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The First Report of Eurasian Bearded Vulture (*Gypaetus Barbatus*) Sex Identification Using CHD1 Gene Markers in Comparison to Alexandrine Parakeet (*Psittacula Eupatria*), Kestrel (*Falco Tinnunculus*) and Ring Necked Parakeet (*Psittacula Krameri*)

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Abstract

Molecular identification methods based on DNA analysis are essential useful techniques with high sensitivity for sex identification of monomorphic birds. One of the most endangered bird species that has no sexually dimorphism is Bearded vulture (*Gypaetus barbatus*) which its abundance and breeding range have drastically declined in recent years. In this study comparative PCR sexing assays based on amplification of CHD-1 encoding sequence including 6 different primer sets were used in AMI molecular biological lab institute in Iran to identify the unknown sub adult Bearded vulture sex that is kept and protected in FPWC center in Armenia, Yerevan. Some of the assays were also used on samples taken from Alexandrine parakeet (*Psittacula eupatria*), Kestrel (*Falco tinnunculus*) and Ring necked Parakeet (*Psittacula krameri*). Results showed that P_2/P_8 and P_2/NP primer sets are useful for sex identification of Bearded vulture (*Gypaetus barbatus*).

Keywords: Bearded vulture; Bird; Sex identification; PCR; CHD-1

Introduction

There are many endangered species of animals including various kind of ornamental poultry and pray birds because of destruction of their natural environment and illegal hunt all over the world. Many of these bird species (almost 60 percent) are sexually monomorphic and there is no way to morphologically distinguish between both sexes [1]. Molecular identification methods based on DNA analysis are essential useful techniques with high sensitivity for sex identification of monomorphic birds [2]. There are useful PCR techniques based on amplification of partial parts of the encoding CHD-1 gene sequence located on both Z and W sex chromosomes in birds, which are two Z chromosomes in male and one Z and one W chromosome in female [3-8]. One of the most endangered bird species is Bearded vulture (*Gypaetus barbatus*) which its abundance and breeding range have drastically declined in recent years [9]. The bird has been classified into two sub species of GB barbatus or the Eurasian Homa which lives in mountainous regions of the Middle East including the Zagros and Alborz Mountains of Iran, the Caucasus region, the Koh-i-Baba in Bamyan, Afghanistan, the Altai Mountains, the Himalayas, Ladakh in northern India, western and central China. And the sub-species of GB meridionalis or the African Homa [10].

The female usually lays a clutch of 1 to 2 eggs (rarely 3) which are incubated for 53 to 60 days and the chicks are dependent to their parents for up to two years. It usually takes 5 years to reach full maturity [11]. Since this species has no morphological sexual dimorphism and according to the low rate of breeding, a reliable sex identification test is essential for breeding strategies of captive birds for recovery process in animal preservation's organizations and further release them into their natural environment.

Materials and Methods

In this study three blood samples were taken from one Bearded vulture (*Gb. barbatus*) or Home bird with sex known as male, one sub mature unknown sex and another unknown sex mature Home bird in FPWC center in Armenia, Yerevan, and sent to the AMI molecular biological lab institute in Iran for sex identification. Two blood samples of Alexandrine parakeet (*Psittacula eupatria*) from both sexes, a male Kestrel and a female Ringed neck Parakeet (*Psittacula krameri*) were already taken from the birds in Eram Zoo in Tehran, and were used as comparative samples in the study. DNA was purified from the blood samples using DNA purification kit (*Sinaclon, Iran*) according to the manufacturer protocol. Since the mature Home with unknown sex was released in nature and the DNA purification process of its blood sample was failed due to clot formation, the sample was eliminated from the study. Primers used in this study for amplification of CHD_1-Z and CHD_1-W encoding gene sequences were as below. PCR amplificationwith two primersets of P2 [5'TCTGCATCGCTAAA TCCTTT-3'] and P3 [5'- AGATATTCTGGATCTGATAGTGA-3'] [4] and P8 [5'-CTCCCAAGGATGAGRAAYTG-3'] and P2 [5'-TCTGCA TCG CTAAA TCCTTT-3'] [7]using annealing temperature 48°C PCR was performed in 50 µL volumes including 10 µg genomicDNA, 250 nM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTP, and 0.5 or 1 unit Taq polymerase (*Sinaclon, Iran, Tehran*). The settings for the thermal cycler were the following: hot start at 94°C (5 min) followed by 40

cycles including denaturing at 94°C (45 s), primer annealing at 46°C temperature (1 min), elongation at 72°C (55 s), and final extension at 72°C (5 min).

All PCR products were stained using DNA safe dye (Sinaclon, Iran, Tehran) and separated by electrophoresis on 1.5% and 3% agarose gel then visualized in illuminator under UV light.

