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Sex Identification in Cinereous Vulture (*Aegypius monachus*) from Feather and Blood Samples: A Case Report

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Abstract : Twenty-four cinereous vultures that had been taken to a wildlife center due to starvation and exhaustion were studied to evaluate approaches for determining sex. Coelioscopy was performed to identify sexes of two vultures, whereas, DNA testing was performed to identify the sexes of the 24 vultures. Testes and ovaries could be unambiguously identified with an endoscope and DNA analyses could identify sex sex in most, but not all of the specimens. Although the coelioscopy examination can unambiguously confirm sex, the approach is invasive and requires anesthesia. Thus, coelioscopic examination should only be performed when sex cannot be determined through DNA analysis.

***Key words* :** cinereous vulture, coelioscopy, genetic analysis, sex identification.

Introduction

The cinereous vulture (*Aegypius monachus*) is South Korea's natural monument and one of the largest and rarest raptors in the world (8). Many of the vultures brought to wildlife centers in Korea are near starvation and exhausted.

The ability to differentiate male and female raptors in the field is important when conducting evolution and conservation biology research on sex distribution, behavior, and breeding status. Many raptor species are sexually dimorphic relative to size, but male and female vultures are very similar in size. Sex can be determined by examining the thoracic air sacs of a captured vulture with an endoscope. However, this method is fairly invasive, requires technical knowledge, and is at times, impossible when the bird is in poor health. Therefore, genetic analysis is a better alternative in such instances.

In birds, female sex chromosomes are heterogametic (Z and W chromosomes), whereas male sex chromosomes are homogametic (two Z chromosomes). A method has been developed for sex identification in birds using DNA primers (P2 and P8), which amplify an intron from the CHD (Chromo Helicase DNA) binging gene (1). This report evaluates the methods for sexing *A. monachus* using coelioscopy and DNA extracted from feathers and blood.

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Case Description

Blood and feather samples were collected from 24 cinereous vultures that were taken to a wildlife center in Korea, among which two were monitored with 5-mm rigid endoscope that was inserted into their caudal thoracic air sacs. Before examining the birds, anesthesia [5% isoflurance (Ifran, hana Pharm, Korea) in 100% oxygen (3 L/min)] was administered to the birds in non-rebreathing apparatus delivered via a mask mask.

Feathers and blood samples were collected to determine the sex via molecular assays. Genomic DNA was obtained from 200ul of whole blood using a DNA using a DNA Micro Kit (QIAGEN Inc., 27220 Turnberry Lane, Valencia, CA). Blood samples were stored at −20°C until analyzed. Collected feather samples were stored in polyethylene bags for over a month at 4°C until analysis. A 0.3-cm segment of feather was cut from the root end of two or three feathers from each vulture and placed in a 1.5-ml Eppendorf tube. Genomic DNA was extracted using i-genomic DNA Extraction mini kit (Intron biotechnology, Inc., Gyeonggi-do, Korea) according to the manufacturer's instructions. A region of the CHD gene was amplified from blood and feathers using PCR and P2 and P8 primers (as described by Griffithes et al., 1998) or P2 (5′-TCTGCATCGCTAAATCCTTT-3′), NP (5′-GAGAAACTGTGCAAAACAG-3′), and MP (5′-AGTCACTATCAGATCCGGAA-3′) primers as described by (4). Both sets of primers produced a single Z-band (P2/P8 and P2/NP) in males,

