Management implications of genetic studies for *ex situ* populations of three critically endangered Asian *Gyps* vultures

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Abstract

Captive breeding and *ex situ* conservation have become important tools in species conservation programmes. The effectiveness of the management of captive populations can be hampered by the absence of pedigree data, but molecular markers can be used to inform conservation objectives and reduce inbreeding in the captive population. Using microsatellite markers, we examined the genetic diversity and relatedness among wild-caught individuals of three critically endangered Asian vulture species, *Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris*, populations established for captive breeding. Estimates of the inbreeding coefficient (*f*) were low, indicating that populations of all three species do not appear to be inbred. Moreover, the data show that a large proportion of wild-taken birds were unrelated individuals (94.6, 87.1 and 85.3% in *G. bengalensis*, *G. indicus* and *G. tenuirostris*, respectively). No significant genetic differentiation (F_{ST}) was observed between historic and 2000/2001 populations of *G. bengalensis* in Pakistan and India. However, the more recent sampling (2002–2006) in Pakistan showed a significant difference from India. A genetic signal of a demographic bottleneck in all three species was found in some (*M*-ratio test), but not all, tests (heterozygote excess and mode shift in allele frequencies). Levels of genetic diversity in *G. bengalensis* and *G. indicus* populations were comparable to levels prior to or soon after the start of their decline, respectively, suggesting that adequate numbers of birds exist in captivity for these two species and reflect neutral diversity levels observed in the wild. The genetic data obtained from this study allow us to minimize the loss of neutral genetic diversity in small wild and captive populations and to identify related pairs to avoid inbreeding depression.

Introduction

Captive breeding and *ex situ* conservation practices have become important tools in species conservation programmes. Species threatened with extinction are maintained in captivity until those factors that have threatened their existence are remedied and individuals can be returned to the wild (i.e. the ark concept; Bowkett, 2009). However, due to limitations associated with resources and space, captive populations are often small, resulting in inbreeding and the loss of genetic diversity (Bouman, 1977; Ralls, Brugger & Ballou, 1979; Ralls & Ballou, 1986). As such, most captive breeding programmes establish a pedigree for managing individuals for breeding purposes to avoid inbreeding depression and maintain equal representation of the founding population, or minimize population mean kinship,

across multiple generations (Foose & Ballou, 1988; Hedrick & Miller, 1992; Lacy, 1994; Ballou & Lacy, 1995). This practice, therefore, allows a much smaller captive population size than if the population reproduced at random and/or unregulated (Foose & Ballou, 1988). It also assumes, however, that founders are unrelated and represent the standing genetic variation in the wild population. To what extent this is true depends upon the demographic history and population structure of the wild population (Leberg & Firmin, 2008; Willi, Griffin & Van Buskirk, 2013; Kennedy *et al.*, 2014).

During recent decades, the availability of highly polymorphic neutral genetic markers, particularly microsatellites, coupled with methods used to provide information concerning familiar relationships within captive populations and their demographic changes has led to an

increasing number of studies on the genetic structure of captive stocks. These studies show that inbreeding can be minimized by using information obtained from genetic methods, and managers can be better informed for identifying adequate population sizes required for reducing genetic diversity loss (for details, see Witzenberger & Hockkirch, 2011).

One such programme where genetic information is of potential use is in the conservation of three critically endangered *Gyps* species endemic to South Asia, which have declined by > 97% over the past two decades (BirdLife International, 2010). In 2007, the oriental white-backed vulture *Gyps bengalensis*, once thought the most common large bird of prey in the world (Houston, 1985), had been reduced to just 0.1% of its population size as recorded in the early 1990s in India (Prakash *et al.*, 2007, 2012). Similarly, populations of long-billed *Gyps indicus* and slender-billed *Gyps tenuirostris* vultures in India had also declined in abundance with levels in 2007 and 2011 at 2.5 and 2.3%, respectively, of those recorded in early 1990s (Prakash *et al.*, 2007, 2012). Significant declines of *G. bengalensis* and *G. indicus* have also been reported in Pakistan (Gilbert *et al.*, 2004; Johnson *et al.*, 2008) and *G. bengalensis* and *G. tenuirostris* in Nepal (Baral, Giri & Virani, 2004).

The primary cause of *Gyps* vulture decline in South Asia was consumption of carcasses of domesticated ungulates that had been administered prior to mortality a non-steroidal anti-inflammatory drug (NSAID) called diclofenac (Green *et al.*, 2004; Oaks *et al.*, 2004; Shultz *et al.*, 2004). Diclofenac is particularly toxic to *Gyps* vultures, with a high probability of death due to kidney failure after feeding on carcasses of ungulates that had died within a few days of treatment (Oaks *et al.*, 2004; Green *et al.*, 2006; Swan *et al.*, 2006). Consequently, a conservation management programme of advocacy measures was developed to implement the ban and raise awareness among veterinarians to pharmaceutical manufacturers (Pain *et al.*, 2008).

