

DNA Sexing of the Philippine Eagle (*Pithecophaga jefferyi* Ogilvie-Grant) in Captivity at the Philippine Eagle Center, Davao City, Philippines

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Abstract

The Philippine eagle is a sexually monomorphic raptor which lacks the sex-linked morphology determining the gender especially in the juveniles. Thus, a PCR amplification technique was used to determine the sex of 24 eagles at different stages of development (2 to 37 years old) in captivity at the Philippine Eagle Center, Malagos, Davao City. Fractions of the sex-linked genes, CHD-W and CHD-Z of each individual were amplified. Ka Brienne (female) and Jag (male) having 9 offspring conceived through artificial insemination were used as positive controls for sex identification of 22 other individuals. Two individuals of *Gallus domesticus* with confirmed genders were also included and run through PCR amplification together with the Philippine eagles using primers CHDFORNEW and CHDREVNEW to test the method. Females revealed two distinct bands (290 bp and 280 bp in size) while the males revealed only a single band of 280 bp. Eleven eagles were found to be females while 13 were found to be males. DNA sexing gave a 100% confirmation of the assigned sexes of the eagles, which were obtained through morphometric analysis done by personnel at the captive breeding center. DNA sexing could be a practical technique in sexing newly hatched eaglet and juveniles, naming of eagles, establishing life history characteristics, and pairing attempt or assignment of partners in the threatened avian species such as the Philippine eagles.

Keywords: sex-linked, CHD gene, PCR amplification, Philippine Eagle

Introduction

The Philippine eagle, the national bird of the Philippines is a giant forest raptor endemic in the Philippines. The species is known to occur only on four of the islands of the Philippine archipelago, namely Luzon, Mindanao, Samar and Leyte. Its usual habitat is the primary forest and also occurs in the secondary and gallery forests (Bueser et al., 1997). The adult individual has an average body length of 1 m and a wingspan of 2 m. This airborne predator is adapted to foraging in the forest with its long tail feathers and broad wings.

In 1965, the status of the Philippine eagle came to the world's attention as its population was found to be declining rapidly. This species is now classified as critically endangered mainly due to deforestation and extensive hunting. It was estimated in 1971 that human persecution of the species had resulted in a mortality rate of 19.3 eagles annually. Drastic measures came mostly from foreign conservationists to save the eagle from the brink of extinction. In 1977, the Philippine Eagle Foundation Incorporated (PEFI) established a captive breeding program which became operational since 1982 (Tadena et al., 1999), wherein eagles in captivity at the Philippine Eagle Center (PEC) were bred to supplement depleted populations in the wild. The captive populations also serve as "genetic insurance" for the entire population. Eagles undergo pairing attempt or imprint process, incubation and chick rearing. The newly hatched eaglet's weight and other important vital statistics are monitored everyday to ensure its survival (Tadena et al., 1999). However, the sex of the eaglet remains unknown until it grows up to a sub-adult stage.

Sex determination is vital in conservation efforts, especially in addressing sex-related differences in dispersal, habitat-use and survival because ecological studies such as population, brood and sex ratios; sex-ratio manipulation; and site fidelity are dependent on sex identification. Determining the sex of juveniles is important because their survival rates are often used to determine the population status (Morrison and Maltbie, 1999).

Available methods to determine the sex of raptors include physical examination of reproductive organs, and, in some cases, surgery (ZI, 1994). Another method is morphometric analysis which is done by using weight and size measurements to distinguish variability among individuals (Morrison and Maltbie, 1999). The latter is used by PEFI in determining the sex of the Philippine eagles in captivity in the PEC (Tubio, 2004) by measuring the tarsus. Conservationists also used behavioral clues because when the eagles reach sexual maturity,

they exhibit breeding behavior unique to each sex (Morrison and Maltbie, 1999). These methods are unreliable in sexing juveniles and newly hatched chicks. Conventional sexing which requires surgery poses risks of anesthetic surgery or death in juveniles (ZI, 1994). Apart from this, surgical sexing is an invasive technique and cannot be applied to threatened species. Morphometric analysis cannot be done in juveniles and newly-hatched chicks, while behavioral clues cannot be observed in them. Furthermore, morphometric analysis is still not accurate for all individuals since males and females may overlap in measurements and errors in classification often reach 20% (Morrison and Maltbie, 1999).

