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SHORT COMMUNICATION

Comparison of different procedures of DNA analysis for sex identification in the endangered bearded vulture (*Gypaetus barbatus*)

Cristina Belén García · Jesús Antonio Insausti · Juan Antonio Gil · Ángel de Frutos · Manuel Alcántara · Javier González · María Rebeca Cortés · José Ignacio Bonafonte · María Victoria Arruga

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Abstract During the last century, bearded vulture populations have declined and are threatened by extinction in Europe. Conservation efforts such as captive-bird breeding programs require the knowledge of the sex of individuals. The bearded vulture is difficult to sex morphologically because it is sexually monomorphic. Until now, there were no published genetic methods to sex this species. In our study, we tested different methods based on polymerase chain reaction analysis of the chromobox-helicase-DNA binding protein gene. This gene is located on both sex chromosomes, but the two copies differ in size depending on chromosomal location. Differences can be detected by digestion with restriction enzymes or with the amplification refractory mutation system technique. These methods are quick, accurate, and inexpensive and allow a large scale sex typing of bearded vultures.

Keywords *Gypaetus barbatus* · Sexing · *CHD* gene · ARMS · Conservation program

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C. B. García · M. V. Arruga (✉)
Laboratory of Cytogenetics and Molecular Genetics,
Faculty of Veterinary Science, University of Zaragoza,
C/Miguel Servet, 177,
50013 Zaragoza, Spain
e-mail: mvarruga@unizar.es

J. A. Insausti · J. González · M. R. Cortés
Centro de Recuperación de Fauna Silvestre La Alfranca,
Zaragoza, Spain

J. A. Gil · Á. de Frutos
Fundación para la Conservación del Quebrantahuesos,
Zaragoza, Spain

M. Alcántara
Departamento de Medio Ambiente, Servicio de Biodiversidad,
Gobierno de Aragón,
Zaragoza, Spain

J. I. Bonafonte
Surgery Unit, Faculty of Veterinary Science,
University of Zaragoza,
Zaragoza, Spain

Introduction

The bearded vulture (*Gypaetus barbatus*) is one of the most endangered species in Europe. Until the nineteenth century, it was distributed along most Southern mountain regions in Europe and in Asia and Africa. Since then, bearded vulture populations have declined, and many of them are now fragmented. The Pyrenean population is the only viable wild population in Europe (119 breeding pairs; FCBV 2008).

A reliable method to determine the sex of birds is important for conservation strategies of the species. Like in many other bird species, there is no appreciable difference between the sexes in bearded vultures. Female birds are heterogametic (ZW), and males have two identical sex chromosomes (ZZ). In monomorphic species and nestlings, polymerase chain reaction (PCR) analysis of the DNA is a fast and useful procedure for gender identification. Other methods like endoscopy, cloacae touch, karyotyping, fecal steroid analysis, and flow cytometry are more stressful for the animals, need higher sample quantity to be analyzed or are more time-consuming. The chromobox-helicase-DNA binding protein (*CHD*) gene was the first W chromosome

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gene discovered and enabled sex identification in most avian species using a rapid method consisting of amplifying *CHD* genes of both sex chromosomes (*CHD-W* and *CHD-Z*) whose lengths are different because of varying size of introns (Ellegren 1996; Griffiths et al. 1998, Kahn et al. 1998). However, this approach to sexing is not applicable to all avian species, and modifications have been developed to solve this problem in Falconiformes by using different primers (Ito et al. 2003), digesting the PCR product with restriction enzymes *Hae*III or *Asp*700I (Sacchi et al. 2004) or using the amplification refractory mutation system (ARMS) technique (Ito et al. 2003; Reddy et al. 2007). ARMS is a PCR-based technique to amplify specific alleles using a 3′-terminal primer that fits to one allele variant and mismatches to the other allowing the detection of allele differences between *CHD-W* and *CHD-Z* genes.

In the present work, we compared different molecular sexing procedures that had been developed for other birds of the same family of the bearded vulture (Accipitidae). Procedures to extract DNA from different kinds of sample were also compared to select the most suitable one for large-scale studies.

Materials and methods

Blood samples kept on FTA® cards and two to three feather quills were collected from 20 bearded vultures (*G. barbatus*) (four nestlings, six chicks, and ten adults) by the staff of the La Alfranca Conservation Center. Fifteen out of 20 samples came from animals found dead by poisoning (4), electrocution (3), shooting (3), accident (3), infection (1), or freezing (1), which could not be cured in the Conservation Center. The Spanish Foundation for the Conservation of Bearded Vultures provided blood samples from eight additional individuals of the same species (six chicks and two adults) spread on FTA® cards for posterior analysis. In total, 28 bearded vultures were analyzed, all of which belonged to the Pyrenean population. Five dead males and two dead females were sexed by autopsy to be used as validation controls for the methods.

