ASSESSING THE DEMOGRAPHY AND CONSERVATION GENETICS OF ASIAN VULTURES USING NON-INVASIVE MOLECULAR TECHNIQUES

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Asian vultures are undergoing widespread population declines and several species are listed as critically endangered. In spite of this conservation crisis, there is little information on the demography and genetics of any species. This is largely because vultures are very difficult to study using conventional methods: individuals breed irregularly, non-breeders are itinerant, and all vultures can travel hundreds of kilometers to forage. To overcome the challenges of studying vultures using traditional methods, I obtained genotypes derived from non-invasively collected feather samples as an alternative means to 'capture' individual vultures for demographic and genetic analyses. I analyzed samples from three species in Southeast Asia, *Gyps bengalensis*, *G. tenuirostris*, and *Sarcogyps calvus*, whose populations have been greatly reduced and geographically restricted in Cambodia. I also collected feathers from two species in Kazakhstan, *Gyps himalayensis* and *Gyps fulvus.* In my first dissertation chapter, I described a restriction endonuclease digest assay that distinguishes the visually similar feathers of the five vulture species*,* and I described a panel of 8 polymorphic microsatellite loci that I used for subsequent analyses. In my second chapter, I analyzed microsatellite loci and mitochondrial DNA to assess the genetic diversity of the three species found in Cambodia. I found that *G. bengalensis*, despite having a small population size, showed relatively high levels of genetic diversity, whereas *G.*

tenuirostris and *S. calvus* had lower levels of genetic variation. In addition, I compared the genetic structuring of *G. bengalensis* in Cambodia with samples collected from individuals in Pakistan in 2000/01 and found that the two populations have significant levels of population differentiation. For my third chapter, I used DNA from feathers for genetic capture-mark-recapture analyses for the three vulture species in Cambodia and for *G. himalayensis G. fulvus* in Kazakhstan. I used closed capture models to generate abundance estimates for *G. bengalensis* and *G. himalayensis* but lacked the requisite statistical power for the remaining three species due to low resampling rates. Overall, my research identified aspects of vulture biology previously not studied and provides the methods that can be used to further investigate the biology of these threatened avian scavengers.

BIOGRAPHICAL SKETCH

Yula Kapetanakos was born in Austin, Texas but spent the formative part of her life in the Washington, DC area. She received a Bachelor's of Science from the Zoology Department at the University of Maryland in 1993. After completing her BS, Yula worked with the US Fish and Wildlife Service Whooping Crane recovery program at the Patuxent Wildlife Research Center. In 1997, she returned to school to complete a Master's of Science in Conservation Biology and Sustainable Development also from the University of Maryland. In 1999, Yula moved to New York City where she worked as a zoo animal curator for the Wildlife Conservation Society. In 2007, she returned to graduate school at Cornell to pursue her Ph.D. in Ecology and Evolutionary Biology.

For my family,

who have supported me in my journey every step of the way

and

for my grandmothers,

who inspired me with their enduring grace and strength

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PREFACE

Evaluating the long-term persistence of wildlife populations requires both accurate demographic estimates, such as population size, and an understanding of the level of genetic diversity within a population. These data are critical for the conservation and management of wildlife species particularly those that are on the threshold of extinction. The generation of accurate population estimates for old-world vultures is now more critical than ever. *Accipitrid* vultures are found throughout Eurasia and Africa and are among the most difficult birds to access in the field. They are, however, of high conservation priority and are becoming increasingly more vulnerable to human pressures. Thirteen of the sixteen old-world vulture species are known to have declining populations and many species are of immediate conservation concern (IUCN 2013).

Vultures are challenging to study because they easily become leery of capture, their nests are often precariously positioned, juveniles are itinerant, and all vultures can travel hundreds of kilometers to forage.

Over the years, a variety of field-based methods have been developed to collect demographic data on vertebrate populations, including direct observation, mark-resight, mark-recapture, and transects (Witmer 2005; Thompson et al.1998). However, more recently, the increasing sophistication of genetic technologies has greatly increased the power of DNA analyses, incentivizing researchers to combine genetics and fieldwork when investigating the ecology and behavior of wildlife. In particular, for species that are difficult to capture or dangerous to handle, DNA from scat, and

shed feathers or hair can be used to address a wide range of ecological or conservation related questions, such as movement/dispersal, relatedness between individuals, and the degree of genetic differentiation between populations. The long-term viability of populations can be examined through estimates of genetic diversity, as can historical events such as bottlenecks (Frankham et al. 2010). Genetic data can also be used within a mark-recapture framework to establish demographic estimates of abundance, survival rates and gender ratios (Lukacs & Burnham 2005; Waits 2004). In contrast to traditional mark-recapture sampling, which requires capturing and physically tagging an animal for individual identification, genetic mark-recapture applies the unique genetic fingerprint of each individual in the population as the identifying "tag". At present, the utility of genetic mark-recapture studies has best been illustrated in population inventories of mammals (Kendall et al. 2008; Boulanger et al. 2004; Boulanger et al., 2008; Bellemain et al. 2005; Guschanski et al. 2009). However, these methods have only rarely been applied to answer ecological questions for bird populations. My conservation-focused research expanded on current mark-recapture methods by using DNA from feather samples collected from vulture feeding sites in Cambodia and Kazakhstan. The ultimate objective was to describe the demographic and genetic attributes of understudied Asian vultures to contribute to future conservation efforts.

In Southeast Asia, White-rumped (*Gyps bengalensis*), Slender-billed (*Gyps tenuirostris*), and Red-headed vultures (*Sarcogyps calvus*) are found in Cambodia, and parts of Laos and Vietnam, where they persist in very low, but seemingly stable numbers (Clements et al. 2013). Southeast Asian vultures have likely been separated

from those in South Asia for at least several decades. In the past 20 years, these species in India, Nepal and Pakistan have declined by as much as 99% (Prakash et al. 2007). As a result, the populations in Cambodia have become the focus of a series of fast-acting initiatives involving several wildlife conservation organizations (Clements et al. 2013). Fragmented populations, such as the vulture populations of Southeast Asia, are particularly vulnerable to population collapse due to genetic complications arising from inbreeding depression and genetic drift; these are processes that accelerate loss of genetic diversity. Small populations lose genetic diversity at a greater rate than larger populations, with a potential reduction in reproductive fitness and an increased threat of extinction (Frankham et al. 2010). Conservation of these birds is crucial because they may be the last remaining sustainable populations of these species in the wild. Likewise, already small populations of other Gyps vultures (*G. fulvus, G. himalayensis*) in central Asia and the Caucasus have experienced population declines that are best explained by increases in adult mortality rates (Katzner et al. unpublished data). However, there are no reliable population estimates for vultures in this region.

My dissertation as a whole examined the population abundance and conservation genetics of these five vulture species using non-invasively collected feathers samples that were shed naturally at vulture feeding sites (i.e. livestock carcasses). Chapter 1 describes the molecular assay used to distinguish the visually similar feathers of the five target species, as well as the development of a panel of polymorphic microsatellite loci used in subsequent analyses. In Chapter 2, I investigated the genetic diversity of the Southeast Asia vulture populations to assess

the genetic impacts of population decline, and separately examined the population structure of *Gyps bengalensis* using samples collected from South (Pakistan) and Southeast Asia (Cambodia). Chapter 3 describes the use of genetic capture-mark-recapture to assess the population size of the vultures in Cambodia and Kazakhstan, while chapter 4 discusses the utility of these non-invasive methods to study bird populations.

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CHAPTER 1

DEVELOPMENT OF MICROSATELLITE MARKERS AND A RESTRICTION ENDONUCLEASE DIGEST ASSAY FOR NON-INVASIVE SAMPLING OF ENDANGERED WHITE-RUMPED, SLENDER-BILLED AND RED-HEADED VULTURES

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Abstract

Southeast Asian vultures have been greatly reduced in range and population numbers, but it is challenging to use traditional tagging and monitoring techniques to track changes in their populations. Genotypes derived from non-invasively collected feather samples provide an alternative and effective means to 'capture' individual vultures for mark-recapture analyses. We describe a restriction endonuclease digest assay that distinguishes the visually similar feathers of three species of critically endangered Asian vultures (*Gyps bengalensis, G. tenuirostris*, and *Sarcogyps calvus*). In addition, we describe a panel of 8 polymorphic microsatellite loci. In combination, the restriction endonuclease assay and microsatellite marker set developed here are powerful molecular tools for investigating the genetic and demographic status of these Asian vultures species.

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Introduction

Of the nine species of vulture found in Asia, seven are undergoing population declines (IUCN 2013) largely due to severe reduction in food resources, habitat loss, and poisoning (Clements et al. 2013; Pain et al. 2003). Within the last two decades, three species found on the Indian sub-continent, White-rumped (*Gyps bengalensis*), Slender-billed (*G. tenuirostris*) and Red-headed (*Sarcogyps calvus*) vultures, were nearly extirpated by secondary exposure to the veterinary pharmaceutical diclofenac (Green et al. 2004) and are now listed as critically endangered (IUCN 2013). The three species also occur in low population densities in Cambodia. Monitoring and research efforts to conserve the Cambodian populations are underway through visual surveys and supplemental feeding programs that began in 2004 (Clements et al. 2013). To estimate abundance and genetic variability, we initiated a non-invasive genetic mark-recapture study using naturally shed feathers in 2008 (e.g. Rudnick et al. 2008). We developed an endonuclease digest assay to identify the vulture species associated with each feather sample and generated a panel of microsatellite markers to identify unique individual genotypes for mark-recapture analyses.

Methods and Results

We used blood samples obtained from wild *G. bengalensis* (n = 4), *G. tenuirostris* (n=5), and *S. calvus* (n=2). Additionally, we collected naturally shed feathers from six supplemental feeding sites in northern Cambodia.

We extracted genomic DNA from both types of samples using a DNeasy Blood and Tissue Kit (QIAGEN) (Horváth et al. 2005). We designed a vulture specific *COI* primer, GypsR1 (5'-CCAAAGCCCGGTAGRATTAGG), from a mitochondrial

cytochrome oxidase (*COI*) sequence from Eurasian Griffon (*Gyps fulvus*) (Mindell et al. 1997; GenBank U83772) to use with a generic avian forward primer, AvianCOIF (5'-CTGTAAAAAGGACTACAGCCTAACGC). We PCR amplified an 806 base pair fragment of *COI* for six vulture species found in Asia and sequenced in both directions using an ABI 3730xl capillary DNA Analyzer (Applied Biosystems). Sequences from vouchered tissues were used to identify diagnostic restriction endonuclease cut sites. Each 20 μL PCR reaction was divided equally into two reactions so that restrictions enzymes *HaeIII* and *RsaI* (New England BioLabs) could be used separately. Digest products were visualized on a TAE buffered 2% agarose (Fisher Scientific) gel stained with ethidium bromide following electrophoresis. Species were differentiated based on the predictable size fragment profiles produced by the enzymatic digest (Figure 1.1). Vouchered samples were used as positive controls to ensure that complete digestion was achieved.

For microsatellite development, we extracted genomic DNA from blood samples collected from *G. bengalensis* and *G. tenuirostris*. DNA libraries enriched for microsatellites were created using a universal linker and ligation process (Hamilton et al. 1999) with modifications (Barnett et al. 2008; Grant & Bogdanowicz 2006). Following PCR amplification of plasmid DNA with universal M13 primers, nucleotide sequences were obtained from 127 *G. tenuirostris* and 26 *G. bengalensis* positive plasmid clones. Primers were designed for 20 *G. tenuirostris* and 7 *G. bengalensis* microsatellite loci and tested for variability on a panel of 16 *G. bengalensis* samples.

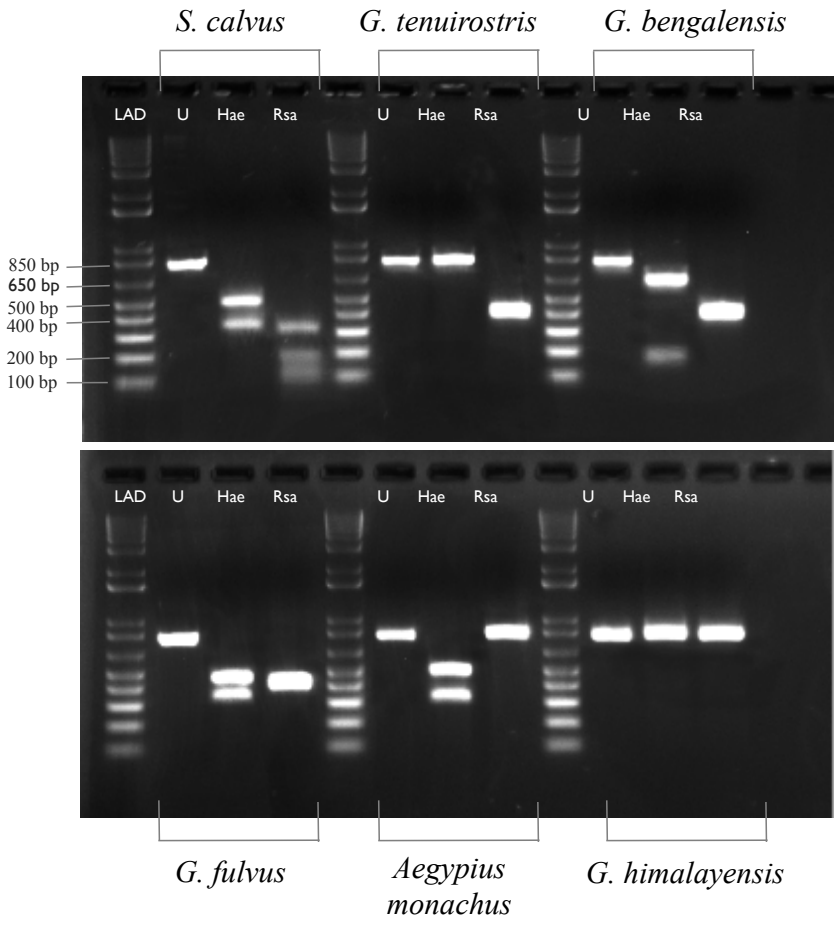

Figure 1.1 - Restriction endonuclease digest of *cytochrome oxidase I* region of mtDNA of six vulture species for species identification from non-invasively collected samples. U= undigested DNA, Hae = *Hae*III enzyme, Rsa = *Rsa*I enzyme, LAD = 1 kb Plus DNA Ladder with fragment standards. We used 2 µL of uncut DNA and 15 µL of digested DNA for visualization on a 2% agarose gel.

We used two methods to determine allele size (Rubin et al. 2009). Initial variability screening involved a 'universal tag' method (Schuelke 2000) using an unlabeled locus-specific forward primer (Waldbieser et al. 2003), a 'universal' primer

containing the same base pairs with the addition of a 5' fluorescent tag, and a modified third locus-specific reverse primer (Brownstein et al. 1996). For the second method of amplification we used a locus-specific forward primer that was modified with a 5' fluorescent label (PET, 6-FAM, VIC, or NED, Applied Biosystems). Labeled PCR products were analyzed on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems), and allele sizes were estimated using the GeneScan 500 (-250) LIZ size standard (Applied Biosystems) and GENEMAPPER® version 3.7 software (Applied Biosystems). We cross-tested each of the loci with blood and feather samples obtained from *G. tenuirostris* and *S. calvus* (Table 1.1).

To test for evidence of genotyping error and the presence of null alleles, we used the program MICRO-CHECKER version 2.2.3 (10,000 iterations) (van Oosterhout et al. 2004). There was no evidence of error due to stutter peaks or dropout of large alleles. We used the program GIMLET version 6.4 (Valiere 2002) to ensure genotypes obtained from feather samples represented unique individuals. We evaluated deviations from Hardy-Weinberg equilibrium (HWE) and linkage-disequilibrium, and derived estimates for observed and expected heterozygosity (H_o and H_E) for each locus using the program GDA (Lewis & Zaykin 2001). We used the program GENALEX version 6.5 (Peakall & Smouse 2006) to calculate the probability of identity (P_{ID}) for loci that amplified (Waits et al. 2001). All 8 loci were polymorphic for *G. bengalensis* with 4–16 alleles/locus. Mean observed (H_o) and expected heterozygosity (H_E) across all loci were 0.74 and 0.70. Two loci showed evidence for null alleles (GB2-4A &

Table 1.1- Characteristics of microsatellite loci in *Gyps bengalensis*, *G. tenuirostris* and *Sarcogyps calvus*. *n* = number of samples, N_A, = number of alleles, H_o = observed heterozygosity; H_E = expected heterozygosity. Annealing temperature = 60˚C, and $MgCl_2$ = 1.5mM (except GT2-28 = 52˚C and 2.25 mM $MgCl_2$); † =possible null alleles.

Locus Name: (GenBank accession #) Primer Sequence (5' – 3')	Repeat motif	*G. bengalensis* P_{ID} = 4.3 x10^{-9}					*G. tenuirostris* P_{ID} = 2.6x10^{-4}					*S. calvus* P_{ID} = 2.7 x10^{-5}				
		n	*Range*	N_A	H_o	H_E	*n*	*Range*	N_A	H_o	H_E	*n*	*Range*	N_A	H_o	H_E
GB2-4A (GenBank Accession # KJ663809) ACATTCATAGATGATCAGCAACCTG GTTTCTCTGTGATCGCTCTAGGATGTTGCTTC	(GA)30	39 †	397-464	16	0.72	0.85	30	—	—	—	—	28	—	—	—	—
GB2-4B (GenBank Accession # KJ663806) CAACTCCACAGTTTAGGCAGATGTACC GTTCTGGTGACTTCACAAGGGACTATCAGAGA	(AC)15	39	341-365	7	0.77	0.74	30	348-365	3	0.53	0.57	27	350-360	4	0.63	0.73
GB3-2C (GenBank Accession # KJ663805) ATGAATCCAGGCTCAGTCAGAAC AGACATGGTAAGGAGTCAGCAGC	(AGA)50	39 †	404-461	14	0.77	0.88	30	410-433	5	0.73	0.73	28 †	404-443	10	0.50	0.77
GB4-4G (GenBank Accession # KJ663810) CGGTGAGCGGCCTCATTATC GCTCAACTTTCAGTTCCACTTC	(GTTT)8	39	164-176	4	0.61	0.57	23	164-172	2	0.57	0.50	17	158	1	—	—
GT2-28 (GenBank Accession # KJ663807) CCATCATCGTGGATGTTAGAAACTA GTTTCTCACTTCTTCATTGCCTGAGATATA	(GT)12	39	286-305	9	0.51	0.48	30	297	1	—	—	28	293-315	11	0.68	0.79
GT3-35 (GenBank Accession # KJ663811) CCCCTTGTATGACAATGGTACAGTAT GTTTCTGTATTCAAAAGACATGACATCCAC	(ATT)10 (ACT)13	39	216-264	12	0.82	0.79	30	252	1	—	—	28	225-240	4	0.57	0.55
GT3-38 (GenBank Accession # KJ663808) CCCGAGCCAAGCCAGTTATTATA GTTTCTCATACAACAATCTCTTGTGCTGAC	(TAA)12 (CAA)5	39	370-385	6	0.615	0.73	30	373-385	4	0.53	0.69	28	358-385	6	0.76	0.76
GT4-20 (GenBank Accession # KJ663812) GTGAGCCCTCCCATTGAGTCAT CTCAAGTGCATGCCCGCTG	(GAAA)12 (GA)8	27	298-334	10	0.815	0.84	24	303-330	8	0.63	0.75	16	299	1	0.63	0.76

GB3-2C). No loci deviated from HWE or linkage disequilibrium following Bonferroni corrections.

The analysis of feather samples dropped at carcass feeding sites provides the opportunity to explore the demographics and genetics of vulture populations. The protocols described here will thereby contribute to future management of these endangered species.

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CHAPTER 2

GENETIC VARIATION IN FRAGMENTED POPULATIONS OF CRITICALLY ENDANGERED SOUTHEAST ASIAN VULTURES (*GYPS BENGALENSIS, G. TENUIROSTRIS, SARCOGYPS CALVUS*)

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Abstract

Vulture populations in Asia began undergoing population declines in the early and mid- twentieth century, and many species are now seriously threatened. As a result of the extirpation of wild ungulates, vultures that were once common throughout Southeast Asia have become largely restricted to Cambodia where they are now critically endangered. We investigated the genetic diversity of the three species presently found in Cambodia: White-rumped (*Gyps bengalensis*), Slender-billed (*G. tenuirostris*), and Red-headed (*Sarcogyps calvus*) vultures. DNA was obtained from feather samples collected from a monitored vulture feeding station in the Preah Vihear Protected Forest. Based on analyses of microsatellite loci and mitochondrial DNA, we found that White-rumped vultures, despite having a small population size, showed relatively high levels of genetic diversity, whereas Slender-billed and Red-headed vultures had lower levels of genetic variation. In addition, we compared the genetic structuring of White-rumped vultures in Cambodia with samples collected from individuals in Pakistan in 2000/01 and found that the two populations have significant levels of population differentiation.

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Introduction

When managing threatened species, it is vital to assess the genetic diversity and structure of their populations (Frankham 2005). The degree of genetic diversity provides insight into a populations' fitness, evolutionary potential, and likelihood of extinction (Booy et al. 2000; Frankham 2003; Reed & Frankham 2003).

Genetic considerations are especially important for rare and geographically isolated species. Declining heterozygosity, an indicator of inbreeding depression, can lead to an increase in deleterious traits and loss off fitness as observed in the Florida panther (Hedrick & Kalinowski 2000; Roelke et al. 1993) and the Greater Prairie Chicken (Westemeier et al. 1998). In the case of the Greater Prairie Chicken, population declines continued despite an increase in protected habitat and intensified conservation efforts. Genetic management became necessary to stem further losses. Populations that suffer from very low levels of genetic variability are highly prone to extinction if genetic management is not implemented (Saccheri et al. 1998).

Asian vultures have undergone massive population declines (Pain et al. 2003). In Southeast Asia, declines have occurred over the past century, driven by losses to habitat and foraging opportunity (Clements et al. 2013; Pain et al. 2003). Three vulture species (*Gyps bengalensis*, *Gyps tenuirostris*, and *Sarcogyps calvus*) are found in small geographic pockets, primarily in Cambodia and Myanmar (Figure 2.1), at very low population abundance (Clements et al. 2013; Hla et al. 2011). The three species

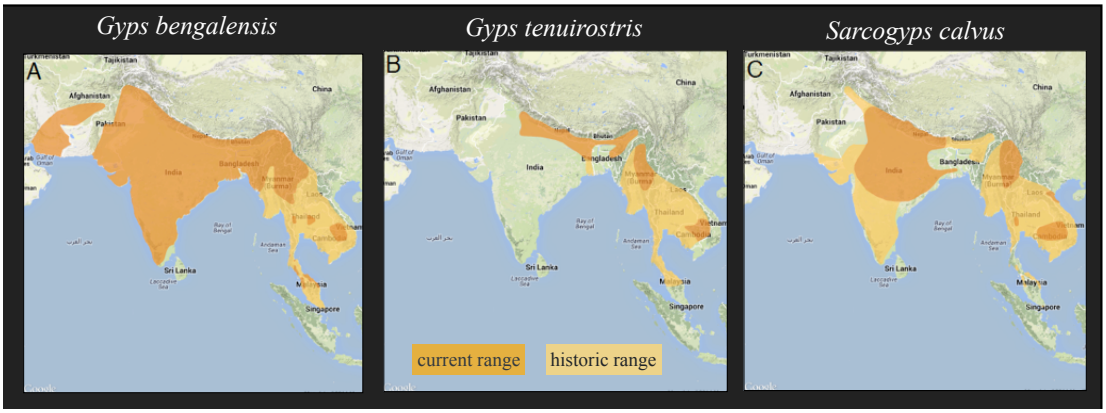

Figure 2.1 - Distribution of (A) White-rumped vulture (*Gyps bengalensis*), (B) Slender-billed vulture (*G. tenuirostris*), and (C) Red-headed vulture (*Sarcogyps calvus*). The darker shading represents the current geographic range, the lighter shade shows where the species have been extirpated.

are also found in South Asia (India, Nepal and Pakistan; Figure 2.1) where, in contrast, declines occurred rapidly over a fifteen-year period after food resources became contaminated with pharmaceutical toxins (Prakash et al. 2003; Prakash et al. 2007; Prakash et al. 2012; Oaks et al. 2004; Green et al. 2004). All three species are listed as critically endangered by the IUCN (IUCN 2013).