Recently, there were methods introduced by a number of scientists to sex birds using DNA markers (Griffiths et al., 1998; and Palma et al., 2001) through the polymerase chain reaction (PCR). A highly conserved region of the W-chromosome called the Chromodomain-Helicase-DNA-binding gene (CHD gene) is unique to female birds. Another version of this gene, the CHD-NW gene is linked to the Z chromosome, present in both sexes. Because they are identical, fractions of these genes could be amplified and used in determining the sex of raptors (Norris-Caneda and Elliot, 1998). This method, on the other hand, is reported by the aforementioned researchers to be very reliable and safe to use in juveniles and newly-hatched eaglets.

This study was conducted to determine the sex of the Philippine eagles in captivity at the PEC using DNA markers, verify the sex identification done using morphological traits, and determine possible implications of DNA sexing technology on the conservation of Philippine eagles.

Materials and Methods

Blood sampling and DNA extraction

Blood samples were extracted from 24 Philippine eagles (2 to 37 years old) found at the PEC, Malagos, Davao City with the assistance of a veterinarian during the annual check-up of the birds. Samples were extracted using a 3-mL syringe with a 25 gauge needle from the brachial vein of the wing after swabbing the area with alcohol. Blood was immediately transferred into heparinized microcentrifuge tubes with screw cap containing 10% ethylenediamine tetraacetic acid (EDTA). The tubes were immediately stored at 4 °C and later on transferred to -20 °C until DNA extraction through phenol/chloroform method (Sambrook et al., 1989). Tris-EDTA (TE) Buffer

pH 8.0 (10 mM Tris; 1 mM EDTA) was added to the dried pellets and the DNA samples were stored at 4 °C until ready for PCR amplification.

PCR amplification of the CHD gene

Prior to PCR amplification, the concentration of the DNA sample was diluted to a final concentration of 5 ng·mL⁻¹ using Eppendorf Biophotometer (AG Hamburg, Germany). Amplification of the CHD-W and CHD-Z gene fractions was done using GeneAmp[®] PCR System 9700 (Applied Biosystems) thermal cycler. This was conducted at the Laboratory of Animal Genetics, Nagoya University, Japan in 2005, and at the Molecular Biology Laboratory, University of the Philippines in Mindanao, Davao City in 2006. The sequence primers used were CHDFORNEW 5'-CAAGGATGAGAACTGTGCAAACAG-3' and CHDREVNEW 5'-CTATCAGATCCAGAATATCTTCTGC - 3' (Raymond et al., 1999), which were designed for *Gallus domesticus*. Each PCR mix contains 1.0 µL of 10X Buffer, 0.8 µL of dNTP, 0.2 µL of each primer, 4.76 µL ddH₂O, 0.04 µL TaKaRa Ex Taq[®], and 3.0 µL template DNA (5 ng·mL⁻¹) to a final volume of 10 µL per reaction. A negative control was included to check the absence of contamination. PCR conditions were 94 °C for 2 min followed by 94 °C for 1 min, 50 °C for 30 sec and 72 °C for 1 min for 40 cycles, then final extension of 72 °C for 5 min.

Immediately after the PCR amplification, 10 µL of each PCR product was run on 3.5% Seaken GTG Agarose (BioScience Rockland Inc.) at 100 V for 1 h and 15 min in 1x TBE buffer. This is to allow separation and measurement of PCR products. A 25 bp DNA Step Ladder (Promega) was used during electrophoresis in order to detect the molecular size of the band of interest. Staining followed for 30 min in 0.5 ng·mL ethidium bromide solution. The gel was subjected to ultraviolet light using Uvisave[™] for visualization.

