Rapid, Easy and Reliable Sexing Method for Falconiformes by PCR

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INTRODUCTION

In recent years, many species all over the world have reached the verge of extinction due to the destruction and fragmentation of their habitats, pollution of their environments, over-hunting and the introduction of alien species. Like other countries, Japan has listed many species as endangered.

Because raptors such as the golden eagle (Aquila chrysaetos), mountain hawk eagle (Spizastus nipalensis) and goshawk (Accipiter gentilis) are located at the top of their respective ecosystems, they are highly vulnerable to environmental changes. Consequently, some raptors are specified as threatened species on the Red List in Japan (Ministry of the Environment, Japan: http://www.biodic.go.jp/rdb/rdb_f.html. 1998) and internationally (International Union for Conservation of Nature and Natural Resources: http://www.redlist.org/, 2000). In particular, golden eagle and mountain hawk eagle fledglings have been recently suffering from severe decreases in numbers, and their futures are bleak. Therefore, immediate conservation and/or management of these species are needed.

Identification of sex is extraordinarily important in wildlife conservation, because the sex of individuals gives information on behavior, ecology, evolution and genetics. However, since many species of birds do not display a high degree of sexual dimorphism in their external morphology, such as size or plumage characteristics, sexing of individuals is very difficult. It has been estimated that the sex of individuals can not be identified in over 50% of the world’s avian species [1]. Establishment of an easy sexing method is essential for...
conservation of birds. So far, various sexing methods, such as flow cytometry, karyotyping and various molecular genetic techniques, have offered a solution to the problem [reviewed in 2]. Specifically, because polymerase chain reaction (PCR) requires only a minute sample, for instance a single feather or a drop of blood, sexing methods based on PCR are very useful. Among the sexing methods using PCR, several are now available for the sexing birds based on PCR that simultaneously amplifies both the W chromosome-linked chromo-helicase-DNA binding protein (CHD1W) gene and the Z chromosome linked CHD1Z gene [1, 3-8]. The most recent methods have exploited the difference in intronic length between the CHD1W and CHD1Z genes [1, 8, 9]. The sexing methods of Griffiths et al. [1] and Kahn et al. [9] targeted the same region in the CHD1 genes, but the method of Fridolfsson and Ellegren [8] amplified a different region in the same genes. We have previously applied the method of Griffiths et al. [1] to Falconiformes [6]. However, because the difference in the length of amplified regions between the CHD1W and CHD1Z genes was so minute, sex identification was impossible. We therefore developed a new sexing method with the amplification refractory mutation system (ARMS) [6]. Since ARMS uses template-primer mismatch, amplification efficiency is relatively low and thus decision of PCR condition is slightly laborious. On the other hand, in the method of Fridolfsson and Ellegren [8], although the size difference of amplified regions between CHD1W and CHD1Z was large and this method is easy and quick to perform, it has proven useful in only a few species.

As mentioned above, each method has its advantages and disadvantages and this implies an easier, quicker and more universal sexing method is required. In this study, we applied Fridolfsson and Ellegren’s sexing method [8], which showed large differences in amplified fragments between CHD1W and CHD1Z, to Falconiformes inhabiting Japan, and examined the usefulness of this method.

**MATERIALS AND METHODS**

In this research, all samples which died under nature or protection were used. Genomic DNA was extracted from kidney or pectoral muscle taken from the black kite (Milvus migrans), goshawk (Accipiter gentilis), marsh harrier (Circus spilonotus), golden eagle (Aquila chrysaetos), Eurasian sparrow hawk (Accipiter nisus), mountain hawk eagle (Spizaetus nipalensis), peregrine falcon (Falco peregrinus) and kestrel (Falco tinnunculus) by phenol-chloroform methods or QIAGen tissue kit (Qiagen, USA). In addition, genomic DNA of eight black kites was extracted from a few feathers by QIAamp tissue kit according to manufacturer’s protocol with some modifications. All samples were pre-sexed by anatomical examination.

Some of the CHD1 gene was amplified using the primer set of 2550F (5'-GGTACTGATTCCGTCTACAGA-3') and 2718R (5'-ATTGAAATGATCCAGCTTTG-3') that was described by Fridolfsson and Ellegren [8]. PCR amplification was carried out in a total volume of 25 μl. The final reaction conditions were as follows: 50 mM KCl; 10 mM Tris-HCl pH 8.3; 1.5 mM MgCl2; 0.2 mM of each dNTP; 0.4 μM of each primer and 0.625 units of Taq polymerase (TaKaRa, Japan). One hundred ng of genomic DNA was used as a template. An initial denaturing step at 95°C for 5 min was followed by 35 cycles at 95°C for 30 sec, 50°C for 1 min, and 72°C for 1 min. A final run at 72°C for 5 min completed the program. PCR products were separated by electrophoresis for 30 min at 100 V in a 1.5% agarose gel in 1× TBE, stained with ethidium bromide and visualized under UV light.

