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Short communication

Temporal genetic analysis of the critically endangered oriental white-backed vulture in Pakistan

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ABSTRACT

Populations of oriental white-backed vultures (*Gyps bengalensis*) in south Asia have declined over 95% since the mid-1990s due to feeding on livestock carcasses that had been treated with diclofenac, an anti-inflammatory pharmaceutical that is fatal to *Gyps* vultures. To prevent extinction, captive breeding efforts have been initiated; however, given the overall decline, it is not known to what extent levels of genetic diversity currently exist in the remaining populations. Here we document temporal genetic diversity levels during the 2000–2006 interval for the last remaining breeding colony of oriental white-backed vultures in Pakistan, and show with simulations that a much larger captive population size is required than currently maintained to prevent further loss of genetic diversity. Before this species is extinct in the wild, it is crucial that additional individuals are included in the captive population.

1. Introduction

Many natural populations have been reduced in size due to increased human activities associated with landscape changes and persecution (Mace et al., 2005). Concern exists because significant reductions in the size of large populations will likely cause a loss of genetic diversity due to drift and increase extinction risk by adversely affecting the ability of populations to adapt to changing environments (Frankham and Kingslover, 2004; Frankham, 2005; Willim et al., 2006).

Populations of oriental white-backed vultures (*Gyps bengalensis*) in south Asia have collapsed by over 95% since the mid-1990s and are continuing to decline at alarming rates (Prakash et al., 2003; Prakash et al., 2007; Green et al., 2004; Green et al., 2007; Gilbert et al., 2006). This is primarily due to vultures feeding on carcasses of livestock that had been treated with diclofenac, a non-steroidal anti-inflammatory pharmaceutical now known to be toxic to *Gyps* vultures (Oaks et al., 2004; Shultz et al., 2004; Swan et al., 2006). Numbering in the millions as little as 15 years ago, the oriental

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white-backed vulture was considered one of the most common raptors in the world (Houston, 1985). This species is now considered Critically Endangered (IUCN, 2007), and extinction in the wild appears imminent. Of 31 former breeding colonies surveyed across Pakistan's Punjab province since 2000, only one persists (Fig. 1, see Asian Vulture Population Project, 2008), and the number of individuals there has steadily declined (Fig. 2; see also Gilbert et al., 2002) with no recruitment during the 2006/07 and 2007/08 breeding seasons. The species has also steadily declined in our study sites in the Indian states of Rajasthan and Madhya Pradesh with no birds observed in the 2006/07 breeding season (see also Prakash et al., 2003). During the 2007/08 season, however, a flock of nearly 70 oriental white-backed vultures was observed in Madhya Pradesh (personal observation).

Although small-scale captive breeding efforts have been initiated, genetic diversity estimates, potentially useful in conservation efforts, are presently lacking. Here we provide such genetic diversity estimates for the last wild oriental white-backed vulture breeding colony in Pakistan during 2000–2006, and compare them with estimates from museum samples collected before 1961 (pre-decline). We also use simulations to assess adequacy of various captive population sizes in preventing loss of genetic diversity. This information is useful for conservation efforts aimed at establishing and maintaining a viable captive population before this species is extinct in the wild.

2. Materials and methods

Intensive population monitoring has occurred within the Punjab province in Pakistan since 2000, which at that time possessed the largest known breeding population of oriental white-backed vultures. The colony at Toawala followed a 6.4 km length of canal bank. Nest activity was monitored weekly starting in December 2000 and continued to May 2004, with no visits between July and October when vultures were not breeding (see Gilbert et al., 2006 for details). For the 2004/05 breeding season onwards, nests at Toawala were monitored on a monthly basis. All vulture nests within Toawala were located at the start of the breeding season and monitored until completion (i.e., abandonment or fledged young).