This led to a ban on veterinary diclofenac in South Asia; however, its use continued with the human version of the drug (Cuthbert *et al.*, 2011a). Studies have shown that the levels of diclofenac in ungulate carcasses have declined substantially in India since the ban of the veterinary form (Cuthbert *et al.*, 2011b), and the rate of population decline of *Gyps* vultures has slowed and may have even stopped (Prakash *et al.*, 2012). However, the rate of population growth for *Gyps* vultures is sensitive to a low prevalence of diclofenac contamination (Green *et al.*, 2004) and the maximum growth rate, even under the most favourable conditions, is slow due to their reproductive life-history constraints (Niel & Lebreton, 2005; Prakash *et al.*, 2012). The Bombay Natural History Society (BNHS) has established a captive breeding programme at three centres: Pinjore (Haryana), Rajabhatkhawa (West Bengal) and Rani Forest (Assam) for all three *Gyps* species. The programme was initiated in 2004 to restore or supplement extinct or depleted wild populations using captive-bred stock. Once diclofenac is no longer a significant cause of mortality in the wild, individuals will be released from captivity.

Removal of diclofenac is a priority across South Asia so there is no need to maintain a large captive population for more than 10 generations. However, it is still vital to identify individuals for breeding purposes that minimize mean kinship within the population and prevent inbreeding as supplementation may be required for an extended period of time after reintroduction. Minimizing mean kinship is accomplished by preferentially breeding genetically under-represented individuals to minimize a population's average co-ancestry in future generations (Ballou & Lacy, 1995; Frankham, Ballou & Briscoe, 2002; Pemberton, 2004). Because pedigrees rarely exist for wild populations, assuming that the founders of the captive population are unrelated may be erroneous and result in inbreeding (Rudnick & Lacy, 2008; but see Ivy *et al.*, 2009). In the absence of pedigree data on wild-taken founders, relatedness coefficients calculated from multi-locus microsatellite genotypes are often used as a surrogate in captive breeding programmes (e.g. Gautschi *et al.*, 2003; Pemberton, 2004, 2008; Russello & Amato, 2004; Ivy & Lacy, 2012; Hammerly, Morrow & Johnson, 2013).

In this study, we use multi-locus microsatellite markers to examine neutral genetic diversity and relatedness among wild-caught individuals of three *Gyps* species captive population established for conservation breeding in India. Analyses were also performed to ascertain whether sufficient levels of genetic diversity were captured in the *G. bengalensis* and *G. indicus* captive populations based upon measures obtained from the wild prior to and during their decline (Johnson *et al.*, 2008; Arshad *et al.*, 2009). While we acknowledge that neutral genetic markers may not necessarily reflect adaptive genetic diversity *per se* (Hedrick, 2001; Reed & Frankham, 2001; Aguilar *et al.*, 2004), the captive breeding programme's primary goal has been to protect as many individual vultures as possible given the constraints on space and resources, and measures of neutral genetic diversity at least provide a reference to ascertain whether current numbers are sufficient to maintain such levels. As such, the results from this study are used to make recommendations for the conservation breeding programme of three critically endangered *Gyps* species in South Asia in a concerted effort to prevent their extinction.

Materials and methods

Captive vultures

The BNHS breeding centres hold the majority of the world's captive stock of the three threatened species. In December 2013, the three centres possessed 121 *G. bengalensis*, 72 *G. indicus* and 55 *G. tenuirostris*, all collected from the wild. In addition, 42 *G. bengalensis*, 33 *G. indicus* and 15 *G. tenuirostris* captive-bred offspring have been produced at the centres.

