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Comparison between DNA and Analyses Morphometrics as Tool for Identification of Sex of the Golden Eagles (*Aquila chrysaetos Canadensis*)

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Abstract

The Golden eagle (*Aquila chrysaetos canadensis)* is an endangered species in Mexico, and the captive breeding is relevant to its conservation. Monomorphic raptor species, such as Golden eagle, are difficult for sex determination based only on morphometric measures and plumage characteristics, and a reliable method to determine the sex of birds is important for conservation strategies. With the purpose of forming new captive breeding couples, the sex of 10 adult Golden eagles was identified using DNA analysis such as CHD gene amplification (P8, P2 primers), and by CHD amplification by ARMS technique. In addition bioinformatic, and PCA statistical analysis of morphometric characteristics were conducted. We confirmed that the P2-P8 pair of primers inside the CHD gene was not a good option for Golden eagles because fragments derived from sex specific alleles CHD-W and CHD-Z differed just in 6 bp, which are not enough to be distinguished in agarose gel electrophoresis. In contrast, sex identification in Golden eagles with ARMS technique was fast, reliable and useful, obtaining a difference of more than 60 bp between CHD-W and CHD-Z that allows discriminating directly the alleles in a simple agarose gel. The PCA analysis of morphometric characteristics indicated three significant morphological patterns that differentiated males from females.

Keywords: *Aquila chrysaetos Canadensis;* Gender identification; PCR; CHD gene; Sexual dimorphism; PCA

Introduction

The golden eagle (*Aquila chrysaetos*) is a member of the family Accipitridae and is widely distributed in open and mountain habitats of the Northern Hemisphere [1]. Although globally widespread and abundant, populations in México (*A. c. canadensis*) have declined due to habitat loss, electrocution, poisoning, illegal traffic and hunting, and at present is a conservation concern [2]. Captive breeding programs are a key for future conservation strategies.

Many monomorphic raptor species such as Golden eagle are difficult for sex determination based only on morphometric measures and plumage characteristics [3,4]. Females tend to be larger than males, but size differences are not always reliable for sex determination [5]. Unfortunately, these characteristics have not traditionally been collected on Golden Eagles, and extracting information on population or fledgling sex ratios from extant data sets is therefore difficult. Furthermore, weight and footpad length are anatomically "soft" and hence are subject to greater potential variability in measurement [6].

Montopoli and Harmata propose used empirical data derived from the path and then DPNB PD models first if necessary to assign sex individuals in the field of *A. c. canadiensis* 3.5 months [7]. The magnitude of the sexual dimorphism of the species may mask progressive differences in measures of hallux claw (HAL) and head length (HEAD) of one sex over the latitudinal range of the species, and these measurements can be accurate indicators of sex.

Other methods like endoscopy, cloacae touch, karyotyping, fecal steroid analysis, and flow cytometry are more stressful for the animals, or need high sample quantity to be analyzed, in addition these methods are expensive, difficult to conduce, results interpretation is uncertain or are excessively time-consuming [8,9].

The repertoire of repetitive DNA in *A. chrysaetos* is limited relative to mammals, but is generally similar to known avian genomes. The *A. chrysaetos* genome does not exhibit substantial variation in repeat content, either in the total proportion of repeats in the genome or in the relative proportions of different superfamilies and/or classes of repetitive elements. The *A. chrysaetos* genome appears to have fewer LINEs than the chicken genome, but this could also be attributable to technical factors such as enrichment of repetitive regions in unassembled portions of the genome and/or incomplete repeat libraries Overall, the lack of variation in repeat contents is consistent with the relative homogeneity of avian [10].

The golden eagle presents 62 chromosomes and the use of data to characterize chromosome sex (ZZ in male and ZW in female) is easy and can be used by conservation programs. Female birds are heterogametic (ZW), and males have two identical sex chromosomes (ZZ). The gene coding for Chromobox-Helicase-DNA binding protein (CHD) was the first W chromosome described, and enabled sex identification in most avian species using a simple method consisting on the amplification of CHD genes of both sex chromosomes (CHD-W, CHD-Z) whose lengths are different because of varying size of introns [11], using a couple of primers P2 and P8 [12]. It has been reported that this technique is not applicable to Accipitridae birds such as eagles, although Xirouchakis and Poulakakis reported the sex identification of 49 individuals of Eurasian griffon vulture (*Gyps fulvus)* using P2-P8 primers [13]. In order to have a reliable method for sex identification in Accipitriformes modifications to the technique have been developed using the Amplification Refractory Mutation System "ARMS" [3,9,14]

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ARMS is a PCR-based technique to amplify specific alleles using a 3'-terminal primer (MP) that fits to one allele variant and mismatches to the other (P2, NP) allowing the detection of allele differences between CHD-W and CHD-Z genes [3].