In the early 2000's, the genetic variability for White-rumped vulture (*Gyps bengalensis*) in South Asia (i.e. Pakistan) was assessed using both mitochondrial and nuclear DNA (Johnson et al. 2008). The study showed that the species maintained high levels of genetic diversity despite recent population declines. However, genetic variability has not been assessed for vulture populations in Southeast Asia. The contrasting rates of declines of vulture populations in Southeast and South Asia presents an opportunity to evaluate the genetic structuring of White-rumped vultures,

and also to contrast the genetics of two differently sized populations of highly vagile birds of prey. In this study, we analyzed mitochondrial and microsatellite markers to address the following two questions: First, what is the genetic diversity of these three critically endangered bird species in Cambodia; second, what does a comparison of genetic diversity of White-rumped vultures tell us about the impacts of different types of population decline on avian population genetics and genetic structure.

Methods

Sample collection

In 2004, in collaboration with Cambodian government agencies, wildlife organizations based in Cambodia initiated a vulture supplemental feeding and population monitoring program. Seven supplemental feeding stations ('vulture restaurants') were distributed throughout the northern and eastern parts of the country in an area covering roughly 300 km east-west by 250 km north-south (Clements et al. 2013). One livestock carcass was placed at each of the sites on a monthly or bi-monthly basis and monitored over the course of several days until the carcass had been consumed. In 2009, to compare count data collected from visual surveys, we initiated a non-invasive genetic capture-recapture study (chapter 3) using feathers gathered from these seven sites. Feathers were also collected from roosting sites situated at the periphery of the feeding sites. A total of 3,258 feathers were collected between January-May, 2009.

To examine the genetic variation of White-rumped (*Gyps bengalensis*), Slender-billed (*G. tenuirostris*) and Red-headed (*Sarcogyps calvus)* vultures in Cambodia, we used a subset of feather samples collected from one site, Preah Vihear

Protected Forest (PVPF), in 2009, and examined both nuclear (microsatellites) and mitochondrial genetic markers. To characterize genetic differentiation between White-rumped vulture populations in Cambodia and South Asia, we compared our microsatellite and mtDNA analyses to blood and tissue samples collected in Pakistan in 2000/01 (Johnson et al. 2008).

Laboratory methods

We divided our genetic analyses into five main steps to achieve the following goals: 1) extract DNA from feather samples; 2) identify the species associated with each sample; 3) ensure that samples used in our analyses represented unique individuals; 4) assess genetic variability in microsatellite loci; and 5) assess genetic variability in mitochondrial DNA. For steps 1-3, we included all samples collected from PVPF in 2009. For steps 4-5, we selected a subset of samples from PVPF to include in our variability analyses.

DNA extraction

We extracted DNA from a total of 725 feathers collected from PVPF using the E-Z 96® Tissue DNA kit (Omega Biotek) (Horváth et al. 2005). DNA was isolated from the calamus tip of each feather, as well as from a residual blood clot in the superior umbilicus. For large flight feathers, the basal tip of the calamus was quartered, and the superior umbilicus located at the upper most portion of the calamus was removed separately (Horváth et al. 2005). We followed the manufacturers DNA extraction protocols for tissue except that samples were incubated in Proteinase K extraction buffer for 48-72 hours (Bayard de Volo et al. 2008).

Species identification

White-rumped, Slender-billed and Red-headed vultures foraged together at the supplemental feeding sites, and we could not reliably distinguish single feathers of these species based on visual differences alone. However, we were able to differentiate feathers in the lab based on diagnostic differences in the mitochondrial region between each of the species. We used a non-vulture specific forward primer (AvianCOI) and a *Gyps* vulture specific reverse primer (GypsR1) to PCR amplify an 806 base pair fragment of the COI gene (Kapetanakos et al. 2014). Each 20 μL PCR reaction included: 1-10 ng of genomic DNA, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 pmoles each of forward and reverse primers, 200 μM dNTP's and 0.5 U Jumpstart ™ *Taq* polymerase (Sigma). The PCR program consisted of one cycle at 95˚C for 4min30sec, followed by 35 cycles of 95˚C for 1min, 54˚C for 1min, and 72˚C for 1min20sec for 35 cycles, with a final extension step of 72˚C for 4min30sec. PCR products were divided equally into two reactions and digested overnight with *HaeIII* and *RsaI* (New England BioLabs). Digest products were visualized on a TAE buffered 2% agarose (Fisher Scientific) gel stained with ethidium bromide following electrophoresis. Each species could be easily identified from distinctive DNA fragment sizes produced by the enzymatic digest. Vouchered samples were used as positive controls to ensure that complete digestion was achieved (Kapetanakos et al. 2014). We repeated any sample that did not initially amplify. If amplification failed on the second attempt, we discarded that sample from all further analyses.

Microsatellite genotyping

We anticipated that individual vultures would be represented by more than one feather in our sample collection; therefore, it was important that we first group our samples according to unique genotypes before continuing with analyses for genetic variability. We initially amplified all samples at nine microsatellite loci in two separate reactions using a combination of loci from two different sources (Table 2.1): GB and GT primers were developed for White-rumped (*Gyps bengalensis*) and Slender-billed (*G. tenuirostris*) vultures respectively (Kapetanakos et al. 2014), and Gf primers were developed for Eurasian griffon (*G.* fulvus) (Mira et al. 2002). Microsatellite loci were combined in two multiplex PCR reactions (multiplex mix A: GT3-35, GT3-38, GB2-4A, GB2-4-4B; GB3-2C; multiplex mix B: Gf11A4, Gf3H3, Gf9C1, GT2-28). PCR's were performed in 10 µl reactions; 0.12 – 0.25 µM fluorescently labeled forward and unlabeled reverse primer (GT3-35, Gf3H3 = 0.12 µM; GT3-38, GB2-4A, GB2-4-4B, GT2-28, Gf9C1, Gf11A4= 0.25 µM), 1x buffer solution (Sigma), 1.5 mM (mix A) or 2.5 mM (mix B) $MgCl_2$, 0.2 mM dNTP's, and 0.05 U/µL of Jumpstart ™ *Taq* polymerase (Sigma). The PCR conditions were as follows: Initial denaturation step at 94˚C for 4min, followed by 35 cycles at 94˚ C for 50sec, 1min at the annealing temperature (60˚C for mix A, 52˚C for mix B), 72˚C for 1min, with a final extension step at 72˚C for 30min. We amplified two additional loci for Slender-billed and Red-headed samples, using primers BV6 and BV20 developed for Bearded vulture (*Gypaetus barbatus*) (Gautschi et al. 2000). Conditions for the BV reactions were the same as those for Mix A except that we used 0.12 µM of the forward and reverse primers, and an annealing temperature of 58˚C.

Table 2.1 - Microsatellite loci used to group unique genotypes and then to assess genetic variation in White-rumped (WR), Slender-billed (SB) and Red-headed (RH) vultures. Red diamonds (◆) in the three final columns indicate loci used to group unique genotypes. Black dots (•) indicate loci used in analyses to quantify genetic variability.

Locus	Label	Species	GenBank Accession No.	Primer sequence (5'-3')	Reference	Loci used in analyses *WR*	*SB*	*RH*
BV6	PET	*Gypaetus barbatus*	AF270732	F: AATCTGCATCCCAGTTCTGC R: CCGGAGACTCTCAGAACTTAAC	Gautschi et al. 2000	•	◆•	◆•
BV11	FAM	*Gypaetus barbatus*	AF270736	F: TGTTTGCAAGCTGGAGACC R: AAAAGCCTTGGGGTAAGCAC	Gautschi et al. 2000	•		
BV12	VIC	*Gypaetus barbatus*	AF270737	F: TCAGGTTTTGACGACCTTCC R: GTGGTAACGGAGGAACAAGC	Gautschi et al. 2000	•		•
BV13	NED	*Gypaetus barbatus*	AF270738	F: TTCAGGAAACAGAAGCATGAAC R: AAAACAGAGTTTTCACATTTTCATAAG	Gautschi et al. 2000	•	•	•
BV14	PET	*Gypaetus barbatus*	AF270739	F: GGCAGTGTGGAGCCTACATC R: CTCCAGGGTCCTTGTTTGC	Gautschi et al. 2000	•	•	•
BV20	FAM	*Gypaetus barbatus*	AF270742	F: GAACAGCACTGAACGTGAGC R: GTTTCTCCTGACAGTGAAATAACTC	Gautschi et al. 2000	•	◆	◆•
Gf11A4	PET	*Gyps fulvus*	AY035858	F: GATCCCTTCCAACCGAAAAT R: TGGTGACCAACGGAAGTGTG	Mira et al. 2002	◆•	◆•	
Gf3H3	NED	*Gyps fulvus*	AY035859	F: GTAGAATAATTTGCTCCTGG R: GTGAAGGCACCTCATAGACA	Mira et al. 2002	◆	◆•	
Gf9C1	FAM	*Gyps fulvus*	AY035855	F: GGTGGACATTACATACACTG R: CAAGGAATCTGGACTACTAA	Mira et al. 2002	◆•		
GB3-2C	NED	*Gyps bengalensis*	KJ663805	F: ATGAATCCAGGCTCAGTCAGAAC R: AGACATGGTAAGGAGTCAGCAGC	Kapetanakos et al. 2014		◆•	◆
GT3-35	NED	*Gyps tenuirostris*	KJ663811	F: CCCCTTGTATGACAATGGTACAGTAT R: GTTTCTGTATTCAAAAGACATGACATCCAC	Kapetanakos et al. 2014	◆•		◆•
GT3-38	PET	*Gyps tenuirostris*	KJ663808	F: CCCGAGCCAAGCCAGTTATTATA R: GTTTCTCATACAACAATCTCTTGTGCTGAC	Kapetanakos et al. 2014	◆•	◆	◆•
GB2-4B	FAM	*Gyps tenuirostris*	KJ663806	F: CAACTCCACAGTTTAGGCAGATGTACC R: GTTCTGGTGACTTCACAAGGGACTATCAGAGA	Kapetanakos et al. 2014	◆•	◆•	◆•
GT2-28	VIC	*Gyps tenuirostris*	KJ663807	F: CCATCATCGTGGATGTTAGAAACTA R: GTTTCTCACTTCTTCATTGCCTGAGATATA	Kapetanakos et al. 2014	◆•		◆

Based on the performance of each of the loci (e.g. low vs. high failure rate, reliability of allele peak interpretation, and monomorphic vs. polymorphic alleles), we ultimately selected seven microsatellites per species for our initial screening of samples (Table 2.1) and subsequent grouping of genotypes to identify individuals within the population. We genotyped 1μL of the labeled PCR products on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems), and allele sizes were estimated using the GeneScan 500 (-250) LIZ size standard (Applied Biosystems) and GENEMAPPER® version 3.7 software (Applied Biosystems).

We eliminated from further analysis any sample that did not amplify at four or more of the seven loci analyzed. Poor amplification typically reflects low DNA sample quality and discarding such samples early in the genotyping process reduces the probability of miscalled alleles (Paetkau 2003). For samples that successfully amplified for five or six loci, we repeated the PCR and genotyping for any of the loci that initially failed until genotypes for all seven loci had been generated. For samples that PCRed but with weak amplification as visualized on a 2% agarose gel, we used a pre-amplification method that included two separate PCR reactions run under the same conditions. We modified the protocol from (Piggott et al. 2004) so that both PCR reactions were in 10ul, and for the second PCR reaction we used 1μl of PCR product from the first reaction. Both PCR reactions were subject to the same thermal cycling conditions. Once all loci were repeated, we eliminated samples that failed at any locus. We then continued analyses for samples that genotyped successfully at all seven loci.

We used the program GIMLET to group identical genotypes (Valiere 2002). GIMLET creates consensus genotypes that are compared with each other to find unique

genotypes. We then pooled identical genotypes and considered each pool as a single individual (Schwartz et al. 2006). Allelic dropout is one of the primary sources of error associated with genotyping samples that yield low quantities of DNA (See chapter 4); therefore, we further scrutinized our pooled genotypes to identify samples that mismatched at one or two loci. As part of this process, we first carefully inspected genotyping results visually to assess the results from the original genotyping electropherogram. If errors were not resolved at this stage, we repeated PCR amplification and genotyping for each of the mismatched loci three additional times (Paetkau, 2003). We eliminated samples that failed to produce consistent results. For homozygous samples, we required all three trials to produce the same single allele. For heterozygous samples we required that two of the trials produce the same two alleles.

Once complete genotypes were assembled, we used MICRO-CHECKER (van Oosterhout et al. 2004) to detect null alleles or scoring errors from all 2009 samples. To verify the power of our selected loci, we used GENALEX 6.5 (Peakall & Smouse 2006) to calculate the observed probability of identity (P_{ID}), the probability that two randomly drawn individuals from the population share the same genotype, and the P_{ID} for siblings ($P_{ID\text{-}SIB}$), a more conservative calculation describing the probability that two full-siblings would have identical genotypes at the markers used (Waits et al. 2001). P_{ID} is calculated from the sum of the squares of expected allele frequencies of all genotypes (Paetkau et al. 1998). The smallest values of P_{ID} can be expected when there are many alleles of roughly equal frequency, while the largest values of P_{ID} occur when there is low genetic variation (Mills et al. 2000). Based on recommendations by Mills et al. (2000) and Waits et al. (2001), a P_{ID} of < 0.01 is

appropriate to discriminate between individuals when estimating abundance, and a P_{ID} of < 0.0001 is sufficient for wildlife forensic analyses (Waits et al. 2001).

After unique genotypes were assigned, we randomly selected 38 White-rumped, 29 Slender-billed, and 28 Red-headed samples collected from PVPF to examine the genetic variation of each species. To increase the power of our microsatellite analyses, we used additional microsatellite loci to assess variability. We added six BV loci for White-rumped vulture (BV6, BV11, BV12, BV13, BV14, BV20); for Slender-billed and Red-headed samples we added 4 loci (BV11, BV12, BV13, BV14) (Gautschi et al. 2000) (Table 2.1). We PCR amplified and genotyped each of these additional loci as separate reactions using the same PCR conditions listed above for BV primers.

Mitochondrial DNA

For mitochondrial DNA (mtDNA) analyses, we sequenced 29 White-rumped, 27 Slender-billed, and 21 Red-headed samples. For White-rumped and Slender-billed samples we analyzed 773 and 784 basepairs (bp) respectively of mtDNA control region (CR) I and II using two primer pairs: GbCR1.L/ GbCR2.H (Johnson et al. 2006) and GbCR4.L/CSB1.H (Johnson et al. 2008). CR II amplified poorly for Red-headed samples. Therefore, in addition to CR I, we opted to sequence a 407 bp region of the *cytochrome oxidase* I (COI) region using Avian COIF/GypsR1 primers (Kapetanakos et al. 2014).

We used between 1–20ng of genomic DNA in 10μL PCR reactions containing: 10mM Tris-HCl (pH 8.3), $MgCl_2$ (4mM for CR I and II primers; 1.5mM for COI primers), 2.5pmol each of the forward and reverse primers, 250μM dNTPs, 0.25U of

Jumpstart™ *Taq* Polymerase (Sigma). The PCR cycling profile consisted of one cycle at 95°C for 4min30sec, 35 cycles of 1 min at 95°C, 1 min at the locus-specific annealing temperature (57˚C for domain I and II primers, 54˚C for *COI* primers), 1min20sec at 72°C, and a final extension of 4min30sec at 72°C. We sequenced PCR products in both directions using ABI Big Dye Terminator chemistry, and aligned sequences using SEQUENCHER™ 5.0.

Genetic Diversity

To assess genetic diversity from microsatellite data, we used the program MICRO-CHECKER (van Oosterhout et al. 2004) to test for the presence of null alleles and problems with scoring due to large allele dropout and stuttering artifacts. We used the program GDA (Lewis & Zaykin 2001) to detect microsatellite linkage disequilibrium (LD) and deviations from Hardy-Weinberg equilibrium (HWE), and to determine the mean number of alleles per locus, and mean observed (H_O) and expected (H_E) heterozygosity. For both LD and HWE we applied a Bonferonni correction for multiple tests.

For White-rumped and Slender-billed samples, we concatenated mtDNA regions into a single alignment. To assess mtDNA diversity for CR I (for White-rumped, Slender-billed, and Red-headed samples), CR II (for White-rumped and Slender-billed), and COI (for Red-headed) we used the program ARLEQUIN version 3.5.1.3 (Excoffier and Lischer 2010) to calculate haplotype (h), nucleotide (π) diversity, and *Tajima's D* test for neutrality.

Comparative analysis of genetic diversity of White-rumped vultures

To contrast the diversity found in Cambodian populations with estimates produced from a larger vulture population outside of Cambodia, we obtained both microsatellite genotyping data and mtDNA CR I and II sequences from 19 White-rumped vultures sampled in 2000/01 from Pakistan and analyzed by Johnson et al. (2008). For these samples, DNA was extracted from liver, kidney, muscle and feathers. Although populations in Pakistan have been declining since the 1990's, historic populations numbered in the 100's of thousands throughout the Indian subcontinent. Thus, we made the assumption that estimates of genetic diversity from 2000/01 from Pakistan would be representative of a larger panmictic population.

The analytical protocols we used to assess genetic diversity in this part of our study followed those used by Johnson et al. (2008). In summary, to maintain uniformity between studies, we used the same microsatellite loci used in the analysis for samples from Pakistan. Loci included: BV6, BV11 BV12, BV13, BV14, BV20, Gf3H3, Gf11A4, and Gf9C1. To compare allelic diversity between White-rumped samples collected in Cambodia to those collected in Pakistan we calculated allelic richness using FSTAT Version 2.9.3 (Goudet 2001), which accounts for variation in sample size. Genetic differentiation between populations was estimated with ARLEQUIN vs. 3.5.1.3 (Excoffier & Lischer 2010) using CR data with 100 random permutations to estimate pairwise F_{ST}, and program GENALEX (Peakall & Smouse 2006) to estimate F_{ST} values for microsatellite data. Genetic data for Slender-billed and Red-headed vultures have not been published; therefore, a similar comparative analysis was not possible for these species.

Imprint of population bottleneck

To test for the imprint of a historic bottleneck, we used BOTTLENECK 1.2.02 (Cornuet & Luikart 1996) The program BOTTLENECK computes the distribution of the expected heterozygosity for each population sample and for each microsatellite locus from the observed number of alleles, given the sample size under the assumption of mutation-drift equilibrium. The distribution is obtained through simulating the coalescent process of *n* genes under the infinite allele model and the single mutation model. This enables the computation of the average expected heterozygosity, which is compared to the observed heterozygosity to establish whether there is an heterozygosity excess or deficit at each locus (Cornuet & Luikart 1996).

We implemented the Wilcoxon's test under the two-phase mutational model (TPM) (Piry et al. 1999), which is appropriate for analysis of microsatellite loci with short repeats (2 base pair repeats such as is the case for the majority of our loci) for fewer than 20 loci (Cornuet & Luikart 1996; Piry et al. 1999).

Results

Species assignment

We extracted DNA from a total of 733 samples collected from PVPH in 2009, of which 605 samples were White-rumped, 86 were Slender-billed, and 42 were Red-headed vultures. We eliminated 246 samples when they did not pass our screening process (% samples eliminated: White-rumped = 33%; Slender-billed = 35%; Red-headed = 33%) leaving a total of 403 White-rumped, 56 Slender-billed and 28 Red-headed samples to be grouped into unique genotypes. Once samples were grouped based on matching genotypes, we identified a total of 146 unique genotypes for

White-rumped, 25 for Slender-billed and 21 for Red-headed vultures.

Probability of Identity

The probability of identity (P_{ID}) and probability of identity for siblings ($P_{ID\text{-}SIB}$) for each species were: White-rumped vulture $P_{ID} = 7.2 \times 10^{-8}$ and $P_{ID\text{-}SIB}$ 2.6×10^{-3}; Slender-billed vulture $P_{ID} = 1.9 \times 10^{-5}$ and $P_{ID\text{-}SIB}$ 9.9×10^{-3}; Red-headed vulture $P_{ID} = 2.7 \times 10^{-6}$ and $P_{ID\text{-}SIB}$ 5.8×10^{-3}. All P_{ID} values fell within the recommended ranges (Mills et al. 2000; Waits et al. 2001); therefore, our microsatellite markers had sufficient power to discern between individuals within the study population.

Genetic diversity

Microsatellite loci

There was no evidence of error due to stutter peaks or large allelic dropout based on results from MICRO-CHECKER for any of the loci. Results from the microsatellite analyses for each species are described below and summarized in Table 2.2.

White-rumped vulture: One locus (GB3-2C) showed evidence for null alleles (N_0 freq. 0.058). Two loci (GF11A4 & GF3H3) appeared to be at linkage disequilibrium (LD) even after Bonferroni correction. None of the loci were out of Hardy-Weinberg equilibrium (HWE) after Bonferroni correction. We eliminated GB3-2C and GF3H3 from the more comprehensive analysis leaving 12 loci for genetic diversity analyses. The number of alleles (A) ranged from 3 to 17 per locus (mean = 8.5); Observed (H_o) and expected heterozygosity (H_E) ranged from 0.50-0.97 (mean = 0.72) and 0.47-0.89 (mean=0.72) respectively (Table 2.2).

Table 2.2 - Polymorphism data for 12 microsatellite loci for White-rumped (*Gyps bengalensis*), 7 loci tested for Slender-billed (*G. tenuirostris*) and 8 loci tested for Red-headed (*Sarcogyps calvus*) vulture populations in Cambodia. Mean number of alleles per locus (*A*), observed heterozygosity (H_O), expected heterozygosity (H_E), Hardy-Weinberg Equilibrium P-value (HW) Asterix indicates significant deviation from HW equilibrium.

