (Ritland 2000). However, both methods were developed for estimating relationships in natural populations and include the population allele frequencies as parameters. Their applicability to an artificially built population with unknown origin of founders such as the captive bearded vulture population remains to be tested.

Therefore the first objective of the present study was to test whether mean relatedness of all known parent–offspring, full-sib and half-sib pairs within the captive population were in agreement with theoretical expectations when using allele frequencies of the total captive population and the method-of-moments estimator of Queller and Goodnight (1989). We further used the information derived from 14 microsatellite loci in 150 individuals to estimate pairwise relatedness among the founders and to test whether the assumption of no relatedness among the founders could be upheld. In addition, we used a maximum likelihood approach (Goodnight and Queller 1999) to discriminate between full-sib and parent–offspring versus unrelated pairs among the founders. To overcome part of a possible bias when inferring relationship among the founders based on the allele frequencies of an artificially built captive population, we further looked at genetic similarity among the founder birds by sequencing a 268 bp fragment of the mitochondrial DNA (mtDNA) control region. As mtDNA is predominately maternally inherited (Dawid and Blackler 1972) full-sib and mother offspring pairs are expected to share the same mtDNA haplotype. By looking at these maternal lines within the captive population we were therefore able to additionally test whether any of the founder birds could be as closely related as two full sibs or a mother and offspring.

A second objective of this study was to explore the possibilities of deducing the geographic origin of the founder birds, as estimation of relatedness among founders would greatly gain in accuracy when allele frequencies of their origin population could be used in the calculations. In the present case only one of the potential source populations, the one from the Spanish Pyrenees, has been characterised genetically (Negro and Torres 1999; Gautschi et al. 2003). At present, we therefore only estimate the probability with which founder birds are derived from this natural population and if this probability is highest for the three individuals indicated in the studbook entries as Pyrenean birds. The comparison with other potential source populations will have to wait until further data become available.

Materialsandmethods

Samplecollectionandmicrosatellitetyping

The studbook of the captive bearded vulture population lists 57 founders, of which 39 had reproduced by the year 2000. Because the offspring of three founder birds died, all captive-born birds so far descend from 36 founder birds (Table 1). We obtained DNA samples from 39 founder individuals (24 males and 15 females) of the captive bearded vulture population, including 11 museum specimens of animals that had died already. Of these, 28 individuals have reproduced successfully by the year 2000 (Table 1). We further collected samples of 111 (62 males and 49 females) individuals born to the founders in captivity and 27 (11 males and 16 females) individuals from the wild population in the Spanish Pyrenees. DNA was extracted as described in Gautschi et al. (2003). We typed all birds for 14 microsatellite loci. The primer sequences, the conditions for the amplification of each locus, and the methods for allele detection were as described in Gautschi et al. (2000).

Relatednessanalyses

We estimated mean genetic relatedness between all parent–offspring, full-sib and half-sib pairs and among

Table1. Individuals listed as founders in the captive bearded vulture studbook which (A) have produced viable offspring so far, and (B) have not reproduced successfully by the year 2000. Listed are studbook number, sex, source used for DNA extraction, and suspected origin (studbook entry) of the founder

	Studbook id	Sex	DNA source	Suspected origin
A	BG002†	Male	(BG044)*	Unknown
	BG003	Female	Blood	Middle-west Asia
	BG004†	Male	Feather	Unknown
	BG009	Male	Blood	FSU
	BG010†	Female	Blood	Pyrenees
	BG014	Male	Blood	FSU
	BG019	Male	Feather	Kopetdag, Turkmenistan
	BG020†	Female	(BG005)*	Kopetdag, Turkmenistan
	BG021	Female	Feather	Unknown
	BG022†	Male	Blood	FSU
	BG023†	Female	(BG070)*	Asia
	BG026†	Female	Feather	FSU
	BG027†	Female	Feather	Unknown
	BG030†	Male	(BG104)*	Unknown
	BG031	Male	Blood	Unknown
	BG034	Male	Feather	FSU
	BG035†	Female	Feather	Unknown
	BG065	Male	Blood	Crete
	BG131	Male	Blood	FSU
	BG132	Female	Blood	FSU
	BG134	Male	Feather	FSU
	BG135	Female	Feather	FSU
	BG150†	Male	(BG107)*	FSU
	BG151	Female	Blood	FSU
	BG152	Male	Feather	FSU
	BG153	Female	Feather	FSU
	BG154†	Male	(BG118)*	FSU
	BG155	Female	(BG118)*	FSU
	BG159	Male	Feather	Caucasus
	BG161	Male	Feather	Kyrgyzstan
	BG162†	Female	(BG105)*	FSU
	BG178	Female	Blood	Greece
	BG199	Male	Blood	Unknown
	BG201	Male	Blood	Tadzhikistan
	BG270	Female	Feather	Altay mountains, Kazakhstan
	BG286	Male	Blood	Andorra, Pyrenees
B	BG001†	Male	Feather	Caucasus
	BG008†	Male	–	Pyrenees
	BG012†	Male	–	Unknown
	BG013†	Male	–	Unknown
	BG016†	Male	Blood	Unknown
	BG024†	Male	–	Asia
	BG025†	Female	–	Asia
	BG028†	Male	–	FSU
	BG029†	Male	Feather	FSU
	BG032†	Female	Feather	Afghanistan
	BG033†	Female	Feather	Afghanistan
	BG036†	Female	–	FSU
	BG037†	Male	–	FSU
	BG038†	Male	Feather	Central Asia
	BG059†	Male	–	Unknown
	BG075†	Female	Feather	FSU
	BG136†	Male	Feather	FSU border to Afghanistan
	BG158	Male	Feather	FSU
	BG204	Male	Blood	FSU
	BG205†	Male	–	Pyrenees
	BG232	Male	Blood	Aragón, Pyrenees

