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A non-invasive test for sex identification in Short-toed Eagle (*Circaetus gallicus*)

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Abstract

Knowledge of the sex of individuals in natural populations greatly facilitates evolutionary ecology, breeding systems and genetics. Therefore, the development of a simple, not stressing and objective sexing test would facilitate conservation of the Short-toed Eagle (*Circaetus gallicus*), an endangered *Accipitridae* species living mainly in southern Europe and Asia. A PCR test was used employing primers that amplify two homologous fragments of both the CHD-W gene, unique of females, and the CHD-Z, occurring in the two sexes. The analysis of the PCR products obtained from blood DNA showed a band of about 380 bp, apparently unique in all individuals. The alignment of the sequences of the two fragments revealed that CHD-W is only 9 bp longer than CHD-Z (387 vs. 378 bp) while CHD-Z lacks the restriction site for *Asp*700I. After digestion male PCR products showed a unique band of 378 bp while fragments belonging to females resolved into three bands (378, 280 and 107 bp). Using feathers as DNA sources, the individual patterns obtained were identical with the corresponding blood DNA samples. This sexing technique is objective and non-invasive and could be useful for verifying the sex ratio theories and improving the management.

Keywords: Sex identification; Short-toed Eagle; CHD gene

1. Introduction

The Short-toed Eagle has a wide distribution throughout southern and central Europe from Spain and Eastern France to Afghanistan and India. In Europe during the 19th century, the Short-toed Eagle has suffered a steep decline in numbers and range; it is now rare and still decreasing in several countries due to human activities affecting natural habitat and food supply. In 2003, a population of 3000–4500 pairs is esteemed in Europe from which 380–415 pairs in Italy [1,2].

It is a medium-sized raptor, the adults are easily recognised by their predominantly white underside, grey to brown upper parts, barred tail, bare tarsus and brilliant yellow eyes [3].

Short-toed Eagle immatures and juveniles, like for many other avian species, do not show sex-linked morphology. As for the adults, authors do not agree upon the development of an unambiguous sexual dimorphism. Nevertheless, a discrimination between male and female is possible based on some discernible plumage traits [3,4]. However, the knowledge of the sex of young individuals in a population greatly facilitates the study of behaviour, evolutionary ecology, breeding system and genetics. Therefore, the development of an objective test to sex this species would undoubtedly facilitate the species management.

In recent years some DNA-based tests have been applied for the detection of sex in few species of birds. In these tests the determination of W-specific DNA sequences has been used [5–7]. Another general approach based on two conserved CHD (chromo-helicase-DNA-binding) genes located on the sex chromosomes of all non-ratite birds has been proposed [8,9]. This PCR test employs a single set of primers that amplifies homologous fragments of both the CHD-W gene, unique of females, and CHD-Z gene, occurring in the two sexes, but introns, whose length is usually different between the genes, are incorporated.

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A crucial point in any kind of studies of free-ranging animals is the sampling, that must be simple, not stressing and non-invasive. In this respect, hairs and faeces have proved to be suitable materials in mammals [10], while bird feathers are rarely used [11,12].

In this paper we describe an adaptation of the CHD gene test developed by Griffiths et al. [13] for an early sex identification in Short-toed Eagle using genomic DNA extracted from feathers.

2. Materials and methods

2.1. Sample collection

Blood samples and some large (scapular and median covers) and small feathers were collected from six known-sex individuals, four females and two males of Short-toed Eagle stabled in wild bird rehabilitation centres. Feathers were put in plastic bags and stored at −20 °C until analysis.

2.2. DNA extraction

Genomic DNA was obtained from 200 μl of whole blood using the Invisorb Spin Blood Mini Kit (Invitek).

From two to three individual feathers, a 0.5 cm segment was cut from the root end and placed in a 1.5 ml Eppendorf tube. Genomic DNA was extracted using the Genomic DNA from Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions with the following pre-lysis step modifications. Digestion was performed at 37 °C overnight using 500 μl of lysis buffer (50 mM Tris-HCl, pH 8, 20 mM EDTA, pH 8, 2% SDS) and proteinase K at a final concentration of 175 μg/ml [11].