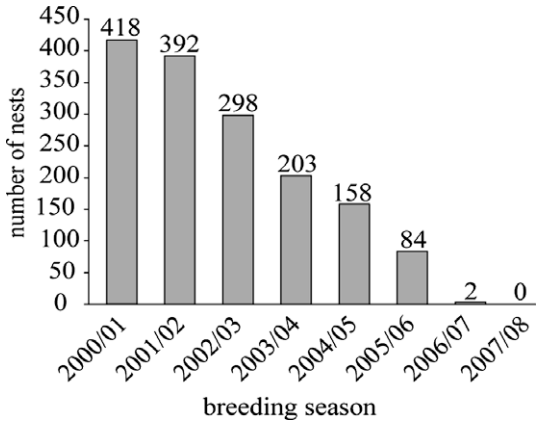

Fig. 2 – Number of nests observed in Toawala, Pakistan between 2000 and 2008.

Tissues from spleen, kidney, liver, muscle, or feathers were sampled from between 19 and 42 adult vultures during each breeding season. A historic pre-decline population sample was also analyzed using toe-pad tissues obtained from museum specimens collected between the years 1893–1960 in Pakistan, India, Nepal and southern China (see Supplementary material Table S1). Total genomic DNA was extracted from contemporary tissues or toe-pad tissue from museum specimens using a DNeasy Tissue Extraction Kit (QIAGEN Inc.). All work with museum samples was conducted in a facility used only for ancient DNA work at the University of Michigan Museum of Zoology and included negative controls and replications.

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

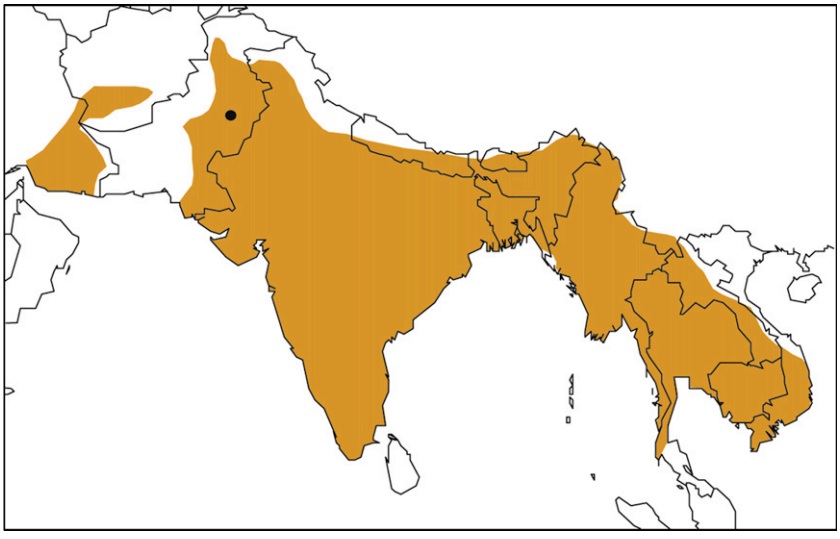

Fig. 1 – Oriental white-backed vulture pre-decline distribution, with the last remaining breeding colony in Pakistan (Toawala) indicated by a solid black circle. Distribution based on del Hoyo et al. (1994) and Rassmussen and Anderton (2005).

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vulture (*Gyps fulvus*; Gf11A4, Gf3H3 and Gf9C1; Mira et al., 2002) were used for the microsatellite analyses. The PCR amplifications were performed using the following reagents in a 10 mL reaction: 50 ng total genomic template, 0.5 mM fluorescently labeled forward and unlabeled reverse primer, 1× buffer solution (Invitrogen), 1.5 mM $MgCl_2$ (except loci Gf11Af, Gf3H3, and Gf9C1 required 2.0 mM), 0.4 mM of each dNTP, and 0.5 units of Taq polymerase (Invitrogen). The PCR conditions consisted of one denaturing cycle at 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 30 s at annealing temperature (58 °C for all primers except Gf11Af, Gf3H3, and Gf9C1 at 50 °C), and a 30 s extension step at 72 °C. This was followed by an extension step at 72 °C for 5 min. For the pre-decline museum samples, 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.