†Founders dead at the time of the study (1997–2000).
– No DNA samples available.
*Founders of which no DNA was available. The genotype of one offspring was used as a replacement in Kinship analysis. For females only, the mtDNA haplotype was inferred from this offspring.
FSU = Former Soviet Union.

the founder birds in the captive population as well as among the birds in the Pyrenean population with the programme relatedness (Queller and Goodnight 1989). This programme uses a regression measure of relatedness, which weights each allele inversely by its frequency in the population, so that rare alleles are given a relatively higher weight (Queller and Goodnight 1989). The expected relatedness values (R) are 0.5 among full sibs, 0.25 among half sibs and 0 among unrelated individuals. Note that negative values of R may occur if the gene frequencies of the two compared individuals differ from the population mean in opposite directions (Queller and Goodnight 1989). The relatedness estimates between individuals may also be biased if their relatives contribute greatly to the calculation of allele frequencies. To correct for this bias all known relatives may be excluded from the data set to calculate population allele frequencies. However, if the data set is large enough and includes many different groups or sets of relatives, the contribution of each single set of relatives is negligible (Blouin et al. 1996), we therefore did not apply bias correction in this study. To calculate pairwise relatedness among the founder birds we used the allele frequency of the total captive population. Pairwise relatedness between all known parent–offspring, full-sib and half-sib pairs was individually estimated using the population allele frequencies of the captive bearded vulture population. We then averaged over all pairs in a group to obtain mean relatedness values for parent–offspring, full-sib and half-sib pairs. These relatedness estimations with pairs of known relationship allowed us to evaluate the usefulness of the applied method (which assumes a random mating population) for the captive bearded vulture population. Relatedness among the Pyrenean birds was calculated using the population allele frequencies of the Pyrenean population. The relative nature of relatedness causes the mean relatedness among all individuals of a population calculated based on the allele frequencies of the same individuals to be zero, regardless of the true level of relatedness (Ritland 1996). We therefore avoided comparisons including the mean relatedness value of the Pyrenean population but compared the distribution of the pairwise values to the one observed in the founders of the captive population.

KINSHIP analysis

We used the programme KINSHIP version 1.2 (Goodnight and Queller 1999) to determine the likelihood

that pairs of founder birds are as closely related as full sibs or parent–offspring. The programme tests pedigree relationships between pairs of individuals and reports for each pair the likelihood ratio for a primary hypothesis vs. a null (Ho) hypothesis (e.g. likelihood of being related/likelihood of being unrelated). These hypotheses are described with the help of two variables, *Rp* and *Rm*, which define the probabilities that two individuals share an allele by direct descent from their father or mother, respectively. We tested the following primary hypothesis: pairs are full sibs and pairs have a mother offspring or a father offspring relationship. *Rp* and *Rm* values were set as described in Goodnight and Queller (1999). These *R*-values, the population allele frequencies, and the genotype combination of the two individuals are used to calculate the likelihood that a genotype combination could have been produced by the hypothesised relationship. To receive significance levels for a given likelihood ratio the programme generates from the allele frequencies in the data set series of pairs which match the null hypothesis and determines the ratio needed to reject the null hypothesis. Then it generates a series of pairs, which match the primary hypothesis to determine the type-II error rate. We used the allele frequency of the total captive population as a base to calculate the likelihood ratios for each pair of founders and the allele frequency of the Pyrenean population to resolve the kinship of the founders originating from the Pyrenees. Of eight founders from which no DNA samples were available, but that had successfully reproduced by the year 2000, we included the genotype of one of their offspring in the KINSHIP analyses (Table 1). To get an empirical estimate of the statistical power achieved, we calculated the likelihood ratios for all known full-sib pairs and recorded whether the true relationship was detected.

Assigningindividualstoasourcepopulation

According to studbook entries, 3 of the founder birds originated from the Pyrenees (Table 1). To verify this information and to explore the possibilities of deducing the geographic origin of founder birds in general, we applied an assignment test. This likelihood-based test assigns individuals to putative source populations based on the expected frequencies of their genotype in those populations (Rannala and Mountain 1997; Waser and Strobeck 1998; Cornuet et al. 1999). We used the software Doh implemented on the web at http://www.biology.ualberta.ca/jbrzusto/Doh.php to calculate "assignment indices" for all founder birds and all birds from the Pyrenees. The assignment index is the highest probability of an individual's genotype in any of the tested populations. The Doh software calculations are based on the descriptions in Paetkau et al. (1995, 1997).