PCR products were cloned with the use of an Original TA Cloning Kit (Invitrogen, USA). Nucleotide sequences were determined using a ThermoSequenase II dye terminator cycle sequencing kit (Amersham Pharmacia, USA) including M13 forward primer and M13 reverse primer (TaKaRa) with an ABI PRISM 377 DNA Sequencer (PE Applied Biosystems, USA).

**RESULTS**

Application of primers 2550F and 2718R to eight raptors including Accipitridae and Falconidae resulted in successful identification of sex for all species tested in this study (Table 1 and Fig. 1). In five of the species (black kite, golden eagle and mountain hawk eagle in Accipitridae and peregrine falcon and kestrel in Falconidae), the males had a single band while the females two. However, in the remaining species (goshawk, marsh harrier and Eurasian sparrow hawk), the females showed only a single band, as did the males as well. In these species, the detected band in females was easily discriminable from that in the males. The results of sex identification using DNA extracted from a few feathers of black kite are shown in Table 1.

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Table 1. Identification of sex using primers 2550F and 2718R in 8 raptors belonging to Accipitridae and Falconidae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Amplified gene copy</th>
<th>Number of animals tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Black kite</td>
<td>Z, W</td>
<td>Z</td>
</tr>
<tr>
<td>Goshawk</td>
<td>W</td>
<td>Z</td>
</tr>
<tr>
<td>Marsh harrier</td>
<td>W</td>
<td>Z</td>
</tr>
<tr>
<td>Golden eagle</td>
<td>Z, W</td>
<td>Z</td>
</tr>
<tr>
<td>Eurasian sparrow hawk</td>
<td>W</td>
<td>Z</td>
</tr>
<tr>
<td>Mountain hawk-eagle</td>
<td>Z, W</td>
<td>Z</td>
</tr>
<tr>
<td>Peregrine falcon</td>
<td>W, Z</td>
<td>Z</td>
</tr>
<tr>
<td>Kestrel</td>
<td>W</td>
<td>Z</td>
</tr>
</tbody>
</table>

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Sexing in Raptors

Fig. 1  Agarose gel electrophoresis of PCR amplification products in CHDI. The products obtained from individuals of known sex are indicated. M: standard marker. For each species female (uneven lanes) and male (even lanes) are shown alternatively. Lane 1 and 2, black kite; 3 and 4, goshawk; 5 and 6, marsh harrier; 7 and 8, golden eagle; 9 and 10, Eurasian sparrow hawk; 11 and 12, mountain hawk eagle; 13 and 14, peregrine falcon and 15 and 16, kestrel.

Fig. 2  Agarose gel electrophoresis of PCR amplification products using DNA derived from a few feathers in the black kites. M: standard marker. Lane 1, 2, 5 and 6 are the PCR product in females. 3, 4, 7 and 8 are that in males.

<table>
<thead>
<tr>
<th>Species</th>
<th>Length (bp)</th>
<th>Accession number</th>
<th>Length (bp)</th>
<th>Accession number</th>
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</thead>
<tbody>
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<td>Black kite</td>
<td>655</td>
<td>AB112957</td>
<td>411</td>
<td>AB112949</td>
</tr>
<tr>
<td>Goshawk</td>
<td>631</td>
<td>AB112952</td>
<td>411</td>
<td>AB112944</td>
</tr>
<tr>
<td>Marsh harrier</td>
<td>628</td>
<td>AB112954</td>
<td>411</td>
<td>AB112946</td>
</tr>
<tr>
<td>Golden eagle</td>
<td>645</td>
<td>AB112951</td>
<td>412</td>
<td>AB112943</td>
</tr>
<tr>
<td>Eurasian sparrow hawk</td>
<td>632</td>
<td>AB112953</td>
<td>412</td>
<td>AB112945</td>
</tr>
<tr>
<td>Mountain hawk eagle</td>
<td>642</td>
<td>AB112958</td>
<td>412</td>
<td>AB112950</td>
</tr>
<tr>
<td>Peregrine falcon</td>
<td>619</td>
<td>AB112955</td>
<td>422</td>
<td>AB112947</td>
</tr>
<tr>
<td>Kestrel</td>
<td>606</td>
<td>AB112956</td>
<td>422</td>
<td>AB112948</td>
</tr>
</tbody>
</table>
in Fig. 2. All the samples were able to be amplified and sexed successfully.

Table 2 shows sizes of PCR-amplified fragment of CHDIW and CHDIZ by sequence analysis in all of the eight species examined, additionally the sequences of region of exon was shown in Appendix by reference. The sequence data were registered in the DDBJ/EMBL/GenBank databases. CHDIW fragments varied between 411 and 422 bp in size, and CHDIZ fragments between 606 and 655 bp (removing primer sites).