The main objective of the present study was to identify the sex by the CHD amplification by ARMS technique, confirming gen identity by sequencing of amplified fragments and Bioinformatic analysis. In addition, having individuals with molecular biology confirmed gender, a PCA (Principal Component Analysis) of morphometric characteristics was conducted, in order to identify the consistency of morphological patterns to distinguish males from females.

Methods

Blood samples and morphological measures were taken from 10 adult Golden eagles (*A. c. canadensis*), which are resident in the "El Nido, Vida Silvestre Jesús Estudillo Lopez" aviary (Mexico State, México).

Seven morphological characteristics in each individual were measured to use as markers to distinguish males from females in this species. The use of head width, head length and, left tarsus thickness as measures were proposed because of the experience obtained from Falconry elaborating Hoods (head measures are different from male to female of the same species) and Jesses (tarsus tend to be thicker in females than males). And these measures were complemented with a more common measures; length of the culmen without cere (bill-cere), left wing length from shoulder to the last primary flight feather, left phalange II length, and weight. All the measures were taken with a thin and soft tape measure, and the weight with a spring scale up to 10 kilograms.

Blood samples were collected on heparin in vacutainers and DNA was extracted from blood samples using Wizard DNA purification Kit (Promega, USA). As a first sex identification approach, a fragment of the CHD gene was amplified by PCR using the primers P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3') [12]. PCR amplification was performed in a final volume of 50 µl containing 2 µl of DNA template, 0.5 µl of each primer (100 nM stocks), 0.5 of dNTPs mix (10 mM stocks) (Promega, USA), 0.25 µl (1.25 U/µl) of Taq polymerase (GoTaq Flexi DNA Polymerase, Promega, USA), 5 µl buffer 10x and $MgCl_2$ 1.5 mM. PCR conditions included an initial denaturing step at 94°C for 1 min 30 s followed by 35 cycles at 94°C for 30 s, 52°C for 1 min, and 72°C for 2 min with a final extension step at 72°C for 5 min. PCR products were resolved by electrophoresis in a 5% agarose gel stained with ethidium bromide, and 8% polyacrylamide silver stained gel. The second approach tested was the ARMS technique, so three primers were used for PCR amplification: P2 described above, NP (5'-GAGAAACTGTGCAAAACAG-3') and MP (5'-AGTCACTATCAGATCCGGAA-3') 3'-terminal mismatch primer (Ito et al. 2003). PCR amplification was performed as described, and the final reactions were conducted using the following conditions: 94°C for 1 min 30 s, followed by 36 cycles at 94°C for 30 s, 52°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 5 min. PCR products were separated by electrophoresis in a 5% agarose gel stained with ethidium bromide and visualized under UV light.

To confirm that the bands observed corresponded to the target CHD fragments, sequence analysis were performed. Purification of PCR products was made using QIAEX II (Gel Extraction Kit, QUIAGEN) and Quantum Prep PCR Kleen Spin Columns (BioRad, EUA). The sequence of the purified PCR products was obtained by M. en C. Rosa Elena Gómez (CISEI-INSP, Mexico). The sequence analysis was performed using BLASTN and CLUSTALW programs from WORKBENCH [15].

To test which morphological patterns could be used to distinguish males from females, a Principal Component Analysis (PCA), was performed using PC Ord [16]. PCA simplifies morphological variation patterns and make them easier to interpret by replacing the original variables with new ones (principal components, PC's) that are linear combinations of the original variables and independent of each other [17].

Results

The results of the sex identification using final point PCR and ARMS techniques are shown in Figure 1 Regular PCR with P2- P8 primers (panel A) was not successful for Golden eagles, because both sexes showed a single band in 5% agarose gel. With ARMS technique (panel B) the males revealed a single band (A1, A3, A8) while the females presented two bands (A2, A4, A5, A6, A7, A9, A10). Resulting in a total of 3 males and 7 females.

Sequence analyses of the purified PCR products confirmed that each band corresponded to the target CHD fragments, with an "E value" accuracy ranging from 5.00E-96 to 3.00E-05. Differences in size between DNA fragments derived from CHD-W and CHD-Z from P2-P8 primers resulted in 6 bp. (Gene Bank ID 1641605). MP 3'-terminal mismatch primer assembly was confirmed and compared with the original sequences from Ito et al. [3] available in *GenBank* (Figure 2). The difference that permits the primer to assemble in CHD-W and not in CHD-Z, is the thymine (T) in the beginning of the insertion site, instead of the cytosine (C) of CHD-Z.