MitochondrialDNA(mtDNA)analysis

From 39 founder birds we sequenced an approximately 270 bp fragment in the left domain of the control region. From four additional founder females (BG020, BG023, BG155 and BG162) we obtained information by sequencing parts of the control region of one of their offspring each (Table 1). Approximately 100 ng of total genomic DNA was used as template for a polymerase chain reaction (PCR) in 50 μl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 150 μM per dNTP (Amersham Pharmacia), 0.5 μM of each primer [QHD1-1F (5′-CCCAGCTATDTATWATTGTAC-3′) and Fbox2-R (5′-GTAGGTTCGACAGGAAATGGC-3′)], and 0.5 U of Taq DNA Polymerase (Amersham Pharmacia). We used the following thermotreatment: 30 to 45 cycles with 30 s at 95 °C, 30 s at 59 °C and 30 s at 72 °C. An initial denaturing step (95 °C, 5 min) was included and the last cycle was followed by an 8 min extension at 72 °C. The PCR product was purified from unincorporated primers and dNTPs using the PCR Purification Kit (QIAGEN) following the supplied protocol. DNA was ethanol precipitated and re-dissolved in 11 μl of double distilled water. We used 5.5 μl of the re-dissolved DNA in a 10 μl sequence reaction containing 0.5 μM primer and 2 μl of ABIPRISM^{TM} Ready Reaction BigDye-Dye-Terminator premix (Applied Biosystems) and 2 μl of 5 $\times$ buffer (400 mM Tris-Cl, 10 mM $MgCl_2$; pH. 9.0). Amplification conditions were 25 cycles of denaturing at 96 °C for 15 s, annealing at 50 °C for 10 s and elongation at 60 °C for 4 min. The sequencing reaction was purified using Sephadex-G50 (Amersham Pharmacia). Sequences were analysed on an ABI Prism310 Genetic Analyzer and data were edited with Sequence Navigator Software (Applied Biosystems) and aligned by eye. We determined the number of polymorphic sites and the number of haplotypes and recorded which founder birds share the same maternal line. Preliminary phylogenetic analysis performed with paup* version 4.0b5 (Swofford 2000) revealed very low resolution due to small numbers of parsimonious informative characters (results not

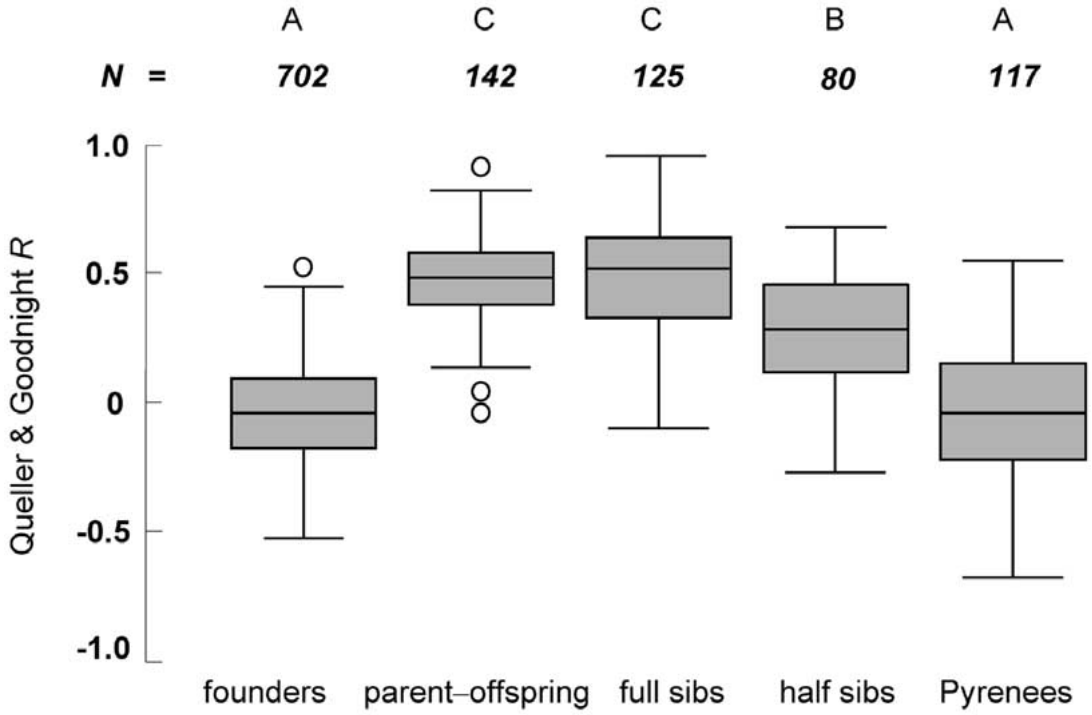

Figure 1. Mean relatedness R (Queller and Goodnight 1998) among founder-, parent–offspring, full-sib, and half-sib pairs in the captive bearded vulture population and among individuals from the Pyrenean population. The boxes represent the lower and upper quartiles, divided by the median. Vertical lines represent the 10 and 90% quantiles and circles indicate outliers. The number of pairs compared per group is indicated above. Groups classified with the same letter above the sample sizes were not significantly different (A = marginally different).

presented). We therefore did not perform any further phylogenetic analyses with this data. Nevertheless, the mtDNA data obtained were valuable for determining whether founder birds share the same haplotype and thus for exploring the possibility that they are as closely related as full sibs or mother and off-spring.