Sex identification and analysis

After electrophoresis, bands were visualized under ultraviolet light and photos of the gels were taken. The bands were measured with the software UviDocMw[®] and molecular markers. The amplification with sequence primers CHDFORNEW and CHDREVNEW (Raymond et al., 1999) was expected to reveal two bands from females and only one band from males. Ka Brianne and Jag, whose sexes were already confirmed (Tables 1 and 2) were used as positive controls for the identification of the genders of the

Table 1. Breeding history of 11 female Philippine eagles in captivity at the Philippine Eagle Center (PEC)

Code No.	Name	Age (year)	No. of eggs	Offspring	Partner
002	Ka Brianne	20	16	Pangarap, Kapayapaan Bayani, Maginoo Mindanao, MiaMacanudo, Chick #15	Jag
004	Marikit	10	3	None	Arakan (natural pair)
006	Princess Maasim	13	6	Gloria Victoria Chick #17	Tsai
008	Thor	37	5	None	None
010	Kapayapaan	5	None	None	None
012	Ellen Therese	4	None	None	None
014	Girlie	25	1	None	Jag (natural pair)
016	BGR	7	None	None	None
018	Pangarap	6	1	None	None
020	Pitha	21	9	Army	Human surrogate-mate
022	Mia	3	None	None	None

Table 2. Breeding history of 13 male Philippine Eagles in captivity at the Philippine Eagle Center (PEC)

Code	Name	Age (year)	Offspring	Partner
001	Jag	21	Pangarap, Kapayapaan Bayani, Maginoo Mindanao, Mia Macanudo, Chick #15	Ka Brianne
003	Freedom	Approx. 2	-	-
005	Magiting	2 Not exact	-	(paired to) Pangarap
007	Bayani	5	-	(paired to) Jean
009	Arakan	7	-	(paired to) Marikit
011	Army	3	-	-
013	Pag-asa	13	-	Imprinted
015	Kabayan	2	-	-
017	Maginoo	4	-	-
019	Gloria Victoria	2	-	-
021	Tsai	24	Gloria Victoria Chick #17	Princess Maasim
023	Macanudo	2	-	-
024	Ka Jag/ Mindanao	4	-	-

22 other individuals. The results were then compared with those of the assigned sexes of the Philippine Eagle obtained through morphometric analysis.

Results and Discussion

CHD gene fractions of the Philippine Eagle

Using the primers CHDFORNEW and CHDREVNEW of Raymond et al., (1999), all female *P. jefferyi* exhibited two bands with 290 bp and 280 bp in sizes (Figure 1). The two fragments differed in size by 10 bp. All males, on the other hand, exhibited a single band 280 bp in size. This allowed discrimination between males and females (Figure 1A and 1B). Eleven of the 24 eagles were found to be females and 13 were found to be males. The females include Mia, Pangarap, BGR, Girlie, Ellen Therese, Kapayapaan, Thor, Marikit, Pitha and Princess Maasim. One of them, Ka Brianne, was used as a positive control in the analysis of the CHD gene fragments. Male eagles include Mindanao, Macanudo, Gloria Victoria, Maginoo, Kabayan, Pag-asa, Arny, Arakan, Bayani, Magiting, Freedom, Tsai and Jag. The last two were also used as positive controls. The DNA sexing confirmed the sex assignments of the eagles done by measuring the tarsus (Tubio, 2004) to be 100 % accurate. Meanwhile, the PCR amplification using primers Palma1 and Palma2 (Palma et al., 2001) did not successfully identify the sexes of the eagles because all eagles, regardless of their sex, revealed a single band.

The sex of the Philippine eagle is difficult to determine because this species is sexually monomorphic. The lack of sex-linked morphology is a characteristic common to raptors (Sacchi et al., 2004). There have been methods suggested to sex the Philippine eagles, namely: behavioral clues and morphometric analyses. The drawback of these methods is that they cannot correctly identify the sex of juveniles and newly-hatched chicks (Morrison and Maltbie, 1999). This is very important since the survival rate of juveniles is very useful in determining the status of the overall population (Fleming et al., 1996).

The CHDFORNEW and CHDREVNEW sexing had correctly identified the sexual category of the Philippine eagles. Its success was due to the difference in sizes of the two gene fractions, CHD-W and CHD-Z. Griffiths et al. (1998) used a different set of primers, namely: P2 and P8, and reported that the comparison of sequence data of CHD genes of chicken and mouse demonstrated the conservation of the genes in bird species. Although the conservation was poor because of the taxonomic distance between the two, the data provided the length difference between CHD-W and CHD-Z that made their DNA sexing successful. The study of Ellegren (1996) with female collared flycatchers pointed out that although length