Species	Sample Size	Locus	*A*	H_O	H_E	HW *P*-value
White-rumped	38	BV6	3	0.605	0.658	0.442
	38	BV11	6	0.684	0.660	0.932
	37	BV12	10	0.757	0.791	0.398
	38	BV13	9	0.868	0.820	0.813
	38	BV14	5	0.500	0.569	0.570
	36	BV20	5	0.667	0.560	0.730
	38	Gf11A4	17	0.974	0.890	0.989
	38	Gf9C1	13	0.868	0.893	0.310
	38	GT3-35	12	0.816	0.804	0.618
	38	GT3-38	6	0.605	0.742	0.047*
	38	GB2-4B	7	0.763	0.740	0.931
	38	GT2-28	9	0.500	0.468	0.938
	Mean		**8.5**	**0.717**	**0.716**	
Slender-billed	30	BV6	2	0.533	0.472	0.699
	30	BV13	2	0.433	0.494	0.702
	29	BV14	3	0.552	0.571	0.214
	30	GF11A4	10	0.833	0.852	0.065
	30	GF3H3	2	0.567	0.508	0.704
	30	GB3-2C	5	0.733	0.726	0.288
	30	GB2-4B	3	0.533	0.571	0.260
	Mean		**3.9**	**0.598**	**0.599**	
Red-headed	28	BV6	6	0.536	0.586	0.114
	28	BV12	2	0.143	0.195	0.250
	28	BV13	5	0.643	0.731	0.363
	28	BV14	2	0.143	0.135	1.00
	28	BV20	5	0.393	0.343	1.00
	28	GT3-35	4	0.571	0.546	0.591
	28	GT3-38	6	0.786	0.758	0.483
	27	GB2-4B	4	0.630	0.725	0.508
	Mean		**4.3**	**0.481**	**0.502**	

Using the same microsatellite loci as Johnson et al. (2008) we found mean A = 8.4, and mean H_o and H_E = 0.70 and 0.69 respectively (Table 2.3b).

The F_{ST} value calculated between Cambodia and Pakistan vulture populations (F_{ST} = 0.034; P = 0.001) suggest that the two populations of White-rumped vultures are significantly differentiated at the microsatellite markers used in the analysis.

Slender-billed vulture: Three loci were monomorphic (BV11, GT3-35 & GT2-28). Locus BV12 did not amplify and GF9C1 amplified too poorly for reliable allele size assessment. Locus BV20 showed evidence of null alleles (N_0 freq. 0.181) and was not at HWE. Locus GT3-38 appeared to be at LD with two other loci (GF11A4 & GB2-4B). Therefore, we eliminated loci BV11, GT3-35, GT2-28, BV12, GF9C1 and GT3-38, leaving seven loci for our final analyses. The number of alleles ranged from 2 to 10 per locus (mean=3.9); Observed (H_o) and expected heterozygosity (H_E) ranged from 0.50-0.97 (mean=0.598) and 0.43-0.83 (0.599) respectively (Table 2.2).

Red-headed vulture: Two loci were monomorphic (BV11 & Gf3H3). Two loci (GF11A4 & GF9C1) amplified poorly. GB3-2C showed evidence of null alleles (N_0 freq. 0.164) and loci GB3-2C and GT2-28 were not in HWE after Bonferroni correction. We eliminated loci BV11, Gf3H3, Gf9C1, GB3-2C and GT2-28. The number of alleles ranged from 2 to 6 per locus (mean=4.3); Observed (H_o) and expected heterozygosity (H_E) ranged from 0.14-0.79 (mean=0.48) and 0.14-0.76 (mean = 0.50) respectively (Table 2.2).

Mitochondrial control and COI regions:

White-rumped vulture: We identified 10 haplotypes for the concatenated mtDNA CR I and II; haplotype diversity (h) was estimated at 0.89 (SD = 0.04) and

nucleotide diversity (π) was 0.003 (SD = 0.002) (Tables 2.3a). Our analyses closely paralleled Johnson et al.'s (2008) estimates of haplotype diversity (number of haplotypes = 8; h=0.85 (SE = 0.05). We also estimated π for the Pakistan samples, which was 0.0021 (SD = 0.001). F_{ST} between Cambodia and Pakistan populations was significant at mtDNA CR (Φ_{ST} = 0.124; P<0.01).

Slender-billed vulture: We found 10 haplotypes for control region; h = 0.85 (SD= 0.05) and π = 0.004 (SD = 0.002) (Table 2.3a).

Red-headed vulture: We identified 2 haplotypes for *COI* region and 3 for CR I; h = 0.44 (SD = 0.08) and π = 0.0007 (SD = 0.0007). For control region I, h = 0.43 (SD = 0.12) *and* π = 0.001 (SD =0.001) (Table 2.3a).

Signature of recent bottleneck

The TPM Wilcoxan sign-rank test showed a significant excess of heterozygotes as compared to expectation under mutational-drift equilibrium for Slender-billed samples (P = 0.002), which may be indicative of a recent genetic bottleneck. This was not the case for Red-headed (P= 0.81), nor for White-rumped samples (P=0.97). Therefore we could not reject the null hypothesis of no bottleneck for these two species.

Discussion

The results from this study represent the first population genetic analysis of White-rumped (*Gyps bengalensis*), Slender-billed (*G. tenuirostris*) and Red-headed vultures (*Sarcogyps calvus*) in Southeast Asia. By using both mitochondrial and microsatellite data we were able to estimate the genetic variation found in these

Table 2.3a. Measures of genetic diversity from microsatellite and mtDNA data for White-rumped, Slender-billed and Red-headed vultures in Cambodia. Number of haplotypes, haplotype diversity (h), Tajima's D, and nucleotide diversity (π). ± indicates standard deviation. Asterisk indicates significance ($P<0.05$) for Tajima's D.

Species	Sample size	mtDNA region	Base pairs	No. haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)	Tajima's D	GenBank Accession No.
White-rumped	38	CR I & II	773	10	0.897 (± 0.038)	0.00334 (± 0.00212)	-0.381 (± 0.408)	KJ506786 - KJ506808
Slender-billed	29	CR I & II	784	10	0.845 (± 0.0453)	0.00356 (± 0.00216)	-1.805 (± 0.019)*	KJ506809 - KJ506836
Red-headed	28	CR I	407	4	0.433 (± 0.117)	0.00138 (± 0.0013)	-0.941 (± 0.217)	KJ506737 - KJ506756
		CO1	662	2	0.443 (± 0.080)	0.00067 (± 0.00069)	1.083 (± 0.885)	KJ506757 - KJ506785

Table 2.3b. Comparison of genetic diversity levels between White-rumped vulture populations in Pakistan and Cambodia based on eight microsatellite loci and mtDNA CR domains I & II (773 bp). Mean number of alleles per locus, allelic richness (A_R), observed heterozygosity (H_O), and expected heterozygosity (H_E), number of haplotypes, haplotype diversity (h) and nucleotide diversity (π). ± indicates standard deviation

	Microsatellites					mtDNA				
Population	Sample size	A	A_R	H_O	H_E	Sample Size	No. haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)	Tajima's D
Pakistan	19	7.4	6.7	0.643	0.669	19	8	0.854 (± 0.012)	0.00210 (± 0.00145)	-1.096 (± 0.967)
Cambodia	38	8.4	7.2	0.702	0.686	29	10	0.897 (± 0.038)	0.00334 (±0.00212)	-0.381 (± 0.408)

critically endangered populations, and to examine the genetic structure of White-rumped vulture populations between Pakistan and Cambodia.

Genetic Diversity

White-rumped vultures appear to have high microsatellite and mitochondrial diversity despite their small population size in Cambodia (Tables 2.2 and 2.3a,b). Without historic samples to serve as a comparison, however, we were not able to assess changes in genetic diversity over time as has been done for this species in South Asia, specifically in Pakistan (Johnson et al. 2008). The breeding population of White-rumped vultures in Towala, Pakistan, was reduced to zero in approximately 15-years. Despite this decline, the population showed only a slight reduction in microsatellite diversity over a sampling period that stretched from prior to the population crash (years 1893-1960 from museum specimens) to 2006. No decline was seen in the diversity of mitochondrial sequences. This suggests that genetic diversity can be maintained in long-lived species such as vultures over multiple decades although diversity will eventually decrease if a small population size persists. This has been demonstrated in fragmented populations of other long-lived vertebrates (Goosens et al. 2005). However, using simulated data, Johnson et al. (2008) showed that both allelic diversity and heterozygosity would diminish for the Towala vultures through year 2100 if populations continued to decline. A simulation modeling 200 individuals showed a decline of 20% and 5% for allelic diversity and heterozygosity respectively over an 80 year time period. When the population was reduced to 30 individuals, there was a drastic loss of > 60% in allelic diversity and a 25% decline in heterozygosity.

The differing rate at which alleles versus heterozygosity was lost is predictable and potentially reflected in our results for Slender-billed and Red-headed vultures in Cambodia (Table 2.2). After a reduction in population size, it is not uncommon for a loss in allelic variation to occur prior to a decrease in heterozygosity, as rare alleles are purged from a population in the early stages of population contraction (Luikart & Cornuet 2008). The distinction between the two indices (heterozygosity vs. allelic diversity) is important when considering both immediate (heterozygosity) and long-term (allelic diversity) evolutionary potential for a species. The number of alleles in a population sets the limit for selection to act; allelic diversity is more sensitive to bottlenecks and is a better indicator of changes in historic population size (Allendorf 1986; Cornuet & Luikart 1996; James, 1970; Nei et al. 1975). However, from a conservation management perspective, it is important to maximize both heterozygosity and allelic diversity for the adaptive potential of a species (Fernandez et al. 2004).

The simulated outcomes observed for the Pakistan samples reflect a present-day scenario for Southeast Asian vulture populations, which have contracted greatly in the last century. Prior to the 1950's, populations of White-rumped, Slender-billed and Red-headed vultures extended east from the Indian subcontinent through Myanmar and China's Yunan Province into Thailand, Cambodia, Vietnam, Laos and Malaysia (Bezuijen et al. 2010; Pain et al., 2003) (Figure 2.1). Surveys in the 1980's and 1990's revealed that the three species had disappeared from Malaysia and China and were limited to parts of Cambodia, with some individual birds possibly wandering into parts of Laos and Vietnam (Pain et al. 2003). Surveys in Myanmar between 2005 – 2009 confirmed the presence of small White-rumped and Slender-billed populations; only a

handful of Red-headed were observed (Bezuijen et al. 2010; Hla et al. 2011). Demographic data collected at vulture monitoring stations in Cambodia between 2004 – 2011, showed a minimum population of 210 White-rumped, 46, Slender-billed, and 45 Red-headed vultures (Clements et al. 2013). The loss of genetic variation for these species could become critical in the coming decades. The relatively lower levels of allelic diversity and heterozygosity we show for Slender-billed and Red-headed vultures (Table 2.2) may indicate an already declining trend in genetic diversity due to genetic drift and other stochastic events, particularly if there is an absence of gene flow (Frankham 2010).

Genetic structuring

An important component to assessing the long-term viability of vultures in Cambodia is determining if geneflow exists between Southeast and South Asian populations. A lack of geneflow, and hence an increased likelihood of inbreeding, may have conservation implications for individual and population level success (Keller & Waller 2002). For instance, Egyptian vultures (*Neophron percnopterus*) on the Canary Islands produce fewer young as compared to mainland conspecifics, possibly a result of inbreeding effects (Kretzmann et al. 2003; Keller & Waller 2002). Despite their ability to disperse widely, the island population showed approximately 40% lower levels of heterozygosity than mainland conspecifics and had 1.5 times fewer alleles (Kretzmann et al. 2003). Low reproductive success is also suspected for Slender-billed vultures in Cambodia although more information on breeding success rates is needed (Hugo Rainey *pers. comm.*). Our preliminary analysis of population differentiation between White-rumped vultures in Pakistan and Cambodia may suggest that geneflow

is negligible and Southeast Asian populations are becoming increasingly isolated by distance; F_{ST} and Φ_{ST} values were significant for nuclear (F_{ST} = 0.034; P = 0.001) and mitochondrial markers (Φ_{ST} = 0.124; P<0.01). Lower genetic differentiation is often characteristic of highly mobile species with wider distributions (Galbusera et al. 2004), and even small migration events can have important consequences for populations with low levels of diversity (Vila et al. 2003). However, depending on the effective size of a population, dispersal between fragments may not be sufficient to maintain the genetic diversity of a population as genetic drift increases and subpopulations become smaller and less connected (Uimaniemi et al. 2000). This may be the case for vultures in Cambodia as populations have become geographically fragmented from northern and western counterparts.

Even though vultures are capable of dispersing widely, their actual ranging distance is influenced by multiple factors. Data from tracking units (Radio, GSM-GPS and satellite transmitters) or from banding studies used to assess vulture movements in Europe and Africa have shown that vulture home ranges can be expansive but can vary greatly between and within populations. Range size can differ based on species, the age and sex of the individual, and by season (*Gyps fulvus:* Xirouchakis & Andreou 2009, García Ripollés et al. 2011; *Sarcogyps calvus:* Clements et al. 2013; *G. coprotheres:* Bamford & Diekmann 2007; Kendall et al. 2014). Adults are more site dependent than juveniles or subadults, which tend to range much farther, covering almost twice the area covered by adults (Bamford & Diekmann, 2007; Zuberogoitia et al. 2012). The distances adults travel to forage is largely defined by proximity to nesting grounds. Adults of *Gyps* species, for example, concentrate foraging efforts to

within 20 km of roosting and breeding sites (Bamford & Diekmann, 2007; García Ripollés et al., 2011; Xirouchakis & Andreou, 2009). Related to this is the ability of vultures to locate suitable food resources efficiently. *Gyps* vultures forage exclusively on medium to large carcasses, which is an unpredictable food source spatially and temporally. Vultures may therefore modify their foraging behavior to optimize encounter rates with this type of food (Kendall et al. 2014; Xirouchakis & Andreou 2009). Wild ungulates in Southeast Asia have been widely eliminated from over-hunting (Pedrono et al. 2009; Steinmetz et al. 2010). As a result, vultures have come to increasingly rely on domestic livestock for sustenance (Pain et al. 2003; Clements et al. 2013). In Cambodia and Myanmar vultures are sighted more frequently in areas associated with farms and domestic livestock than in forested areas where wild ungulate populations have been severely diminished (Hla et al. 2011; Clements et al. 2013). Therefore, the localized availability of food in Cambodia may in part be limiting dispersal. In addition, unlike other Asian vultures, such as Himalayan griffon (*Gyps himalayensis*) and Eurasian griffon (*Gyps fulvus*), which disperse over long distances, (Bunnat & Rainey 2009; McGrady & Gavashelishvili 2006), White-rumped, Slender-billed and Red-headed vultures are more sedentary (Clements et al. 2013; Prakash et al. 2007). Juvenile vultures in Cambodia have been observed dispersing farther than adults (Hugo Rainey pers. comm.), however, more information on the ranging behavior of these species is needed.

Conservation Implications

Vultures are long-lived birds but have low reproductive rates; recovery from a population crash can take many years (Newton 1979). The critically endangered status

of White-rumped, Slender-billed and Red-headed vultures is cause for concern. Since 2004, wildlife groups in Cambodia have been concentrating conservation efforts to prevent further population losses of vultures in Cambodia by providing uncontaminated carcasses as a supplemental food source and by protecting nesting sites. A more difficult challenge is stemming mortality from secondary poisoning. The use of poisons is having a devastating effect on vultures Asia (Clements et al. 2013; Green et al. 2007). Although populations currently appear stable (Clements et al. 2013), the persistence of vultures in Cambodia will depend heavily on the success of conservation strategies to mitigate further population losses. Genetic considerations are an important aspect of management efforts to maximize the adaptive potential of these species. In particular, Slender-billed and Red-headed vultures may be approaching a critical point at which diversity will be lost rapidly due to their very small population sizes. Based on the results of this study, we recommend that periodic genetic monitoring of these populations be conducted to identify trends in diversity over time. Feathers collected from feeding stations or nesting sites is an efficient means to acquire samples (chapter 4). We also recommend genetic analyses for populations outside of Cambodia, such as in Myanmar and India, to further explore levels of connectivity between groups. Fitting vultures with satellite transmitters or other tracking devices will also help inform on the movement of individuals and geneflow between populations.

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CHAPTER 3

NON-INVASIVE GENETIC SAMPLING TO CHARACTERIZE THE DEMOGRAPHY OF ASIAN VULTURES

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Abstract

Quantifying the population size of bird populations can be difficult especially when species are elusive or rare. However, an assessment of the number of individuals in a population forms the basis for most conservation efforts and is essential information for managing species that are threatened or endangered. Many vulture species throughout Asia are undergoing population declines yet there is little information on the population status of most vultures there. The ability to obtain DNA from numerous naturally shed feathers can greatly facilitate the generation of population estimates when variable microsatellite loci are used in conjunction with genetic capture-mark-recapture (CMR) analyses. We used DNA from feathers to generate abundance estimates for five species: White-rumped (*Gyps bengalensis*), Slender-billed (*G. tenuirostris*) and Red-headed (*Sarcogyps calvus*) vultures in Cambodia, and Himalayan (*Gyps himalayensis*) and Eurasian (*G. fulvus*) griffons in

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Kazakhstan. Feather samples in Cambodia were collected at monitored supplemental feeding stations, whereas samples from Kazakhstan were collected opportunistically from livestock carcasses distributed more naturally. We compared our abundance estimates derived from genetic samples to minimum population estimates obtained from visual surveys conducted during the same periods. We used diagnostic DNA sequence differences to assign feathers to the associated vulture species, and a suite of microsatellite markers to identify individuals via their unique genotypes. We then used closed capture models to generate abundance estimates for White-rumped vulture and minimum abundance estimates for Himalayan griffon. However, we lacked the requisite statistical power for the remaining three species due to low resampling rates. Therefore, we address the conservation implications for these two species and provide suggestions for how sampling efforts might be tailored to improve abundance analyses of less common species. This study demonstrates that feathers sampled non-invasively can be used to monitor the abundance of vulture populations through genetic CMR even in the absence of established long-term monitoring programs.

Introduction

Conservation measures intended to foster the long-term persistence of wildlife populations require accurate demographic information, with the most fundamental such estimate being the population size of the target species (Williams et al. 2002). Estimates of population size serve as a basis for the conservation management of wildlife species, particularly for those that are in decline or jeopardized by extinction, and provide a metric for conservation-based legislation (Luikart et al. 2010).

A measure of total population size, or census, accounts for every individual in the population. Census counts are only feasible in exceptional cases, typically when populations are small, geographically restricted, and easily monitored (Mills 2009). Instead, abundance estimates are often obtained by sampling from a subset of the population, with density estimates providing more detailed information on the number of individuals per unit area (Williams et al. 2002). On an even finer level, the effective population size (N_e) is the size of an ideal population that has the same rate of change of allele frequencies or heterozygosity as the observed population (Fisher 1930; Wright 1931). Loosely, N_e, which is often smaller than the census size, reflects the number of breeding individuals in the population that produce offspring that live to reproductive age (Schwartz et al. 2006).

Estimating abundance can be difficult for species that are elusive, wide-ranging, or rare (Petit & Valiere 2006). There are both direct and indirect field methods that can produce count statistics for a population although transect counts and capture-mark-recapture (CMR) are among the most commonly used (Williams et al. 2002; Burnham et al. 1980).

In the last two decades, wildlife biologists have incorporated non-invasive sampling and genetic analyses into CMR models to estimate abundance (Palsbøll 2008; Petit & Valiere 2006; Waits & Paetkau 2005). The utility of non-invasive sampling has been demonstrated in wildlife studies involving a wide range of taxa and research challenges: from mammalian carnivores that exist at low abundance and are shy of capture (Kelly et al. 2012; Williams et al. 2007) to cetaceans and forest ungulates where visual counts are hampered by environmental conditions (Brinkman

et al. 2011; Palsbøll et al. 1997). Eliminating the need to capture or observe an animal, non-invasive sampling can produce much larger sample sizes, and hence increase the probability of detection. The majority of genetic CMR studies have used DNA from hair collected from baited, barbed snags (Kendall et al. 2008), or from fecal droppings collected opportunistically or from transects from areas known to be occupied by the target species (Poole et al. 2011; Brinkman et al. 2011; Lampa et al. 2013). Genetic CMR, however, has only rarely been applied to bird populations through the collection of naturally dropped feathers (see Rudnick et al. 2008 for the exception). This is true even though DNA extracted from dropped feathers has been successfully used to identify individuals, examine genetic variation, sex ratios, dispersal, and relatedness among individuals (Booms et al. 2008; Hogan & Cooke 2010; Miño & del Lama 2009; Johansson et al. 2012; Oyler-McCance & St John 2009; Segelbacher 2002; Seki 2006; Vili et al. 2013).

There are potential challenges associated with using non-invasively collected samples (Bonin et al. 2004; Mckelvey & Schwartz 2004; Paetkau 2003; Pompanon et al. 2005; Roon et al. 2005). Hair, feces, and feathers collected from the field often yield low concentrations of DNA; furthermore, DNA can become degraded if samples are exposed to environmental conditions such as moisture, UV, and temperature extremes (Johansson et al. 2012; Vili et al. 2013; Brinkman et al. 2009). The identification of animals for genetic CMR is accomplished by analyzing multiple microsatellite loci with enough variability to discriminate differences between individuals. Genetic samples that are of sub-optimal quality due to degradation or low-yields are more prone to genotyping errors stemming from allelic dropout or through

the creation of false alleles (Waits & Paetkau 2005). These types of errors can influence the proper identification of individuals within a population leading to an over- or under-estimation of population size (Creel et al. 2003; Knapp et al. 2009; Lampa et al. 2013; Kohn et al. 1999). Genotyping errors can be greatly reduced by using laboratory protocols designed to cull samples that amplify poorly (Paetkau 2003). In addition, incorporating a certain level of genotyping error into CMR statistical analyses can help prevent a potential loss of valuable information in lieu of using protocols that are too stringent (Lukacs & Burnham 2005).

The widespread decline of old world vulture populations across Eurasia and Africa has heightened the need for reliable methods to estimate their population sizes (IUCN 2013; Ogada et al. 2012; Donázar et al. 2002; Hla et al. 2011; Pain et al. 2008; Margalida et al. 2011). Live capture—a prerequisite for traditional mark-recapture methods—is far more difficult to achieve for vultures than for most other birds, and even with substantial field effort, live capture methods applied to vultures often result in insufficient sample sizes for reliable inferences about population size. As a result, researchers studying vultures have primarily relied on remote observation methods, such as transect surveys or counts conducted at management sites where vultures are provisioned with carcasses (so called "vulture restaurants") (Clements et al. 2013; Margalida et al. 2007; Prakash et al. 2003). These observational surveys have been important in assessing the conservation status of vulture populations at risk (Clements et al. 2013; Prakash et al. 2003) but they have limited power for estimating population size. Transect counts are only effective at producing a count statistic for vultures seen at a particular time and place, and observational counts at feeding sites cannot usually

differentiate between individuals that are inadvertently counted multiple times (Margalida et al. 2011). Feathers collected non-invasively provide an alternate method to identify individuals, particularly for those species (e.g. *Gyps spp.*) that aggregate in large numbers at feeding and roosting sites.