Morphometrical measures are presented in the Table 1. Only by the weight and size of each individual, 5 males (A1, A3, A4, A8, A10) and 5 females (A2, A5, A6, A7, A9) were erroneously expected. It was necessary to consider all the morphometric values under PCA analysis to clarify the sexual dimorphism patterns.

PCA analysis graphic is presented in Figure 3, where the males A1, A3, A8 are clearly grouped in the superior-right portion of the graph, and are differentiated from the rest of the females due decrease in phalange II length and weight, and proportional increase in wing chord and lateral skull length. Phalange II length was the character that most differentiated females from males.

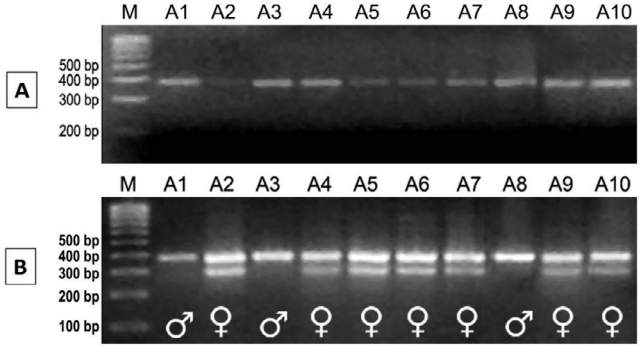

Figure 1: Sex identification bands. Panel a) PCR amplification of CHD Z and CHD W genes using P2 – P8 primers [12] both sexes have a single band, and sex determination was not possible. Panel b) ARMS-PCR method with primers P2-NP-MP [3] showed individuals with two bands (female) and with one (males). M.- 100 bp Molecular size Marker (Promega, USA).

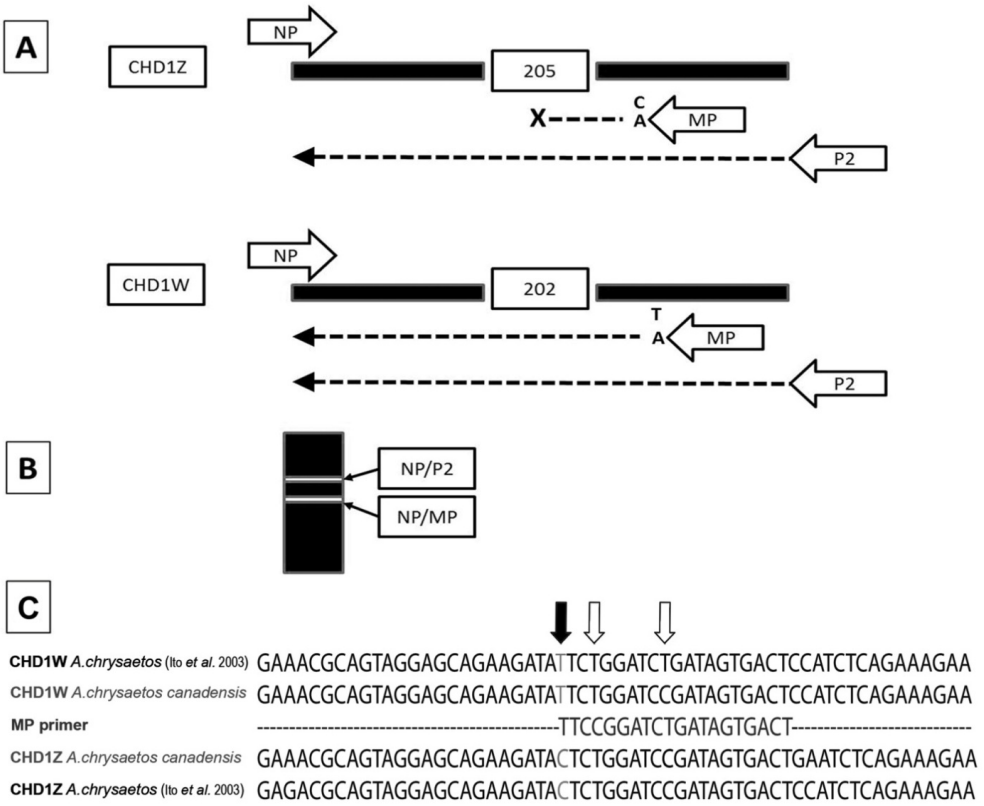

Figure 2: Principle of ARMS method and alignment of experimental sequences from W and Z chromosomes. Panel A.- Differential amplification of target sequence from Z and W using the ARMS system, distinguishing chromosomes using three NP, MP and P2 primers.NP primer (forward) anchors at the 5´ extreme of the target region of both sexes. The MP primer (reverse) have a terminal T that match with the extreme of the W sequence but not with the Z.The P2 primer match with sequence from both sexes. Panel B.- W chromosome products expected in a gel electrophoresis. Panel C.- Alignment of sequences of the region around the MP primer obtained from both W and Z amplification fragments show that in position 11 could be either a C or a T but it did not disturb the amplification, in contrast to the key position 20 where the C is specific for Z chromosome and T for W.