DNA was extracted from blood samples on FTA® cards or from feather tips (fresh samples and those stored at −20°C for 1 year) using Chelex (Jensen et al. 2003) and DNeasy® tissue kit (Qiagen, Valencia, USA), respectively. When using FTA® cards, only two or three drops of blood are required, minimizing the contact and disturbance to the animals. The samples are stored at room temperature (Gutierrez-Corchero et al. 2002; García and Arruga 2006).

A fragment of the *CHD* gene was amplified by PCR using the primer pairs P2 and P8 (Griffiths et al. 1998) and P2 and NP (Ito et al. 2003). PCR amplification was performed in a final volume of 25 µl containing 5 µl of DNA, 0.75 units of Taq polymerase (Ecogen, Madrid, Spain), 500 nM of each primer, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$ and PCR buffer with 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH=8), and 0.01% Tween-20. PCR conditions included an initial denaturing step at 94°C for 4 min followed by 43 cycles at 94°C for 30 s, 48°C for 45 s, and 72°C for 45 s with a final extension step at 72°C for 5 min. PCR products of both pairs of primers were analyzed by electrophoresis directly after PCR and after digestion with *Hae*III or *Asp*700I (Roche Applied Science Penzberg Germany) restriction endonucleases, which had previously been described as effective enzymes in sex determination in other species (Sacchi et al. 2004). Digestions were performed in 20 µl final volume containing 4.5 U of enzyme and 1× reaction buffer and incubated at 37°C for 3 h. Digestion products were then separated by electrophoresis.

In the ARMS technique, three primers were used in the PCR amplification: P2, NP and MP (3′-terminal mismatch primer; Ito et al. 2003). The final reaction conditions were as follows in 25 µl total volume: 5 µl of DNA, 0.625 units of Taq polymerase, 800 nM of P2 and 400 nM of NP and MP, 200 µM of each dNTP, 1.5 mM $MgCl_2$, and 1× PCR buffer. PCR reactions were performed using the following conditions: 94°C for 3 min 30 s, 36 cycles at 94°C for 30 s, 48°C for 45 s, and 72°C for 45 s, and 1 cycle at 94°C for 30 s, 48°C for 1 min, and 72°C for 5 min. PCR products were analyzed by electrophoresis. All electrophoreses were carried out in 3% low EEO agarose (Ecogen, Madrid, Spain) in 1× Tris–borate–EDTA. Amplicons were stained with ethidium bromide and visualized under UV light.

Results

There were no differences between the analysis of the DNA extracted from blood on FTA® cards and from tips of feathers.

The analysis of P2/P8 and P2/NP PCR products on agarose gels showed an apparent single band of equal size in both sexes, so these methods were discarded. Digestions of P2/P8 and P2/NP PCR products with *Hae*III and *Asp*700I restriction enzymes showed differences between both sexes. *Hae*III digestions produced two bands in males and three bands in females. After digestion with *Asp*700I, PCR-amplified DNA from males generated a single band, while that of females was resolved into three bands (Fig. 1).

The ARMS technique was also successfully applied to determine the sex of bearded vulture individuals. Males were recognized by one visible band, and female individuals showed two bands of a very similar length. Gels had to be run for a long time in order to discriminate the sex.

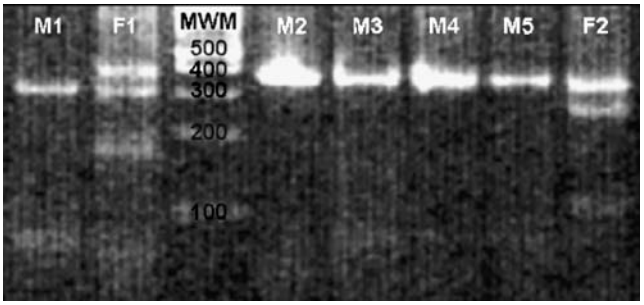

Fig. 1 Identification of sex of *G. barbatus* by *Hae*III and *Asp*700I digestions of *CHD* gene amplification using P2/P8 primers. *M* male, *F* female. *MWM* molecular weight marker (size in base pairs). *Hae*III digestions produce two bands in males (*M1*) and three bands in females (*F1*). *Asp*700I digestions produce one band in males (*M2*, *M3*, *M4*, and *M5*) and three bands in females (*F2*)

Sexing results from *Hae*III and *Asp*700I digestions and from ARMS were consistent with autopsies of the seven validation control samples. These methods were applied to the remaining samples and identified 17 males and 11 females.