2.3. PCR

A region of the CHD gene was amplified by PCR from blood and feathers using the P2 and P8 primers proposed by Griffiths et al. [13] (Roche Diagnostics). PCR reactions were performed using 5 μl of DNA solution obtained from feathers or 2.5 μl of DNA solution obtained from blood in 25 μl of final volume containing PCR Buffer 1X (QIAGEN), 0.2 mM/each dNTPs, 1.5 mM $MgCl_2$, 0.5 μM primers and 0.015 U/μl HotStarTaq (QIAGEN). Different PCR programs were assessed to optimise reactions because the original protocol showed to be suitable for DNA extracted from blood but failed to give any products when applied to feathers DNA. Finally, assay conditions were standardised at 95 °C for 5 min followed by 42 cycles of 94 °C for 30 s, 48 °C for 45 s and 72 °C for 45 s. A final extension step of 72 °C for 5 min was carried out for all reactions. PCR was performed in a Gene Amp PCR System 2400 thermal cycler (Applera Biosystems).

To verify whether contamination of samples with exogenous DNA or PCR products had occurred, tubes without samples were included in the DNA extraction and PCR amplification procedure as negative controls. This is important as the human CHD-1 gene can be a contaminant [9].

2.4. RFLP analysis

PCR products (15 μl) were digested with *Hae* III and *Asp*700I (Roche Diagnostics). The reactions were performed in 20 μl final volume containing 0.3 U/μl of enzyme, 1X Buffer and incubated for 3 h at 37 °C.

Digestion products were analysed by agarose gel electrophoresis (2% in TBE) and visualised under UV light after ethidium bromide staining. GeneRuler 50 bp DNA Ladder (Fermentas) was used as size standard.

2.5. Sequencing

P2–P8 amplified fragments from a female were cloned into the pDRIVE Cloning Vector using QIAGEN PCR Cloning Kit (QIAGEN). Clones were then submitted to RFLP analysis using *Hae* III to choose a clone containing the CDH-W fragment and a clone containing the CDH-Z fragment [9]. These cloned fragments were cycle sequenced on an ABI PRISM 310 Genetic Analyser (Applera Biosystems) by the dideoxy chain termination method with fluorescence dye terminators (Applied Biosystem). The sequencing on both strands was performed using the two M13 vector primers. The resulting sequences were compared and aligned with 'ClustalW' program [14].

3. Results and discussion

The analysis of P2–P8 PCR products from blood DNA on a 3% agarose gel did not allow to sex the Short-toed Eagles but showed an apparently unique band of about 380 bp in all samples regardless of the gender (Fig. 1). In contrast, Griffiths et al. [13] using this method were able to sex Marsh Harrier (*Circus aeruginosus*) belonging, as the Short-toed Eagle, to the *Accipitridae* family.

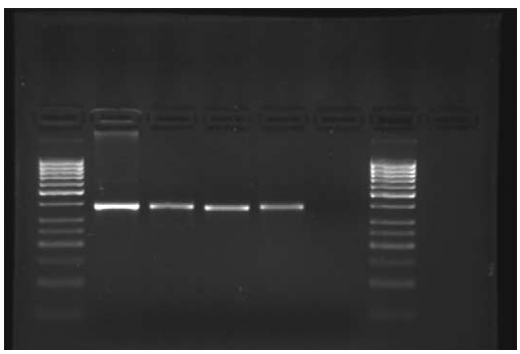

Fig. 1. P2–P8 PCR products. Lanes 2 female (DNA from blood); lanes 3 and 5 females (DNA from feathers); lane 4 male (DNA from feathers); lane 6 blank; lanes 1 and 7 GeneRuler 50 bp DNA Ladder (Fermentas).

To investigate the reason of the failure of the PCR test in Short-toed Eagle, PCR products obtained from a female were cloned and sequenced. Nucleotide sequence data are available in the EMBL and GeneBank databases (accession numbers AY313609 and AY313610 for CHD-W and CHD-Z, respectively). The comparison of the obtained sequences showed that the CHD-W fragment was only 9 bp longer than CHD-Z fragment (387 vs. 378 bp): such a difference in size is too small to be detectable on a 3% agarose gel.

Then we considered some PCR-RFLP methods in order to test their applicability to sex Short-toed Eagle.

The restriction sites of two enzymes, *Mae* II and *Hae* III, unique to CHD-W and CHD-Z respectively, were observed by Griffiths in many avian species [9,13].