We developed protocols that could be used to estimate the abundance of vultures using naturally shed feathers under two different sampling scenarios; 1) where vultures were known to predictably congregate to feed (i.e. vulture restaurants) and feathers could therefore be collected at regular intervals from the same sites; and 2) where food resources are less predictable and more randomly distributed, and hence feather collection must be opportunistic. Here, we explore our ability to estimate demographic parameters from feathers sampled over two years from vulture restaurants in the northern and eastern territories of Cambodia, where a long-term vulture-monitoring program for White-rumped (*Gyps bengalensis*), Slender-billed (*G. tenuirostris*) and Red-headed (*Sarcogyps calvus*) vultures has been underway since 2004. We compare our population estimates derived from genetic and mark-recapture techniques to those obtained from visual observations over the same time period.

We also generate demographic estimates using vulture feather samples collected in Kazakhstan at domestic livestock carcasses that we located opportunistically; this represents the first attempt to produce a minimum population size estimate for Himalayan griffon (*Gyps himalayensis*) and Eurasian griffon (*G. fulvus*) in this region.

Study species and area

Cambodia. -- The Northern Plains of Cambodia are among the largest remaining

intact blocks of Indo-Chinese Dry Forests. There are two distinct seasons in Cambodia: a dry season, which extends from November to May, and a wet season from June to October. The average temperature is 27.0˚C. Dipterocarpaceae dominate these deciduous forests that once harbored large herds of grazing ungulates such as Asian elephant (*Elephas maximus*), banteng (*Bos javanicus*), kouprey (*Bos sauveli*), gaur (*Bos gaurus*), wild water buffalo (*Bubalus arnee*), Eld's deer (*Cervus eldii*) and Sambar (*Rusa unicolor*) (Gray et al. 2012). In the last 50 years or more, wild ungulate densities have been severely depressed throughout southeast Asia, likely impacting the ecology of the forests through reduced seed dispersion and a loss of food resources for predators and scavengers (Corlett 2007; O'Kelly et al. 2012).

Three resident vulture species are found in Southeast Asia: White-rumped (*Gyps bengalensis*), Slender-billed (*Gyps tenuirostris*) and Red-headed (*Sarcogyps calvus*) vultures. Vultures in Southeast Asia declined in abundance as their ranges contracted over the twentieth century. Prior to the 1950's, populations of White-rumped, Slender-billed and Red-headed vultures extended east from the Indian subcontinent through Myanmar and China's Yunan Province into Thailand, Cambodia, Vietnam, Laos and Malaysia (Bezuijen et al. 2010; Pain et al. 2003) (Figure 3.1). Surveys in the 1980's and 1990's revealed that these three species had been extirpated from Malaysia and China and were limited to parts of Cambodia, with some individual birds moving through bordering areas of Laos and Vietnam (Pain et al. 2003). Minimum population estimates based on observational counts conducted over eight years (2004-2011) in Cambodia for White-rumped, Slender-billed and Red-headed vultures were 201, 48 and 47 respectively (Clements et al., 2013). Surveys in

Myanmar between 2005–2009 confirmed the presence of the three species in that country as well (Bezuijen et al. 2010; Hla et al. 2011). As in Indochina, vulture

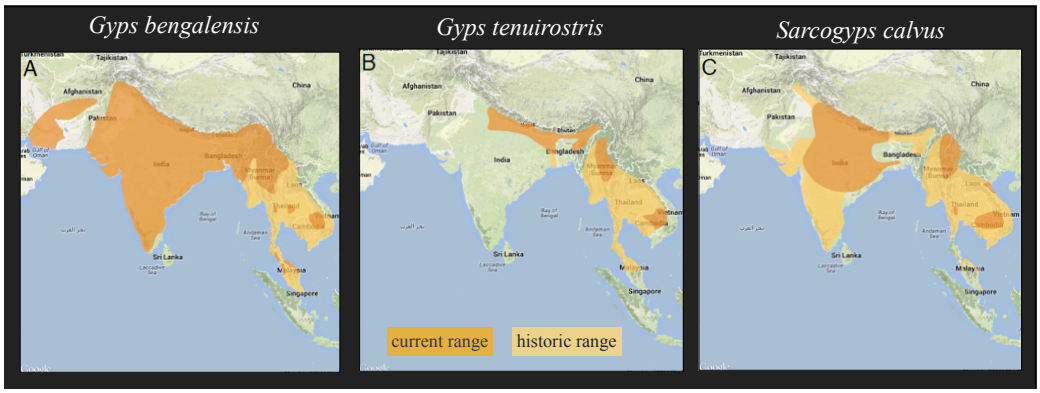

Figure 3.1 - Distribution of (A) White-rumped vulture (*Gyps bengalensis*), (B) Slender-billed vulture (*G. tenuirostris*), and (C) Red-headed vulture (*Sarcogyps calvus*). The darker shading represents the current geographic range, the lighter shade shows where the species have been extirpated.

populations in Myanmar have undergone major population and range contractions (Hla et al. 2011). Although these populations are all small, survey trends suggest that they may currently be stable, with possible population expansion for White-rumped and Slender-billed vultures (Clements et al. 2013).

In contrast, vulture populations in South Asia (India, Nepal, Pakistan), which include White-rumped, Slender-billed and Red-headed vultures as well as four other resident species, had flourished throughout the twentieth century due to the abundant availability of domestic livestock carcasses, a consequence of religious and cultural practices (Grubh 1993; Pain et al. 2003). However, in the mid-1990's and early 2000's, these populations underwent a precipitous decline, being reduced to less than 10% of their former abundance. Declines were most catastrophic for White-rumped vultures, which was once the most abundant vulture species throughout Asia before

falling to less than 1% of their historic population size (Prakash et al. 2003). Slender-billed and Red-headed vultures were similarly reduced to less than 3% and 9% of their former numbers (Cuthbert et al. 2006; Prakash et al. 2007). These vulture population declines were directly attributed to the veterinary use of a non-steroidal anti-inflammatory drug (diclofenac) used to treat ailing livestock, and to which the vultures showed extreme sensitivity (Prakash et al. 2003). White-rumped, Slender-billed, and Red-headed vultures are now categorized as 'Critically Endangered' on the IUCN Red List (IUCN 2013).

The diclofenac-induced declines in South Asia prompted conservation groups in the early 2000's to initiate a vulture monitoring program in Cambodia as part of a broad strategy to prevent the extinction of these species (Clements et al. 2013). Due to the low density of vultures in southeast Asia, transect surveys were deemed an inappropriate method for surveying the populations (Clements et al. 2013). Instead,

Site Latitude/Longitude
Preah Vihear Protected Forest (PVPF) 13°55'48.0"N 105°29'59.7"E
Western Siem 14°8'14.5" N 106°22'58.2"E
Seasan River 13°39'42.5"N 106°31'41.3"E
Lumphat Wildlife Sanctuary (LWS) 13°27'16.2"N 106°54'35.5"E
Mondulkiri Protection Forest (MPF) 12°57'58.2"N 107°9'52.0"E
Phnom Prich Wildlife Sanctuary (PPWS) 13°57'25.4"N 106°59'27.9"E
Kulen Promtep Wildlife Sanctuary (KPWS) 13°57'54.6" N 104°51'40.5"E

Figure 3.2 – Cambodian Sampling sites: Triangles indicate location of supplemental feeding stations ('vulture restaurants) in Cambodia. Orange triangles represent the sampling sites used for genetic and capture-mark-capture analyses for this study. Yellow lines are country borders. The white dotted line shows the approximate range for the three vulture species based on satellite tracking (Clements et al. 2013).

surveys based on visual observations were begun in 2004 in the northern and eastern parts of the country where the vultures were known to breed and forage (Clements et al. 2013). Wildlife organizations, in collaboration with the Cambodian Government Department of Nature Conservation and Protection and Forestry Administration, distributed livestock carcasses at seven designated supplemental feeding sites (Figure 3.2) on a monthly or bi-monthly basis. These monitoring efforts are still underway. Restaurant sites include: Preah Vihear Protected Forest, Western Siempang, Phnom Prich Wildlife Sanctuary, Lomphat Wildlife Sanctuary, Mondulkiri Protected Forest, Kulen Promtep Wildlife Sanctuary, and the Seasan River. Restaurant dates were staggered to maximize the effectiveness of the feeding stations by providing vultures the opportunity to move between sites across their entire range. These feeding events also afforded us the opportunity to collect feathers non-invasively and in great quantity at pre-determined time intervals.

Kazakhstan. -- The Assy Plateau in the Tian Shan Mountains of southeastern Kazakhstan has been used for centuries by Kazak pastoralists in the summer months to graze livestock. Himalayan (*Gyps himalayensis*), Eurasian griffons (*G. fulvus*) and Cinereous vultures (*Aegypius monachus*) are known to breed in the Tian Shan (Figure 3.3). The mountain range borders with Kyrgyzstan to the south and China to the east. The most widespread habitat in this region is steppe, which occurs at elevations between 1,066 - 3,300m. We originally identified two primary sites in Kazakhstan for this study. Both are located just south of Kazakhstan's largest city, Almaty. The Assy Plateau (Figure 3.4) located in the Zailyisky Alatau ridge in the northern Tian Shan

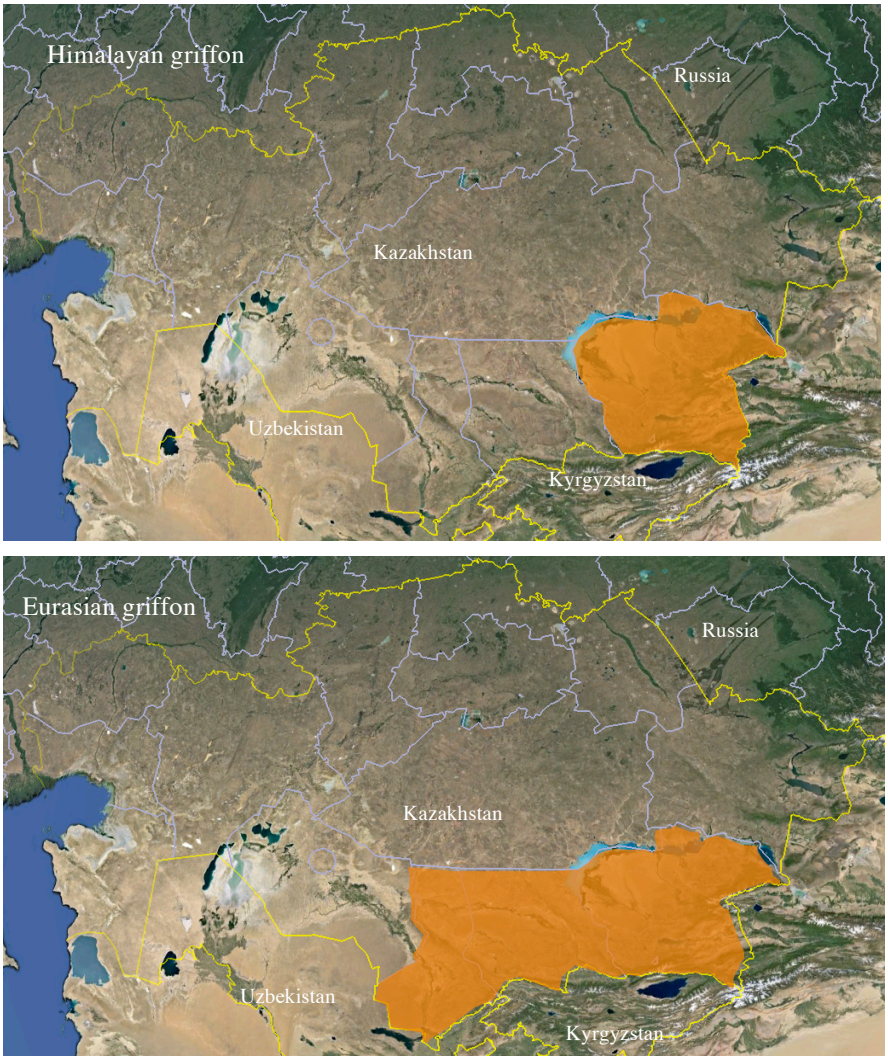

Figure 3.3 - Distribution of Himalayan Griffon (top) and Eurasian Griffon (bottom) in Kazakhstan. Yellow lines define country borders. Orange shading shows approximate range for each species.

has an area of approximately 400 km^2. Located at an elevation of 1500m, the plateau is characterized by expansive grasslands punctuated by steep cliffs and large gorges that provide both good nesting habitat for all four species as well as up-slope drafts that enable efficient soaring for these large birds of prey (Wassink & Oreel 2007). The

second is Ushkanoor, another plateau approximately 75 miles (121 km) west of Assy. Both sites are used as summer grazing pastures for large numbers of sheep, cattle, goats and horses. Livestock herders will often leave dead livestock in the field providing foraging opportunities for vultures. A third site, Jalanash, is located approximately 74 km east of Assy. Although livestock are not grazed here during the summer, up to ten active Himalayan griffon nests were confirmed during our visits to the site in the 2009. In past years, Eurasian griffons and Cinereous vultures (*Aegypius monachus*) have nested at this site indicating a recycling of nests by different species (Katzner, *personal communication.*). The population status of vultures in Kazakhstan has never been assessed for any species yet populations there may be at risk. As in southeast Asia, it is possible that vultures in Kazakhstan may be affected by a reduction in wild food sources as a result of overhunting of ungulates, in particular the Saiga (*Saiga tatarica tatarica* -- Robinson et al. 2003). However, the prevalence of domestic livestock during the summer months may afford vultures ample feeding opportunities in that season. The ingestion of diclofenac may be another factor impacting mortality rates if vultures migrate from Kazakhstan to South Asia during the winter (Wassink & Oreel 2007).

Transect counts conducted approximately annually since the early 2000's have been the only means used to evaluate vulture population trends in Kazakhstan. However, these counts have been largely uninformative due to low observation rates coupled with an inability to definitively identify the particular species of many vultures seen only at great distances from the observer.

METHODS

Sample collection

Cambodia. -- We collected feather samples from the seven vulture supplemental feeding stations established as part of the Vulture Conservation Program in Cambodia (Figure 3.2). Samples were collected between January to May 2009 and January to June 2011. Restaurants were provisioned monthly by slaughtering one 150-250 kg cow or buffalo purchased from local villages at each of the research sites. Once the vultures had consumed the carcass (typically after 1-3 days), we exhaustively collected all contour and flight feathers from the feeding site. We did not collect down feathers as these were too numerous and were unlikely to contain sufficient amount of DNA for robust CMR analyses. We stored feathers in paper envelopes, with 5-20 feathers per envelope depending on the size of the feathers. Feathers were stored at room temperature until DNA extraction. Ultimately, we decided to focus our analyses on samples collected at four sampling locations: Preah Vihear Protected Forest, Western Siempang, Lomphat Wildlife Sanctuary, and the Seasan River. We selected these locations to ensure a spatially broad distribution of sites. From these sites, we further subsampled from feathers collected in January, March and May 2009, and from March, May, and June in 2011 in addition to those collected in January from Western Siempang and in February from LWS (Table 3.1). Our decision to sub-sample was based on a practical need to economize our laboratory costs given the very large number of collected feathers available for analysis.

Kazakhstan.—In Kazakhstan, we sampled in Assy, Ush-Kanoor and Jalanash from July 15-30, 2009 (Table 3.1). Himalayan and Eurasian griffon range in the

southern part of the country with Eurasian griffon extending farther west (Figure 3.3). We frequently relied on local pastoralists for information on the location of livestock carcasses. We also searched for congregations of vultures, which would lead us to potential feeding sites. When we found an older carcass site we could not with certainty identify when that the animal had died; therefore, most of the Kazakhstan feather samples were exposed to weather conditions for an unknown duration. We recorded GPS locations at each sampling location. As in Cambodia, we collected all feathers from around the carcasses apart from down feathers. If the carcass had not been completely consumed during the time we sampled, we returned for subsequent days on the chance that the same or additional vultures would visit the site on subsequent days. If the carcass was completely consumed, we did not return. Feathers were collected and stored in the same manner as in Cambodia.

For our genetic analyses, we decided to focus on those samples collected on July 10-11, 2009 from five carcass locations (Figure 3.4). One site was sampled twice, one time on each of these two days. Although our initial goal was to analyze samples obtained from each of three species most likely to be found at the carcass sites (Himalayan and Eurasian griffon and Cinereous vulture), we decided for the preliminary aspect of this project to focus on those collected from Himalayan and Eurasian griffon.

Table 3.1 - Sampling dates, sites, number of feathers collected (n_f) and sampling occasion (occ) for Cambodia 2009, 2011 and Kazakhstan 2009. Sites in Cambodia include Western Siempang (Siem), Seasan River, Preah Vihear Protected Forest (PVPF), and Lumphat Wildlife Sanctuary (LWS). The sites in Kazakhstan are locations where we collected feathers from livestock carcasses.

Country year	Sampling month / date	Site	n_f	occ.
Cambodia 2009	January 10	Siem	91	1
	January 23	Seasan	51	2
	January 24	PVPF	319	3
	January 30	LWS	22	4
	March 6	LWS	25	5
	March 12	Seasan	47	6
	March 12	Siem	59	7
	March 25	PVPF	138	8
	March 28	LWS	13	9
	May 8	LWS	32	10
	May 10	Siem	41	11
	May 19	Seasan	40	12
	May 25	PVPF	299	13

Country year	Sampling month / date	Site	n_f	occ
Cambodia 2011	January 13, 15	Siem	24	1
	February 28	LWS	60	2
	March 7	PVPF	257	3
	March 11	Siem	140	4
	March 27	PVPF	20	5
	March 27	Seasan	84	6
	March 31	LWS	62	7
	May 13	PVPF	82	8
	May 21, 22, 23	Siem	161	9
	May 26	LWS	64	10
	May 28	Seasan	84	11
	June 13,14	PVPF	68	12
	June 13	Siem	27	13
	June 15	Seasan	16	14
	June 26	PVPF	29	15
	June 28	LWS	39	16

Country year	Sampling month / date	Site	n_f	occ.
Kazakhstan 2009	July 10	C 2 09	74	1
	July 10	C 3 09	203	2
	July 10	H 3 09	34	3
	July 10	H 4 09	207	4
	July 10	S 4 09	21	5
	July 11	C 3 09	506	6

Figure 3.4 - Map of Kazakhstan and a close-up of sampling site in the Assy Plateau located in the Zailyisky Alatau ridge of the Tian Shan Mountains. Orange triangles identify the five sampling locations used for this study (GPS coordinates for triangle on right [C209]: 43^{0}13'59.94"N; 078^{0}59'58.5"E; sites upper left [H409, S409]: 43^{0}18'27.24”N, 078^{0}07'22.44"E; lower far left triangles [H309, C309]: 43°15'0.88"N; 78° 0'22.59"E)

Genetic analysis

DNA extraction -- We extracted DNA using the E-Z 96® Tissue DNA kit (Omega Biotek) following suggestions by Horváth et al. 2005. DNA was isolated from the calamus tip of each feather, as well as from a residual blood clot in the superior umbilicus. For large flight feathers, the basal tip of the calamus was quartered, and the superior umbilicus located at the upper most portion of the calamus was removed separately (Horváth, et al. 2005). We followed the manufacturers DNA extraction protocols for tissue except that samples were incubated in Proteinase K extraction buffer for 48-72 hours (Bayard De Volo et al. 2008).

Species identification --Vultures gather in mixed-species groups when feeding and shed feathers are not visually identifiable to the species. Therefore, our first step after extracting DNA from each feather was to identify the species associated with each sample based on DNA differences in the mitochondrial cytochrome oxidase I (COI) region between each of the species. Based on known vulture range distributions during our sampling period, we expected to only encounter White-rumped, Slender-billed and Red-headed vultures in Cambodia, and Himalayan griffon, Eurasian griffon, and Cinereous vulture in Kazakhstan. Although Bearded vulture is also seen in Kazakhstan, and is known to visit the carcasses or large animals, they do not engage competitively as do the other more gregarious species; in addition, their diet is composed primarily of bone and marrow, which enables them to visit a carcass after the soft tissue has been consumed by other vulture species (Mundy et al. 1992).

We used a non-vulture specific forward primer (Avian*COI*) and a *Gyps* vulture specific reverse primer (GypsR1) to PCR amplify an 806 base pair fragment of the COI gene following methods in Kapetanakos et al. (2014). Each species could be easily identified from distinctive DNA fragment sizes produced by the enzymatic digest. Vouchered samples were used as positive controls to ensure that complete digestion was achieved (Kapetanakos et al. 2014). We repeated any sample that did not initially amplify. If amplification failed on the second attempt, we discarded that sample from further analysis. Using mitochondrial primers as a preliminary step prior to genotyping has the additional advantage of identifying which samples have low quantity or quality DNA providing an opportunity to weed out such samples at an early stage (Morin et al. 2001). We also eliminated from all further analysis all

samples found to derive from Cinereous vulture.

Microsatellite genetic analysis--To identify individuals, we initially amplified a subset of samples from each species at nine microsatellite loci in two separate reactions, using a combination of loci from two different sources (Table 3.2): GB and GT primers were developed for White-rumped (*Gyps bengalensis*) and Slender-billed (*G. tenuirostris*) vultures respectively (Kapetanakos et al. 2014), and Gf primers were developed for Eurasian griffon (*G. fulvus*) (Mira et al. 2002). Microsatellite loci were combined in two multiplex PCR reactions (multiplex mix A: GT3-35, GT3-38, GB2-4A, GB2-4-4B; GB3-2C; multiplex mix B: Gf11A4, Gf3H3, Gf9C1, GT2-28).

PCR's were performed in 10 µl reactions; 0.12 – 0.25 µM fluorescently labeled forward and unlabeled reverse primer (GT3-35, Gf3H3 = 0.12 µM; GT3-38, GB2-4A, GB2-4-4B, GT2-28, Gf9C1, Gf11A4= 0.25 µM), 1x buffer solution (Sigma), 1.5 mM (mix A) or 2.5 mM (mix B) $MgCl_2$, 0.2 mM dNTP's, and 0.05 U/µL of Jumpstart ™ *Taq* polymerase (Sigma). The PCR conditions were as follows: Initial denaturation step at 94˚C for 4min, followed by 35 cycles at 94˚ C for 50sec, 1min at the annealing temperature (60˚C for mix A, 52˚C for mix B), 72˚C for 1min, with a final extension step at 72˚C for 30min. We amplified two additional loci for Slender-billed and Red-headed samples, using primers BV6 and BV20 developed for Bearded vulture (*Gypaetus barbatus*) (Gautschi et al. 2000). Conditions for the BV reactions were the same as those for Mix A except that we used 0.12 µM of the forward and reverse primers, and an annealing temperature of 58˚C.

Table 3.2 - Microsatellite loci used to group unique genotypes and then to assess genetic variation in White-rumped (WR), Slender-billed (SB) Red-headed (RH) vultures in Cambodia and Himalayan griffon (HG) and Eurasian griffon (EG) in Kazakhstan. Black dots (•) indicate the loci selected to group genotypes for individual identification and for analysis of genetic variation.