The PCR amplification with four different primer sets (Table 1) were done on DNA samples taken from the two mentioned Home birds, a male Kestrel (*Falco tinnunculus*) and one female Ringed parakeet (*Psittaculata krameri*) for comparison the results. In addition to P_2 and P_8 primers, the two primer sequences were FNP [5'-GAGAAACTGTGCAAAACA-3'] and Rmp [5'-AGTCACTATCAGATCCGGAA-3'] [7,12]. The later primer sets were used to determine the female bird because the primer set can only detect female-specific CHD1W gene by including 3'-terminal mismatch primer MP [13]. Alternatively, another primer set of P_2 and P_8 (5'-CTCCCAAGGA TGAGRAAYTG-3') was used for bird species whose sex cannot be determined by the former primer set [7,13]. Four different PCR amplification was done as shown in Table 1 in an automated thermo cycler under PCR program as following: 50 µL volumes including 10 µg genomic DNA, 250 nM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTP, and 0.5 or 1 unit Taq polymerase (*Sinaclon, Iran, Tehran*). The settings for the thermal cycler were the following: hot start at 94°C (5 min) followed by 40 cycles including denaturing at 94°C (45 s), primer annealing at 46°C temperature (1 min), elongation at 72°C (55 s), and final extension at 72°C (5 min). All PCR products were stained using DNA safe dye (*Sinaclon, Iran, Tehran*) and separated by electrophoresis on 3% agarose gel then visualized in illuminator under UV light.

Results and Discussion

In contrast with mammals, the sex determination system in birds is different and the females have heterogametic chromosomes (ZW) in comparison to male birds whose sex chromosomes are identical (ZZ). As more than 60% of all bird species are sexually monomorphic, and the necessity of precise method for sex identification of birds, today various molecular techniques based on sex-specific genetic markers are used due to sex identification of birds [13-16]. Griffiths reported that 27 species of birds could be sexed successfully using one set of primers, P_2/P_8 by changing PCR conditions annealing temperature and $MgCl_2$ concentration. In this study six different PCR primer sets were tested for amplification of CHD_1-Z and CHD_1-W encoding gene sequence for sex identification of Eurasian Bearded vulture for the first time.

Samples from Alexandrine parakeet, Ringed neck parakeet and Kestrel were also tested. As shown in Figure 1, PCR amplification of male Alexandrine parakeet genomic DNA sample with P_2/P_8 and P_2/P_3 primer sets led to a PCR product length of about 400 bp and 150 bp respectively. In this study in spite of changes in PCR program and PCR set concentrations no PCR product was amplified using DNA of Female Alexandrine parakeet as template.

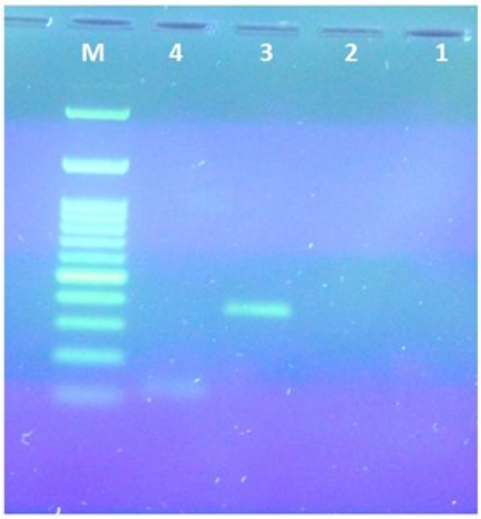

Figure 1: PCR sexing using P_2/P_8 and P_2/P_3 primer sets: 1) Female Alexandrine parakeet (*Psittacula eupatria*) P_2/P_8; 2) Female Alexandrine parakeet (*Psittacula eupatria*) P_2/P_3; 3) Male Alexandrine parakeet (*Psittacula eupatria*) P_2/P_8; 4) Male Alexandrine parakeet (*Psittacula eupatria*) P_2/P_3.

Figure 2 and Figure 3 shows PCR sexing assays of male and sub adult Bearded vulture in comparison with male Alexandrine parakeet using P_2/P_8 and P_2/P_3 primer sets. As shown below, the sub-adult Bearded vulture determined as female bird because of two different PCR products on 1.5% agarose gel electrophoresis.

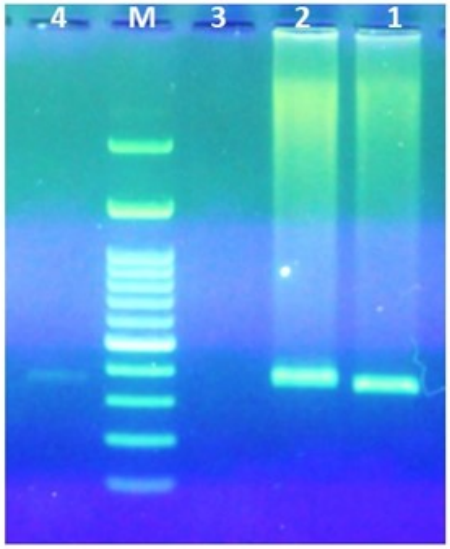

Figure 2: PCR sexing assays P_2/P_8 primer sets: 1) Male Bearded vulture (*Gypaetus Barbatus*) P_2/P_8; 2) Male Bearded vulture (*Gypaetus Barbatus*) P_2/P_8; 3) Failed due to imperfect DNA Purification; 4) Male Alexandrine parakeet (*Psittacula eupatria*) P_2/P_8.

