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Sex Identification by Alternative Polymerase Chain Reaction Methods in Falconiformes

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ABSTRACT—A number of avian species are difficult to sex morphologically, especially as nestlings. Like other avian species, many species of Falconiformes are sexually monomorphic. Therefore, it is desirable that new methods based on DNA analysis are established in Falconiformes and other sexual monomorphic species. We identified sex in Falconiformes by two alternative methods. First, we used a sexing method based on the intronic length variation between *CHD1W* and *CHD1Z* using primers flanking the intron. In this method, two species of Falconidae could be identified for sexing. However, six species of Accipitridae could not, because they have few length variations. The second method used was based on differences in sequences between *CHD1W* and *CHD1Z*. From sequence analysis, a 3'-terminal mismatch primer on point mutation conserved among Falconiformes was designed, and identification of sex with the amplification refractory mutation system (ARMS) was performed. This method could identify sex in all species tested. In addition, because the 3'-terminal mismatch primer was designed on a point mutation conserved among Falconiformes, ARMS with these primers may identify sex in all Falconiformes. These are simple and rapid sexing methods, since only polymerase chain reaction (PCR) and agarose electrophoresis are required. In conclusion, sex identification by an alternative PCR approach based on intronic length variation and on differences in sequences between *CHD1W* and *CHD1Z* proved applicable to and useful for Falconiformes.

Key words: Falconiformes, sex identification, chromo-helicase-DNA binding protein (CHD) gene, PCR, amplification refractory mutation system (ARMS)

INTRODUCTION

It is difficult to sex many avian species from external morphology. It has been estimated that the sex of adult individuals unidentified in over 50% of the world's avian species, and in nestlings this rate is even higher (Griffiths *et al*., 1998). Sex identification is of considerable importance for studies of avian behavior, ecology, evolutionary biology and genetics, and for the conservation of endangered avian species.

Like many other avian species, raptors do not display a high degree of sexual dimorphism either in size or plumage characters even as adults. Since diurnal birds of prey (raptors) such as the golden eagle or mountain hawk-eagle are located at the top of an ecosystem, they tend to be readily impacted by contamination and destruction of their habitat. With the proliferation of human activities, the number of individuals decreases remarkably. Some species are already classified as endangered by the Red List in Japan (Ministry of the Environment, 1998), and about 40 species of raptors are on the verge of extinction (IUCN, 2002). Therefore, conservation of these species is desirable, and the need for scientific studies, including sexing, is urgent.

Because male birds have two identical sex chromosomes (ZZ), whereas females are heterogametic (ZW), sex identification can be made by detection of the W chromosome or DNA sequences present on the W chromosome (Tone *et al*., 1984). So far, cytogenetic approaches like karyotyping or flow cytometry (Nakamura *et al*., 1990; De Vita *et al*., 1994) and various molecular genetic techniques have been used in sex identification of birds. The latter includes two general strategies; one is hybridization with oligonucleotide or W-specific DNA probes (Dvorak *et al*., 1992; Millar *et al*., 1992, 1996; Longmire *et al*., 1991; Griffiths and Holland, 1990; Rabenold *et al*., 1991), and the other is a

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method based on polymerase chain reaction (PCR) such as random amplified polymorphic DNA (RAPD) (Griffiths and Tiwari, 1993; Lessells and Mateman, 1998; Bello and Sánchez, 1999), amplified fragment length polymorphism (AFLP) (Griffiths and Orr, 1999), and amplification of microsatellite locus (Wink *et al*., 1998; Nesje and Røed, 2000). PCR is particularly useful in sex identification because it requires only a minute DNA sample from a single feather or a drop of blood. The discovery of a chromo-helicase-DNA binding protein (CHD1) gene that was conserved on an avian W chromosome enabled sex identification in most avian species. In paticular, a method with PCR primers flanking introns which vary in size between *CHD1W* and *CHD1Z* (Ellegren, 1996; Griffiths *et al*., 1998; Kahn *et al*., 1998; Fridolfsson and Ellegren, 1999) is simpler, quicker, and more valuable than other methods. However, to date this method is not applicable to all species. Thus, when this method is applied to untested species an investigation of utility is called for.