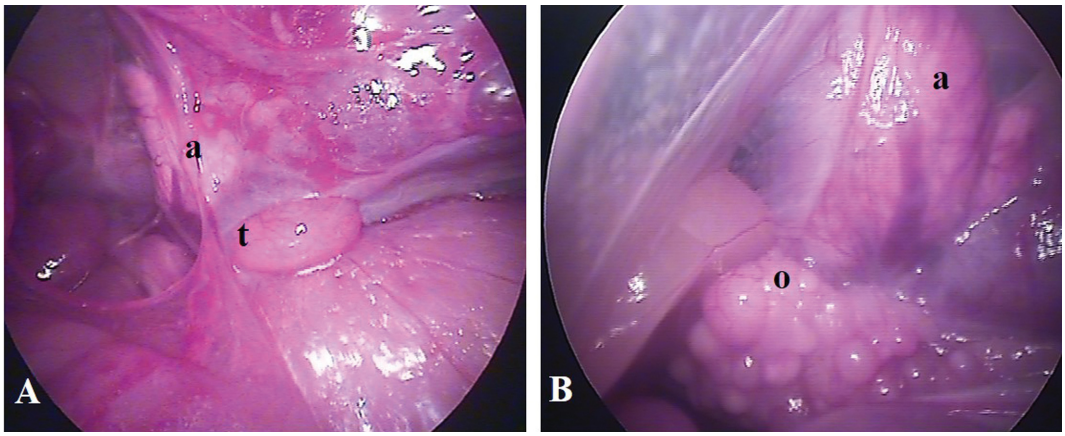

Fig 1. Coelioscopic view of gonadal structures within the left abdominal air sac in cinereous vulture *Aegypius monachus*. (A) testis (t) at maturity and adrenal gland (a); (B) ovary (o) at maturity and adrenal gland (a).

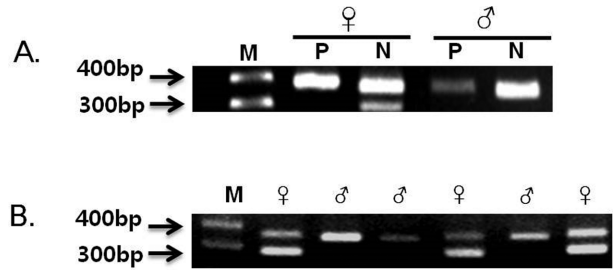

Fig 2. Molecular sex identification of *A. monachus* by analyzing a fragment of CHD, amplified with P2/P8 primers and P2/NP/MP (A, B) primers. Electrophoresis was performed on a 2% agarose gel and visualized with ethidium bromide (M = 100 bp maker). (A) P2/P8, showing a single (390 bp) band for both sexes. (B) P2/NP/MP, showing a single (390 bp) band for males and double (310 bp and 390 bp) bands for females.

and Z- and W-bands (NP/MP) in females. Because the primer sets can only detect the female-specific CHD-W (NP/MP) gene by including the 3′-terminal mismatch MP primer, another primer set of P2 and P8 were also used when sex could not be determined with the first primer pair. The CHD genes of *A. monachus* were also sequenced with BLAST and compared those results with BLAST results of five closely related raptor species (i.e., those with similar DNA sequences): *Gyps himalayensis*, *G. bengalensis*, *G. fulvus*, *Circaetus gallicus*, and *G. indicus*.

Pictures of testes and ovaries obtained via coelioscopy are provided in Fig 1. Multiplex PCR, using the P2/NP/MP primer set, resulted in one single band (390 bp) for male cinereous vultures and two bands (310 bp and 390 bp) for females (Fig 2, Table 1). Used together, primers P2, NP, and MP enabled us to amplify two specific fragments, one with approximately 390 bp (P2/NP) and the other with approximately 310 bp (NP/MP). Table 1 shows the result of our PCR analysis performed on 24 *A. monachus* individuals. However, five of the cinereous vultures we tested could not be sexually differentiated with PCR.

Discussion and Conclusion

Determining sex by molecular analysis in sexually mono-

Table 1. List of collected sample (*A. monachus* feathers and bloods) number of GNWLC and the result of PCR amplification using P2/NP/MP primer sets

GNWLC No.	Sample	Results of PCR	GNWLC No.	Sample	Results of PCR
GW174	Feather	Female	GW201	Feather	-
GW176	Feather	Male	GW213	Blood	Male
GW187	Blood	Male	GW227	Blood	-
GW188	Blood	Male	GW238	Blood	Male
GW189	Feather	Male	GW240	Blood	Male
GW190	Feather	-	GW243	Blood	-
GW191	Blood	Female	GW246	Blood	Male
GW192	Blood	Female	GW249	Feather	Male
GW194	Blood	Female	GW251	Blood	Male
GW195	Blood	Female	GW253	Blood	-
GW199	Feather	Female	GW262	Blood	Female
GW200	Feather	Male	GW264	Blood	Female

*GNWLC No.: Sample number of GyeongNam Wildlife Center.
-: Non-successful of PCR.

morphic birds has been shown to be as reliable as conventional coelioscopy methods, but the feasibility of the latter method is influenced by body condition and size (1,8). In this study, we used the intraperitoneal approach (coelioscopy) to accurately identify male testes and female ovaries. In coelioscopic approaches, it is necessary to access a caudal thoracic air sac using the last intercostal space and flexor cruris medialis muscle as markers (8).