Results

Meanpairwiserelatedness

The relatedness values of all founder, parent–offspring, full-sib, and half-sib pairs analysed are shown in Figure 1. Mean relatedness among parent–offspring pairs and full-sib pairs was 0.468 (SE $\pm$ 0.012) and 0.470 (SE $\pm$ 0.020), respectively. These relatedness values were not significantly different from the expected 0.5 and were significantly larger than the mean relatedness found among half-sib pairs (Figure 1). Relatedness among half-sib pairs was on average 0.259 (SE $\pm$ 0.022) and not significantly different from the expected 0.25 (Figure 1). The relatedness among the founder birds was -0.051 (SE $\pm$ 0.007) which was significantly different from zero ($t_{1,703} = -7.373$; $P < 0.001$) and significantly smaller than the mean relatedness found among parent–offspring, full- and half-sib pairs (Figure 1). Female founder birds tended to be on average less related than male founders, but not significantly so (data not presented). The distribution of pairwise relatedness values of the founders did only differ marginally from the one found in the natural population of the Pyrenees (Kolmogorov-Smirnov goodness-of-fit test, Chi2 (2df) = 5.21; $P = 0.07$) (Figure 1). No difference was found between the mean relatedness of male and female individuals in the total captive and the wild Pyrenean birds, respectively (data not presented).

KINSHIP results

The founder pairs observed as having a likelihood ratio value exceeding 95% of all ratios of simulated unrelated pairs are given in Table 2. The corresponding type-II error probability (i.e. not detecting the hypothesised relatedness among the birds when

Table2. Pairs of founders with high probability of being full-sibs (A), mother–offspring (B) and father–offspring (C) according to KINSHIP analysis

	Founder 1 (studbook id)	Founder 2 (studbook id)	Sig. level[‡]	Origin[°]	mtDNA haplotype[Δ]
A	BG004	BG010	*	Unknown / Pyrenees	4 / 9
	BG009	BG010	**	FSU / Pyrenees	12 / 9
	BG010	BG029	*	Pyrenees / FSU	9 / 3
	BG010	BG132	*	Pyrenees / FSU	9 / 18
	BG016	BG131	**	Unknown / FSU	6 / 5
	BG022	BG158	*	FSU / FSU	8 / 2
	BG026	BG152	*	FSU / FSU	9 / 22
	BG026	BG161	**	FSU / Kyrgyzstan	9 / 12
	BG027	BG033	*	Unknown / Afghanistan	10 / 12
	BG027	BG134	*	Unknown / FSU	10 / 13
	BG027	BG151	*	Unknown / FSU	10 / 21
	BG029	BG158	*	FSU / FSU	3 / 2
	BG031	BG153	*	Unknown / FSU	22 / 10
	BG031	BG270	*	Unknown / Kazakhstan	22 / 15
	BG033	BG178	***	Afghanistan / Greece	12 / 25
	BG033	BG199	**	Afghanistan / Unknown	12 / 26
	BG034	BG038	*	FSU / Central Asia	(20) / 15
	BG034	BG158	*	FSU / FSU	(20) / 2
	BG038	BG135	*	Central Asia / FSU	15 / 3
	BG131	BG270	*	FSU / Kazakhstan	5 / 15
	BG134	BG151	*	FSU / FSU	13 / 21
	BG134	BG161	*	FSU / Kyrgyzstan	13 / 12
	BG134	BG201	**	FSU / Tadzhikistan	13 / (12, 13)
	BG152	BG159	*	FSU / Caucasus	22 / 24
	BG152	BG161	*	FSU / Kyrgyzstan	22 / 12
	BG152	BG270	*	FSU / Kazakhstan	22 / 15
	BG159	BG199	***	Caucasus / Unknown	24 / 26
	BG159	BG201	**	Caucasus / Tadzhikistan	24 / (12, 13)
	BG161	BG201	*	Kyrgyzstan / Tadzhikistan	12 / (12, 13)
	BG201	BG286	*	Tadzhikistan / Pyrenees	(12, 13) / 29
	BG232	BG286	*** (n.s.)[§]	Pyrenees / Pyrenees	28 / 29
	(BG020) BG005[#]	BG003	**	(Turkmenistan) / Middle-west Asia	(2, 3) / 2
	(BG020) BG005[#]	BG022	*	(Turkmenistan) / FSU	(2, 3) / 8
	(BG020) BG005[#]	BG132	*	(Turkmenistan) / FSU	(2, 3) / 18
	(BG020) BG005[#]	BG151	*	(Turkmenistan) / FSU	(2, 3) / 21
	(BG162) BG105[#]	BG026	*	(FSU) / FSU	(23) / 9
	(BG162) BG105[#]	BG152	*	(FSU) / FSU	(23) / 22
	(BG030) BG104[#]	BG270	*	(Unknown) / Kazakhstan	– / 15
	(BG150) BG107[#]	BG065	*	FSU / Crete	– / 16
B	BG270	BG004	**	Kazakhstan / Unknown	15 / 4
	BG270	BG131	***	Kazakhstan / FSU	15 / 5
	(BG020) BG005[#]	BG003	**	(Turkmenistan) / Middle-west Asia	(2, 3) / 2
C	BG034	BG038	**	FSU / Central Asia	(20) / 15
	BG034	BG158	*	FSU / FSU	(20) / 2
	BG134	BG201	***	FSU / Tadzhikistan	13 / (12, 13)
	BG004	BG270	**	Unknown / Kazakhstan	4 / 15
	BG131	BG270	***	FSU / Kazakhstan	5 / 15