The wild-taken *G. bengalensis* individuals in India were collected from seven states (Haryana, Rajasthan, Madhya Pradesh, Maharashtra, Gujarat, West Bengal and Assam). For *G. indicus*, individuals were obtained from six states
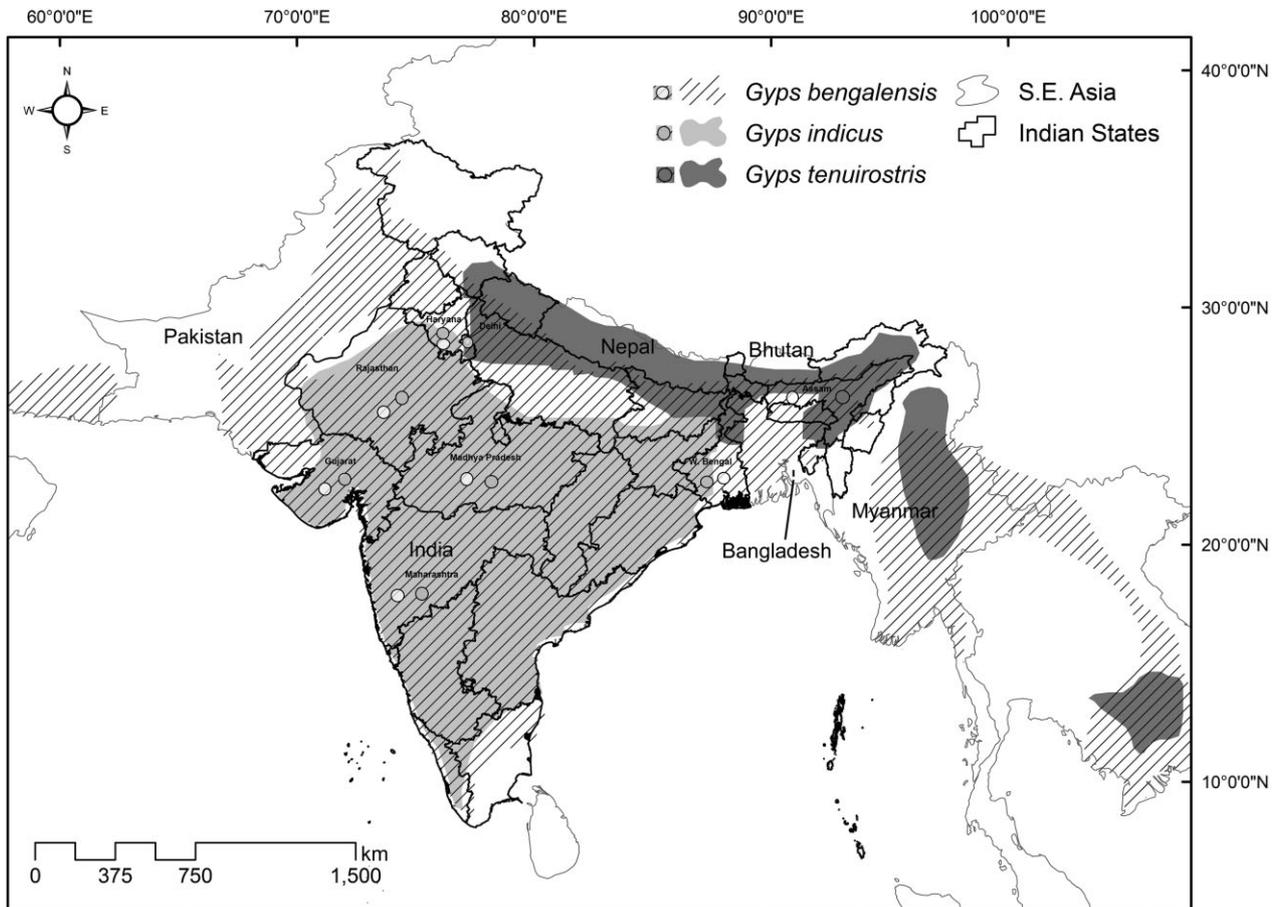
Figure 1 Historic distribution of *Gyps* species in South East Asia. Sample collection states in India are marked with circle for each species.

(Haryana, Rajasthan, West Bengal, Madhya Pradesh, Maharashtra and Gujarat), whereas all *G. tenuirostris* were collected from Assam (Fig. 1). Collection of injured and sick vultures and trapping started in 2001, but most were taken during the period 2004–2008. During this period, many of the birds were taken as nestlings, especially for *G. indicus*, although full-grown birds were also trapped or obtained as sick or injured birds. While many of the vultures taken into captivity in the same year are probably unrelated because breeding pairs rear only a single chick per year, a number of individuals were also obtained from the same breeding colony in different years. Therefore, the possibility exists that some captive birds may be full or half-siblings.

Sampling and DNA extraction

Blood samples were collected from wild-caught individuals in the captive population from all three species (*G. bengalensis*, *n* = 82; *G. indicus*, *n* = 56; *G. tenuirostris*, *n* = 34) in 2009–2010. The percentages of the wild-taken stock that were sampled for this study were 68, 78 and 81% for each of the three species, respectively. DNA extraction was performed by the ammonium acetate salt extraction

method, as described elsewhere (Nicholls *et al.*, 2000). In addition to the samples collected in this study, we used genetic data previously reported for 113 wild *G. bengalensis* from Pakistan that were collected before and during their recent decline (Johnson *et al.*, 2008) and 55 wild *G. indicus* also in Pakistan (Arshad *et al.*, 2009).

Marker selection and amplification

Nine microsatellite loci originally developed for the bearded vulture *Gypaetus barbatus* (BV6, BV11, BV12, BV13, BV14 and BV20; Gautschi *et al.*, 2000) and the Eurasian griffon vulture *Gyps fulvus* (Gf11A4, Gf3H3 and Gf9C1; Mira *et al.*, 2002) were used for the microsatellite analyses. The polymerase chain reaction (PCR) amplifications were performed using Qiagen multiplex PCR kit (Hilden, Germany) and protocols therein. We multiplexed nine loci in three groups: BV6, BV11 and BV13 at 50°C; BV14, BV20 and BV12 at 58°C; and Gf11A4, Gf3H3 and Gf9Cl at 50°C. All markers were florescent-labelled. Each sample for each locus was amplified a minimum of four times to control for allelic dropout. Amplified products were diluted and run on an ABI 3730 automated sequencer and analysed in GeneMapper version 3.7 (Applied Biosystems, Foster City, CA, USA).

Data analyses

Genetic diversity

All loci were examined for the presence of null alleles and allelic dropout within each species using the program MICRO-CHECKER (Van Oosterhout *et al.*, 2004). The departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (exact tests) were compared among loci for each population using the program GDA (Lewis & Zaykin, 2001). Mean number of alleles per locus (N_A) and mean observed (H_O) and expected (H_E) heterozygosity values were calculated using GDA, and allelic richness was calculated using the program FSTAT version 2.9.3 (Goudet, 2001). Measures of allelic richness were standardized for differences in sample size among populations (Leberg, 2002).