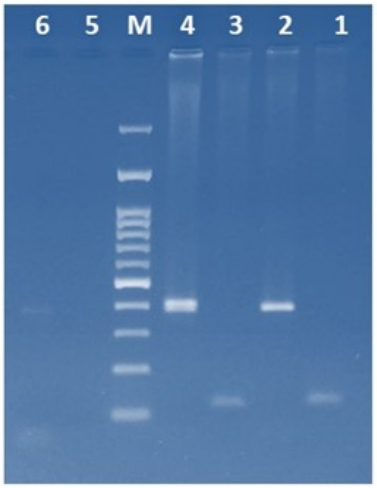

Figure 3: PCR sexing assays P_2/P_3. P_2/P_8 primer sets: 1) Male Bearded vulture (*Gypaetus Barbatus*) P_2/P_3; 2) Male Bearded vulture (*Gypaetus Barbatus*) P_2/P_8

3) Female Bearded vulture (*Gypaetus Barbatus*) P_2/P_3; 4) Female Bearded vulture (*Gypaetus Barbatus*) P_2/P_8; 5) Female Alexandrine parakeet (*Psittacula eupatria*) P_2/P_8; 6) Male Alexandrine parakeet (*Psittacula eupatria*) P_2/P_8.

Since the sub-adult's two PCR products were blur, another PCR assay were done using four different primer sets on two male and sub-adult Bearded vulture, Female Ringed neck Parakeet, and male Kestrel. The PCR products were electrophoresed on 3% agarose gel (Figure 4). The comparative results are summarized in Table 1.

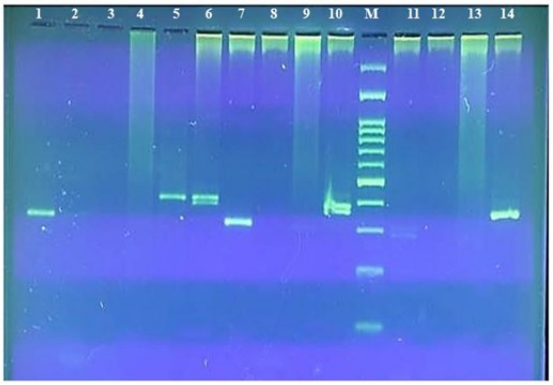

Figure 4: PCR sexing assays using four sets of primer pairs: 1) Female Ring necked parakeet (*Psittacula krameri*) P_8/RMP; 2) Male kestrel (*Falco tinnunculus*) P_8/RMP

3) Male kestrel (*Falco tinnunculus*) P_2/RMP; 4) Male kestrel (*Falco tinnunculus*) FNP/RMP; 5) Male kestrel (*Falco tinnunculus*) FNP/P_2; 6) Female Bearded vulture (*Gypaetus Barbatus*) P_2/P_8; 7) Female Bearded vulture (*Gypaetus Barbatus*) P_8/RMP; 8) Female Bearded vulture (*Gypaetus Barbatus*) P_2/RMP; 9) Female Bearded vulture (*Gypaetus Barbatus*) FNP/RMP; 10) Female Bearded vulture (*Gypaetus Barbatus*) FNP/P_2; 11) Male Bearded vulture (*Gypaetus Barbatus*) P_8/RMP; 12) Male Bearded vulture (*Gypaetus Barbatus*) P_2/RMP; 13) Male Bearded vulture (*Gypaetus Barbatus*) FNP/RMP; 14) Male Bearded vulture (*Gypaetus Barbatus*) FNP/P_2.

Sex	Species	P_2/P_3	P_2/P_8	P_8/MP	P_2/MP	NP/Mp	Np/P_2
M	Bearded vulture	+	+	+	-	-	+
F	Gypaetus barbatus	+	+	+	-	-	+
M	Alexandrine parakeet	Not tested	+	Not tested	Not tested	Not tested	Not tested
F	Psittacula eupatria	Not tested	-	Not tested	Not tested	Not tested	Not tested
M	Kestrel	Not tested	Not tested	-	-	-	+
	Falco tinnunculus						
F	Ring necked Parakeet	Not tested	Not tested	+	Not tested	Not tested	Not tested
	Psittacula krameri						

Table 1: Summarized results of PCR sexing assays using six different primer sets based on amplification CHD1-Z and CHD1-W encoding gene sequences.

Conclusion

PCR assays using P_2/P_8 and NP/P_2 primer sets led to amplification of two PCR product length in female Bearded vulture DNA samples in comparison to one PCR product in male bird as expected. These results shows that P_2/P_8 and NP/P_2 primer sets are useful for sex determination of male and female Bearded vulture bird.

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