[‡]Likelihood ratios exceeding that of 95% (*), 99% (**) and 99.9% (***) of simulated unrelated pairs.
[°]Studbook information about the origin of the founders.
FSU = Former Soviet Union.
[§]Not significant when allele frequencies of the Pyrenean population were used for the simulations.
[#]Non-sampled founders (studbook id in brackets) were represented in the analyses by the genotypes of one offspring, studbook id of offspring given.
[Δ]mtDNA haplotype numbers according to Appendix I.

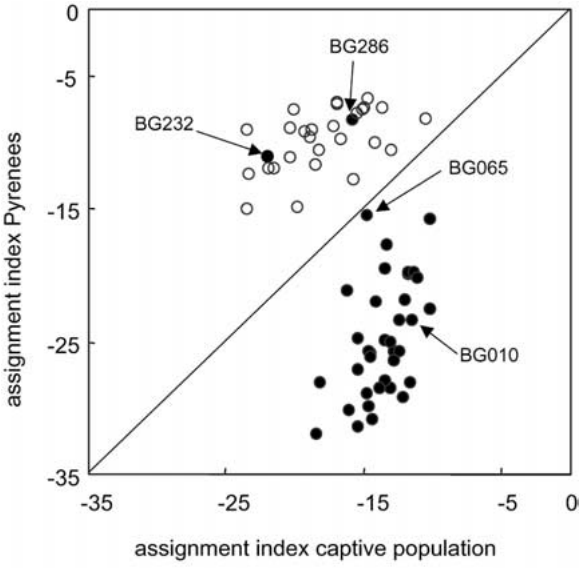

Figure2. Log expected frequencies ("assignment indices") of genotypes drawn from the captive (solid circles) and the Pyrenean (open circles) population. Each point represents a genotype. Founders BG232, BG286 and BG010 according to the studbook had Pyrenean origin. Individual BG065 is recorded to come from Crete.

it in fact occurs) for full-sibs was 0.043 and 0.155 when using the captive and Pyrenean population allele frequencies as a reference, respectively. The power predicted by the programme was supported by empirical data evaluation. Of 136 known full-sib pairs within the captive population, 129 (i.e. 94.8%) were detected. The type-II error for both father offspring and mother–offspring relationships within the captive population was <0.001. The two founders originating from the Pyrenees, BG232 and BG286, had a significant likelihood ratio only when the simulations were based on the allele frequencies of the captive population (Table 2). Based on the Pyrenean allele frequencies no significant ratio value was detected for this pair (Table 2). Included in Table 2 are the significant likelihood ratio values produced by the genotypes of the offspring of non-sampled founders and the genotypes of sampled founders. The significant values produced by the offspring and their parents are not included.

Assignmenttest

The log expected frequencies (i.e. assignment indices) of genotypes from the founders and the Pyrenean individuals in these two populations are plotted in Figure 2. Two individuals from the captive population were assigned to the Pyrenean population. These are the founders BG232 and BG286, both known to originally come from the Pyrenees (above the 45° line in Figure 2). The genotype of the founder BG010, also recorded to come from the Pyrenees, however, had a higher probability to come from another source population, as it clearly groups with the captive population. Individual BG065, a founder bird from Crete, is intermediate between the two groups (Figure 2).

MitochondrialDNA(mtDNA)analysis

We identified 27 clearly distinguishable mtDNA haplotypes among the nucleotide sequences of mtDNA in 43 of the founder birds (Appendix I). The 268 bp fragment contained 19 variable sites (Figure 3). Nucleotide sequence substitutions were responsible for 17 of these sites whereas two sites were indels (insertions/deletions) in a short $(AT)_n$ microsatellite region (Figure 3, sites 18 and 19). The mtDNA sequences of six founders contained a sequence ambiguity at variable position 2 (Appendix I). Because of these ambiguities, the haplotypes of four founders could not be identified distinctively (e.g. the haplotype of founder BG201 is either identical to haplotype 12 or 13; Appendix I). Only 15 of the variable characters were parsimoniously informative (i.e. sites that have a minimum of two nucleotides that are present at least twice).