For the mtDNA analysis, 19–26 individuals were sequenced from each of the sampling locations used in the microsatellite analysis. Two primer pairs, GbCR1.L/GbCR2.H (Johnson et al., 2006) and GbCR4.L (5′-CGATTCATGGTAGCAGGTCA)/CSB1.H (5′-AACATGTCCAACAAGCATTCA) were used to amplify approximately 790 basepairs (bp) of mtDNA control region domains I and II sequence. PCR amplification was performed in 25 mL reaction volumes using 50 ng/mL genomic template, containing 0.5 mM of each primer, 1.9 mM $MgCl_2$, 1× buffer solution (Invitrogen), 0.8 mM of each dNTP, and 1.25 units of Taq polymerase. The PCR conditions were one denaturing cycle at 94 °C for 2 min, followed by 32 cycles at 94 °C for 25 s, 58 °C for 25 s, and an extension step for 1 min at 72 °C. This was followed by an extension step at 72 °C for 7 min. PCR products were directly sequenced in both directions with ABI Big Dye Terminator chemistry. All sequences were aligned using Sequencher™ 4.2.2, and verified for accuracy. Unique sequences were deposited in GenBank (accession numbers EU752258-EU752279).

Microsatellite linkage disequilibrium and Hardy–Weinberg equilibrium within each sampling period were assessed using the program GDA (Lewis and Zaykin, 2001). The program Micro-checker (Van Oosterhout et al., 2004) was used to test for null alleles and scoring errors due to large allele dropout and stutter peaks. Mean number of alleles per locus (allelic diversity) and mean observed (H_o) and expected (H_e) heterozygosity values were calculated using gda, and measures of allelic richness were calculated using the program Fstat vs.2.9.3.2 (Goudet, 1995) to account for differences in sample sizes across sampling periods (Leberg, 2002). Differences in microsatellite genetic diversity estimates between time periods were tested for significance using a Wilcoxon signed rank test.

Mitochondrial DNA control region diversity was determined by calculating population estimates of haplotype diversity (h) and Tajima's D using the program Arlequin 3.11 (Excoffier et al., 2005). Because we sequenced only the first 396 bp of the mtDNA control region domain I from the pre-decline museum samples, we only report diversity estimates based on homologous sequence from all other samples. All of the nucleotide variability observed among the contemporary samples was present in this smaller fragment. Estimates of haplotype diversity were compared between time periods using the t-test described by Nei (1987). Bonferonni corrections were applied to correct for multiple simultaneous comparisons (Rice, 1989).

To investigate loss of genetic diversity due to random genetic drift, we used the program BottleSim (Kuo and Janzen, 2003) to simulate population bottlenecks of varying sizes and durations. Using both the empirical allelic frequency data and census estimates from the past six years for the Toawala breeding colony, we ran simulations with different post-bottleneck population sizes to investigate the degree of loss over time in both observed number of alleles and heterozygosity measures. We chose the minimum size of 30 individuals to reflect the maximum size that the current breeding facility in Pakistan can support due to space constraints (C. Murn, personal communication). Similarly, we determined via simulation the minimum size required to retain 90% of the genetic diversity observed prior to decline over a 100-year period, a common goal under these circumstances (Frankham et al., 2002). For each simulation based on 1000 iterations, we used the settings for maximum generation overlap (100%), random mating, five years for age at first breeding, 20-year longevity, and equal sex ratios. Our measures of age at first breeding and longevity are based on observations made both in the field and with captive individuals (M. Gilbert and C. Murn, personal observations). However, it is possible that average longevity is lower for this species, especially in light of diclofenac exposure, and in this case our estimates of genetic diversity are conservative because diversity estimates will decline at a faster rate with decreased longevity (data not shown).