Inbreeding coefficients and relatedness

To determine inheritance patterns among sampled vultures, individual inbreeding coefficients (*f*) were calculated using the method of Weir & Cockerham (1984) as implemented by GDA software (Lewis & Zaykin, 2001). Estimates of 95% confidence intervals around inbreeding estimates were generated using 10 000 bootstrap replicates. Pairwise values of genetic relatedness (r_{xy}) values were calculated using two estimators: Queller & Goodnight (1989) and Lynch & Ritland (1999) indices (hereafter named *Q&Gr* and *L&Rr*, respectively) and best performed simulations selected based upon the criteria described in Blouin *et al.* (1996). We applied the cut-off values method (Blouin *et al.*, 1996), based upon previously calculated pairwise r_{xy} values, to classify individuals into relationship categories: unrelated (UR), half-siblings (HS), full siblings (FS) and parent–offspring (PO). Using this method, there is a greater than zero probability of misclassifying individuals if observed values of relatedness fall outside theoretically expected values (Russello & Amato, 2004). To minimize this error, we calculated the cut-off values specific for our samples using the Monte Carlo simulation procedure recommended in Blouin *et al.* (1996). For this approach, we used *iREL* (Gonçalves da Silva & Russello, 2009) to randomly generate 1000 dyads in each of the four relatedness categories using the population allele frequencies estimated for each sample, and then computed *Q&Gr* and *L&Rr* for simulated dyads. The midpoints between the means of the distributions of pairwise relatedness estimates of each simulated relationship category were taken as cut-off values (Blouin *et al.*, 1996). The relationship category compatible with the observed r_{xy} value was then determined for each individual pair.

Estimation of population structure

We used two approaches to evaluate population structure. The first was the index of population differentiation (F_{ST}) and was calculated using the method of Weir & Cockerham (1984) as implemented in FSTAT version 2.9.3 (Goudet, 2001) and significance was assessed by 1000 randomizations after Bonferroni correction (Rice, 1989). Intra- and inter-specific pairwise F_{ST} comparisons within and between the three species were conducted depending upon sampling.

A Bayesian clustering approach implemented in the program STRUCTURE, version 2.3.3 (Pritchard, Stephens & Donnelly, 2000), was also used to assess the presence of distinct genetic subgroups within the wild-taken captive populations for each of the three species. We ran the program without including information on the geographical location where the bird was collected. For *G. bengalensis*, we ran STRUCTURE for the combined data for samples collected in Pakistan (Johnson *et al.*, 2008) and the captive population in India. STRUCTURE was run with 10 independent simulations, each one using 5×10^5 iterations following a burn-in of 5×10^4 while assuming the admixture model with correlated allele frequencies. The optimal value of the number of subgroups *K* was selected from a possible range of $K = 1$–8 by the method of Evanno, Regnaut & Goudet (2005).

Bottleneck detection

Genetic signatures of demographic contraction were assessed using three different approaches: (1) the heterozygote excess test; (2) the mode-shift test, both implemented in the program BOTTLENECK version 1.2.02 (Cornuet & Luikart, 1996; Piry, Luikart & Cornuet, 1999), and (3) the Garza–Williamson index or *M*-ratio implemented in the program ARLEQUIN (Excoffier, Laval & Schneider, 2005). The heterozygote excess test is to determine whether the captive *Gyps* vulture populations possessed a genetic signal associated with a significant change in population size. For example, a rapid decline in population size often causes rare alleles to be lost more rapidly than common alleles, which can then lead to an expected heterozygosity excess when compared to a population at equilibrium with the same number of alleles (Cornuet & Luikart, 1996; Piry *et al.*, 1999). Using the above approach, equilibrium conditions were simulated using 1000 replications assuming a stepwise model (SMM) or a two-phase model (TPM, with 30% multi-step mutation events) of mutation. Significant departure from equilibrium conditions was tested using Wilcoxon signed-rank tests. The *M*-ratio test uses data on the frequency and total number of alleles and the allelic size range to investigate population decline. In a reducing population, the expectation of the reduction of number of alleles is much higher than the reduction of allelic size range. Thus, the ratio between the number of alleles and the allelic size range is expected to be smaller in recently reduced populations than in equilibrium populations. Datasets using seven or more loci and the appropriate mutation model can be assumed to have experienced a reduction in population size with an $M \leq 0.68$ (Garza & Williamson, 2001).

Results

A total of 153 individuals (74 *G. bengalensis*, 46 *G. indicus* and 33 *G. tenuirostris*) were successfully genotyped at seven to nine microsatellite loci, depending upon the species. Of

the nine loci, locus BV12 did not amplify in *G. tenuirostris* and locus Gf9C1 did not amplify in *G. indicus* and *G. tenuirostris*. All loci analysed, with the exception of the two loci above for the respective species, were polymorphic with two to seven alleles per locus. Allelic richness and heterozygosity (*H*) are given in Table 1. No deviations from HWE or linkage disequilibrium were detected in any of the species and loci, and no indication of null alleles or scoring errors were observed based upon the results from MICRO-CHECKER.