Combiningmicrosatelliteanalyses,mtDNAresults, andstudbookentries

None of the sampled founder birds sharing one of the clearly distinct mtDNA haplotypes (Appendix I), revealed a significant likelihood ratio value in the three pedigree relationships tested with KINSHIP (Table 2), and with the exception of BG003 and BG158, founders sharing the same haplotype were not located on the same clade in a neighbour-joining tree based on the proportion of shared alleles (data not presented). BG003 and BG158, however, did not have a significant likelihood ratio based on KINSHIP analysis (Table 2). Further, we can reject the primary hypothesis of three genetically likely father–offspring pairs (Table 2) due to studbook information. BG270, a founder caught at an age of approximately 10 years in 1995, can not be an offspring of BG004 that died in 1994 after living 30 years in captivity. Similarly, neither BG131 nor BG134 can be the fathers of BG270 and BG201 (the latter was born around 1988), because both of these potential fathers have been living in captivity

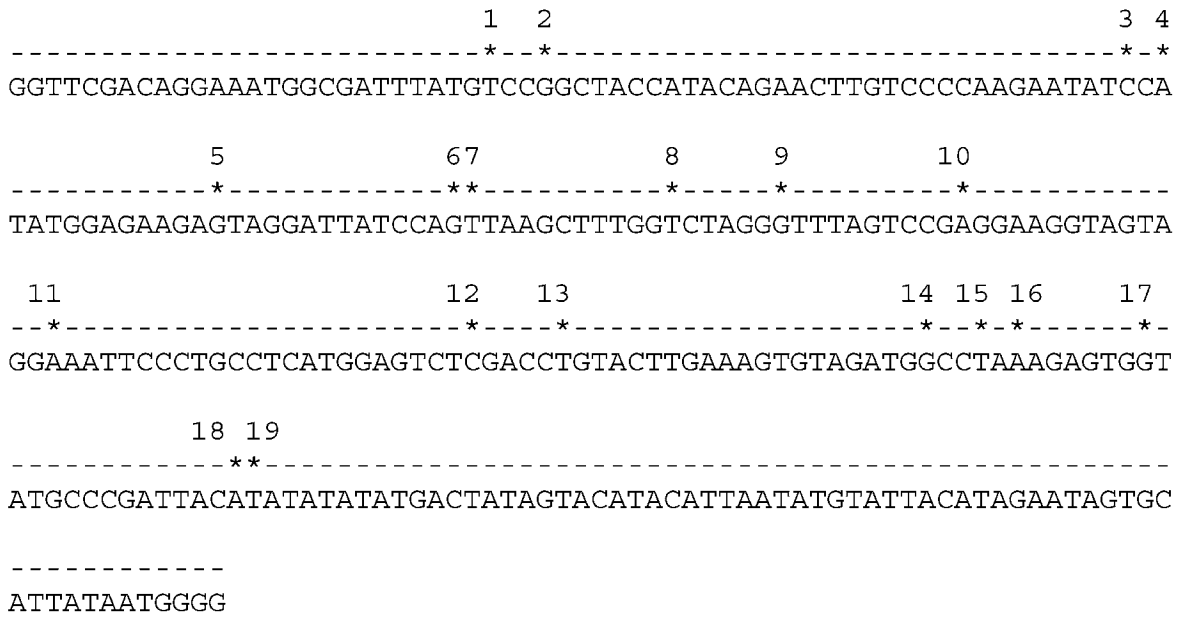

Figure 3. 268 bp nucleotide sequence of the control region in the bearded vulture (5′-3′ orientated). Stars above letters indicate variable positions, consecutively numbered from 1 to 19.

since before mid 1970s. The other two genetically likely father offspring pairs have no impact on the genetic composition of the captive population, as BG038 has died without reproducing (Table 1) and BG158 is unlikely to reproduce due to its advanced age (H. Frey, personal communication). However, we can not exclude a close relationship between founder BG201 and either founder BG161 or BG134. They have a high probability of being as closely related as full sibs according to KINSHIP (Table 2A) and BG201 has an ambiguous mtDNA sequence at variable position 2, indicating either an identical haplotype to BG161 or BG134 (Appendix I). In addition, when including genotype information from the offspring of non-sampled founders, we can not exclude a close relationship between the founders BG003 and BG020, and BG152 and BG162. The genotypes of BG003 and BG005 (offspring of the founders BG020 and BG019) and the ones of BG152 and BG105 (offspring of the founders BG161 and BG162) produced a significant likelihood ratio value (Table 2A, B), and there exist ambiguities at variable position 2 in the mtDNA sequences of BG005 and BG105 (see BG020 and BG162, Appendix I). Furthermore, because of lacking mtDNA information of the non-sampled male founders, we can not exclude a close relationship between BG030 and BG270, and between BG150 and BG065. Of the possibly related founder pairs, only BG009 and BG010 were paired in captivity but were separated after having only produced unfertile eggs (Frey et al. 1995). In addition, BG005 (the offspring of founder BG003) was paired with BG015 (an offspring of BG020) for only one year and without successful reproduction.