Locus	Label	Species	GenBank Accession No.	Primer sequence (5'-3')	Reference	Loci used in analyses				
						WR	*SB*	*RH*	*HG*	*EG*
BV6	PET	*Gypaetus barbatus*	AF270732	F: AATCTGCATCCCAGTTCTGC R: CCGGAGACTCTCAGAACTTAAC	Gautschi et al. 2000		•	•		
BV20	FAM	*Gypaetus barbatus*	AF270742	F: GAACAGCACTGAACGTGAGC R: GTTTCTCCTGACAGTGAAATAACTC	Gautschi et al. 2000		•	•		
Gf11A4	PET	*Gyps fulvus*	AY035858	F: GATCCCTTCCAACCGAAAAT R: TGGTGACCAACGGAAGTGTG	Mira et al. 2002	•	•		•	•
Gf3H3	NED	*Gyps fulvus*	AY035859	F: GTAGAATAATTTGCTCCTGG R: GTGAAGGCACCTCATAGACA	Mira et al. 2002	•	•		•	•
Gf9C1	FAM	*Gyps fulvus*	AY035855	F: GGTGGACATTACATACACTG R: CAAGGAATCTGGACTACTAA	Mira et al. 2002	•				•
GB3-2C	NED	*Gyps bengalensis*	KJ663805	F: ATGAATCCAGGCTCAGTCAGAAC R: AGACATGGTAAGGAGTCAGCAGC	Kapetanakos et al. 2014		•	•		
GT3-35	NED	*Gyps tenuirostris*	KJ663811	F: CCCCTTGTATGACAATGGTACAGTAT R: GTTTCTGTATTCAAAAGACATGACATCCAC	Kapetanakos et al. 2014	•		•	•	
GT3-38	PET	*Gyps tenuirostris*	KJ663808	F: CCCGAGCCAAGCCAGTTATTATA R: GTTTCTCATACAACAATCTCTTGTGCTGAC	Kapetanakos et al. 2014	•	•	•	•	•
GB2-4B	FAM	*Gyps tenuirostris*	KJ663806	F: CAACTCCACAGTTTAGGCAGATGTACC R: GTTCTGGTGACTTCACAAGGGACTATCAGAGA	Kapetanakos et al. 2014	•	•	•	•	•
GT2-28	VIC	*Gyps tenuirostris*	KJ663807	F: CCATCATCGTGGATGTTAGAAACTA R: GTTTCTCACTTCTTCATTGCCTGAGATATA	Kapetanakos et al. 2014	•		•	•	•

Based on the performance of each of the loci (e.g. low vs. high failure rate, reliability of allele peak interpretation, and monomorphic vs. polymorphic alleles), we ultimately selected seven microsatellites per species for Cambodia and six microsatellites for the Kazakhstan samples (Table 3.2).

We genotyped 1μL of the labeled PCR products on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems), and estimated allele sizes using the GeneScan 500 (-250) LIZ size standard (Applied Biosystems) and GENEMAPPER® version 3.7 software (Applied Biosystems).

Quality control.--After we initially screened our loci, our subsequent genotyping protocol followed that of Paetkau (2003) to assess and minimize genotyping errors. In summary, we first attempted to genotype all of the samples at the selected loci for each species. We eliminated from further analysis any sample that did not amplify at four or more of the loci for Cambodia, and at three or more for Kazakhstan. Poor amplification typically reflects low DNA sample quality and discarding such samples early in the genotyping process reduces the probability of miscalled alleles (Paetkau 2003). For samples that successfully amplified at our designated number of loci, we repeated the PCR and genotyping for any of the loci that initially failed until genotypes for all target loci had been generated (seven loci for Cambodia and six for Kazakhstan). For samples collected in Cambodia in 2009, we used a pre-amplification method that included two separate PCR reactions run under the same conditions for samples with weak amplification as visualized on a 2% agarose gel. We modified the protocol from (Piggott et al. 2004) so that both PCR reactions were in 10ul. For the second PCR reaction we used 1μl of PCR product from

the first reaction. Both PCR reactions were subject to the same thermal cycling conditions. For samples collected in Cambodia in 2011 and in Kazakhstan, we eliminated the pre-amplification step because of the mounting laboratory costs associated with this process. We felt that scrutinizing samples for quality after the second round of genotyping was sufficient to reduce genotyping error. Once all loci were repeated, we eliminated samples that failed at any locus so that only complete genotypes were included in CMR analyses.

We used the program GENECAP (Wilberg & Dreher 2004) to identify and pool identical genotypes and then considered each pool as a single individual (Schwartz et al. 2006). Allelic dropout is one of the primary sources of error associated with genotyping samples that yield low quantities of DNA. Therefore, we further scrutinized our pooled genotypes to identify samples that mismatched at one or two loci (1MM- and 2MM-pairs respectively; Paetkau 2003; Wilberg & Dreher 2004). Most genotyping errors create pairs of genotypes that match at all but one (1MM) or two markers (2MM) and can thus be corrected by re-analyzing the mis-matching markers (Kendall et al. 2000). As part of this process, we first carefully inspected genotyping results visually to assess the results from the original genotyping electropherogram to rule out human error as the cause of the inconsistency. If errors were not resolved at this stage, we then repeated PCR amplification and genotyping for each of the mismatched loci three additional times. We culled samples that failed to produce consistent results. For homozygous samples, we required all three trials to produce the same single allele. For heterozygous samples we required that two of the trials produce the same two alleles. Any samples with more than two inferred alleles

were discarded.

We used results from the analysis of mis-matched pairs to estimate genotyping error rates associated with allelic dropout and false alleles (Pompanon et al. 2005) using the software GIMLET (Valiere 2002). However, we could not assume that these error rates were representative of our entire data set since the repeated samples were likely of lower quality and therefore more prone to error.

Once complete genotypes were assembled and individual identification was complete, we used MICRO-CHECKER (van Oosterhout et al. 2004) to detect null alleles or scoring errors that may have resulted from stutter peaks.

Statistical genetic analysis -- We obtained estimates of allele frequency and measures of genetic diversity (observed and expected heterozygosity) using the program GENALEX (Peakall & Smouse 2006). We estimated measures of deviation of population genotype frequencies from Hardy-Weinberg expectations (HWE) and Linkage Disequilibrium (LD) using the program GDA (Lewis & Zaykin 2001). For both HWE and LD we applied a Bonferonni correction for multiple tests.

To verify the power of our selected loci, we used GENALEX 6.5 (Peakall & Smouse 2006) to calculate the observed probability of identity (P_{ID}), the probability that two randomly drawn individuals from the population share the same genotype, and the P_{ID} for siblings ($P_{ID\text{-}SIB}$), a more conservative calculation describing the probability that two full-siblings would have identical genotypes at the markers used (Waits et al. 2001).

Population estimation

Although we had large sample sizes for White-rumped vulture and Himalayan

griffon, sample sizes for Slender-billed, Red-headed vultures and Eurasian griffon were considerably smaller. As a result, we recognized that the probability of recapture was likely to be very low for these species, which would influence our ability to estimate population size using the statistical approaches implemented by CMR estimation methods.

We used Program MARK (White & Burnham 1999) to compare standard closed capture-mark-recapture (CMR) models to closed CMR models that account for genetic misidentification (Lukacs & Burnham 2005). We initially explored closed models incorporating heterogeneity in capture probability but probability parameters were not estimable due to low recapture rates.

Population estimates obtained using closed CMR models are based on the assumption that an animal's mark cannot be lost and that it is recorded correctly when an animal is trapped (Otis et al. 1978). However, when genetic markers are used, there is some likelihood that individuals have been erroneously marked as a result of genotyping errors. This is not a problem if the individual is represented by only one sample even if there are errors in the genotype (Paetkau 2003). However, estimates can become biased if the genetic markers used in the study do not have enough variability to distinguish between individuals (i.e. different individuals *appear* to share the same genotype and so are grouped together as the same individual; this is also known as a 'shadow effect'), or if multiple samples representing the same individual are erroneously reported as two or more distinct individuals. In MARK, there are models designed to incorporate mis-identification that use capture histories to account for genotyping error when such errors are not directly estimated from the data set by

repeating each sample multiple times (i.e. a multiple tubes approach as describe by Navid et al. 1992). The process of analyzing the same sample repeatedly to control for genotyping error becomes less realistic when a large number of samples are used in CMR analyses, or when control samples are unavailable from sampled individuals from which to create a consensus genotype (e.g. tissue samples collected from harvested animals, see Dreher et al. 2007 for example). The mis-identification models include an additional parameter 'α' to account for the probability that an individual was correctly genotyped at the initial capture. These models are robust to estimating abundance, but increase in bias when there is low capture probability ($p \leq 0.1$) or when genotyping error rates exceed 10% (Lukacs & Burnham 2005; Lukacs *personal communication*). Despite these shortcomings we felt that incorporating error in our models was a conservative approach considering our large sample size and our potential inability to eliminate all sources of error.

We assessed variation in detection probability using basic models in MARK. We used an 'α' value of 0.9 to account for some degree of genotyping error in closed CMR models. We felt an error rate of 10% was a conservative assumption given our protocols designed to minimize genotyping error. An 'α' value of 1.00 was equivalent to no misidentification and thus served as a comparison to models incorporating a probability of error. We constructed four basic models using the full-likelihood *p* and *c* mis-identification models. We included the derived 'α' parameter for all models except for the constant capture probability model (model M_0) which assumes both incorrect and correct genotypes are included in initial captures, but assumes no misidentification for recaptures (Lukacs & Burnham 2005). The three additional

models included M_t, which accounted for the probability of time variation across sampling occasions (where p and c were set to be equal in order to constrain p), model M_{t+z}. which included an additive effect of time and group (p and c), and model M_B which further constrains p and c to model for potential variation in a behavioral response (White & Cooch 2008).

Using Program MARK, we ranked competing models based on Akaike's Information Criterion (AIC_c, adjusted for sample size) to assess which models were best supported by the data. We used AIC_c weights to derive average population estimates to account for uncertainty in model selection (Burnham & Anderson 2002; White & Cooch 2008).

Results

Feather collection and Genetic Analyses

We collected 3,258 feather samples from the seven sampling sites in Cambodia in 2009, and 2,021 samples in 2011. From the feathers collected, we subsampled 1,159 feathers in 2009 and 1,219 feathers in 2011 from the four sampling sites selected for the mark-recapture analysis (PVPF, Siempang, LWS, Seasan). The number of feathers we collected varied by site: The sites with the highest number of shed feathers were Preah Vihear Protected Forest and Siempang (605 and 117 feathers collected respectively in 2009, and 353 and 263 respectively in 2011) (Figure 3.5). Feathers from each of the three species were found at all sampling sites. We had a total of 13 sampling occasions in 2009 and 16 in 2011 (Table 3.1).

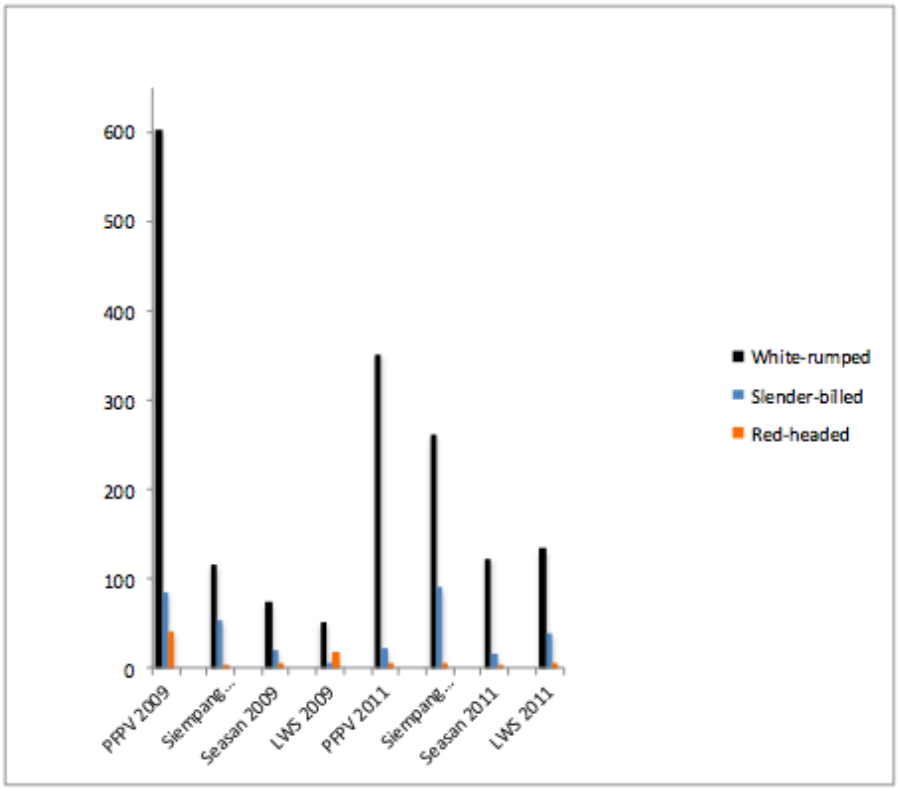

Figure 3.5 - Total numbers of feathers collected from Preah Vihear Protected Forest (PVPF), Siempang, Seasan River, and Lumphat Wildlife Sanctuary (LWS) in Cambodia (2009 and 2011) for White-rumped, Slender-billed and Red-headed vultures.

Our COI endonuclease protocol allowed us to successfully determine the vulture species for 96% (Cambodia 2009), 86% (Cambodia 2011) and 95% (Kazakhstan 2009) of the samples analyzed. From Cambodia, 849 and 875 samples were identified as White-rumped vulture in 2009 and 2011 respectively, 168 and 169 samples were Slender-billed, and 72 and 24 were Red-headed vulture. We eliminated 8% of samples (70 samples from 2009 and 151 from 2011) when they did not amplify using the COI primers and therefore could not be grouped based on species.

In Kazakhstan, we collected 3,991 feathers from 27 different sampling sites in Assy, Ush Kanoor and Jalanash. For this study, we extracted DNA from 946 samples

from 5 sites over 6 days (6 sampling occasions) (Table 3.2). We eliminated 5% of samples (49 samples) when they did not amplify using the COI PCR protocol; despite the potentially longer period of environmental exposure prior to collection in the field, the amplification success of the Kazakhstan samples was slightly higher than that of the Cambodian samples. We did not analyze Cinereous vultures samples beyond this point.

The microsatellite loci we used to group samples into unique genotypes and to assess genetic variability were polymorphic for each of the five species (Table 3.3). We eliminated samples that did not pass our screening process either due to genotyping failure or when 1MM or 2MM loci could not be resolved by repeating genotyping reactions (see Table 3.4 for numbers of samples eliminated at each stage of the screening process). Our genotyping success rate varied between 49% to 79% depending on species and year (Table 3.4). We used genotyping results from the 1MM and 2MM locus repeats to assess genotyping error rates. The number of samples we repeated varied by locus as did rates for allelic dropout (1.3% to 34%), and false allele (9% to 13%) (Table 3.5).

From the 2009 Cambodia samples, we identified 217 unique genotypes for White-rumped vulture, 63 for Slender-billed, and 28 for Red-headed vulture. In 2011, we found 283 unique genotypes for White-rumped, 64 for Slender-billed and 10 for Red-headed vulture; 83 White-rumped vultures, 15 Slender-billed vultures, and 1 Red-headed vulture were sampled from both 2009 and 2011. From Kazakhstan, we grouped 173 genotypes for Himalayan griffon and 8 for Eurasian griffon. The

Table 3.3 - Polymorphism data for microsatellite loci tested for White-rumped (*Gyps bengalensis*), Slender-billed (*G. tenuirostris*) and Red-headed (*Sarcogyps calvus*) vulture populations in Cambodia, and Himalayan griffon (*Gyps himalayensis*) in Kazakhstan. Mean number of alleles per locus (A), allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), Probability of identity (P_{ID}) and probability of identity for siblings ($P_{ID\text{-}SIB}$).

	Year: 2009									Year: 2011						
Species	Sample Size	Allelic range	Locus	A	A_R	H_O	H_E	P_{ID}	$P_{ID\text{-}SIB}$	Sample size	A	A_R	H_O	H_E	P_{ID}	$P_{ID\text{-}SIB}$
White-rumped	212	216-264	GT3_35	14	14	0.722	0.783	0.077	0.378	281	13	12.7	0.758	0.797	0.070	0.369
	212	365-388	GT3_38	8	8	0.604	0.720	0.108	0.417	281	8	7.7	0.630	0.740	0.096	0.404
	212	340-364	GB2_4B	7	7	0.637	0.712	0.133	0.428	281	8	7.7	0.623	0.723	0.125	0.420
	212	286-305	GT2_28	9	9	0.443	0.432	0.334	0.618	281	9	9.0	0.413	0.421	0.348	0.626
	212	131-183	Gf11A4	20	20	0.910	0.881	0.025	0.317	281	20	19.8	0.904	0.876	0.027	0.319
	212	138-158	Gf3H3	9	9	0.439	0.444	0.324	0.609	281	9	8.9	0.431	0.422	0.348	0.626
	212	255-287	Gf9C1	17	17	0.821	0.892	0.020	0.310	281	18	17.8	0.833	0.897	0.018	0.307
			Mean	**12.0**	**12.0**	**0.654**	**0.695**	**All loci = $6.37\text{x}10^{-8}$**	**All loci = $2.51\text{x}10^{-3}$**	**Mean**	**12.1**	**11.9**	**0.656**	**0.697**	**All loci= $5.32\text{x}10^{-8}$**	**All loci= $2.42\text{x}10^{-3}$**
Slender-billed	62	373-388	GT3_38	6	6	0.597	0.695	0.148	0.442	62	6	6	0.613	0.677	0.159	0.454
	62	348-364	GB2_4B	4	4	0.565	0.598	0.245	0.514	62	3	3	0.645	0.618	0.231	0.501
	62	400-464	GB3_2C	8	8	0.694	0.744	0.106	0.407	62	8	8	0.613	0.751	0.098	0.402
	62	136-183	Gf11A4	10	10	0.823	0.849	0.043	0.339	62	9	9	0.903	0.828	0.054	0.353
	62	140-142	Gf3H3	2	2	0.581	0.502	0.376	0.595	62	2	2	0.516	0.491	0.381	0.601
	62	133-141	BV20	4	4	0.339	0.502	0.301	0.576	62	4	4	0.339	0.399	0.412	0.655
	62	123-125	BV6	2	2	0.452	0.470	0.393	0.614	62	2	2	0.258	0.352	0.484	0.696
			Mean	**5.1**	**5.1**	**0.578**	**0.623**	**All loci = $7.60\text{x}10^{-6}$**	**All loci = $6.66\text{x}10^{-3}$**	**Mean**	**4.8**	**4.8**	**0.555**	**0.588**	**All loci = $1.51\text{x}10^{-5}$**	**All loci = $8.88\text{x}10^{-3}$**

Table 3.3 (Continued)

Species	Year: 2009 Sample Size	Allelic range	Locus	A	A_R	H_O	H_E	P_{ID}	$P_{ID\text{-}SIB}$	Year: 2011 Sample size	A	A_R	H_O	H_E	P_{ID}	$P_{ID\text{-}SIB}$
Red-headed	29	225-252	GT3_35	5	4.3	0.517	0.574	0.229	0.525	11	3	3	0.455	0.602	0.271	0.530
	29	373-385	GT3_38	5	4.7	0.724	0.760	0.106	0.403	11	5	5	0.636	0.684	0.172	0.466
	29	350-365	GB2_4B	5	4.5	0.586	0.746	0.120	0.413	11	4	4	0.636	0.693	0.163	0.460
	29	404-443	GB3_2C	10	6.4	0.483	0.796	0.077	0.378	11	6	6	0.455	0.814	0.084	0.382
	29	293-315	GT2_28	13	8.9	0.655	0.819	0.054	0.361	11	8	8	0.818	0.844	0.063	0.363
	29	133-141	BV20	4	3.3	0.379	0.328	0.485	0.709	11	3	3	0.273	0.385	0.441	0.676
	29	122-150	BV6	8	5.7	0.586	0.596	0.194	0.505	11	6	6	0.727	0.727	0.123	0.433
			Mean	**7.1**	**5.4**	**0.562**	**0.660**	**All loci= 1.18×10^{-6}**	**All loci = 4.30×10^{-3}**	**Mean**	**5.0**	**5.0**	**0.571**	**0.678**	**All loci = 2.25×10^{-6}**	**All loci = 4.64×10^{-3}**
Himalayan	173	216-273	GT3_35	14	---	0.780	0.815	0.058	0.358							
	173	373-394	GT3_38	9	---	0.717	0.804	0.061	0.364							
	173	343-364	GB2_4B	8	---	0.410	0.522	0.335	0.573							
	173	125-208	GF11A4	20	---	0.925	0.890	0.023	0.312							
	173	291-323	GT2_28	16	---	0.792	0.839	0.045	0.343							
	173	131-165	GF3H3	10	---	0.584	0.654	0.152	0.462							
			Mean	**12.8**		**0.701**	**0.754**	**All loci = 1.94×10^{-7}**	**All loci = 3.70×10^{-3}**							

Table 3.4 - (A) Number of DNA extractions from feathers, (B) samples that did not amplify with *COI* primers and therefore could not be grouped by species, (C) total samples genotyped, (D,E,F) samples eliminated during screening process – (D) samples discarded after initial genotyping reaction, (E) samples discarded after repeating samples at microsatellite loci that failed during initial genotyping, (F) samples discarded if they could not be definitively resolved at 1-MM or 2 –MM loci, (G, H) number of samples mis-matching at 1 and 2 loci in the final analyses, (I) samples successfully genotyped, (J) number of unique genotypes used in capture-mark-recapture (CMR) analysis.

	A. DNA extractions	B. no. that did not PCR at *COI*	C. no. genotyped	D. discarded after 1st genotyping	E. discarded after repeat genotyping	F. discarded: 1-MM, 2-MM	G. mismatching at 1 locus (1-MM)	H. mismatching at 2 loci (2-MM)	I. successfully genotyped (%)	J. unique genotypes
Cambodia 2009										
All species	1159	70	1089	44	240	116			689	
White-rumped			849	34	174	89	1	4	550 (65%)	217
Slender-billed			168	7	34	25	1	3	101 (61%)	63
Red-headed			72	3	32	2	0	1	35 (49%)	28
Cambodia 2011										
All species	1219	151	1068	107	251	87			623	
White-rumped			875	106	188	59	13	19	521 (60%)	283
Slender-billed			169	1	51	28	7	8	88 (53%)	64
Red-headed			24	0	12	0	0	0	12 (50%)	10
Kazakhstan 2009										
All species	947	50	756	67	84	24			581	
Himalayan			712	61	71	21	13	14	559 (79%)	173
Eurasian			44	6	13	3	1	0	24 (55%)	8

Table 3.5 - Rates of allelic drop out (ADO) and false alleles (FA) determined from 1-MM and 2-MM error checking from Cambodia 2009 and 2011. Years and species were combined for each locus. *N* = sample size; White-rumped (WR), Slender-billed (SB), Red-headed (RH).