Inbreeding coefficient and relatedness

Estimates of inbreeding coefficient (*f*) for *G. tenuirostris* were lower than estimates obtained for *G. bengalensis* and *G. indicus*. However, none of the estimates of *f* differed significantly from zero (Table 1). Comparison of the relatedness indices of Queller & Goodnight (1989) and Lynch & Ritland (1999) suggested that the latter performed better at discriminating between unrelated and first-order individuals, with fewer unrelated dyads being misclassified as first-order dyads following Blouin *et al.* (1996) (Supporting Information Appendix S1). All further analyses were therefore based upon the index of Lynch & Ritland (1999). The distribution of observed relatedness values for all sampled individuals for each species was log normal with a peak around zero (Fig. 2). Using the empirical distribution derived from 1000 simulated unrelated dyads, we determined that a dyad with a relatedness value of ≥ 0.2201 had a ≤ 0.05 probability of being unrelated (Fig. 2).

Proportions of dyads of wild-taken birds estimated to fall into different categories of kinship (Fig. 3; Supporting Information Appendix S2) indicated that a high proportion of the dyads comprised unrelated individuals in all three species (94.6, 87.1 and 85.3% in *G. bengalensis*, *G. indicus* and *G. tenuirostris*, respectively). Proportions estimated as parent–offspring were 0.0, 0.0 and 0.8% (same species order, respectively); full siblings were 0.3, 1.2 and 4.0%; and half-sibling dyads were 5.1, 11.7 and 10.0%.

Population structure

No significant genetic differentiation (F_{ST}) was observed between historic and 2000/2001 populations of *G. bengalensis* in Pakistan and India. However, the more recent sampling in Pakistan (2002, 2003/2004 and 2005/2006) showed significant differentiation with India, whereas all three *Gyps* species were significantly different from one another (Tables 2 and 3). Results observed with STRUCTURE also indicated a single contiguous population (*K* = 1) for *G. tenuirostris* and *G. indicus*. *Gyps bengalensis* populations in India and Pakistan combined showed similar results (Fig. 4). These results suggest that the individuals sampled from the captive breeding populations all originated from a single population for each species.

Bottleneck detection

No evidence of a significant population decline in the past was observed in the captive population based upon genetic

Table 1 Genetic diversity indices for South Asian *Gyps* species

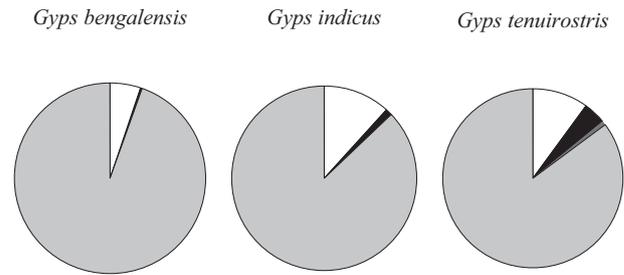| Species	Location	Timing	Mean alleles/locus	Number of loci	Sample size	Allelic richness	H_E	H_O	*f* (95% CL)	Study
bengalensis	South Asia[a]	Pre-decline	7.3	9	22	7.1	0.673	0.652	0.0345 (− 0.007–0.0943)	Johnson *et al.* (2008)
bengalensis	Pakistan	Decline	8.2	9	113	7.1	0.679	0.656	0.031 (− 0.002–0.0633)	Johnson *et al.* (2008)
bengalensis	Pakistan	Decline	6.0	8	32	4.4	0.589	0.583	0.01 (NC)	Arshad *et al.* (2009)
bengalensis	India	Decline	9.4	9	74	9.0	0.670	0.620	0.072 (− 0.045–0.234)	This study
indicus	Pakistan	Decline	4.0	8	55	2.6	0.475	0.467	0.07 (NC)	Arshad *et al.* (2009)
indicus	India	Decline	4.6	8	46	4.6	0.540	0.510	0.055 (− 0.018–0.130)	This study
tenuirostris	India	Decline	4.0	7	33	4.0	0.470	0.500	− 0.070 (− 0.206–0.009)	This study

The values for *Gyps bengalensis* from Pakistan during the population decline are means of the annual values for 2000/2001 to 2005/2006 from table 1 of Johnson *et al.* (2008) weighted by the sample size in each year.
[a]Pre-decline museum specimens collected throughout South Asia (Pakistan, India, Nepal and South China).
CL, confidence level; NC, not calculated.