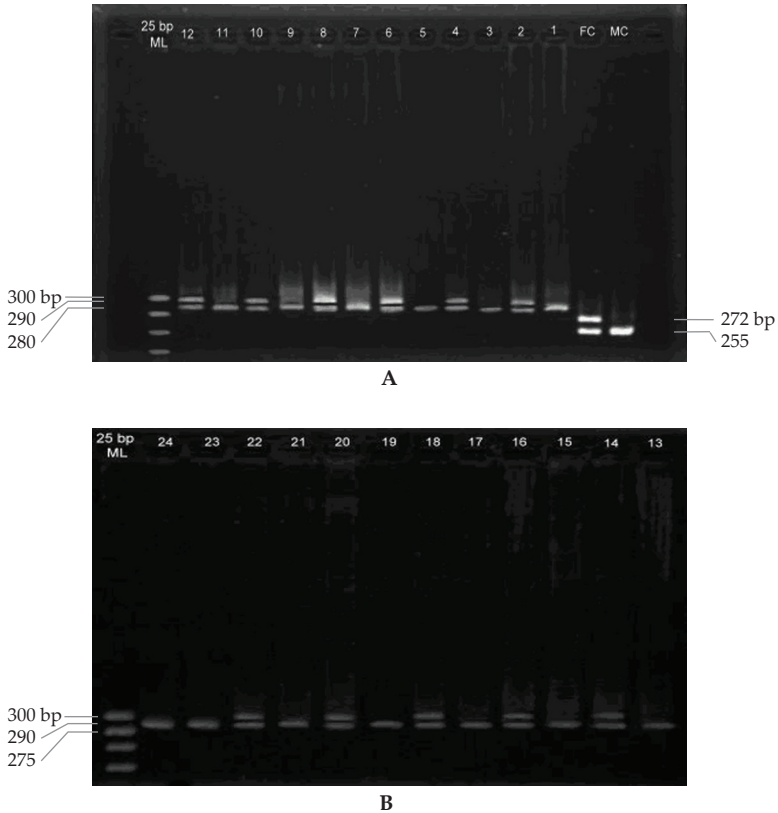


Figure 1. Chromodomain-Helicase-DNA-binding gene fractions of Philippine eagles amplified using primers CHDFORNEW and CHDREVNEW of Raymond et al. (1999). **A:** MC- male chicken, FC- female chicken, Eagle samples 1-12; and **B:** Eagle samples 13 to 24.

difference in two copies of a highly conserved coding sequence was rare, such difference may exist within introns. Thus, the CHDFORNEW and CHDREVNEW primers were able to amplify two specific introns with a significant length difference.

The presence of the two bands in females has already been observed in other avian species such as in: *Struthio camelus* (ostrich), *Gallus domesticus* (domestic chicken), *Cygnus odor* (mute swan), among others in the study of Griffiths et al. (1998); *Circus gallicus* (short-toed eagles) (Sacchi et al., 2004); *Hieraaetus fasciatus* (Bonelli's eagle) (Palma et al., 2001); *Hieraaetus pennatus* (Booted eagles) (Balbontin et al., 2001); and the Japanese cormorants (*Phalacrocorax capillatus*) published by Inoue-Murayama et al. (2002).

Amplification of the sex-linked gene fractions confirmed all the gender assignments of the eagles established through morphometric analyses. Two Philippine eagles: Jag and Ka Brienne (Figure 1A, lanes 1 and 2), which were used as positive controls, had 9 offspring conceived through artificial insemination, namely: Pangarap, Kapayapaan, Bayani, Maginoo, Mindanao, Mia, Macanudo, chick #15 and chick #18. Besides the pair, there is another eagle pair, Tsai and Princess Maasim, who have had two offspring, Gloria Victoria and chick #17. With this, the gender of some females, such as Marikit, Thor, Kapayapaan, Ellen Therese, Girlie, BGR, Pangarap and Mia were confirmed, even though these females have not yet laid an egg.

The failure to discriminate the two bands of the PCR amplification using primers Palma 1 and Palma 2 could be attributed to the very small difference in size of the two gene fragments in the case of the Philippine eagle. According to Palma et al. (2001), the primers were designed to amplify fragments both from CHD-Z and CHD-W genes that differ in size of only 5-10 bp. Thus, the two bands, if present, could not be distinguished from each other using 3.5% agarose gel during electrophoresis. This result was also observed in the study of Griffiths et al. (1998). One of the test organisms, the tawny owl, could not be sexed because of the similarity in size of the two gene fragments.