3. Results

The number of occupied nests in the Toawala breeding colony has steadily declined from 418 in the 2000/01 breeding season to only two nests in 2006/07 and no nests in 2007/08 (Fig. 2). Only 33 oriental white-backed vultures were observed in total during the 2006/07 breeding season and no vultures were observed in 2007/08. Despite a dramatic decline in abundance, no significant decrease in microsatellite genetic diversity measures was observed within the 2000/01 to 2005/06 interval, or in comparing these estimates with pre-decline diversity (Table 1). Allelic richness and expected heterozygosity (H_e) ranged from 7.0 to 7.3 alleles per locus and 0.664–0.690, respectively. No deviations from Hardy–Weinberg equilibrium or linkage disequilibrium were recovered in any of the temporal datasets, and no indication of null alleles or scoring errors were observed based on the results from Micro-checker.

For the mitochondrial control region, the number of haplotypes varied from seven to 13 haplotypes with no obvious temporal decline during the sampling period. In contrast, estimates of haplotype diversity (h) for our most recent sampled breeding season (2005/06) was significantly lower than that obtained from the earlier breeding seasons, including the historic dataset (Table 2). In fact, h appears to decrease over time, with five out of the ten pairwise comparisons possessing significantly lower h after adjusting for multiple comparisons (Table 2; p-value < 0.005 indicates statistical significance).

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Table 1 – Measures of genetic diversity estimates (microsatellite and mtDNA) for oriental white-backed vultures in Pakistan

	Pre-decline	2000/01	2002/03	2003/04	2005/06
Microsatellite DNA					
Sample size	22	19	33	42	19
Mean alleles/locus	7.3 (1.5)	7.6 (1.3)	8.4 (1.7)	8.7 (2.0)	7.3 (1.4)
Allelic richness	7.1 (1.4)	7.3 (1.2)	7.0 (1.3)	7.0 (1.3)	7.1 (1.4)
Exp. heterozygosity	0.673 (0.071)	0.669 (0.071)	0.664 (0.074)	0.690 (0.065)	0.690 (0.074)
Obs. heterozygosity	0.652 (0.078)	0.643 (0.076)	0.673 (0.074)	0.618 (0.071)	0.725 (0.072)
mtDNA control region[a]					
Sample size	26	19	20	25	19
No. of haplotypes	13	8	10	11	7
Haplotype diversity	0.846 (0.013)	0.854 (0.012)	0.837 (0.017)	0.830 (0.014)	0.760 (0.021)
Tajima's D[b]	−1.319	−1.096	−1.779	−1.112	−1.362

Standard error values are given in parentheses.
a Mitochondrial diversity estimates are based on 396bp from control region domain I (see methods).
b Tajima's *D* statistic, $p > 0.05$ in all cases (significance indicates the violation of neutral expectations).

Table 2 – t-Values from haplotype diversity (h) pairwise comparisons using the t-test described by Nei (1987)

	Pre-decline	2000/01	2002/03	2003/04	2005/06
Pre-decline	–				
2000/01	−1.597 (43)	–			
2002/03	1.248 (44)	2.534 (37)	–		
2003/04	2.499 (49)	4.116 (42)*	0.896 (43)	–	
2005/06	9.615 (43)*	11.002 (36)*	7.700 (37)*	7.420 (42)*	–

An asterisk (*) indicates that haplotype diversity estimates are significantly different from each other ($p < 0.005$), and degrees of freedom are given in parentheses.

Simulations for population sizes of 30 individuals (the maximum capacity for the Pakistan breeding facility), with proposed captive breeding strategies based on random pairings, show autosomal microsatellite allelic diversity declining significantly after 2006, with microsatellite heterozygosity declines beginning after 2015 (Fig. 3). However, the breeding facility in Pakistan currently only possesses 11 vultures, three of which are confirmed females (C. Murn, personal communication). Therefore, projected estimates of genetic diversity over time for 11 individuals would be even less than that reported here. Based on simulations, a minimum of 300 individuals is required to retain 90% of the current level of allelic diversity in the captive population over a 100-year period (Fig. 3), while 100 individuals are required to retain 90% of the current level of heterozygosity.