a

b

c
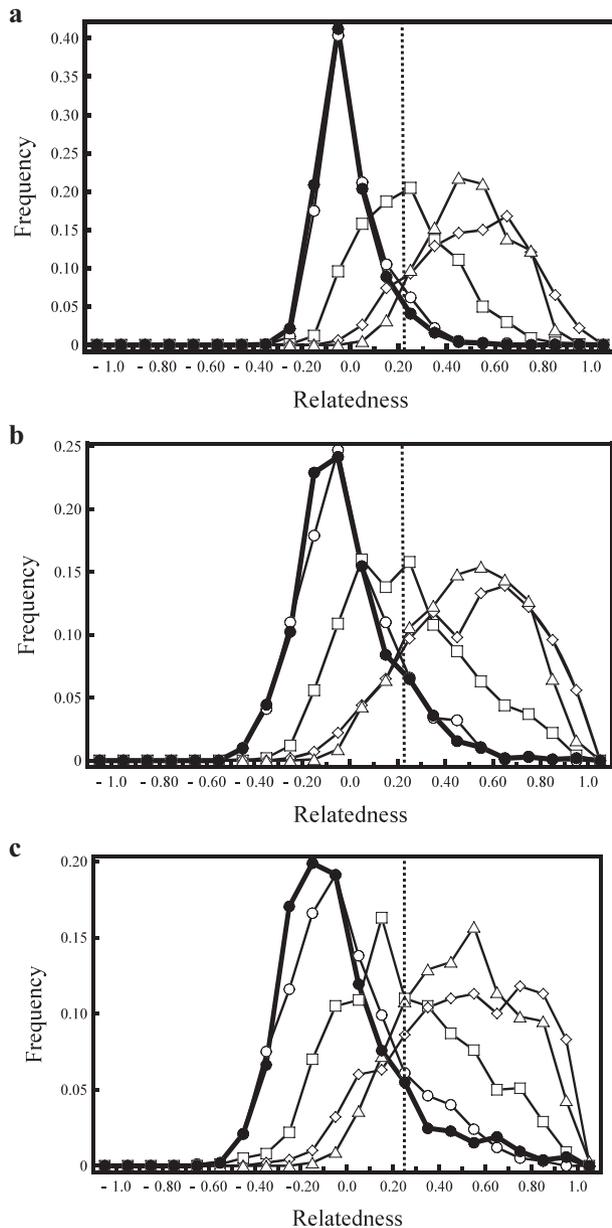
Figure 2 Frequency distributions of Lynch and Ritland relatedness values (●) for all dyads of wild-taken individuals of (a) *Gyps bengalensis*, (b) *Gyps indicus* and (c) *Gyps tenuirostris* in conservation breeding centres in India. Also shown are simulated relatedness distributions for unrelated (○), half-siblings (□), full siblings (◇) and parent–offspring (△) dyads. The dashed vertical line indicates the cut-off value of 0.2201 that separates related from unrelated dyads (see text).

Gyps bengalensis   *Gyps indicus*   *Gyps tenuirostris*

Figure 3 Proportions of all dyads classified as being comprised of unrelated birds (light grey), full siblings (black), half-siblings (white) and parent–offspring (mid grey) for wild-taken vultures of each of the three vulture species in conservation breeding centres in India.

Table 2 Pairwise fixation indices (F_{ST}) between *Gyps* species (below diagonal)

	Population	N	1	2	3
1	*G. bengalensis* (India)	74	–	0.002	0.001
2	*G. indicus* (India)	46	0.232	–	0.001
3	*G. tenuirostris* (India)	33	0.326	0.272	–

Uncorrected *P* values above the diagonal. All F_{ST} values were significant after Bonferroni corrections. *N*, sample size.

historic samples. For *M*-ratio test, we obtained contrasting results. Garza & Williamson (2001) suggested that values of *M* lower than 0.7 would indicate evidence of a bottleneck, whereas values greater than 0.8 would suggest no bottleneck history. In our dataset, mean *M*-ratio for white-backed vultures (0.46 SD 0.02) was lower than mean *M* for long-billed vultures (0.52 SD 0.23) and slender-billed vultures (0.53 SD 0.15), indicative of a recent reduction in population size. The BOTTLENECK tests are sensitive to specific demographic scenarios (Williamson-Natesan, 2005) and its ability to identify a bottleneck event depends upon multiple parameters such as sample size, number of loci, and bottleneck size and duration (see Cornuet & Luikart, 1996; Peery *et al.*, 2012; Hoban, Gaggiotti & Bertorelle, 2013). It is possible that the number of loci used in the BOTTLENECK test in this study lacks the power to detect a decline in our populations.

Discussion

Founder populations

Based upon the genetic management of captive breeding programmes, a general rule is to establish at least 20–30 unrelated founders (Foose *et al.*, 1986; Lacy, 1987, 1989; Foose & Ballou, 1988; Willis & Willis, 2010). However, such programmes rely upon an accurate pedigree to minimize mean kinship and avoid inbreeding. In the case of *Gyps* vultures, a much larger number of individuals were used for establishing the captive population. This was carried out for two reasons. The first was to protect as many vultures as possible from exposure to diclofenac given the severity of the problem and overall rate of decline (> 97%; BirdLife International, 2010), whereas the second reason was to

data using BOTTLENECK. The allele frequencies of all three species sampled had an L-shaped distribution and no significant observed heterozygosity excess for *G. bengalensis* (*P* > 0.85), *G. indicus* (*P* > 0.38) and *G. tenuirostris* (*P* > 0.81) under either SMM or TPM. Sample sizes were too small for the BOTTLENECK test to be applied to the

Table 3 Pairwise fixation indices (F_{ST}) between *Gyps bengalensis* in India and Pakistan sampled temporally, pre- (historic) and post-decline (2000–2006) periods