**DISCUSSION**

Our results show that 2550F and 2718R primers targeting CHDI genes are useful for identification of sex in Falconiformes. In theory, the sexing method using these primers expects that the males have a single band while the females have two bands because of difference in length between CHDIW and CHDIZ [8]. This study also demonstrates that amplification by these primer pairs produced one band in males and two bands in females (black kite, golden eagle, mountain hawk eagle, peregrine falcon and kestrel). However, in the result of the species (goshawk, marsh harrier and Eurasian sparrow hawk), unexpected results were obtained and only a single band was detected in the female as well. In these species, the PCR product derived from CHDIZ was not detected in the females. This may be because the shorter CHDIW gene was amplified preferentially compared with the longer CHDIZ or because amplification efficiencies of CHDIZ were lessened by the mutations on primer sites in CHDIZ. In these species, however, individuals could be sexed easily, because the difference of the size between the CHDIW and CHDIZ genes was large (Fig. 1).

In the case where DNA was extracted from a few feathers, sexing was successful in all the individuals (Fig. 2). This method had high amplification efficiency at PCR, perhaps because of independency from template/primer mismatches unlike the conditions seen in the ARMS. Therefore, it was thought that the favorable results were obtained even from a small DNA source. This indicates that PCR with 2550F/2718R primers is a notably effective method for conservation of rare raptors, particularly for studies on wild raptors.

Table 2 shows length of products amplified by 2550F/2718R primers from sequence analysis. With 2550F/2718 primers, the differences in size of amplified fragments between the CHDIW gene and CHDIZ gene in eight species was large (184 bp in peregrine falcon to 244 bp in black kite), compared with that with P2/P8 primers, which was reported to be 2 bp in the black kite to 20 bp in the peregrine falcon and kestrel [6]. The difference in size between the products amplified by the 2550F/2718R primers should normally make it feasible to use agarose gels. Moreover, it has been reported that there are length polymorphisms in amplified regions using P2/P8 primers [10], and these polymorphisms make it difficult to sex individuals. On the other hand, the large difference between the sizes of the two PCR products obtained using 2550F/2718R primers may have minimized errors in identifying sex of individuals, although the amplified region was larger and thus more polymorphisms could be detected than when using P2/P8 primers.

Although the very small number of individuals examined in this study may show the necessity for further investigation on a larger scale, this method has proven useful for sex identification in many species across a wide range of genera [8]. Moreover, the fact that the sex identified by this method was in agreement with that defined anatomically in the wide range of Falconiformes tested demonstrates that this method may be applicable to many raptors.

Our ultimate goal is conservation of endangered raptors. Major causes for reduction of population size are destruction of habitat and environmental deterioration by human disturbance, thus, habitat conservation is the most important factor for species management. However, conservation of each individual and/or population may also contribute much to species conservation. Our result, therefore, provides useful information for any future research and conservation efforts for rare raptors, since sex of individuals offer varied and valuable knowledge. The method based on PCR using 2550F/2718R primers may serve as a useful tool to carry out any future research aimed at conservation of rare raptors.

**ACKNOWLEDGEMENTS**

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**要旨**

タカ目の多くの種は他の多くの鳥類と同様に外部形態から性判断をすることが困難である。この問題は、タカ目に関する生態学的研究を妨げ、保存のための計画を作製することを困難にする。そのため、タカ目におけるルート判別方法の開発が望まれていた。我々は、CHDIW と CHDIZ の遺伝子間のイントリューチャングの選び方を利用する方法を用いて、日本に生息する8種類のタカ目の性判断を試みた。今回用いた方法は、これまでに開発された他の性判断方法よりも容易で迅速に行うことができる。また、今回調査したすべての種においてルート判別が可能であった。さらには、わずかなサンプルから抽出したDNAからでも性判断が可能であり、この方法が野生個体群の研究に適用することが可能であることが示唆された。結論として、この研究において用いた方法は、タカ目の性判断に非常に有用であり、希少タカ目の将来の保存に大きな価値があると考えられた。

**キーワード**: 性判断, タカ目, Polymerease Chain Reaction (PCR), Chromo-helicase-DNA binding protein gene (CHD)

**REFERENCES**

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Appendix  Sequences of CHD1 gene. The illustrated sequences include exons while the poorly conserved intron has been removed; N (nucleotide) and a number indicate the size of this region. Underlined sequences indicate primer sequences. Asterisks indicate conserved sequences among CHD1W and CHD1Z of eight raptors. BK: black kite; MH: marsh harrier; GE: golden eagle; SP: Eurasian sparrow hawk; MHE: mountain hawk eagle.

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