4. Discussion

Despite a dramatic decline in oriental white-backed vulture abundance in the study area, autosomal DNA (i.e., microsatellite) diversity showed no indication of this decline with reduced levels of diversity, at least prior to our most recent sampled breeding season (2005/06). Mitochondrial diversity, however, has started to decline based on estimates of haplotype diversity (*h*), and autosomal DNA genetic diversity declines are anticipated within the next decade at current population sizes in both captivity and the wild as shown with simulations. These results agree with theoretical expectations that long-lived species can retain levels of genetic diversity in small populations over short time periods (see also Kuo and Janzen, 2004; Hailer et al., 2006), yet levels will decline in both markers provided the population bottleneck is maintained for an extended period of time.

As our results indicate, the current sizes for captive breeding stock are underestimated for those necessary to maintain pre-decline levels of genetic diversity when assuming a breeding strategy based on random pairings. The captive breeding facilities currently possess 11 and 70 oriental white-backed vultures in Pakistan and India, respectively (C. Murn and C. Bowden, personal communication). In contrast, our simulations indicate a minimum of 100 and 300 vultures are required to retain 90% of the current heterozygosity and allelic diversity, respectively, over the next 100 years. Although, the total number of vultures in captivity is slightly less than that required to maintain current levels of heterozygosity, the difference in the number of breeding vultures necessary to maintain allelic diversity is much greater. The current captive breeding population constitutes only 27% of this number, and political and logistical barriers separating the captive breeding facilities potentially hamper exchange of individuals and the maintenance of genetic diversity even further.

Following a significant decline in population size, it is not uncommon to observe a reduction in allelic variation before a loss of heterozygosity (Maruyama and Fuerst, 1985; Allendorf, 1986). In fact, heterozygosity can actually increase following a population bottleneck (e.g., Leberg,

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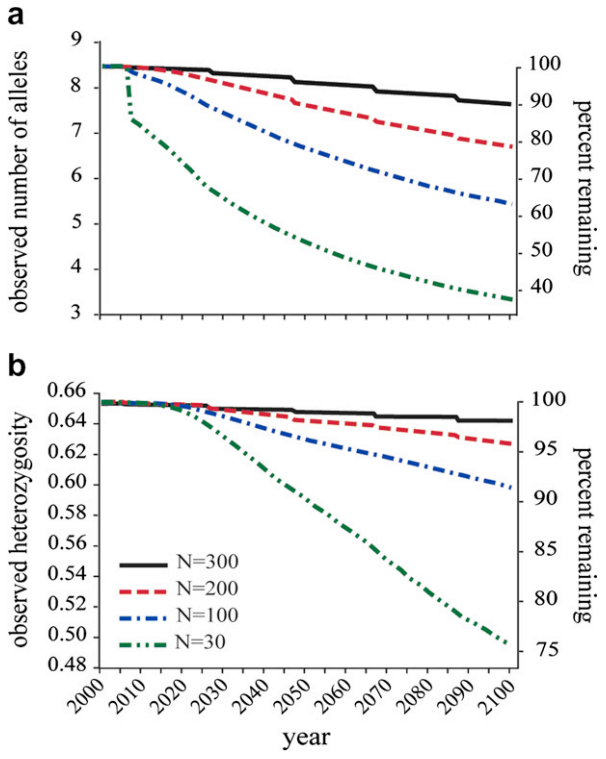

Fig. 3 – Simulations of changes in autosomal microsatellite (a) observed number of alleles and (b) heterozygosity over a 100-year period based on actual census data from Toawala for the first six years and assuming constant, though different, population sizes thereafter.