Locus	*N*	ADO	FA	Species
BV6	38	0.013	0.096	SB, RH
Gf11A4	38	0.048	0.067	WR, SB
Gf3H3	42	0.098	0.013	WR, SB
BV20	53	0.121	0.081	SB, RH
GT2_28	32	0.132	0.044	WR, RH
Gf9C1	67	0.173	0.121	WR
GB2_4B	81	0.185	0.08	WR, SB, RH
GT3_35	69	0.188	0.074	WR, RH
GT3_38	95	0.341	0.134	WR, SB, RH
Mean		0.144	0.0788	

percentage of individuals captured more than once was low for all species. Because of the small samples sizes for Red-headed vulture and Eurasian griffon, we could not generate robust abundance estimates and did not include those species in our CMR analyses. Only one individual Red-headed vulture was sampled more than once in both sampling years, and two Eurasian griffon were sampled more than once in 2009. This is in contrast to White-rumped vulture in which >30% of individuals were resampled more than once in the same year and Himalayan griffon in which 23% were sampled more than once.

Levels of genetic variability as assessed through observed and expected heterozygosity and number of alleles are described in Table 3.3 for all species except Eurasian griffon. We did not detect any deviations from Hardy-Weinberg or linkage equilibrium after applying a Bonferoni correction. In summary, mean H_E for White

rumped vulture was 0.695 and 0.697 in 2009 and 2011 respectively, 0.623 and 0.588 for Slender-billed, and 0.660 and 0.678 for Red-headed vultures. Mean allelic richness for samples obtained from Cambodia in both years varied from 4.8 for Slender-billed vultures, to 5.0 for Red-headed vultures, and 11.9 for White-rumped. Mean H_E for Himalayan griffon was 0.754 with a mean number of alleles of 12.8. P_{ID}, the probability that two randomly selected individuals shared the same genotype, ranged between 6.37×10^{-8} (White-rumped 2009) to 1.51×10^{-5} (Slender-billed 2011); $P_{ID\text{-}SIB}$ varied from 8.88×10^{-3} to 2.42×10^{-3}.

Abundance estimation

Models with the highest support (Δ AIC_c <2) incorporated variation of capture probability over time (M_{t+z} and M_t) both with and without mis-identification (Table 3.6). Although we ultimately used model averaging to estimate abundance, the best supported models for White-rumped vultures in 2009 and 2011, with approximately 2.5 times the support from the second most supported model, were those with mis-identification (ω_i = 0.62 in 2009 and 0.67 in 2011). In contrast, estimates for Himalayan griffon were equally supported by models with and without mis-identification (ω_i = 0.43 and 0.40). Model M_B, which incorporates a behavioral effect resulting from a possible response to trapping effect (i.e. trap happy / trap shy) was not supported for any species. Capture probability was low overall and ranged between 0.06 (SE = 0.018) to 0.44 (SE = 0.13) in 2009, and 0.007 (SE =0.004) to 0.15 (SE =0.03) in 2011 for White-rumped, and 0.04 (SE = 0.03) to 0.61 (SE = 0.21) for Himalayan Griffon. Capture probabilities for Slender-billed in 2009 ranged from 0.00 (SE = 0.00) to 0.79 (SE = 0.17); occasions where p=0 reflected time periods when this

species was not detected (occasions 4,5,6). Although we recovered feather samples for Slender-billed vulture during sampling occasions 4 and 6 (Table 3.7), we eliminated those genetic samples during our screening process. Model averaging resulted in an abundance estimate of 241 (95% CI = 220-426) and 404 (95% CI = 327 to 616) for White-rumped vulture in 2009 and 2011 respectively, and 207 (95% CI = 176 to 504) for Himalayan griffon (Table 3.8). Although abundance for Slender-billed vulture was estimated at 63, 95% confidence intervals were very wide due to the low probability of capture (95% CI = 63 to -8.07).

Discussion

There are advantages to using supplemental feeding stations to survey vultures especially for smaller, localized populations. However, implementing long-term monitoring programs may not be feasible in every situation. The decline in vulture numbers in Eurasia and Africa (Ogada et al. 2012) signals the need to develop methods where population trends can be monitored efficiently with minimal on the ground investment. Although there are few studies to date that use DNA from feathers for CMR studies, these methods are potentially efficient and statistically powerful, especially when numerous samples can be collected efficiently, as is the case with many species of vulture. These indirect methods are also appropriate for monitoring multiple vulture species in the course of a single study, a useful attribute since vultures interact in mixed species groups when feeding. Our study shows that the combination of simple species-specific diagnostics and hypervariable markers for identifying individuals can be used effectively to 'capture' and distinguish between species and individuals for abundance estimation. Feather samples collected non-invasively for

Table 3.6 - Model selection results from mark-recapture analysis of White-rumped vultures (Cambodia) from 2009 and 2011, Slender-billed vultures (Cambodia) from 2009 and Himalayan griffon (Kazakhstan) from 2009 based on non-invasively collected feather samples. Models were structured using closed full-liklihood with and without genotyping error.

Species/Year	Model[A]	α[B]	*Ng*[C]	AICc[D]	ΔAICc[E]	w_i[F]	*K*[G]	*Deviance*	*N*[H]	SE[I]
White-rumped vulture 2009	M_{t+z} - misID	0.9	217	-418.68	0.00	0.62	15	160.87	221.96	14.69
	M_{t+z}	1.0	217	-416.88	1.80	0.25	15	162.67	247.21	16.54
	M_t - misID	0.9	217	-415.02	3.66	0.10	14	166.56	308.48	22.28
	M_t	1.0	217	-412.28	6.40	0.03	14	169.29	366.80	27.71
White-rumped vulture 2011	M_t - misID	0.9	283	-233.33	0.00	0.67	17	376.16	394.25	23.24
	M_{t+z} - misID	0.9	283	-231.34	1.99	0.25	18	376.14	410.13	116.70
	M_t	1.0	283	-228.67	4.66	0.06	17	380.82	465.57	28.69
	Mt+z	1.0	283	-226.68	6.66	0.02	18	380.80	449.15	121.61
Slender-billed vulture 2009	M_{t+z} – misID	0.9	63	-72.14	0.00	0.54	11	37.55	57.29	30.75
	Mt+z	1.0	63	-71.75	0.39	0.44	11	37.95	63.68	28.07
	M_t - misID	0.9	63	-64.67	7.47	0.01	10	47.08	162.72	43.53
	M_t	1.0	63	-64.11	8.03	0.01	10	47.64	198.99	54.28
Himalayan griffon 2009	M_{t+z} - misID	0.9	173	-680.48	0.00	0.43	8	74.70	173.32	12.77
	M_{t+z}	1.0	173	-680.34	0.14	0.40	8	74.83	194.30	15.49
	M_t - misID	0.9	173	-677.24	3.24	0.09	7	79.97	293.76	32.32
	M_t	1.0	173	-677.17	3.31	0.08	7	80.04	350.81	39.77

A. Model notation: Models incorporating genotyping error are identified with the notation –misID. The only models which received support with M_{t+z} and M_t with and without mis-identification. M_{t+z} :structured for an additive relationship (z) between time(t) and group (p and c). M_t : time variance across occasions.
B. Alpha mis-identification parameter: 0.9 denotes a maximum of 10% genotyping error while 1 removes all error.
C. Number of unique genotypes used in the analyses.
D. Akaike's Information Criteria for small sample size.
E. The difference in AICc value between the ith model and the model with the lowest AICc value.
F. Akaike's weight used in model averaging
G. The number of parameters incorporated into each model
H. Abundance estimation
I. Standard Error

Table 3.7 - Comparison of number of vultures observed during observational census counts versus number of individuals identified from feather samples used in genetic capture-mark-recapture (CMR). Probability of capture is based on results from CMR analysis. The number of feathers extracted and unique genotypes observed are specific to each species and sampling year. White-rumped vulture 2011 sampling occasion 5, samples were collected 10 days after census.

White-rumped vulture 2009

Sampling occasion	1	2	3	4	5	6	7	8	9	10	11	12	13
Sampling site	Siem	Seasan	PVPF	LWS	LWS	Seasan	Siem	PVPF	LWS	LWS	Siem	Seasan	PVPF
# individuals observed at census	41	15	33	4	8	22	23	26	1	10	31	11	39
# feathers extracted	63	26	216	11	19	30	30	98	8	14	24	19	246
# unique genotypes	27	15	74	4	8	12	12	54	4	2	19	6	68
probability of capture (*p*)	0.1	0.06	0.32	0.02	0.04	0.07	0.07	0.31	0.03	0.01	0.13	0.04	0.44

White-rumped vulture 2011

Sampling occasion	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Sampling site	Siem	LWS	PVPF	Siem	PVPF	Seasan	LWS	PVPF	Siem	LWS	Seasan	PVPF	Siem	Seasan	PVPF	LWS
# individuals observed at census	58	9	117	35	45*	24	7	50	40	18	7	39	78	11	65	20
# feathers extracted	36	42	215	105	3	66	37	73	106	37	47	28	16	11	20	19
# unique genotypes	14	9	66	58	3	43	27	32	55	19	18	22	12	8	15	15
probability of capture (*p*)	0.03	0.02	0.15	0.13	0.007	0.099	0.063	0.075	0.13	0.045	0.042	0.052	0.03	0.019	0.036	0.036

Slender-billed vulture 2009

Sampling occasion	1	2	3	4	5	6	7	8	9	10	11	12	13
Sampling site	Siem	Seasan	PVPF	LWS	LWS	Seasan	Siem	PVPF	LWS	LWS	Siem	Seasan	PVPF
# individuals observed at census	25	5	5	2	2	7	18	5	0	6	17	5	5
# feathers extracted	17	0	48	2	0	7	27	10	1	2	11	13	29
# unique genotypes	7	0	17	0	0	0	19	3	1	1	11	3	11
probability of capture (*p*)	0.11	0	0.28	0	0	0	0.44	0.13	0.05	0.05	0.52	0.26	0.78

Himalayan griffon 2009

Sampling occasion	1	2	3	4	5	6
Sampling site	C 2 09	C 3 09	H 3 09	H 4 09	S 4 09	C 3 09
# feathers extracted	58	70	12	161	13	397
# unique genotypes	26	34	0	75	4	78
probability of capture (*p*)	0.12	0.18	0.03	0.41	0.04	0.6

Table 3.8. Model Averaging results for models presented in Table 3.6. Estimated abundance (*N*), unconditional standard error (Unc. SE), 95% confidence interval (CI).

Species	Year	*N*	Unc. SE	95% CI
White-rumped vulture	2009	240.6	36.9	220 to 426
White-rumped vulture	2011	404.01	66.9	327 to 616
Slender-billed vulture	2009	62.8	19.5	63 to -8.07
Himalayan griffon	2009	206.6	57.3	176 to 504

genetic CMR studies can also impart information on the genetic variability of that population, adding an additional and important component to conservation management. Our study is the first to use feathers to estimate abundance and genetic diversity for old-world vulture species and demonstrates that these methods can be used in addition to, or in lieu of, counts based on observation alone.

Our research sites in Kazakhstan and Cambodia provide test cases of two distinct methods for sample collection. Our sampling methods in Cambodia were made possible by feathers deposited at pre-existing supplemental feeding sites. In Kazakhstan, we relied on local pastoralists or the behavior of vultures to opportunistically collect feathers from more naturally distributed feeding sites.

In Cambodia, we were able to compare our abundance estimates from genetic CMR to the observational counts that occurred monthly at each sampling site and to the simultaneous counts conducted in May or June at each site on an annual basis. The simultaneous counts were designed to eliminate the possibility of vultures moving between feeding stations so as to prevent the double counting of individuals (Clements et al. 2013). From these counts, the minimum number of individuals was derived

based on the total number of each species recorded across all census sites. Minimum population size in 2009 was estimated at 182 White-rumped, 41 Slender-billed, and 43 Red-headed vulture. Estimates in 2011 were similar with 183 White-rumped, 45 Slender-billed and 39 Red-headed vultures. Our CMR analyses could not produce abundance estimates with high confidence for Slender-billed and Red-headed vultures. However, the number of individual genotypes detected for Slender-billed vulture exceeded that observed through visual census with 63 individuals detected in 2009 and 64 in 2011. Our final grouping of genotypes for Slender-billed vultures had few samples with 1MM or 2MM loci (Table 3.4), indicating that our samples had been thoroughly screened, and further suggesting that observational counts may be under-detecting the number of individuals for this species. However, we recovered even fewer samples from Red-headed vultures, with roughly half the number of unique genotypes as the number of individuals estimated through observation. This discrepancy resulted from the overall small number of samples collected coupled with the subsequent elimination of >50% of samples during our screening process (Table 3.4). We were therefore unable to produce abundance estimates for this species.

Our sample sizes were much more substantial for the White-rumped vulture. Our 2009 abundance estimates were marginally larger than the number of individuals observed in the field (217 unique genotypes vs. 183 individuals estimated from observational counts). However, our 2011 CMR estimates appeared to be inflated with twice as many genotypes (404) as individuals estimated from observational counts (183). Genotyping success was lower for the 2011 samples (60%) than for those analyzed in 2009 (65%), and there was a larger percentage of individuals

represented by a single sample in 2011 (59%) than in 2009 (42%). A related factor is the greater number of 1-MM and 2-MM loci in the final representative genotypes in 2011 as compared to 2009 samples (Table 3.4), potentially indicative of higher rates of genotyping error than represented by our constrained alpha value of 0.9. In addition, our average probability of capture (p) was 0.06 across the 16 sampling occasions in 2011, while that in 2009 for 13 sampling occasions was 0.28. The mis-identification models available in MARK perform well when capture probabilities are between 0.2 and 0.5, but estimation of genotyping error declines in accuracy and the percent bias increases when capture probability is 0.1 or less (Lukacs & Burnham 2005). This is also true for the alpha mis-identification parameter. As alpha declines (i.e. an increase in genotyping error), bias increases. Therefore, we believe that our 2011 estimates are biased high, an assumption that is reinforced by estimates produced from observational counts, which remained constant between the two years. Although it would not be surprising for population estimates obtained from genetic CMR to be larger than those produced by observational counts, we would expect for the Cambodia population that the genetic estimates between the two years would be on par with differences observed through visual census. Our expectations are based on the knowledge that the populations in Cambodia are small and restricted by food resources (Clements et al. 2013; Pain et al. 2003). Densities of wild ungulates within and surrounding the study sites have been estimated at < 4 animals km^{-2}; domestic livestock exist at higher densities of 7 animals or more per km^{-2} (Clements et al. 2013). Although once more widespread, husbandry practices permitting livestock to roam freely in the forests have changed as a result of the intensification of agriculture. The

areas in Cambodia where vultures still persist correspond to those where livestock are still allowed to range. The vulture restaurants, which are located in the same areas, provide approximately 70 carcasses per year and presumably draw in a large fraction of the local vulture population. This is especially true in the wet season when livestock are not permitted to roam freely. Therefore, the monthly and annual census in Cambodia, although imperfect for detecting all individuals, are likely establishing reasonably accurate population estimates, and provide a valuable measure to compare the effectiveness of our genetic CMR methods.

Although we were not able to rigorously estimate abundance for Slender-billed or Red-headed vultures, it would be straightforward for future sampling efforts to obtain adequate sample sizes of these species by more frequently targeting the most visited feeding sites, thereby improving the probability of recapture. We initially selected our four sampling sites in an attempt to cover a broad geographical range. However, results from our capture occasions showed that individual vultures foraged at multiple feeding stations and so a more targeted approach would not necessarily introduce a high degree of sampling bias.

In the absence of a formal vulture monitoring program in Kazakhstan, transect counts have been conducted sporadically on the Assy Plateau between 2003 and 2009. Counts ranged from 6-21 individuals for Himalayan griffon, and 2-11 for Eurasian griffon (Table 3.9). Although we extracted DNA from only 947 of the nearly 4,000 feathers samples we collected in 2009, we identified 173 unique genotypes for Himalayan griffon with a minimum abundance estimate of 207 individuals, more than eight times greater than observed through transect counts. Wassink & Oreel (2007)

describe Himalayan griffon as a rare resident in Kazakhstan and estimated breeding pairs at 10 individuals based on visual observation. Similar discrepancies between observational counts and the number of genetic profiles produced from feathers have

Table 3.9 - Number of Eurasian and Himalayan griffon visually counted during a seven point transect count on the Assy Plateau in Kazakhstan. Table B shows GPS coordinates for transect points.

A.

Transect point	GPS coordinates	
1	N43°12'59.3"	E077°52'44.3"
2	N43°14'44.9"	E077°55'27.6"
3	N43°15'10.6"	E077°59'17.3"
4	N43°15'43.7"	E078°02'56.2"
5	N43°16'39.3"	E078°06'15.4"
6	N43°18'36.2"	E078°08'48.5"
7	N43°18'59.9"	E078°11'10.3"

B.

Date	Species	Total
8/8/03	Eurasian griffon	11
7/9/06	Himalayan griffon	9
7/17/06	Himalayan griffon	6
7/22/06	Himalayan griffon	21
7/31/06	Himalayan griffon	5
7/27/08	Himalayan griffon	8
	Eurasian griffon	2
7/29/09	Himalayan griffon	7

been seen with Eastern Imperial Eagles (*Aquila heliacal*) in Kazakhstan (Katzner et al. 2011).

The number of Eurasian griffons detected in our sample set was low. However, this species has a wider distribution extending farther west in Kazakhstan (Figure 3.3) and has been observed in higher densities outside of the Assy Plaeau (*person. obs.*). As in Southeast Asia, vultures in Kazakhstan rely heavily on domestic livestock for food. The Saiga antelope is the only wild ungulate found in large numbers in Kazakhstan but antelope populations have plummeted in the last two decades (Milner-Gulland et al., 2001; Robinson & Milner-Gulland, 2003). Therefore feather samples collected from the carcasses of domestic livestock may provide ample data to assess the total population size of vultures in Kazakhstan. The majority of Himalayan griffon genotypes identified in our study were sampled from just two carcasses. Therefore, limiting sample collection to just the largest carcasses (i.e. horses and cattle) may be an efficient strategy for optimizing sample collection.

Genetic sampling also provided for us an opportunity to assess the genetic variability of populations in both Cambodia and Kazakhstan. The indices we used (heterzyogosity and allelic diversity) suggest that the three species in Cambodia have not undergone substantial loss of genetic variation as might be predicted for small populations, although allelic diversity for Slender-billed and Red-headed vultures are lower than for White-rumped vulture. This is not surprising considering the smaller population size for the two species. Although the three species are at low abundance, we cannot exclude the possibility that there is genetic connectivity with other populations outside of Cambodia. Genetic diversity for Himalayan griffon was high both for both allelic diversity and heterozygosity, which we would expect for a larger panmictic population.

Non-invasive sampling for genetic CMR may be the most reliable method to estimate vulture populations, especially when heavily managed monitoring programs are not in place. Because our study was the first large-scale application of genetic CMR methods using vulture feathers we can suggest ways to improve study design for future application. Maximizing recapture rates should be a high priority and can be accomplished by sampling intensively at the most heavily visited sites. Reducing genotyping error can also improve confidence in abundance estimates particularly by eliminating microsatellite markers with the highest genotyping error rates. This can be accomplished with pilot studies using a nominal number of representative samples from each species.

We chose to incorporate genotyping error into our CMR models by using the mis-identification models developed by Lukacs and Burnham (2005). These models perform optimally when genotyping error is less than 5% and when the probability of recapture is between 0.2-0.5. Because we had a high percentage of individuals identified by a single sample, we felt that it was more prudent to include error in our analysis rather than cull more samples from our study, especially since we had taken care through our screening process to mitigate error. Other methods to account for uncertainty due to genotyping errors have been developed although they are not yet available in modeling programs such as MARK (Link et al. 2010; McClintock et al. 2014).

Conservation Implications

Cambodia -- Genetic CMR using naturally shed feathers presents exciting opportunities to study and monitor vultures. Field sampling of feathers can be

accomplished at low cost by individuals with no formal biological training. If kept under dry conditions and protected from UV, the DNA in feather samples can persist for several years in storage until a means to analyze them genetically becomes available. Genetic monitoring of populations can be used to supplement existing conservation efforts or can be developed quickly for regions where monitoring programs do not currently exist. . These methods can contribute to long-term monitoring of populations by providing base-line data on population sizes, or used to gather more substantial demographic information such as total population size, dispersal patterns, or survival rates. The conservation status for all vulture species found in Cambodia is currently precarious. Conservation organizations in Cambodia have responded quickly and effectively to prevent the further decline of vultures. A comparison of our genetic results to the visual surveys suggests that the use of feeding stations may be an appropriate method to derive population counts despite the limitations of visual surveys (e.g. double counting or not counting individuals that are not seen). Vultures in Cambodia rely heavily on carcasses provided at the restaurants, and the small population size and restricted geographic range of the species may make them more tractable for observational counts. However, the addition of genetic monitoring can lead to more comprehensive management by providing estimates of abundance and effective population size, recruitment, survival rates, relatedness between individuals, changing trends in genetic diversity, and the genetic connectivity between populations (Schwartz et al. 2006). In addition, archived samples can be revisited to address previously unanswered questions especially as genetic analytical methods provide deeper coverage of a species' genome (Allendorf et al. 2010).

Kazakhstan -- Apart from population estimates produced by this study for Himalayan griffon, the status of vultures found in Central Asia is virtually unknown. Vultures that breed in Kazakhstan likely migrate to south Asia in the non-breeding seasons. The continued use of diclofenac on the Indian sub-continent, despite a legal ban, may pose a serious threat to these migrants. Genetic CMR can be used effectively to document vulture population trends in this region, as well as throughout other parts of Europe, Africa and Asia. The results produced by this study were an initial attempt to assess the effectiveness of genetic mark-recapture techniques. Based on the number of samples analyzed we were only able to obtain an estimate for a portion of the population. We would need to analyze samples from a more extensive part of the species range for more a robust estimate of population size. However, given that transect surveys have been an unreliable method to assess population size in Kazakhstan, the use of genetic mark-recapture may be much more informative to assess population trends. Otherwise, more intensive visual surveys are needed. For example, transect surveys were used in the upper Mustang of Nepal to monitor population trends of Himalayan griffon from 2002-2005. Between 22-24 days of surveys during each sampling year showed that populations were likely declining by as much as 70% (Arachaya et al. 2009). In this situation, transect surveys provided valuable information for the conservation status of the species in Nepal and drastic population declines were detected. Transect surveys may also be appropriate if financial constraints prohibit costly genetic analyses. Genetic CMR, however, may provide more robust long-term information such as those mentioned above for Cambodia.

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CHAPTER 4

THE UTILITY OF NON-INVASIVE GENETIC SAMPLING FOR STUDIES OF BIRDS

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Abstract

The estimation of demographic parameters using mark-recapture analyses based on identities derived from non-invasive samples of DNA is becoming increasingly common, particularly in studies of mammals. However, the use of naturally shed feathers to study birds is still rare even though feathers can be a robust source of genetic material. Yet as is the case with other types of non-invasive samples such as hair and feces, feathers can yield low or degraded DNA, which can lead to low amplification success rate and genotyping errors. For reliable estimates of population size, appropriate field sampling methods and laboratory protocols must be implemented to reduce analytical biases. Here we provide an overview of non-invasive genetic capture-mark-recapture (CMR) methods with a focus on naturally shed feather samples. We further discuss other best practices with genetic CMR using as a case study our research on vultures in Asia.