Extracting DNA from hair or feathers is a much less invasive approach for sexing birds than coelioscopy and the approach has greatly enhanced genetic studies of wild bird species (9). Feathers provide a large quantity of genetic material of high quality for use in molecular assays. Sex determination using feathers has been reported for other species of birds (7) as well; however, to the best of our knowledge, no studies have determined the sex of *A. monachus* from their feathers until now.

Traditionally, the protocol for molecular sex determination of birds using the P2/P8 primers relies on chromosome-specific intron size differences in avian CHD genes (2). However, this method has encountered numerous problems depending on the species being evaluated. For example, in the tawny owl (1) and kiwi (3), evaluating CHD-Z and CHD-W intron size through electrophoresis on PCR can be difficult. In our study, it was difficult to determine length differences for the P2/P8-amplified CHD genes of the five species of eagles we compared with *A. monachus*. The differences in intron length between CHD-Z and CHD-W genes ranged from 3 to approximately 10 bp when using P2/P8 primers in all of the species tested. However, such small differences in distance are very difficult to distinguish using PCR. To resolve problems with this sex analysis method, the PCR-RFLP approach (5,7) or single strand conformation polymorphism approach (6) could be used; however, such methods are expensive and time consuming.

We applied the PCR-based protocol in our study (with primers P2/NP/MP) and used the results to compare DNA sequences among six raptor species (including A. *monachus*). Although the length difference between the P2/P8 products is about 3-10 bp, the length difference between P2/NP/MP products is about 80 bp (i.e., 390 to 310 bp). Such a large difference in length between amplified PCR products facilitates sex identification by agarose gel electrophoresis. Furthermore, a high yield of pure genomic DNA derived from blood and muscle is required in the ARMS technique (4). However, in the present study, the genetic material obtained from *A. monachus* feathers yielded genomic DNA of the quality similar to the results derived from blood and muscle samples.

We demonstrated that PCR alone is useful to efficiently and rapidly determine the sex of cinereous vultures in the field populations. However, coelioscopy can be used if PCR techniques fail to clearly discriminate between males and females.

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References

1. Dubiec A, Zagalska-Neubauer M. Molecular techniques for sex identification in birds. Biol Lett 2006; 43: 3-12.
2. Griffiths R, Double MC, Orr K, Dawson RJ. A DNA test to sex most birds. Mol Ecol 1998; 7: 1071-1075.
3. Huynen L, Miles J, Lambert D. Unusual electrophoretic mobility of a DNA fragment of the universal 'non-ratite' sexing marker CHD allows sexing of New Zealand's endangered kiwi ratite *Apteryx* spp. Ibis 2006; 148: 167-168.
4. Ito H, Sudo-Yamaji A, Abe M, Murase T, Tsubota T. Sex identification by alternative polymerase chain reaction methods in falconiformes. Zool Sci 2003; 20: 339-344.
5. Reddy A, Prakash V, Shivaji S. A rapid, non-invasive, PCR-based method for identification of sex of the endangered Old World vultures (white-backed and long-billed vultures)--Implications for captive breeding programmes. Curr Sci 2007; 92: 659-662.
6. Reynolds RT, Topinka JR, May B, Antolin MF. Population genetics and genotyping for mark-recapture studies of Northern Goshawks (*Accipiter gentilis*) on the Kaibab Plateau, Arizona. J Raptor Res 2005; 39: 286-295.
7. Sacchi P, Soglia D, Maione S, Meneguz G, Campora M, Rasero R. A non-invasive test for sex identification in Short-toed Eagle (*Circaetus gallicus*). Mol Cell Probe 2004; 18: 193-196.
8. Seok SH, Jeong DH, Lee HC, Hong IH, Yeon SC. Evaluation of diagnostic coelioscopy including liver and kidney biopsies in cinereous vultures (*Aegypius monachus*). Vet Med-Czech 2016; 12: 689-700.
9. Taberlet P, Waits LP, Luikart G. Noninvasive genetic sampling: look before you leap. Trends Ecol Evol 1999; 14: 323-327.