ID	Sex	Weight, kg	Wing chord, cm	Tarsus, cm	Phalange II, cm	Bill-cere, cm	Head width, cm	Head length, cm
A1	M	2.8	74.2	6	4.9	2.3	7.3	7
A2	F	4.2	76.5	7.1	5.7	2.9	6.9	8
A3	M	3.2	70.5	6	4.3	2.2	6.1	6.2
A4	F	2.9	71	7	6	2.3	6.1	8.2
A5	F	4.5	82.4	8.2	4.2	2.8	7.1	7.4
A6	F	4	77	8	6.8	2.6	7.1	7.3
A7	F	3.8	65	7.1	6.6	2.4	6.5	7.6
A8	M	2.2	71.5	6.4	4.1	1.8	6.4	6.3
A9	F	4	70.5	7.7	3.2	2.5	7.6	8.2
A10	F	3.8	75	8.1	5	2.9	7	7.8

Table 1: Morphometrical measures taken from each individual.

Discussion

A reliable method to determine the sex of monomorphic species is important for captive breeding programs. In this work two sex identification methods were tested. The final point PCR with a the P2-P8 pair of primers inside the CHD gene was not a good option for Golden eagles because fragments derived from sex specific alleles CHD-W and CHD-Z differed just in 6 bp, which are not enough to be distinguished in agarose electrophoresis. This result is similar to those obtained by Ito et al. [3] who reported unsuccessful sex identification in 6 species of Accipitridae because of differences between 2-8 bp of fragments from CHD-W and CHD-Z. In contrast, Xirouchakis and Poulakakis reported sex identification of individuals of Eurasian griffon vulture using P2-P8 primers, CHD segments with differences of 9 bp that could be differentiated using polyacrylamide gels; although, could not be applied in this case with Golden eagle, the resulting 8% polyacrylamide gels also displayed a virtual single band, like in the agarose gels (data not shown) [13].

In contrast, sex identification in *A. c. canadensis* with ARMS technique was fast, reliable and useful. With this method a difference of more than 60 bp between CHD-W and CHD-Z, allowed discriminate directly the alleles in a simple agarose gel, without the need of digestion enzymes.

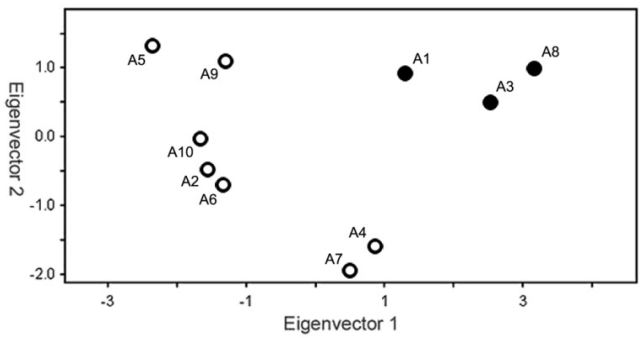

Figure 3: PCA graphic. Each dot represent an individual, white dots represent females, black dots represent males. First two axes (Eigenvector) that represent 73% of the total data.

With the correct gender identification of the individuals, we proceeded to compare it with the morphological data. The use of head width, head length and, left tarsus thickness as measures, were proposed because of the experience obtained from Falconry elaborating Hoods and Jesses, and were complemented with length of the culmen (bill-cere), left wing length, left phalange II length and weight. A PCA analysis was conducted in order to validate which of them could be used for an immediate sex determination. This analysis shows that the males A1, A3, A8 are congregated in the superior-right portion of the graph (Figure 3) and are differentiated from the rest of the females due lower values in phalange II length and weight, and proportional higher values in wing chord and lateral skull length. Phalange II length was the character that most differentiated females from males.

These results differ from a previous study on the morphological variation on the Golden eagle [5], which propose the use of a function that describes the relationship of hallux claw length to culmen length to sex individuals, although, the author also express that immature Golden Eagles appear to have slightly shorter culmens and hallux claws than adult birds. It is important to take on account that dimensions in claws and bill may be subject to wear and overgrowth from use, that's why phalange and skull length can be more reliable measures, because they don't tend to vary on habits, environment and nutrition. Even though, to be able to propose a function or measure useful to distinguish males from females, in field situations, based on phalange and skull length, it would be necessary a bigger population analysis to confirm this sexual dimorphism pattern.

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