Discussion

Founderrelatednessandkinship

The microsatellite-based methods used in the present study were developed for inferring relatedness among individuals from natural populations and may lead to biased estimations when being applied to captive breeding programmes based on founders of unknown origin. Within the captive population, mean relatedness values for parent–offspring, full-sib and half-sib pairs were all in agreement with theoretical distributions (Lynch and Walsh 1998). In addition, 95% of all known full-sib pairs were detected with the maximum likelihood approach, confirming the power of the analyses predicted by the programme. These results strongly support the applicability of the chosen approaches within the captive population. Among the founder birds, however, relatedness values have to be viewed rather as relative measurements of genetic

similarity than in terms of identity-by-descent. A bias in inferring relationships among the founders was apparent in the case of BG232 and BG286. These two founder birds originally come from the Pyrenees (Table 1) (Frey et al. 1995), and kinship analysis based on the allele frequencies of the captive population revealed a high probability of a full-sib relationship between these birds. However, when the analysis was based on the allele frequencies of the Pyrenean population this relationship was clearly not significant. In addition, these birds strongly differed in their mtDNA haplotypes. We therefore have a risk of wrongly identifying other founders as full-sibs because they may have originated from other common source populations and thus have appeared more closely related than most other pairs originating often from different and genetically distinct populations. In contrast, the risk of not detecting full-sib relationships among other founders unknowingly originating from the same and genetically distinct source population must have been very small, because they would have likely formed significant pairs in our analyses.

While the exclusive use of microsatellite information may leave many uncertainties about relationships of founders in an artificially built population, the combination of microsatellite and mtDNA markers makes it possible to obtain a stronger inference of relationships among the founders. Only three of the 40 founder pairs, that were assigned a high probability of being full-sib or parent–offspring pairs according to likelihood ratio tests shared the same mtDNA haplotype. A similar approach, with a combination of DNA fingerprinting and mtDNA analysis, enabled pedigree inferences in a captive-breeding colony of lion-tailed macaques (Morin and Ryder 1991). We could exclude the existence of full-sib and mother offspring relationships among the majority of the founders of the captive bearded vulture population. All genetically likely father–offspring pairs could either be excluded due to studbook information, or had no impact on the captive population so far, as the individuals involved had died without reproducing or are unlikely to reproduce in the future. Unfortunately, we were unable to obtain DNA samples from eight founder individuals that had reproduced successfully within the captive population. However, we included the genotype of one of their offspring in the analyses. Thus, we could exclude a close relationship between any of the sampled founders and five non-sampled founders. The relationship among five founder pairs remains unresolved because of ambiguous or lacking mtDNA data, and special attention needs to be paid to a possible close relationship between the following founders: BG201 and either BG161 or BG134, BG003 and BG020, and BG152 and BG162. Nevertheless, we conclude that close relatedness among the founder birds is not a major concern in the genetic management of the captive bearded vulture population because it could be excluded in the majority of the cases. In addition, the distribution of pairwise relatedness values among the founders did only differ marginally from the one found in the natural population of the Spanish Pyrenees.

Founderorigin

The clear division between the Pyrenean and the captive population allows inferences about the origin of some of the founder birds. BG232 and BG286 both were assigned to the Pyrenean population, verifying the studbook information. The studbook information about the origin of BG010, however, could not be confirmed. This individual was clearly assigned to the captive population overall representing Asian origin (Table 1) (Frey et al. 1995). Although little is known about the population genetic structure of wild bearded vulture populations this result suggests that no further founder individuals, analysed here, originated from the Pyrenees and that this wild population is genetically distinct from the remaining populations contributing founders to the captive population. The intermediate position of BG065 from Crete in the assignment test even suggests an effect of isolation by distance, with all other captive individuals originating from Asian populations, but this certainly needs further investigations (Gautschi 2001). Further data would also be needed to validate studbook entries on the origin of the founder birds with Asian background. The results of the assignment test presented here, in line with others (Kyle and Strobeck 2001; Nielsen et al. 1997; Paetkau et al. 1995; Polziehn et al. 2000), clearly show that microsatellite-based data can provide a high resolution in determining the source of individuals. However, usually large numbers of individuals are needed to achieve high accuracy (Cornuet et al. 1999), especially if differentiation among populations is small (Davies et al. 1999). To validate the studbook entries of all the founders in the captive bearded vulture population a mtDNA-based method might prove to be less costly both in terms of samples required and time and money needed than

a microsatellite-based analysis. In addition, important sources of samples for such a large-scale investigation are museum collections (Brooke 2000). High copy numbers make mtDNA fragments more likely to be amplified from DNA extracted from museum skins than nuclear markers such as microsatellites with only two copies per cell. In addition, mtDNA analysis might reveal a higher genetic differentiation among populations than microsatellites (Eizirik et al. 2001). The 268 bp fragment of the control region used in this study revealed only little resolution within the captive population. The division between the captive and Pyrenean population, however, was supported with high bootstrap values (J.A. Godoy, J.J. Negro and F. Hiraldo, manuscript in preparation). This indicates that while the 268 bp fragment might not be variable enough to resolve within-population relationships, it should be useful to reveal between-population differentiation. Assignment tests based on microsatellites may nevertheless be valuable for the future monitoring of the European bearded vulture populations. Potential gene flow between the Pyrenean and the released population may be detected (Kyle and Strobeck 2001; Polziehn et al. 2000). The detection of immigrants is further facilitated by the possibility of individual identification of all birds released in the Alps, based on the microsatellites used in this study (Gautschi et al. 2000).