Discussion

Although DNA extraction both from quills and from blood stored on FTA® cards worked well in our studies, we recommend the use of FTA® cards as a blood collection method, as they can be stored at room temperature for a long time (Gutiérrez-Corchero et al. 2002; García and Arruga 2006). Extraction of DNA from these cards is also faster and cheaper than extracting it from feathers. With this collection method, we have a duplicate DNA bank stored at room temperature both in the laboratory and in the La Alfranca Centre. Although not faced with that problem, other studies have shown that DNA from feathers was not suitable for the ARMS technique (Reddy et al. 2007).

In the comparison of the different methodologies used previously to determine the sex of birds of the same family (Accipitridae) in which the bearded vulture (*G. barbatus*) belongs to, we found that agarose gel electrophoresis of P2/P8 or P2/NP PCR products does not allow for the differentiation of sexes. Acrylamide gels were discarded for large-scale analysis because of their intricateness. Regarding the application of *Hae*III and *Asp*700I, some previous studies obtained the same results in other Accipitridae species (Table 1). These results demonstrate that *Hae*III and *Asp*700I sites are conserved among many species in the Accipitridae family. The ARMS technique was also successfully used to sex *G. barbatus* individuals and thus corroborated results in all species of the family previously checked. Male bearded vultures showed one single band, and females had two bands. These results are more similar to those observed by Ito et al. (2003) than to the ones by Reddy et al. (2007).

Table 1 Comparison of different procedures for sexing Accipitridae

Species	Sexing with P2/P8	Sexing with P2/NP	Sexing with *Hae*III digestion	Sexing with *Asp*700I digestion	Sexing with ARMS	Reference
Marsh harrier (*Circus aeruginosus*)	Yes (M: 1 band, F: 2 bands)	–	–	–	–	Griffiths et al. (1998)
Black kite (*Milvus migrans*)	–	No	–	–	Yes (M: 1 band, F: 2 bands)	Ito et al. (2003)
Northern goshawk (*Accipiter gentilis*)	–	No	–	–	Yes (M: 1 band, F: 2 bands)	Ito et al. (2003)
Eastern marsh harrier (*Circus spilonotus*)	–	No	–	–	Yes (M: 1 band, F: 2 bands)	Ito et al. (2003)
Golden eagle (*Aquila chysaetos*)	–	No	–	–	Yes (M: 1 band, F: 2 bands)	Ito et al. (2003)
Eurasian sparrowhawk (*Accipiter nisus*)	–	No	–	–	Yes (M: 1 band, F: 2 bands)	Ito et al. (2003)
Mountain hawk eagle (*Spizaetus nipalensis*)	–	No	–	–	Yes (M: 1 band, F: 2 bands)	Ito et al. (2003)
Short-toed eagle (*Circaetus gallicus*)	No	–	Yes (M: 2 bands, F: 3 bands)	Yes (M: 1 band, F: 3 bands)-	–	Sacchi et al. (2004)
Slender-billed vulture (*Gyps indicus*)	–	No	Yes (M: 2 bands, F: 3 bands)	–	Yes (M: 1 band, F: 3 bands)	Reddy et al. (2007)
Indian white-backed vulture (*Gyps bengalensis*)	–	Yes (M: 1 band, F: 2 bands)	Yes (M: 2 bands, F: 3 bands)	–	Yes (M: 1 band, F: 3 bands)	Reddy et al. (2007)
Bearded vulture (*Gypaetus barbatus*)	No	No	Yes (M: 2 bands, F: 3 bands)	Yes (M: 1 band, F: 3 bands)-	Yes (M: 1 band, F: 2 bands)	This study

No It is not possible to sex the species with this method. (–) This method has not been tried in this species, *M* male, *F*, female

We have found that ARMS is a reliable technique in the search for a quick, accurate, and easy procedure for large-scale sex typing of bearded vultures, although the two female bands are very similar in length and one of them is faint. Post-PCR digestions with *Hae*III or *Asp*700I are also suitable, but an additional step is needed in handling the PCR product.

Both methods are equally useful for the gender identification of bearded vultures in the first days of nestling life and in captive breeding programs or to determine the sex ratio in populations in the wild. Finally, it is very important in conservation management of this endangered species.

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