	Populations	N	1	2	3	4	5	6
1	India	74	–	0.366	0.078	< 0.001	<0.001	0.002
2	Pakistan: historic	22	0.002	–	0.902	0.994	0.794	0.351
3	Pakistan: 2000–2001	19	0.017	– 0.009	–	0.813	0.581	0.771
4	Pakistan: 2002	33	0.019	– 0.011	– 0.005	–	0.968	0.241
5	Pakistan: 2003–2004	42	0.018	– 0.004	0	– 0.005	–	0.271
6	Pakistan: 2005–2006	19	0.019	0.001	– 0.006	0.002	0.004	–

Uncorrected *P* values are shown above the diagonal and significant F_{ST} values (after Bonferroni corrections) are underlined.
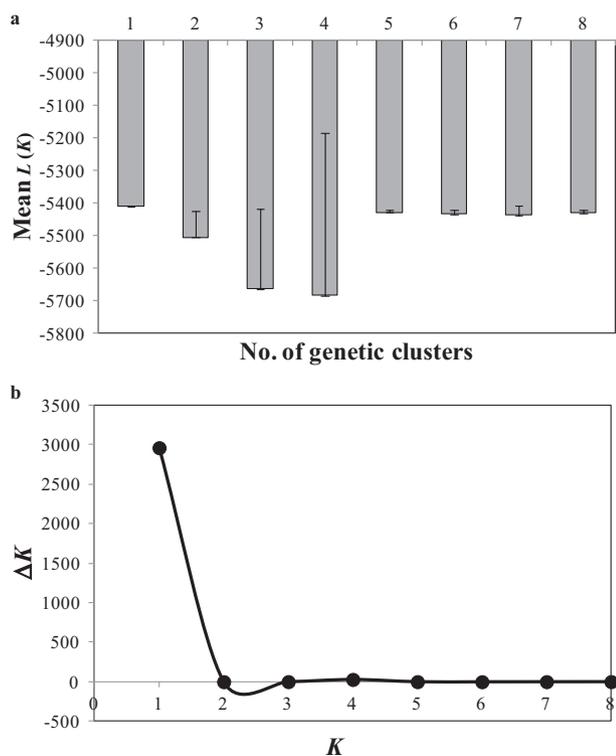
Figure 4 Results from STRUCTURE showing the most likely value of *K* for sampled *Gyps bengalensis* populations in India and Pakistan combined. (a) Plot of the mean posterior log probabilities of the data *L*(*K*). Standard deviation of *L*(*K*) for the five runs of each *K* are plotted only above the mean, (b) the change in the log probability of data between successive *K* values Δ*K*, showing that *K* = 1 is the modal value of the distribution and therefore the most likely number of population clusters.

dependent upon both financial and resource availability (e.g. food and space).

Genetic differentiation

With the exception of more recent periods (2002–2006), these data suggest that the risk of outbreeding depression arising from the breeding of individuals from different geographic areas following release back into the wild is low because the populations do not appear to be genetically differentiated (Edmands, 2007; Frankham *et al.*, 2011). Given their precipitous decline, the observed population genetic differentiation among *G. bengalensis* populations sampled in India and Pakistan (2002–2006) was likely due to reduced population size and the effects of genetic drift. This latter result is worth noting because monitoring changes in population differentiation (e.g. F_{ST}) can be a more sensitive indicator of recent population decline than measures of allelic diversity (Luikart, Cornuet & Allendorf, 1999; Spencer, Neigel & Leberg, 2000; see also Landguth *et al.*, 2010). Within Pakistan, we do not see the same effect between sampled years (Johnson *et al.*, 2008); however, the samples obtained from India were more recent and therefore possessed a larger time frame between sampling periods.

While genetic data are not available for comparing population genetic differentiation among populations of *G. indicus* and *G. tenuirostris*, they likely possessed similar population genetic patterns within their sampled distributions. Dispersal distances tend to be large in large-bodied birds of prey (Newton, 1979), and long-range dispersal had likely maintained large panmictic populations for all three vulture species (see Gilbert *et al.*, 2007). More work is warranted to determine if similar patterns of connectivity existed for *G. tenuirostris* in India with populations in South East Asia such as Cambodia (Clements *et al.*, 2013).

Genetic diversity

The genetic data generated from the captive populations show similar genetic diversity levels for *G. bengalensis* and *G. indicus* as those sampled in Pakistan, India, Nepal and China prior to their decline (Johnson *et al.*, 2008), and in Pakistan between 2005 and 2007 (Arshad *et al.*, 2009), respectively. Finding high levels of neutral genetic diversity for the captive *G. bengalensis* population is encouraging given their significant decline in abundance. Similar patterns

maintain as much standing genetic variation that existed in the wild population prior to their decline and maximize the potential for successful re-introduction (Robert, 2009). To protect as many vultures as possible, several limitations exist for maintaining a large captive population, particularly of species of large body size (e.g. Balmford, Mace & Leader-Williams, 1996; Snyder *et al.*, 1996; Bowkett, 2009), and as such, the current size of the population is largely