CHD gene fractions of the Philippine eagle and chicken

Two *G. domesticus* individuals, whose genders were confirmed, were also sampled and run through PCR amplification together with the Philippine eagles using primers CHDFORNEW and CHDREVNEW. The female domestic chicken revealed two bands of 272 bp and 255 bp in size (Figure 1A, lane FC), while the male revealed a single band with a length of 255 bp (Figure 1A, lane MC). The two bands in the female chicken differed in size by about 17 bp.

Although both the female domestic chicken and female Philippine eagle showed two bands, the bands of the Philippine eagle were larger in size. The difference between the two bands is smaller in female Philippine eagles, which is 10 bp, than that of the domestic chicken, which is 17 bp. This shows that even if the CHD gene is highly conserved, there is still a difference in fragment length between species (Philippine eagle and chicken), even though these fragments are amplified by the same primer set. This observation

was also found in the study of Griffiths et al. (1998), which amplified CHD gene fragments from 27 different species of birds. They discovered that the fragments from different bird species have varying lengths. Despite this, the presence of two bands in females is still the distinguishing characteristic between sexes.

Implications of DNA sexing to conservation

For the Philippine eagle, PCR amplification as a tool for the determination of sex opens many possibilities such as DNA sexing of juveniles, naming of eagles, sex-ratio manipulation, pairing attempt or assignment of partners, and assessing of the practicality of the techniques used.

DNA sexing of juveniles

PCR amplification is not an invasive technique. It does not cause too much stress on the eagle during gender determination, because blood and even feathers can be used as source of DNA. This method, therefore, can be used in juveniles and even in newly-hatched eaglets, whose gender is difficult to determine. Sex determination does not have to wait until the eaglet grows into a sub-adult, which is done using morphometric identification. Tubio (2004) in her study found that eaglets and juvenile whose age ranged from five months to two years were misclassified using the K-means clustering and discriminant analysis based on tarsus d-ventral, tarsus lateral, and tarsus circumference. However, adult eagles ranging from seven to eighteen years old were grouped according to their corresponding gender on the basis of the measurements using the clustering method.

Naming of eagles

Determining the sex of the eagles right after hatching or rescue and donation will enable naming of the eagle accurately, to avoid confusion, as in the case of Gloria Victoria (a female name), which was found to be a male.

Life history characteristics

Establishing sexes of newly-hatched chicks enables biologists to study the basic life history characteristics associated with sex since birth (Ellegren, 1996). Because their life history characteristics can be documented, the difference between habitat-use, behavior and other ecological features of the different sexes can be established. This will help the conservation efforts of the biologists since it enables them to resolve sex-related issues.

Sex-ratio, pairing attempt, and assignment of partners

Since Philippine eagles are monogamous, a fixed sex ratio is preferred to ensure that the captive breeding program will produce more offspring. Donated or rescued eagles are placed in cages at the PEC for possible partnerships with other captive eagles. However, these eagles may still be experiencing trauma early in captivity, depending on what happened to them in the wild. Sex determination using PCR technique can be used as basis for assigning them to a partner belonging to the opposite sex earlier.

Because of the applicability of this method even to newly-hatched chicks, pairing attempts can be planned earlier. Pairing attempt is the first phase of captive breeding (Tadena et al., 1999). In this phase, adult male and female eagles are introduced to each other by placing them in an adjacent cage. Thus, if eaglet's sex is known earlier with the use of this method, cage construction and pairing scheme for the eaglet can be prepared. Introduction of eagles at an early stage heightens the likelihood of compatibility. Creation of communal breeding systems can be made possible if sex is known at an early stage (Ellegren, 1996). Pairing the eagles can also be patterned according to their genetic differences. Eagles that are less related are best paired since to increase the genetic diversity in the next generation.