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Introduction

Many bird species are undergoing unprecedented population declines, and it is predicted that 10% of avian species will become extinct in a century or less (Sekercioğlu et al. 2004). The criteria used to assess the conservation status of a species, such as those applied by the International Union for the Conservation of Nature's (IUCN) Redlist, are primarily based on changes in a species' population size. Accurate estimates of population abundance are therefore essential for ensuring that declining species receive appropriate conservation attention. A variety of approaches are used to investigate the demography of bird populations, most typically transect surveys or other observation-based counts, and capture-mark-recapture/resight methods (Sutherland et al. 2004). However, these field techniques may be less effective or efficient for species that are cryptic or highly vagile, particularly hard to capture or recapture, or for those that inhabit environments less conducive for visual surveys (those found in dense forest, for example). In such situations, the inability to detect or sample from a sufficient number of individuals can lead to poor precision in demographic estimates. Small sample sizes can also diminish the power of genetic analyses, which are now a standard component of conservation directed research for assessing the long-term viability of a population (Schwartz et al. 2007).

Over the past two decades, the genetic analysis of non-invasively collected samples has been increasingly used as an alternative method for detecting animals for ecological and demographic research (Waits & Paetkau 2005). In particular, DNA extracted from shed hair or feces has been used effectively in numerous studies of mammals to discriminate between individuals (Lampa et al. 2013). When non-

invasive genetic samples are collected at multiple time points they can also provide the necessary material for capture-mark-recapture (CMR) analyses used to estimate important demographic parameters such as population size and survival rates. Demographic studies using naturally molted feathers, however, are still uncommon (for exceptions see Hogan & Cooke 2010 and Rudnick et al. 2008) yet may provide opportunities for genetic CMR for a wide range of species (Horváth et al. 2005; Miño & del Lama 2009; Johansson et al. 2012; Kapetanakos et al. 2014; Segelbacher 2002; Seki et al. 2006; Vili et al. 2013).

There are two principle benefits to using non-invasive sampling over more traditional field census or survey methods: 1) individual birds do not have to be handled or even directly observed, and 2) in some situations numerous samples can be collected, often with minimal field effort. There are, however, important assumptions and caveats that must be incorporated into the design of feather-based CMR studies along with a series of related laboratory and analytical precautions. First, an appropriate sampling protocol is necessary to obtain the necessary number of samples while also taking into account the assumptions of CMR modeling (e.g. population closure). Second, non-invasively collected feathers can often yield low quality and low quantity DNA, and resulting genotyping errors can potentially influence the reliability of the genetic profiles used to discriminate among individuals. Different challenges are encountered at each step, and identifying ways to overcome potential sources of error prior to the start of a study can substantially improve its chances of overall success. It is not always conducive to use genetic CMR for population estimation, and its utility depends on the study species (i.e. the number of feathers that can be

collected), as well as the financial and technical expertise available to the project.

Here we review in more detail the methodological and analytical issues that should be considered when using molted feathers for genetic CMR to estimate population size. We specifically target the use of feathers since studies using samples collected non-invasively from birds are relatively uncommon, yet there is substantial potential to use feathers for population studies (Smith et al. 2003). Finally, we provide as a case study our prior genetic CMR research with Asian vultures.

Field Sampling

Genetic CMR projects are only as robust as their sampling designs. Understanding potential sources of sampling bias - which can profoundly impact the accuracy of estimates - is an essential first step to successfully developing a sound sampling plan. Population abundance estimates obtained from genetic CMR are generally most precise when sampling strategies are designed for theoretically closed populations during the sampling period; that is, populations that experience no recruitment via the addition of young birds, no mortality, and no migration of individuals in or out of the population. As a result, population size can be assumed to be constant across the sampling period (Otis et al. 1978). To be consistent with this assumption of population closure, feather-sampling occasions must be spaced close together in time, yet frequent enough so that feathers from individual birds have a high probability of being sampled in more than one sampling interval.

The basic mathematical underpinnings of CMR analyses illustrate how sample size and recapture probability from feather sampling, and genotyping errors in labwork (discussed in detail below), can influence the accuracy of demographic

estimates. In its simplest form, an estimate of population abundance (*N*) is derived from the number of unique individuals sampled (*n*) and the probability that those individuals are encountered at least once (*p*) (Otis et al. 1978):

$$N = \frac{n}{p}$$

Although one can directly obtain the value of *n* based on counts of individuals derived from the number of unique genotypes recovered from feather samples, the value of p, in virtually all real-world cases, must be estimated – and doing so typically requires multiple sampling occasions. In the simplest case consisting of two sampling occasions, *p* is derived from the ratio of individuals sampled on both occasions (m_2) to those captured only during the second sampling occasion (n_2). Assuming population size remains constant (which for a closed population must be true), the ratio of individuals in the total population captured the first time should be proportional to the number of individuals captured during the second occasion:

$$\frac{n_1}{N} = \frac{m_2}{n_2}$$

Therefore,

$$N = \frac{n_1 n_2}{m_2} = \frac{n}{p}$$

The latter equation demonstrates that as the probability of recapture declines, *N* can becomes larger, eventually reaching a point where it is no longer informative (Lukacs & Burnham 2005a). To increase the probability of recapture it is important that a sufficient number of feather samples are collected at each sampling occasion. Feather samples collected during a pilot study can be used to assess the number of individuals 'captured' during a single session and thereby provide an estimate of how

many feathers are left behind by each individual present at the site in each interval. Sampling frequency and intensity can then be adjusted accordingly to suit the needs of the project.

The number of feathers shed by birds will depend in part on the pattern and timing of their molt, which varies by species, age, season, migration, and availability of food resources (Greenberg & Marra 2005). Large soaring birds that require flight ability to feed during molt replace feathers gradually over many months or even years. Accipitrids, for example, molt primary and secondary feathers in several consecutive molt waves (termed serial molt) (Edelstam 1984); large vultures can take up to 2-5 years to replace all flight feathers (Snyder et al. 1987; Zuberogoitia et al. 2013). Molt for such species may be suspended when food is less available such as in winter months (Snyder et al. 1987). Other groups of birds such as waterfowl may have two distinct annual molting periods (Pyle 2005). Understanding when and where birds are likely to shed feathers can improve sampling success. However, a 'sampling occasion' is more difficult to define than a 'capture occasion' (Ebert et al. 2010). If the timing of when the feather sample was dropped cannot be pinpointed to a reasonably short time interval, then the assumption of population closure may be violated (Lukacs & Burnham 2005b).

During a CMR study, it may be feasible to have birds in hand during one or more sampling periods, in which case a combination of genetic samples taken at capture (usually blood or feather samples) and naturally shed feathers may be available. For example, this may be the case when chicks are handled at the nest. Collecting genetic material directly from chicks, as well as shed feathers from adults

around nests, can provide information on relatedness in addition to population size (Rudnick et al. 2005).

The methods used to optimize the numbers of feathers sampled will depend on the biology of the bird species. For example, it may be possible to use baited stations for certain species such as vultures, which shed numerous feathers as they compete for optimal feeding positions at a carcass (chapters 2 and 3). Gebhart et al. (2009) collected macaw feathers from clay licks, whereas at other times is it possible to collect feathers around nesting sites (Rudnick et al. 2005; Hogan & Cook 2010; Miño & del Lama 2009). It is also feasible to use a combination of methods such as taking samples from hunted birds in conjunction with collecting feathers naturally shed in the field (Johansson et al. 2012). Whichever sampling method is chosen, it must be conducted in a way that minimizes capture heterogeneity among individuals (Petit & Valiere 2006) . Passive sampling of feathers (collecting molted feathers) has the advantage of minimizing behavioral response to capture. However, heterogeneity in sampling may be introduced if some individuals are molting during the sampling period while others are not. Or, if baited stations are used, heterogeneity may arise from behavioral differences if some individuals are more likely to visit the stations than others (Boulanger et al. 2006).

During a sampling session, it is highly likely (and generally desirable) that one will collect multiple feathers from the same individual bird (Marucco et al. 2011). Although it might be tempting to collect only a subset of the feathers available from the field site, it is worth remembering that genotyping success can vary dramatically between samples. Therefore, a thorough collection of samples can help ensure that

individuals are not missed due to genotyping failure alone. The advantage of genetic CMR as compared to conventional mark-recapture is that a subset of samples can be randomly selected for analyses after they have been collected. If low recapture rates are observed as analyses are progressing, additional samples can be added to boost the power of CMR analyses. As a principle of precaution, we therefore recommend a thorough collection of feathers during each sampling period.

DNA from feathers: size matters a little, but condition matters more

The amount of DNA that can be extracted from a feather is loosely correlated with its size, as larger feathers yield a higher concentration of DNA (Segelbacher 2002). However, a sufficient quantity of DNA can be successfully extracted from feathers of all sizes (primaries, secondaries, contours, and even down feathers) (Vili et al. 2013) (Hogan et al. 2007; Bayard del Volo et al. 2008); therefore whenever possible, feathers should be collected in the field regardless of their size.

The physical condition of the feather at the time of collection has more of an effect on DNA yield and quality than does its size; feathers that have become degraded from exposure to the elements are likely to perform poorly in laboratory analyses (Hogan et al. 2007). DNA is damaged by environmental conditions such as heat, humidity, UV light, and repeated freezing and thawing (Vili et al. 2013; Ravanat et al. 2001; Pompanon et al. 2005). If a feather appears matted, dirty, and has separated barbs, it has probably been sitting around for a long period (Booms et al. 2008). Collecting feathers shortly after they are shed will reduce their exposure to damaging elements and also prevent further degradation by decomposing organisms (Segelbacher 2002).

It is good practice to wear gloves when collecting feathers, or alternatively to not directly handle the feather below the feather vane since DNAases found on human fingers can degrade DNA. Once feathers have been collected, they should be stored dry and shielded from UV light. If feathers are wet during collection, they should be dried before storage. Based on our experience, feathers can be collected and stored in paper envelopes at room temperature for several months. Plastic bags are poor options for feather storage as trapped moisture can damage the DNA. The optimal storage conditions are at -20˚ C or lower temperatures, in a non-frost-free freezer. Freezers that automatically defrost, as in many home freezers, do so by cycling above freezing at least once per day; because feathers heat and cool very rapidly, this repeated temperature cycling above and below the freezing point can cause rapid DNA degradation. For longer-term storage it is important to keep feathers at -20˚ C or lower until they can be processed in the lab (Hogan et al. 2008).

Laboratory Methods

Once feathers have been brought into the lab, there are several steps that must be completed to acquire the necessary genetic data for abundance estimation. First, DNA must be extracted from collected samples. In some cases it may then be necessary to use genetic methods to verify that the samples collected originated from the target species. The next step requires the selection and subsequent screening of genomic loci that uniquely ‘‘mark’’ and reliably ‘‘recapture’’ individuals. This is the most challenging aspect of this overall approach since feathers often yield small quantities of potentially degraded DNA. Low quantity and poor quality DNA can result in genotyping errors, which can confound the interpretation of genetic CMR

analyses and subsequently present complications for population estimation (Waits and Paetkau 2005). Therefore, a critical component of genetic CMR study design is the mitigation of potential sampling errors through a series of screening protocols.

DNA extraction from feathers

There are two primary sources of DNA in feathers: 1) the pulp caps and feather follicle cells found on the external tip of the quill, and 2) the superior umbilicus, a remnant blood clot formed during feather growth and found inside the base of the feather vane (Horváth et al. 2005). To prepare a feather for DNA extraction, the tip of the feather quill is generally removed and diced into small pieces. For medium and large feathers, the superior umbilicus can be removed separately. We recommend using a new cutting implement (e.g. sterile razor blade) for processing each feather tip to avoid contamination among samples. DNA can be extracted most easily using standard tissue extraction kits, although other methods are also successful (Horváth et al. 2005; Johansson et al. 2012; Rudnick et al. 2005; Bayard del Volo et al. 2008). To improve DNA yield, feathers can be submerged in a protease buffer solution for an extended time period. Especially high quantities of DNA (~115 ng/µl) were recovered from Roseate Spoonbill and Jabiru Stork feathers after a 1 week long Proteinase K digestion, which was then followed by a phenol-chloroform extraction (Miño & del Lama 2009). As a final step, the DNA should be eluted in a volume that will provide sufficient substrate for multiple genotyping reactions (and other analyses depending on the scope of the project), while not over-diluting the sample.

Species identification from feathers

In some cases it may be necessary to confirm whether a feather sample was dropped by the target study species. There are various molecular methods that can facilitate species differentiation. A simple approach is to sequence a diagnostic portion of the genome such as the cytochrome oxidase I (COI) region of mitochondrial DNA, which has often been used to differentiate between avian species (i.e. DNA barcoding) (Valentini et al. 2008). However, this is also one of the more expensive methods. Other approaches include: 1) using a species-specific microsatellite marker in a standard PCR reaction (Palomares et al. 2002); 2) using a rapid classificatory protocol PCR (RCP-PCR) which combines primers from multiple species in a single reaction (Dalén et al. 2004); and, 3) implementing a restriction enzyme assay that will digest DNA into species specific fragments (Kapetanakos et al. 2014; Paxinos et al. 1997).

An added benefit to incorporating one of the above methods into a genetic CMR protocol is that it also provides an initial indicator of sample quality. If a sample performs poorly in this species-identification protocol, there is high probability it will not produce reliable downstream genotypes. Therefore, samples that fail to amplify early on in the analytical process can be identified as low quality and eliminated from further analysis.

Genotyping feather DNA

A fundamental component of genetic CMR is the selection of genomic loci that uniquely ‘‘mark’’ and reliably ‘‘recapture’’ individuals. Any set of genetic markers that are unique to individuals can be used for this purpose, but to date microsatellite loci have been the most common genetic ‘tags’ for such demographic

analyses. Individuals in the study population are differentiated through the analysis of multiple variable loci (Taberlet & Luikart 1999). The power of the selected loci to discriminate among individuals should be assessed early on in the genotyping process. Unfortunately, the performance of the selected markers and the number of individuals used in the study will not be known until the analyses are complete. Waits and Paetkau (2005) recommend genotyping approximately 30 individuals from the population using 10-15 microsatellite markers. The markers can then be ranked according to genetic variation (allelic variation and heterozygosity), and ease of scoring (e.g. minimal stutter peaks, low failure rate, etc.). There are recommended guidelines based on estimated population size and levels of heterozygosity to narrow down how many markers to use in the final analysis (Waits et al. 2001; Paetkau 2003; Paetkau 2004).

Combining multiple markers in one genotyping PCR reaction (multiplex PCR amplification) can reduce cost, increase efficiency, and minimize the volume of DNA consumed (Guichoux et al. 2011; Beja-Pereira et al. 2009). Commercial kits are now available that facilitate the optimization of multiplex PCR conditions (e.g. Qiagen multiplex PCR kit or Life Technologies Platinum® multiplex PCR Kit among others). Once the DNA samples have been genotyped at the chosen microsatellite loci, matching genotypes can be grouped, with each group presumably representing a unique individual. Software programs that facilitate this process include GIMLET (Valiere 2002), GENECAP (Wilberg & Dreher 2004) and ALLELEMATCH (Galpern et al. 2012).

Sources of genotyping error

The potential sources of error in genetic CMR studies and methods to mitigate the impact on population studies have been extensively reviewed (Lampa et al. 2013; Paetkau 2003; Pompanon et al. 2005; Taberlet & Luikart 1999; Waits & Paetkau 2005; Bonin et al. 2004). There are three primary sources of genetic error that can arise when genotyping samples containing low concentrations of DNA: shadow effects, allelic dropout, and false alleles. Human error in sample handling, genotype scoring, or data recording is a fourth potential source (Gagneaux et al. 1997; Schwartz et al. 2006), but not specifically addressed here since it is general to all genetic laboratory analyses.

'Shadow effects' occur when samples originating from separate individuals appear to come from the same individual. This error usually results from using a panel of microsatellite markers that does not have sufficient discriminatory power, either because too few markers are used, and/or because the markers lack variability. Shadow effects result in an underestimation of population abundance. A statistical parameter referred to as the 'probability of identity', or P_{ID}, can be used to assess and minimize this type of error. P_{ID} is the probability that two randomly drawn individuals from a population share the same genotype (Woods et al. 1999), and therefore this statistic is used to assess the overall discriminatory power of a panel of microsatellite markers. For example, a $P_{ID} = 0.20$ implies that 20% of the individuals in the population have matching genotypes. Increasing the number of variable microsatellite markers used in a study will decrease P_{ID} until it is sufficiently small that shadow effects become negligible. A larger value for P_{ID} can be expected for a population

consisting of closely related individuals, a likely situation for species that are highly endangered and persisting in geographic isolation. In such cases it would be more informative to calculate the probability of identity for siblings ($P_{ID\text{-}sib}$), a more conservative estimate that considers the relatedness of individuals. $P_{ID\text{-}sib}$ can be used to set the lower limits for the number of loci used for populations that are suspected of having a high degree of relatedness (Waits et al. 2001). $P_{ID\text{-}sib}$ may be an overly conservative estimate, however, which may lead one to use more loci than actually necessary (Rew et al. 2011).

In an effort to minimize P_{ID}, it may be tempting to use a large suite of microsatellite markers. However, one must also consider that overall genotyping error rate is a product of the per-locus error rate, the number of loci used, and the number of samples analyzed (Mckelvey & Schwartz 2004). By reducing the number of markers used, the less prone a data set will be to error (Lukacs & Burnham 2005b; Waits & Paetkau 2005).

Allelic dropout, when one allele fails to amplify in a heterozygous locus (i.e. false homozygote), is the most common kind of scoring error in genotyping analysis (Mckelvey & Schwartz 2004; Taberlet & Luikart 1999). Studies using non-invasively collected hair and feces from mammals have shown that error rates due to allelic dropout can be as high as 30% (Lampa et al. 2013; Gagneux et al. 1997).

A less common source of error is the amplification of false alleles, which can make a homozygote appear to be a heterozygote (Taberlet & Luikart 1999; Lampa et al. 2013). False alleles usually account for <5% of total genotyping error (Taberlet et al. 1996). Allelic dropout and false alleles both create 'false' genotypes; genetic

samples originating from one bird falsely appear to have originated from two or more individuals thereby contributing to an overestimation of abundance. In a frequently cited case, Creel et al. (2003) demonstrated that genetic CMR analyses that do not account for genotyping error, particularly those stemming from allelic dropout, can produce population estimates up to five times greater than the true population size. In their study, bias was most pronounced when 13 loci were used in the analysis. Errors associated with false genotypes can go on to influence the outcomes of CMR statistical models by giving the appearance of increased capture heterogeneity and lowering the probability of recapture (Lukacs & Burnham 2005a).

False alleles can be largely eliminated through careful scrutiny of genotyping results, as errors often appear as irregular alleles that fall out of the normal range characteristic for each locus. Conversely, allelic dropout is more difficult to spot and requires that samples be repeated several times to provide an opportunity for "hidden" alleles to reveal themselves.

Ideally, it would be informative to know the error rates for each locus ahead of time. However, genotyping errors are not randomly distributed between PCR's; rather, they vary by the amplification success of each sample (Pompanon et al. 2005). As a result, genotyping error rates can only be determined by genotyping every sample at each locus multiple times to create a consensus genotype (Navidi et al. 1992). This is not a practical solution for most genetic CMR studies. Alternatively, one can generally assess the quality of markers by subjecting a randomly selected group of samples to a repeated round of genotyping. Markers that appear to have higher error rates can be subjected to more intense scrutiny when screening genotypes (see next section below),

or eliminated.

Screening genetic samples for allelic dropout

In the last decade, improvements have been made to lab protocols to identify and screen for genotyping errors (Paetkau 2003; Valiere et al. 2006; Dewoody et al. 2006; Wilberg & Dreher 2004; Mckelvey & Schwartz 2005; Miller et al. 2002). A combination of these different screening methods achieves the lowest rates of error (Marucco et al. 2011).

Genetic samples that are degraded or that have a low concentration of DNA are most likely to produce unreliable genotyping results (Taberlet et al. 1996). Assessing the quality of samples prior to genotyping will help identify those of lowest quality early in the screening process. Targeting specific regions of nuclear DNA using species-specific microsatellite primers, or amplifying a portion of mitochondrial DNA (mtDNA) can be an effective and efficient means for identifying the lowest quality samples (Morin et al. 2001). Those samples that do not amplify at this stage can be culled from further analysis, saving time and money for subsequent genotyping analyses. One disadvantage of using mtDNA as an evaluation of DNA quality is that it is present in many more copies per cell than are nuclear DNA loci; therefore, positive results from mtDNA analyses may over-predict the genotyping success of nuclear markers (e.g. microsatellites). Alternatively, real-time quantitative (RTQ) PCR has excellent potential to improve non-invasive studies through the quantification of DNA at targeted sites. Morin et al. (2001) greatly increased the efficiency of their genotyping protocol by using RTQ-PCR to quantify 'amplifiable' DNA concentrations from chimpanzee fecal and hair samples. DNA extracts containing 25-

100 pg/μL of DNA were highly susceptible to allelic dropout whereas those samples containing less than <25 pg were likely unusable. In their study, samples with a concentration of zero were discarded, and new extractions were prepared for those samples that showed low concentrations of DNA. These findings reinforce those of an earlier study demonstrating that samples containing <56 pg of template DNA were highly prone to allelic dropout (Taberlet et al. 1996). However, unlike hair and fecal samples collected from mammals, which can provide enough substrate for multiple DNA extractions, feathers quills typically suffice for only one extraction; therefore, some forethought is necessary when determining the final volume (and hence concentration) of DNA samples during the extraction process, while also taking into account that multiple PCR reactions may be necessary for screening genotypes.

Another important consideration prior to genotyping is the design and subsequent selection of microsatellite primers. The number of repeating units and the size of the amplified sequence can influence the frequency of allelic dropout and overall amplification success. To improve amplification, dinucleotide markers should be selected over tri-or tetranucleotide markers. In addition, microsatellite markers that amplify sequences of >200-300 base pairs can produce higher allelic dropout rates; therefore, primers should be designed to target shorter region of DNA (Broquet et al. 2006). Advancing genetic technologies, such as Next-Generation Sequencing (NGS) techniques, have greatly improved our ability to design primers, particularly for non-model organisms (Malausa et al. 2011). With these methods, a large number of potential microsatellite loci can be identified, allowing for more stringent selection of microsatellite loci (Guichoux et al. 2011).