The analysis of our sequences showed that in Short-toed Eagle the *Mae* II restriction site is not conserved on CHD-W, then this enzyme can not be used for the sex determination in this species. On the other hand, CHD-Z sequence conserves the restriction site for *Hae* III in position 313. In addition, on CHD-W we observed a restriction site for *Asp*700I in position 280; this site is absent on CHD-Z.

In order to test these two PCR-RFLP sexing methods in Short-toed Eagle, blood and feathers DNA sample from six known-sex individuals were used.

Hae III cuts CHD-Z but not CHD-W. After digestion, male PCR products are resolved into two bands (303 and 75 bp) while fragments belonging to females into three bands (387, 303 and 75 bp) (Fig. 2).

*Asp*700I cuts CHD-W but not CHD-Z. After digestion male PCR products show a unique band of 378 bp while fragments belonging to females are resolved into three bands (378, 280 and 107 bp) (Fig. 3).

The enzymes both allowed to sex Short-toed Eagle and all individual samples were correctly assigned to their sex. Nevertheless using *Hae* III as proposed by Griffiths et al. [9,13], if a portion of undigested male PCR products is present, the interpretation of RFLP results could be difficult due to resemblance of restriction patterns of the two sexes. On the contrary, no ambiguity is possible using *Asp*700I, even in the case of incomplete digestion, because CHD-Z cannot be cut in any case.

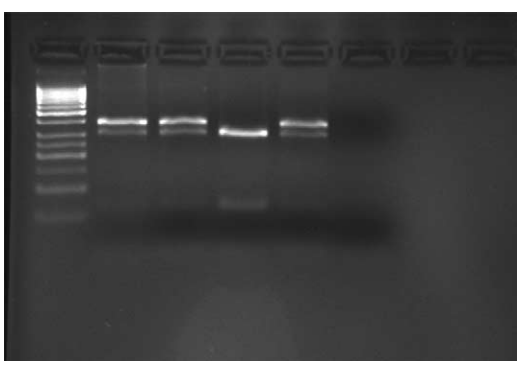

Fig. 2. *Hae* III RFLP analysis of P2–P8 PCR products. Samples as in Fig. 1.

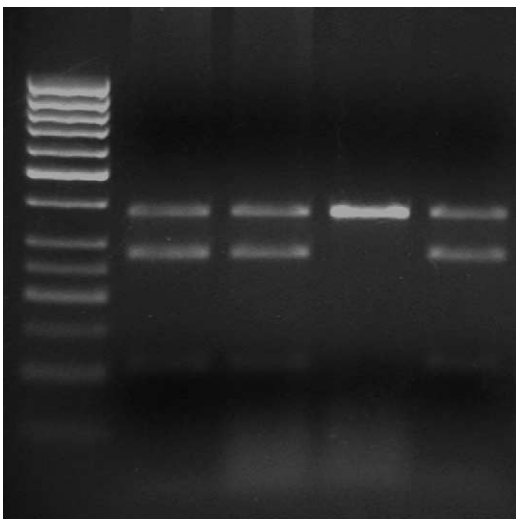

Fig. 3. *Asp*700I RFLP analysis of P2–P8 PCR products. Samples as in Fig. 1.

The alignment of EMBL/GenBank sequences from eight different species, that is *Milvus migrans*, *Accipiter nisus*, *Accipiter gentilis*, *Circus spilonotus*, *Spizaetus nipalensis*, *Aquila chrysaetos*, *Falco tinnunculus*, and *Falco peregrinus*, indicated that the *Asp*700I restriction site on CHD-W is highly conserved across the Order of Falconiformes. Consequently, any restriction site variability within species seems to be unlikely.

Using feathers as DNA source, the individual patterns obtained were identical with the electrophoretic patterns obtained from the corresponding blood DNA samples. Due to the lower recovery of DNA from feather extraction, when feathers instead blood as genomic DNA source are used a larger amount of DNA solution and an increased number of PCR cycles are needed.

In conclusion, we propose the *Asp*700I RFLP protocol for sexing Short-toed Eagle as an improvement over other methods. In fact, the PCR test alone and the PCR-RFLP method based on *Mae* II are not efficient and PCR-RFLP method based on *Hae* III may suffer from incomplete digestion.

Our sexing technique, which is objective, simple and non-invasive, could be useful for verifying the sex ratio in a population and, as a consequence, for improving the management of this endangered species.

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