In conclusion, our study shows the usefulness of relatedness estimates based on microsatellite allele frequency data even in an artificially built, captive population. In combination with mtDNA data, the microsatellite results support the assumption that the majority of founder birds of the captive bearded vulture population are unrelated. A previous study showed that allele frequencies of neither the founders nor the subsequent generations within the captive population did significantly deviate from Hardy-Weinberg expectations (Gautschi et al. 2003). In addition, there was no indication of inbreeding as measured by the inbreeding coefficient F_{IS} (Gautschi et al. 2003). Certainly, unresolved relationships among the founders need to be taken into account in future managing strategies, including a possibly close relationship between the founders BG003 and BG020, BG152 and BG162, and BG201 and either BG161 or BG134. Nevertheless, we conclude that the bearded vulture breeding network does not have to deal with hidden problems of possible inbreeding and inbreeding depression arising from undetected relatedness among the founders. Important estimations of inbreeding coefficients, mean kinship, and effective population size based on pedigree data are therefore not in danger of being erroneous. In addition, knowledge about founder relatedness will help to correctly estimate population viability (Bustamante 1996, 1998). However, given the relatively small number of reproducing founders (e.g. 36 by the year 2000), inbreeding may play an important role in the future if the currently applied strategy of avoiding inbreeding is abandoned, and/or if the effective population size remains small (Gautschi et al. 2003). The clear genetic division between the captive population mainly of Asian origin and the Pyrenean population revealed by the assignment test, implies substantial genetic differentiation among natural populations of the bearded vulture. While this study shows that the captive population might not be in immediate danger of suffering from inbreeding, genetic differentiation among the origin populations enhance the risk of outbreeding depression (Gautschi 2001). Although outbreeding depression is thought to be less common than inbreeding depression it has been observed in several populations (Fischer and Matthies 1997; Knowlton and Jackson 1993) including captive ones (Lacy et al. 1993; Marshall and Spalton 2000). The possibly negative impact of cross-breeding between populations of the bearded vulture therefore needs to be examined.

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AppendixI. Base substitutions at the 19 variable positions (Figure 3) of the 29 different mtDNA haplotypes found among 43 founders (GenBank Accession numbers: AY097433-AY097475). The nucleotides at each position in haplotype 1 are indicated. For all subsequent haplotypes substituted nucleotides are given only and dots indicate identity. Site 18 and 19 represent a microsatellite (AT) repeat, which was not present in BG159. H: Haplotype (ambiguous haplotypes are given in brackets). N: ambiguity G, C, A or T; R: ambiguity A or G; Y: ambiguity C or T. Founders that are represented by one of their offspring are given in brackets

			Variable position																		
H	Founder, Studbook id	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	BG001	1	T	G	C	A	G	G	T	T	g	A	A	C	C	A	T	G	A	A	T
2	BG003, BG158	2	.	A	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.
3	BG029, BG135	2	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.
(2, 3)	(BG020)	1	.	R	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.
4	BG004	1	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	G	.	.
5	BG014, BG131	2	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.
6	BG016	1	.	A	.	.	.	A.	.	.	.	.	.	T	G	.	.	G	.	.	
7	BG021	1	.	.	.	.	.	A.	.	.	.	.	.	T	G	C	.	.	.	.	
8	BG022	1	.	R	T	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.
9	BG026, (BG023), BG010	3	.	A	.	.	.	.	.	.	.	.	.	.	T	G	.	A	.	.	.
10	BG027, BG153	2	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	A	.	.	.
11	BG032	1	N	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.
12	BG033, BG161, BG009	3	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
13	BG134, (BG155)	2	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
(12, 13)	BG201	1	.	R	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
14	BG035	1	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.
15	BG038, BG270	2	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	A	G	.	.
16	BG065	1	.	.	.	.	.	.	.	C	.	.	.	.	T	.	.	.	G	.	.
17	BG075	1	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.
18	BG132, BG019	1	C	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.
19	BG136	1	.	.	.	.	.	.	.	.	A	.	.	.	T	.	.	.	G	.	.
(20)	BG034	1	.	R	.	.	.	.	.	.	A	.	.	.	T	.	.	.	G	.	.
21	BG151	1	.	A	.	.	.	.	.	.	.	.	.	T	T	G	.	.	.	.	.
22	BG152, BG031	2	.	.	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
(23)	(BG162)	1	.	R	.	G	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
24	BG159	1	.	A	.	.	.	.	.	.	.	.	.	T	T	.	.	.	.	–	
25	BG178	1	Y	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.
26	BG199	1	.	A	T	G	A	.	.	.	.	G	.	T	T	G	C	A	T	.	.
27	BG204	1	C	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
28	BG232	1	.	R	.	.	A	.	.	C	.	.	.	.	T	.	.	.	G	.	.
29	BG286	1	.	A	.	G	A	.	.	.	.	G	.	T	T	G	C	A	T	.	.

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