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TECHNICAL NOTE

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 eight 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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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 subcontinent, 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.

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) (Hovarth et al. 2005). We designed a vulture specific *COI* primer, GypsR1 (5′-CCAAAGCCCGGTAGRATTAGG), from a mitochondrial *cytochrome oxidase* (*COI*) sequence from

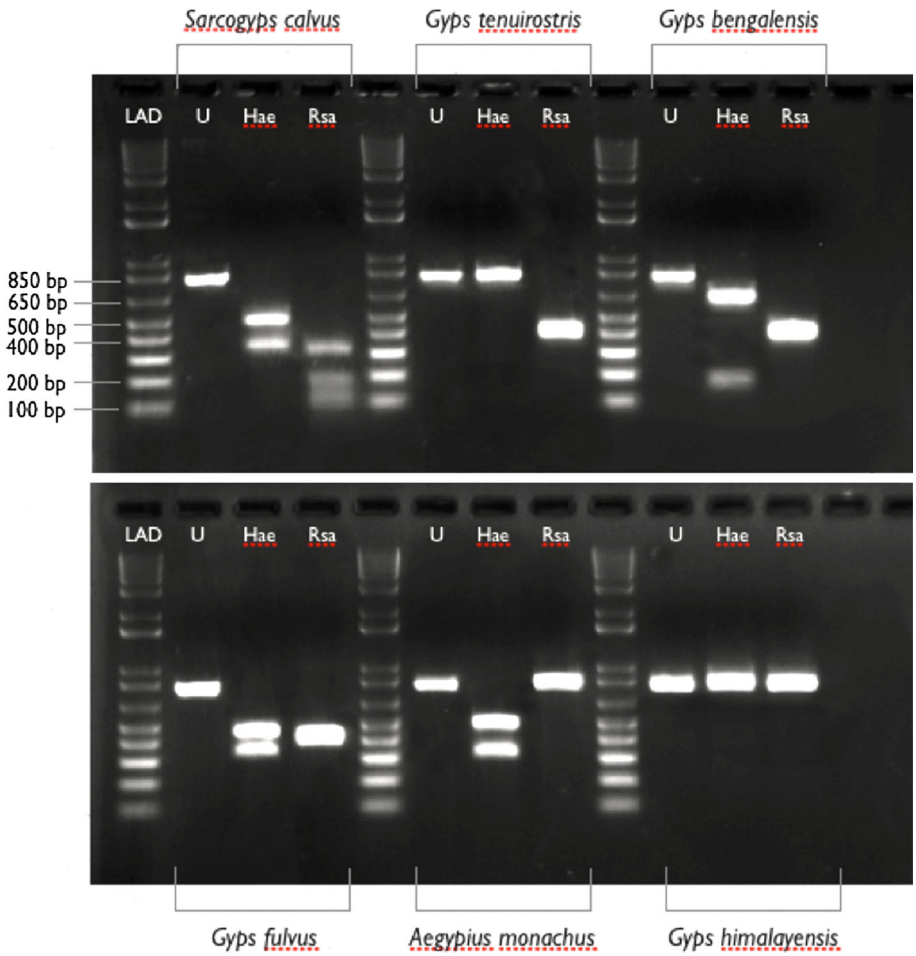

Fig. 1 Restriction endonuclease digest of *cytochrome oxidase I* region of mtDNA of six vulture species for species identification from non-invasively collected samples. U = undigested DNA, Hae = *Hae*III enzyme, Rsa = *Rsa*I enzyme, LAD = 1 kb Plus DNA Ladder with fragment standards. We used 2 µL of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

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 bp 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 (Fig. 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 and 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.

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®

Table 1 Characteristics of microsatellite loci in *Gyps bengalensis*, *G. tenuirostris* and *Sarcogyps calvus*

Locus name: (GenBank accession # XXX) Primer Sequence (5′–3′)	Repeat motif	*G. bengalensis* $P_{ID} = 4.3 \times 10^{-9}$					*G. tenuirostris* $P_{ID} = 2.6 \times 10^{-4}$					*S. calvus* $P_{ID} = 2.7 \times 10^{-5}$				
		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	0.718	0.852	30	–	–	–	–	28	–	–	–	–
GB2-4B (GenBank Accession # KJ663806) CAACTCCACAGTTTAGGCAGATGTACC GTTCTGGTGACTTCACAAGGGACTATCAGAGA	(AC)15	39	341–365	7	0.769	0.744	30	348–365	3	0.533	0.571	27	350–360	4	0.630	0.725
GB3-2C (GenBank Accession # KJ663805) ATGAATCCAGGCTCAGTCAGAAC AGACATGGTAAGGAGTCAGCAGC	(AGA)50	39†	404–461	14	0.769	0.883	30	410–433	5	0.733	0.729	28†	404–443	10	0.500	0.770
GB4-4G (GenBank Accession # KJ663810) CGGTGAGCGGCCTCATTATC GCTCAACTTTCAGTTCCACTTC	(GTTT)8	39	164–176	4	0.605	0.5663	23	164–172	2	0.565	0.496	17	158	1	–	–
GT2-28 (GenBank Accession # KJ663807) CCATCATCGTGGATGTTAGAAACTA GTTTCTCACTTCTTCATTGCCTGAGATATA	(GT)12	39	286–305	9	0.513	0.476	30	297	1	–	–	28	293–315	11	0.679	0.790
GT3-35 (GenBank Accession # KJ663811) CCCCTTGTATGACAATGGTACAGTAT GTTTCTGTATTCAAAAGACATGACATCCAC	(ATT)10 (ACT)13	39	216–264	12	0.82	0.789	30	252	1	–	–	28	225–240	4	0.571	0.546
GT3-38 (GenBank Accession # KJ663808) CCCGAGCCAAGCCAGTTATTATA GTTTCTCATACAACAATCTCTTGTGCTGAC	(TAA)12 (CAA)5	39	370–385	6	0.615	0.739	30	373–385	4	0.533	0.685	28	358–385	6	0.757	0.758
GT4-20 (GenBank Accession # KJ663812) GTGAGCCCTCCCATTGAGTCAT CTCAAGTGCATGCCCGCTG	(GAAA)12 (GA)8	27	298–334	10	0.815	0.8407	24	303–330	8	0.625	0.747	16	299	1	0.630	0.725

Annealing temperature = 60 °C, and $MgCl_2$ = 1.5 mM (except GT2-28 = 52 °C and 2.25 mM $MgCl_2$)

n number of samples, N_A number of alleles, H_o observed heterozygosity; H_E expected heterozygosity

† Possible null alleles

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).

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 and Zaykin 2001). We used the program Genalex version 6.5 (Peakall and 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 and 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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