In this study, we established a new method of sex identification in Falconiformes by alternative PCR methods; one based on intronic length variation and the other on differences in sequences between *CHD1W* and *CHD1Z*, the latter referred to as amplification refractory mutation system (ARMS). ARMS is a technique based on PCR, and can only amplify specific alleles by using a 3'-termial mismatch primer that pairs with only specific alleles (Newton *et al*., 1989).

MATERIALS AND METHODS

Genomic DNA was extracted from kidney or pectoral muscle taken from the black kite (*Milvus migrans*: number of females, f=10; number of males, m=10), Northern goshawk (*Accipiter gentilis*: f=2, m=4), Eastern marsh harrier (*Circus spilonotus*: f=1, m=1), golden eagle (*Aquila chrysaetos*: f=2, m=2), Eurasian sparrowhawk (*Accipiter nisus*: f=1, m=1), mountain hawk eagle (*Spizaetus nipalensis*: f=2, m=1), peregrine falcon (*Falco peregrinus*: f=2, m=1) and common kestrel (*Falco tinnunculus*: f=3, m=3). All samples were presexed by anatomical examination.

A part of the *CHD1* gene was amplified using the primer set of P2 and P8 which was described by Griffiths *et al*. (1998) or P2 and NP (5'-GAGAAACTGTGCAAAACAG-3'). PCR amplification was carried out in a total volume of 25 μl. The final reaction condition were as follows: 50 mM KCl; 10 mM Tris-HCl pH 8.3; 1.5mM $MgCl_2$; 0.2 mM of each dNTP; 0.4 μM of each primer and 0.625 units of *Taq* polymerase (TaKaRa). 100 ng of genomic DNA was used as a template. An initial denaturing step at 94°C for 1 min 30 s was followed by 35 cycles at 45–48°C (P2 and P8) or 48–52°C (P2 and NP) for 45 s, 72°C for 45 s, and 94°C for 30 s. A final run at 48–50°C for 1 min and 72°C for 5 min completed the program. PCR products were separated by electrophoresis for 60–80 min at 100 V in a 3% agarose gel in 1×TBE stained with ethidium bromide and visualized under UV light.

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

From obtained sequence data, a 3'- terminal mismatch primer (MP; 5'-AGTCACTATCAGATCCGGAA-3') was developed which detected only female-specific fragment-derived W chromosome. For sex identification, ARMS using three primers, P2, NP and MP, was performed. 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; 0.8 μM of P2 primer and 0.4 μM of NP and MP primer; and 0.625 units of *Taq* polymerase (TaKaRa). 100 ng of genomic DNA was used as a template. PCR reaction was performed as above. PCR products were separated by electrophoresis for 60 min at 100 V in a 3% agarose gel in 1×TBE stained with ethidium bromide and visualized under UV light.

RESULTS

The results of the sexing method based on intronic length polymorphism were shown in Fig. 1. This method was successful for two species of Falconidae, kestrel and falcon. In these species, the males have a single band while the females have two bands. Six species in Accipitridae (black kite, goshawk, marsh harrier, golden eagle, sparrowhawk and mountain hawk-eagle) could not easily sexed by this method, because only a single band in both sexes was detected by PCR with P2/NP primers (Fig. 1).