Once genotyping markers have been designed and selected based on their variability, the next step is to implement protocols to detect allelic dropout and false alleles. This usually involves establishing a protocol for repeating all, or a subset of samples based on sample quality (Lampa et al. 2013; Marucco et al. 2011). The multiple-tubes approach was one of the first techniques used to screen for genotyping errors (Taberlet et al. 1996). However, the numerous PCR replicates required for each sample (7 identical genotyping results for homozygous samples and a minimum of 2 for heterozygous ones) can quickly overwhelm both lab resources and DNA supplies (Taberlet et al. 1999). Variations on the multiple-tubes approach are now more commonly used. For example, the number of replicate PCRs can be reduced (e.g. 3 to 4 replicates to accept a homozygous result and 2 for heterozygotes (Bellemain et al. 2005; Frantz et al. 2004). Alternatively, if the target DNA has been quantified, PCR's can be replicated only for those samples with low concentrations of DNA (Morin et al. 2001). Paetkau (2003) used a progressively stringent culling process: after an initial genotyping pass, those samples that did not produce high-confidence genotypes for a subset of markers (a minimum of 4 out of 7 markers) were culled. Those that passed this initial screening step were reanalyzed to improve ambiguous or missing results. Paetkau showed in his study that virtually all samples that escaped culling produced complete genotypes. Subsequently, after grouping samples that shared identical genotypes, samples that matched at all but one (1-MM) or two (2-MM) markers were reanalyzed. This protocol was based on the assumption that errors are unlikely to be present at >2 markers in a sample, and that there is a very low probability of having the same error at multiple markers in multiple samples from the same individual

(Paetkau 2004). Creel et al. (2003) used a more conservative approach when markers differed at only one locus (1-MM) by treating these samples as the same individual. We find this to be overly conservative, however, since in many studies it is often likely that some individuals will indeed be represented by only one sample. In those cases, the lumping of samples will lead to an underestimation of population size (Lampa et al. 2013).

A final approach to minimize genotyping error in the lab is to use software tools that can identify whether genotyping errors persist after the above screening mechanisms have been used. Some of these methods are based on deviations from Hardy-Weinberg equilibrium (van Oosterhout et al. 2004), while others detect those samples that differ at 1-3 loci (Mckelvey & Schwartz 2005; Valiere 2002; Wilberg & Dreher 2004). Beja-pereira et al. (2009), Lampa et al. (2013) and Pompanon et al. (2005) provide thorough reviews on these screening tools and a description of the software programs that can be employed.

Regardless of the screening methods applied, it is worth considering that eliminating a large percentage of samples due to genotyping uncertainty can result in a loss of population information, which can impact encounter rates or the detection of new individuals (Lukacs & Burnham 2005a). Therefore, it may be prudent to accept a certain level of genotyping error rather than trying to eliminate it completely (Lukacs & Burnham 2005b). Studies have shown that bias in population estimates can be significantly reduced and CMR model assumptions met when genotyping error rates are <0.005 (Paetkau 2003; Waits & Leberg 2000). However, when misidentification occurs, the constraint that population size is larger than the number of unique

genotypes ($N \geq M_{t+1}$) no longer holds. That is, it is possible that enough animals are misidentified such that the number detected (M_{t+1}) is greater than the number that actually exists in the population (N). Second, this increase in the numbers of animals supposedly encountered causes the estimated probability of detection to be smaller than it should be. The effect of these two factors is to cause the estimated abundance N to be too high.

Incorporating Genotyping Errors in CMR Models

Although every effort should be made to eliminate genotyping errors through stringent laboratory protocols, it is reasonable to expect that errors are present in CMR datasets (Bonin et al. 2004). Population estimates derived from conventional CMR analyses are based on the assumption that the dataset is error free (Mills et al. 2000; Waits & Leberg 2000). New models, however, are being implemented that incorporate the mis-identification of individuals into statistical analyses (Lukacs & Burnham 2005). These 'mis-identification' models, which are for closed populations and robust sampling designs, are based on full-likelihood (Otis et al. 1978), conditional (Huggins 1991) and mixture (Pledger 2000) models. To account for the possibility of genotyping error, a parameter 'α', which is the probability that a genotype is correctly identified the first time it is encountered, is incorporated into the probability function (Lukacs & Burnham 2005a). The models incorporating error are contingent on three assumptions: 1) an erroneous genotype will not be identical to any other genotype in the population; 2) two genotyping errors associated with two different sampling occasions will not produce the same genotype; and 3) there is no shadow effect

(Lukacs & Burnham 2005a). Assumption 1 presumes that the presence of 'false' genotypes is a consequence of genotyping error (i.e. mis-identification). Therefore, capture histories that have only one encounter are assumed to result from misidentification. In contrast, conventional CMR models do not account for misidentification and each observed encounter history is analyzed as a distinct individual. In assessing the performance of their mis-identification models, Lukacs & Burnham (2005) only considered constant capture probability with genotyping error rates of 0.01 to 0.05. The models failed to converge to a reasonable population estimate when capture probability was $\leq$0.1 (Lukacs & Burnham 2005a), or when genotyping error was greater than 0.10 (Lukacs *personal communication*). In addition, these models cannot distinguish between a violation of population closure and the impacts of genotyping error, both of which can result in overestimates of abundance (Marucco et al. 2011). Although the Lukacs and Burnham models are readily accessible in program MARK, they have only been used in a handful of CMR studies on wild populations (Lampa et al. 2013).

Yoshizaki (2007) and Yoshizaki et al. (2011) also used an extension of conventional CMR models to address mis-identification of natural tags. Although the methods proposed by these studies also include the parameter 'α' and are largely based on the same assumptions as the Lukacs and Burnham models, they differ in the assumption that 'ghost' genotypes are present from the start of the study, and that the fates of existing and non-existing genotypes are not independent (Yoshizaki 2007). In contrast to Lukacs and Burnham, Yoshizaki et al. created two separate encounter histories – a real and a 'ghost' history – both having the same probability of

occurrence. Similar to Lukacs and Burnham, these models fared best when capture probability was high and the probability of error was low (Yoshizaki et al. 2011).

The program GUAVA (Genotyping Uncertainty Added Variance Adjustment) (Knapp et al. 2009) uses known information about allele frequencies and Hardy-Weinberg equilibrium to incorporate the probability of genotyping error (Link et al. 2010). The program allows for two types of error - allelic dropout and shadow effect - and calculates the probability that two samples are from the same individual. GUAVA creates pseudo-capture history matrices by relaxing the assumption that identical genotypes come from the same individual. The program requires known error rates that the authors suggest can be obtained through a pilot study by replicating amplifications of each locus an average of three times.

Link et al. (2010) and Wright et al. (2009) both applied a Bayesian approach to incorporate mis-identification. The Link model also includes parameter 'α' but relaxes assumptions about initial detection probabilities. The Wright model, on the other hand, relaxes the assumption that error results in a new genotype. It only accounts for allelic dropout and requires knowledge of the number of alleles in the population. In addition, two positive PCR's per locus are required. Software to fit this model has recently been written in R, with an extension that includes heterogeneous capture probabilities. The modified program also allows for sampling from an open population (Wright et al. 2012). To our knowledge, neither the Link nor the Wright models have been applied by other authors to wild animal populations.

Eventually our technical capabilities in the lab will minimize genotyping error to such an extent that the use of non-invasive samples in population studies will

become the norm rather than the exception. Until that point, we recommend both thorough screening of genotypes and the use of statistical models that incorporate mis-identification. The CMR models currently available seem to perform well when genotyping error is present but at very low rates. If genotyping error rates are high, then it is advisable to revisit the data set for further scrutiny. At present, only the Lukacs and Burnham model is available as part of an easy to access software package. However, this is likely to change as the use of genetic population monitoring continues to increase (Schwartz et al. 2006).

CMR after microsatellite markers

Microsatellites have thus far been the genetic marker of choice in non-invasive CMR studies (Sethi et al. 2014); their high mutation rate provides the essential variability needed to discriminate among individuals. However, as discussed earlier and as demonstrated by a wide range of genetic CMR studies (Lampa et al. 2013; Beja-Pereira et al. 2009), there are some considerable challenges to using microsatellites when genotyping low concentration or degraded DNA. Genotypes produced from microsatellites require a thorough screening to mitigate genotyping error (Kelly et al. 2011; Lampa et al. 2013). An additional problem can arise when working with small, endangered populations - it may not be possible to develop microsatellite loci that have enough variation for distinguishing between individuals.

In contrast to microsatellites, single nucleotide polymorphisms (SNP's) are bi-allelic markers that may be less prone to genotyping error, particularly for low quality DNAs (Campbell & Narum 2008). Sequences targeted in SNP-based genotyping are shorter than those of microsatellites (Morin et al. 2004). Although microsatellite

markers have up to four times more power to discriminate between individuals than SNP's on a locus by locus basis (Morin et al. 2004), technological improvements in SNP discovery, namely through Next Generation Sequencing (NGS), can be used to identify and screen thousands of SNP markers, which when applied to a population study can provide much higher power than a small panel of microsatellite loci (Davey et al. 2011; Mardis 2008). Some methods of SNP genotyping also have low PCR and genotyping failure rates even when low quality samples are genotyped (Morin & McCarthy 2007). The main disadvantage to using these more advanced genomic screening technologies is that cost can be prohibitively expensive (Beja-Pereira et al. 2009). However, as costs decrease over time it is probable that NGS will entirely replace the use of microsatellite loci in CMR research.

Case study: genetic CMR of vultures using shed feathers

We provide a detailed account of our genetic CMR methods in the third chapter of this thesis. To summarize, we used feather samples to estimate the abundance of three vulture species in Cambodia: *Gyps bengalensis*, *Gyps tenuirostris*, and *Sarcogyps calvus*. We sampled feathers from January to June in 2009 and 2011 from seven supplemental feeding stations. The stations were established in 2004 to monitor the populations of these critically endangered species through visual surveys conducted monthly (Clements et al. 2013). Each month, one livestock carcass was placed at each feeding site on an alternating basis. Ranging data acquired from satellite tagged vultures was used to assess the placement of feeding sties to optimize their use by vultures in the region (Clements et al. 2013). As a result of survey information collected at each station, we knew that the three species overlapped in

ranges and foraged together.

Our reasons for using genetic CMR to estimate abundance for these species were 1) vultures are very difficult to capture, 2) conventional methods to count vultures such as transect survey are ineffective for population estimation, and 3) vultures shed numerous feathers while feeding at animal carcasses. Vultures will lose feathers from molt (Zuberogoitia et al. 2013), but also as a consequence of their agonistic interactions while they feeding. Juvenile vultures do not begin molt until their second year. However, birds not undergoing molt are still likely be represented in our sampling due to feather loss from the competitive feeding interactions between individuals. On the other hand, the behavior of individual birds could lead to capture heterogeneity if some individual vultures competed more aggressively for feeding spots than others and hence were represented by more feathers. Individual heterogeneity in capture has been shown to be problematic in some CMR studies (Ebert et al. 2010). However, based on our visual observations of vultures at the stations, it appeared that most individuals engaged in some form of competitive interaction while feeding.

We collected 3,258 feather samples from the seven sampling sites in Cambodia in 2009, and 2,021 samples in 2011. From the feathers collected, we subsampled 1,159 feathers in 2009 and 1,219 feathers in 2011 from four sampling sites. Our motivation to subsample was driven by the cost associated with analyzing each sample (approximately $10 / sample). We decided to sub-sample from the beginning, middle and end of the field season and selected the four sites that spanned the geographic range of the species. We then targeted sampling dates where the most number of

feathers had been collected. There were a total of 13 sampling occasions in 2009 and 16 in 2011. Based on the life history traits of vultures and their restricted range in Cambodia, we could assume that there were no substantial additions to, or losses from, the population over this period.

We stored the feather samples dry in paper envelopes. Samples remained in Cambodia for several months at room temperature until they were shipped to the United States for analysis. At that point, the feathers were kept at -4˚ for up to 2 years, during which time we developed our genetic protocols for species ID and gentoyping.

DNA extraction -- We extracted DNA using the E-Z 96® Tissue DNA kit (Omega Biotek) following recommendations by Horváth et al. 2005. DNA was isolated from the calamus tip of each feather, as well as from a residual blood clot in the superior umbilicus. For large flight feathers, the basal tip of the calamus was quartered, and the superior umbilicus located at the upper most portion of the calamus was removed separately (Horváth et al. 2005). We followed the manufacturers DNA extraction protocols for tissue except that samples were incubated in Proteinase K extraction buffer for 48-72 hours (Bayard De Volo et al. 2008). We estimated the concentration of DNA for a small subset of samples and found that DNA concentration ranged from 1.0 to 4.3 ng/μL (N=13; mean concentration = 2.5 ng/μL), while by comparison, DNAs extracted from blood samples of Slender-billed vulture (N=3) ranged from 2.1 to 30.9 ng/μL (mean = 16.9).

COI for Species ID --Vultures gather in mixed-species groups when feeding, and shed feathers cannot be easily differentiated visually. Therefore, our next step after extracting DNA was to identify the species associated with each sample. To do

so, we used a restriction enzyme to digest PCR amplification products of a portion of the cytochrome oxidase I (COI) region of mtDNA (Kapetanakos et al. 2014). We were able to successfully determine the species for 96% of the samples analyzed from 2009 and 86% from 2011. Samples that did not amplify at using these COI primers after two attempts were discarded. From 2009 and 2011 respectively, we identified 849 and 875 samples as White-rumped, 168 and 169 samples as Slender-billed, and 72 and 24 as Red-headed vulture.

Genotyping for individual ID -- We initially tested > 25 microsatellite primer pairs that we developed from White-rumped (*Gyps bengalensis*) and Slender-billed (*G. tenuirostris*) sequences using conventional primer development methods (Kapetanakos et al. 2014). Eventually, we narrowed our selection to eleven microsatellite loci from three different sources (chapter 3, Table 3.2): those created from two of our study species (Kapetanakos et al. 2014), and ones previously published for Eurasian griffon (*G. fulvus*) (Mira et al. 2002) and Bearded vulture (*Gypaetus barbatus*) (Gautschi et al. 2000).

For each locus that amplified, we obtained initial estimates of allele frequency and measures of genetic diversity (observed and expected heterozygosity) using the program GENALEX (Peakall & Smouse 2006). We estimated measures of deviation of population genotype frequencies from Hardy-Weinberg expectations (HWE) and linkage disequilibrium (LD) using the program GDA (Lewis & Zaykin 2001) (see chapters 2 and 3 for results). To assess the power of each locus, we used GENALEX 6.5 (Peakall & Smouse 2006) to calculate the observed probability of identity (P_{ID}). We also calculated the P_{ID} for siblings ($P_{ID\text{-}SIB}$) because of the potential for inbreeding,

particularly for Slender-billed and Red-headed vultures which are found in very low population numbers (Waits et al. 2001). Following recommendations by Paetkau (2003), we ultimately selected seven loci for each species based on levels of genetic diversity and estimated population size for each of species (40-200 individuals based on survey counts; Clements et al. 2013). The P_{ID} and $P_{ID\text{-}SIB}$ for all species fell within suggested guidelines (P_{ID} ranged between $6.37\text{x}10^{-8}$ to $1.51\text{x}10^{-5}$; $P_{ID\text{-}SIB}$ ranged between $6.66\text{x}10^{-3}$ to $2.42\text{x}10^{-3}$) (Waits et al. 2001; Waits et al. 2005). We combined samples in multiplex PCR reaction to reduce the volume of DNA used and to reduce costs (see chapter 3 for details on multiplex).

We used the protocol established by Paetkau (2003) to screen samples for genotyping error. We selected this method in lieu of the multiple tubes approach (Navidi et al. 1992) given our large sample size. Our genotyping screening protocol involved 3 steps:

1) After all samples were genotyped once at all loci, we discarded those that amplified at fewer than 4 markers.
2) For samples that passed step 1, we re-genotyped samples at those loci that did not amplify or that produced ambiguous allele results. After this round of repeats, we discarded samples that did not produce a complete genotype at all 7 markers.
3) We used the program GENECAP (Wilberg & Dreher 2004) to group samples that had matching genotypes. Samples that differed at 1-MM or 2-MM loci, were repeated three times at those markers. We discarded homozygous samples that did produce consistent results after 3 repetitions, and

heterozygous samples that could not be confirmed after 2 repetitions. Samples that passed step 3 were used in the final estimate for population abundance. For steps 1-3, we visually re-examined the genotyping results for each sample in question to verify that we had not made an error in our data entry.

4) Once complete genotypes were assembled and individual identification was complete, we used the program MICRO-CHECKER (van Oosterhout et al. 2004) to confirm the absence of null alleles and scoring errors that may have resulted from stutter peaks.

The highest genotyping success rate was achieved for White-rumped vultures in both years and the lowest for Red-headed vultures (Table 4.1).

Table 4.1- Percentage of samples discarded after each step of genotype screening for White-rumped, Slender-billed and Red-headed vultures in 2009 and 2011. The final column shows the total number of samples that were successfully genotyped and which were used in the estimation of population abundance. The percentage of samples in each column was calculated from the initial number of samples genotyped.

	No. samples genotyped	% samples discarded after initial genotyping	% samples discarded after repeat genotyping	% samples discarded after 1-MM and 2-MM error check	No. (%) samples successfully genotyped
2009					
White-rumped	849	4.0	20.4	10.5	550 (64.8%)
Slender-billed	168	4.2	43.5	14.9	101 (60.1%)
Red-headed	72	4.2	44	2.8	35 (48.6%)
2011					
White-rumped	875	12.1	21.5	6.7	521 (60%)
Slender-billed	169	0.59	30.2	16.6	88 (53%)
Red-headed	24	0	50.0	0	12 (50%)

We do not know why success rates varied among species (>10% difference), although the fact that the majority of loci were designed from *Gyps* species may have played a role.

We used the program GENECAP (Wilberg & Dreher 2004) to organize individual capture histories in a format usable for CMR analyses. Based on a visual inspection of the capture histories for each species, we determined that recapture rates for both Slender-billed and Red-headed vultures were too low to be estimable (Lukacs & Burnham 2005a). We did not include Red-headed vultures samples in further analyses because there was only one individual with a capture history >1 in both years. We did, however, include the 2009 Slender-billed samples in our population analyses so that we could statistically assess capture probability and explore how the CMR models would behave.

Using program MARK (White & Burnham 1999), we estimated population abundance separately for each year using five basic closed models with and without mis-identification (Lukacs & Burnham 2005a) (See chapter 3 for details on each model used). We fixed α to 0.9 to account for a genotyping error of 10%. For comparison, we also fixed α to 1 in separate models, indicating no genetic error.

Models with the highest support (Δ AIC_c <2) incorporated variation of capture probability over time (M_{t+z} and M_t) both with and without mis-identification. Although we ultimately used model averaging to estimate abundance, the best supported models for White-rumped vultures in 2009 and 2011, with approximately 2.5 times the support from the second most supported model, were those with mis-identification (ω_i = 0.62 in 2009 and 0.67 in 2011). Model M_B, which incorporates a

behavioral effect resulting from a possible response to trapping effect (i.e. trap happy / trap shy), was not supported for any species. We incorporated this model even though the birds were not directly handled; we reasoned that our use of bait at the stations may have impacted the birds' behavior. Capture probability was low overall and ranged between 0.06 (SE = 0.018) to 0.44 (SE = 0.13) in 2009, and 0.007 (SE =0.004) to 0.15 (SE =0.03) in 2011 for White-rumped. Capture probabilities for Slender-billed in 2009 ranged from 0.00 (SE = 0.00) to 0.79 (SE = 0.17); occasions where p=0 reflected time periods when this species was not detected (occasions 4,5,6). Although we recovered feather samples for Slender-billed vulture during sampling occasions 4 and 6 (chapter 3, Table 3.7), we eliminated those genetic samples during our screening process. Model averaging resulted in an abundance estimate of 241 (95% CI = 220 to 426) and 404 (95% CI = 327 to 616) for White-rumped vulture in 2009 and 2011 respectively (chapter 3, Table 3.8). The mis-identification models available in MARK perform well when capture probabilities are between 0.2 and 0.5, but estimation of genotyping error declines in accuracy and the percent bias increases when capture probability is 0.1 or less (Lukacs & Burnham, 2005a). Therefore, we believe the results obtained for this species from 2009 are more reliable, and more biologically reasonable given the estimates produced by the visual surveys (182 in 2009 and 183 in 2011; (Clements et al. 2013). In general, we would expect that visual surveys would underestimate the number of individuals in the population because it is unlikely that every vulture that visits the feeding station will be documented. This may be balanced out somewhat by the fact that some individuals will be double-counted (Margalida et al. 2011). The degree to which each of these events occurs is unknown. However,

given that vultures in Cambodia are geographically restricted and heavily dependent on the supplemental feeding stations, the surveys are probably a good lower bound approximation of the total population size. In addition, because of the endangered status of these species, it is more prudent to accept the lower estimate produced from our CMR analyses.

Although abundance for Slender-billed vulture was estimated at 63 individuals, 95% confidence intervals were very wide due to the low probability of capture (95% CI = 63 to -8.07). We were, however, able to glean information from the unique genotypes we obtained in both years. We identified 63 unique Slender-billed vulture genotypes in 2009 and 64 in 2011. We observed one 1-MM sample in 2009 and seven 1-MM in 2011 leading us to believe that our genotyping error rate was low (Waits & Paetkau 2005). Estimates obtained from visual counts, 41 and 45 in 2009 and 2011 respectively (Clements et al. 2013), are likely underestimating the number of individuals in the population by at least 35%.

In theory, we could have increased capture rates for Slender-billed and Red-headed vultures by increasing the total number of feathers analyzed in the lab. We did not know *a priori* how many samples to expect from each species, although we could roughly gauge proportions based on estimated population sizes from the visual counts. However, extracting DNA from an additional 2,000 feathers to filter out the two species with lower abundance may not be worth the extra effort or expense, particularly if visual surveys can serve as proxy to assess population trends over time even though total abundance may be underestimated.

In conclusion, our results demonstrate the importance of designing a CMR

study that will achieve the highest capture probability possible. If a capture rate between 0.2 – 0.4 cannot be achieved, it may be best to resort to other methods to monitor species (Ebert et al. 2010). However, non-invasively collected feathers can still be an important component to population studies of birds if measures of genetic variability are incorporated into the overall conservation plan (see chapter 2).

Weighing the benefits of genetic CMR

There are several published reviews that address the limitations of genetic CMR (Beja-Pereira et al. 2009; Lampa et al. 2013; Taberlet et al. 1999; Waits & Paetkau 2005). Prior to planning a genetic CMR project to study birds, it is vital to assess whether a sufficient number of feather samples can be collected to fulfill the statistical requirement of repeated captures. If samples cannot be collected in multiple occasions and in enough abundance to estimate population size then another survey method may be warranted. This might be the case for small and isolated populations, where levels of genetic diversity are low. Unfortunately, such species are often most in need of close monitoring.

Another point to consider is the expense of fieldwork versus laboratory costs. Costs associated with genetic analyses can quickly mount, especially with the need to repeatedly screen for genotyping error. The total cost for DNA extractions, PCR for COI restriction enzyme digest, and two genotyping reactions (2 multiplex reactions /sample) totaled more than $8.00 per sample. For the bare minimum analysis, without repeating samples for our screening process, we spent >$20,000 for the combined sampling years.

However, it is also worth considering that genetic samples hold a wealth of

information beyond individual identification for abundance estimation, such as the relationship between individuals, or how populations respond genetically to population declines (Schwartz et al. 2007; Morin et al. 2004; Rudnick et al. 2005; Rudnick et al. 2008). Genetic samples, which can be stored for many years under appropriate conditions, can offer insight into the ecology and evolution of populations long after their samples are collected. As genetic methodologies advance, our coverage of the genome will become deeper, and genetic material from non-model-organisms will become increasingly more informative.

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