have also been observed in a few other threatened species, suggesting that a long lifespan coupled with the short time elapsed since the decline can result in a relatively minimal loss of genetic diversity (Hailer *et al.*, 2006; Lippé, Dumont & Bernatchez, 2006; Lawrence *et al.*, 2008; Bourke *et al.*, 2010; Pittman *et al.*, 2011). Estimates of inbreeding coefficient (*f*) were also low, indicating that the wild-taken populations of all three species do not appear to be inbred. However, concerns still exist about the viability of the remaining populations. While there appears to have been the retention of neutral diversity, it cannot be assumed that diversity among genes under selection has also been retained. For example, recent findings suggest that losses of genetic variability in bottlenecked populations can be more dramatic in immune-related genes, such as the major histocompatibility complex, than at neutral markers (Eimes *et al.*, 2011; Ejsmond & Radwan, 2011; Sutton *et al.*, 2011). It would be useful to conduct further research into how the severity and duration of the bottleneck experienced by the three *Gyps* species may influence genetic diversity associated with fitness and for identifying any additional measures that can be taken to maximize the likelihood for successful reintroduction.

Relatedness and conservation implementation

The Lynch & Ritland (1999) relatedness values indicated a high proportion (85–95%) of unrelated dyads among the wild-taken captive vultures for all three species. However, the relatedness values also identified some probable close relatives within the captive populations. We did not find first-order relatives from the same breeding colonies or even states in *G. bengalensis* and *G. indicus*. However, *G. tenuirostris* collections were made from one state (Assam) only and therefore a few individuals show first-order relatives.

Mainly due to logistical reasons and their social behaviour, most of the captive adult vultures are kept in large colony aviaries, where the birds are allowed to select their own mates and form pair bonds. There are some disadvantages, of course, to such an approach, one of which is the need for a large number of individuals in the population (> 100 individuals) to prevent the random loss of genetic diversity over time by drift (Johnson *et al.*, 2008). Another disadvantage is that related individuals may breed with one another and thereby produce inbred offspring with the potential of reduced fitness (e.g. Hammerly *et al.*, 2013). For example, a lethal form of dwarfism call *chondrodystrophy* was identified and subsequently managed in the California condor *Gymnogyps californianus* captive population. Individuals that possessed putative alleles associated with the condition were prevented from breeding, thereby reducing its impact on the population (Ralls & Ballou, 2004).

All captive-bred young to date have known parentage and will be managed using a pedigree to prevent mating among close relatives. Among founder birds, DNA-based relatedness values will be used to minimize the potential for allowing breeding among related individuals. If this approach proves successful and offspring are produced, it will allow managers to follow standard captive breeding protocols by assigning breeding pairs based upon a pedigree while minimizing mean kinship to maintain levels of genetic diversity and founder genome equivalents (Lacy, 1989; see also Ivy & Lacy, 2012).

In total, there are currently 201 wild-taken *G. bengalensis*, 72 *G. indicus* and 42 *G. tenuirostris* in five captive breeding centres in India, Nepal and Pakistan combined. The organizations responsible for captive vultures in these three countries are collaborating in the consortium Saving Asia's Vultures from Extinction (SAVE) on captive management, re-introduction and *in situ* conservation. Seventy-nine per cent (248/315) of the captive vultures are maintained in three centres in India. While it is difficult for logistic reasons to transfer birds between them, the subdivision of breeding programme and partial genetic isolation of populations is advantageous for genetic, disease and cost considerations. Several small isolated populations with occasional exchange of genetic material should have greater genetic diversity, less inbreeding and less genetic adaptation to captivity than a single large population of equivalent total size, provided there are no population extinctions (Margan *et al.*, 1998; Frankham, 2008).

Recent success with breeding of all three species in captivity, decrease in diclofenac incidence in ungulate carcasses (Cuthbert *et al.*, 2011a) and significant slowing of vulture declines in the wild (Prakash *et al.*, 2012) suggest that large-scale re-introductions of *Gyps* vultures may very soon be possible. Assuming the release programme is initiated and rate of increase for all three species in the wild is 7–12% per year (Niel & Lebreton, 2005; Prakash *et al.*, 2012), then a large decline in genetic diversity might be avoided. As other studies have shown, the shorter the time in captivity, the higher the probability for successful re-introduction (Frankham, 2008; Williams & Hoffman, 2009). If diclofenac and other toxic NSAIDs are removed from the environment, captive birds can be released within the next five years, which will minimize the loss of diversity.

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Rate of misclassification of unrelated (UN) to first-order relatives (FS: full sibs and PO: parent–offspring) for the relatedness indices of Lynch and Ritland (1999, rxyLR) and Queller and Goodnight (1989, rxyQG) based on the midpoint between the distributions of relatedness values calculated from 1000 simulated dyads of each relatedness category using the population allele frequencies estimated for each sample, as described in Blouin *et al.* (1996). Numbers in bold indicate the index with lowest misclassification rate for each comparison.

Appendix S2. The midpoints between the means of the distributions of pairwise relatedness estimates of each simulated relationship category were taken as cut-off values (Blouin *et al.*, 1996). The relationship category compatible with the observed (Lynch & Ritland, 1999), $r_{xy}LR$ value was then determined for each individual-pair.