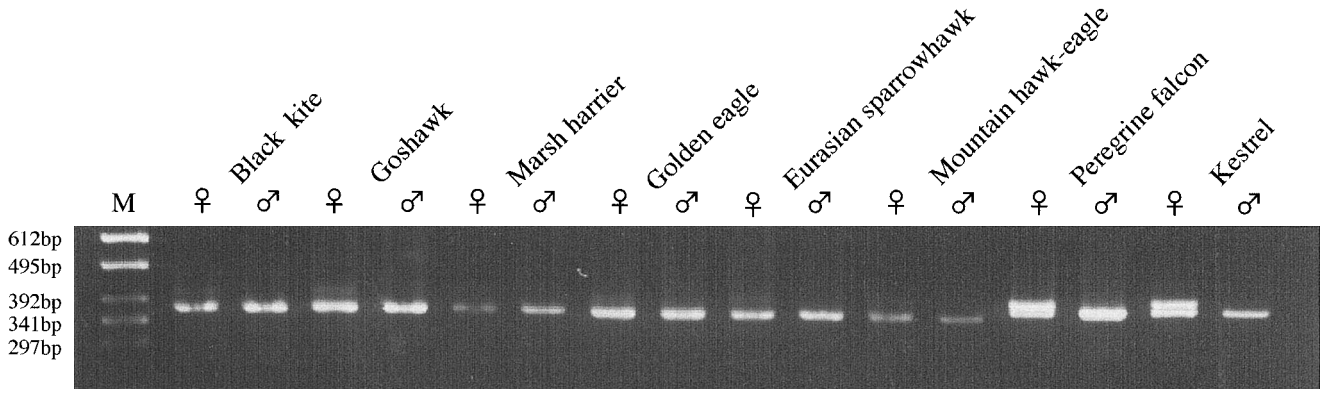

Fig. 1. Identification of sex by the methods based on intronic length polymorphism. The known-sex individuals are indicated. Falconidae, females have two bands, males have one. In Accipitridae, both sexes have a single band. P2/NP primer pair was used. M: Marker (Marker 5, Wako)