1992; Kaeuffer et al., 2007). Heterozygosity is a measure that describes the evenness of allele frequencies in a population (e.g., average proportion of loci that possess two different alleles) and is useful for describing the potential for *immediate* adaptive response to selection. In contrast, the average number of alleles (e.g., alleles per locus or allelic richness) represents the *long-term* evolutionary potential of a population because the initial number of alleles determines the limit of selection regardless of the allelic frequencies (James, 1971; Frankham, 2005). Although both high levels of heterozygosity and allelic diversity are desirable, the latter is especially important for the long-term survival of a species by allowing a population to respond to a changing environment (Frankham, 2005; Swindell and Bouzat, 2005; Willim et al., 2006; Bell and Collins, 2008).

To provide the species with the highest likelihood of persisting in the wild after reintroduction, the captive population of oriental white-backed vultures should be increased in size to at least a minimum of 300 birds. Ideally, this should be done immediately not only to forestall further loss of genetic diversity, but also to protect them from exposure to diclofenac until the general public no longer uses this pharmaceutical for livestock. Despite identifying a suitable alternative drug to veterinary diclofenac (meloxicam, see Swan et al., 2006; Cuthbert et al.2007; Swarup et al., 2007), including banning its manufacture and importation in 2006 by governments of India, Pakistan and Nepal, the availability of diclofenac and hence vulture mortality continues (see also Prakash et al., 2007). In a recent study, Prakash et al. (2007) documented a decline of 99.9% between 1992 and 2007 in the oriental white-backed vulture population based on road transects located throughout northern, central, western and northeastern India. The use of diclofenac for veterinary purposes has also affected other *Gyps* species in south Asia. A reduction in population size > 95% has been documented in the critically endangered slender-billed (*Gyps tenuirostris*, see Johnson et al. 2006) and long-billed (*Gyps indicus*) vultures in both India (Prakash et al., 2003, 2007) and Pakistan (Asian Vulture Population Project, 2008). Of course, this recommendation to increase size of the captive breeding flock by capturing wild birds will be difficult to implement given the expense and current political climate. However, scientific justification for increasing its size is crucial for efforts to raise funds and build political will to cooperate.

The obvious decline in *Gyps* vultures throughout south Asia has significant implications. They not only play an important ecological role in which their absence will likely influence the biology of other organisms (e.g., Gaston and Fuller, 2008), but they are also valuable to human society (Markandya et al., 2008). *Gyps* vultures are extremely efficient scavengers and able to dispose of carcasses quickly thereby limiting access to other potential scavengers such as feral dogs (*Canis familiaris*) that are more likely to spread disease (Pain et al., 2003; Prakash et al., 2003; Markandya et al., 2008). Although we do not know the exact number of *Gyps* vultures remaining, we do know that their numbers have become extremely low and individual vultures are increasingly difficult to observe in the wild throughout south Asia (see also Prakash et al., 2007). Such low numbers spread over a large geographic area may also further intensify the rate of decline in abundance due to reduced foraging success (Jackson et al., 2008) and other potential Allee effects (Berec et al., 2007).

Therefore, it is essential that additional vultures be brought into captivity as soon as possible to ensure that birds exist for reintroduction once diclofenac is no longer available. The captive populations should also be monitored using genetic methods to ensure that diversity levels are maintained (e.g., Schwartz et al., 2007). Additional genetic monitoring may be crucial before this species is extinct in the wild as our last sampling period used to assess genetic diversity levels was over two years ago, and we do not know the current status of genetic diversity levels for either wild or captive oriental white-backed vulture populations. It is also important that the captive breeding facilities cooperate and share birds for breeding purposes, which will reduce the potential for genetic adaptation to captivity (e.g., Frankham, 2008) and help maintain a viable population by increasing the total size of the captive population. As broadly acknowledged, breeding strategies minimizing the pairing of close relatives will also aid in the maintenance of genetic diversity (e.g., Ballou and Lacy, 1995; Lacy, 2000). Similar conservation measures should also be imposed to prevent the extinction of both slender-billed and long-billed vultures in south Asia. The adherence to the above recommendations will be invaluable for reintroduction purposes once diclofenac is no longer present in the environment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocon.2008.07.001.

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