Practicability of the techniques used

The molecular methods used in this study are now used worldwide. In the Philippines, there are other institutions that can do PCR analysis. Thus, application of this technique on the eagles is very feasible. DNA sexing was proven to be very reliable with 100% accuracy. DNA sexing, thus, is more appropriate for the threatened avian species such as the Philippine eagles, compared to the morphometric analysis (Morrison and Maltbie, 1999), which was found to be only 20% accurate.

Acknowledgment

We thank Director Dennis Salvador, Mr. Domingo Tadeña (PEC Manager) and their staff at the Philippine Eagle Center, Malagos, Davao City for providing us blood samples used in this investigation. Special thanks also goes to Dr. Adelina A. Barrion, Dr. Orville L. Bondoc, and Dr. Angel L. Lambio for their comments and suggestions that helped in the improvement of this paper. This study was partly funded through the project of Prof. T. Namikawa under the Japan Society for the Promotion of Science (JSPS) grant.

References

- Balbontín, J., M. Ferrer, and E. Casado. 2001. Sex determination in booted eagles (*Hieraaetus pennatus*) using molecular procedures and discriminant function analysis. *J. Raptor Res.* 35(1):20-23.
- Bueser, G. L., D. S. Afan, G. A. Balaquit, K. M. Gatil and J. C. Ibañez 1997. Distribution and conservation of the Philippine eagle (*Pithecophaga jefferyi*) in Davao Oriental and Davao province. *J. Phil. Ecosyst. & Nat. Resources* 8(1&2):116-118.
- Ellegren, H. 1996. First gene on the avian W-chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *BioScience* 263(1377):1635-1641.
- Fleming, T. L., J. B. Buchanan, and J. L. Halverson. 1996. Use of DNA analysis to identify sex of northern spotted owls (*Strix occidentalis caurina*). *J. Raptor Res.* 30(3):118-121.
- Griffiths, R., M. C. Double, K. Orr, and R. G. Dawson. 1998. A DNA test to sex most birds. *Mol. Ecol.* 7:1071-1075.
- Inoue-Murayama, M., Y. Ueda, T. Yamashita, C. Nishida-Umehara, Y. Matsuda, T. Masegi, and S. Ito. 2002. Molecular sexing of the Japanese cormorants used for traditional fishing on the Nagara River in Gifu City. *An. Sci. J.* 73:417-120.
- Morrison, J. L. and M. Maltbie. 1999. Methods for gender determination of crested caracaras. *J. Raptor Res.* 33(2):128-133.
- Norris-Caneda, K. H. and J. D. Elliot, Jr. 1998. Sex identification in raptors using PCR. *J. Raptor Res.* 32(4):278-280.
- Palma, L., S. Mira, P. Cardia, P. Beja, T. Guillemaud, N. M. Ferrand, M. L. Cancela, and L. C. Fonseca. 2001. Sexing Bonelli's eagles nestlings: Morphometrics versus molecular techniques. *J. Raptor Res.* 35(3):187-193.
- Raymond, C. S., J. R. Kettlewell, B. Hirsch, V. J. Bardwell, and D. Zarkower. 1999. Expression of Dmrt1 in the genital ridge of mouse and chicken embryo suggests a role in vertebrate sexual development. *Dev. Biol.* 215:208-220.
- Sacchi, P., D. Soglia, S. Maione, G. Meneguz, M. Campora, and R. Rasero. 2004. A non-invasive test for sex identification in short-toed eagle. *Mol. & Cell Probes* 18:193-196.
- Salvador, D. I., and T. O. Tadena. 2001. Captive breeding operations 2001 edition. Philippine Eagle Foundation, Davao City Philippines. pp.3-4.
- Sambrook, N.B., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Lab Press, New York.

- Tadena, D. O., A. Aya-Ay, H. C. Miranda, Jr., and D. I. Salvador. 1999. Captive breeding by natural pairing, incubation and chick-rearing methods for Philippine eagles. The Philippine Eagle Foundation, Davao City, Philippines.
- Tubio, E.S. 2004. Approaches on the sex determination of the Philippine eagle (*Pithecophaga jefferyi* Ogilvie-Grant). University of the Philippines in Mindanao. Mintal, Tugbok District, Davao City, Philippines. Undergraduate Thesis.
- [ZI] Zoogen Incorporated. 1994. Breeder's guide to DNA sexing and DNA fingerprinting. Zoogen Incorporated, California.