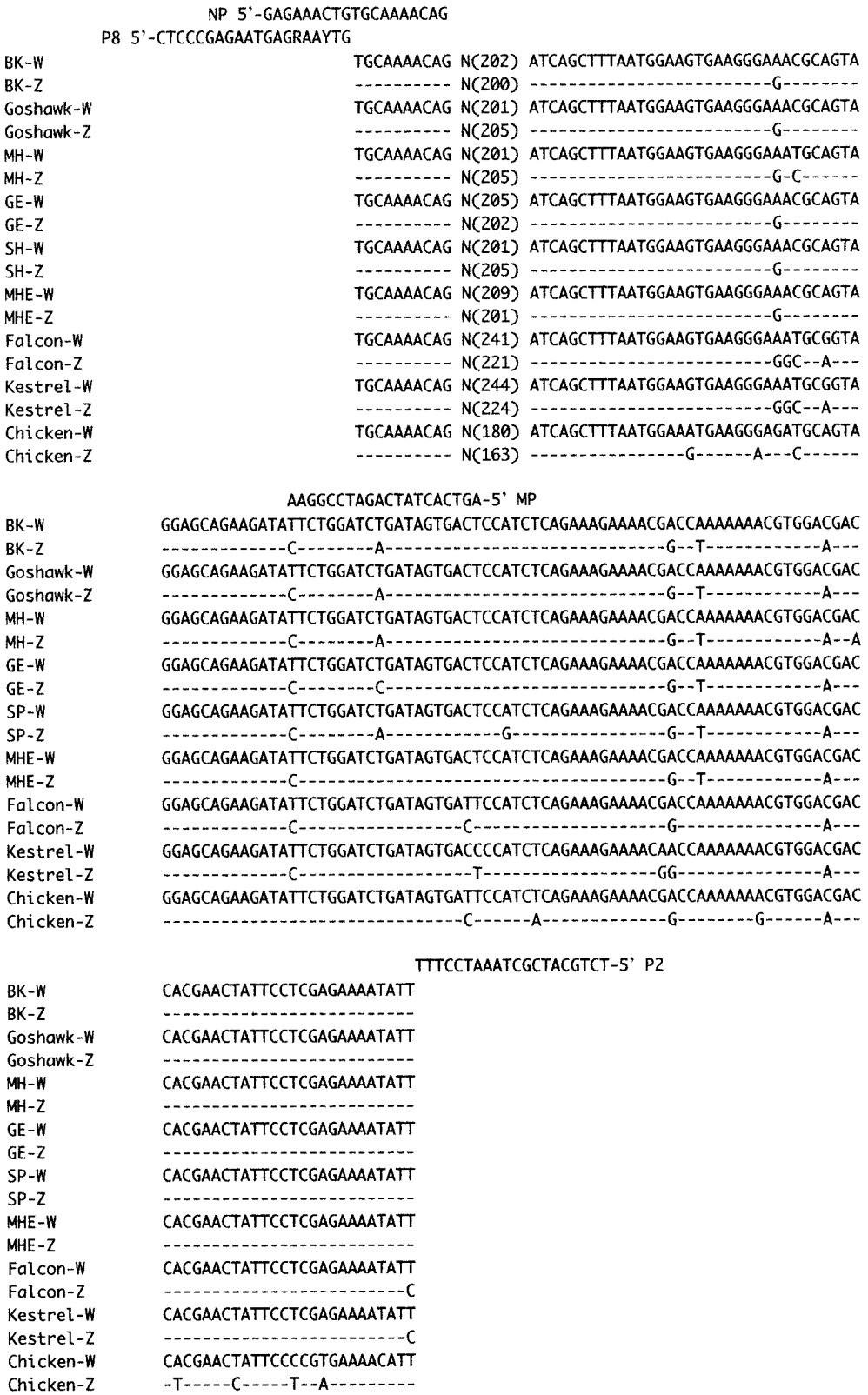

Fig. 2. Position and sequences of primers and sequences of *CHD1* gene. The illustrated sequences include exons while the poorly conserved intron has been removed; N (nucleotide) and a number indicate the size of this region. BK: Black kite; MH: Marsh harrier; GE: Golden eagle; SP: Sparrowhawk; MHE: Moutain hawk-eagle. P2, P8: primers was described Griffiths *et al.* (1998). NP, MP: primers was developed in this study. The respective Accession Numbers of *CHD1W* and *CHD1Z* in each species are as follows: *M.migrans* AB096141, AB096142; *A. gentilis* AB096143, AB096144; *C. spilonotus* AB096145, AB096146; *A. chrysaetos* AB096147, AB096148; *S. nipalensis* AB096149, AB096150; *A. nisus* AB096151, AB096152; *F. peregrinus* AB096153, AB096154; *F. tinnunculus* AB096155, AB096156.

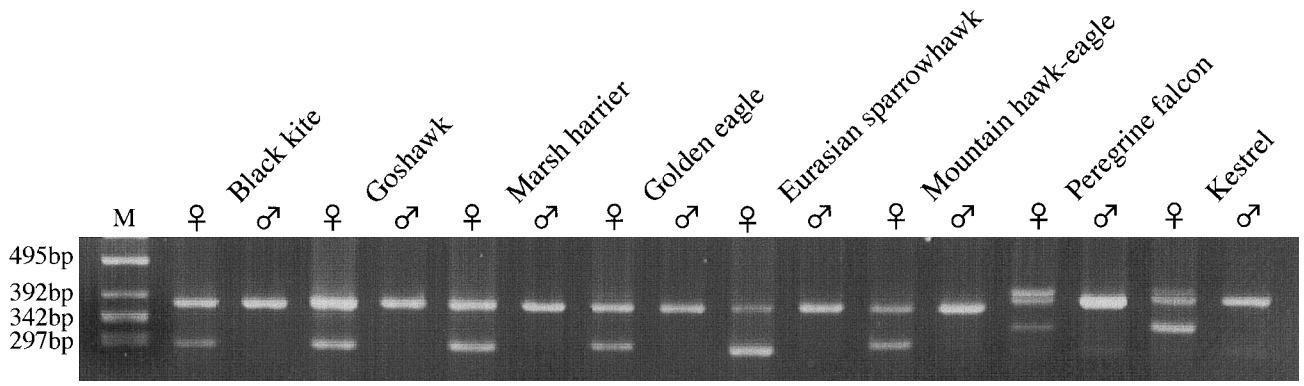

Fig. 3. Identification of sex with ARMS. The known sex of each individual is indicated. In Accipitridae, females have two bands and males have one. However, in Falconidae, females have three bands and males have one. P2/NP/MP primers were used. M: Marker (Marker 5, Wako)

To investigate the reason for the failure of the test in Accipitridae, sequence analyses were performed (Fig. 2). In Accipitridae, differences in size between DNA fragment-derived *CHD1W* and *CHD1Z* were 2-8 bp, against 20 bp in Falconidae.

In order to sex Accipitridae, we focused on the nucleotide difference between fragment-derived *CHD1W* and *CHD1Z*. There were three point mutations. A new primer (MP) that its 3' terminus is on point mutation conserved amongst Falconiformes was developed. ARMS with three primers (P2, NP and MP) expected that NP/MP detect female specific fragment derived *CHD1W*, and NP/P2 amplify fragment derived *CHD1Z* works as internal control. Sex identification by ARMS with these primers was successfully performed in all species tested (Fig. 3). The males have a single band in all species, while the females have two in Accipitridae or three in Falconidae.

DISCUSSION

A sex-determination gene such as *SRY* in mammals has not yet been found in birds. Indeed it is not even clear whether avian sex is determined by a dominant factor on the W chromosome or by a dosage-sensitive factor on the Z chromosome (Ellegren, 2001). Recent studies in molecular biology support both hypotheses; *PKCIW* that is W-linked supports the notion that the existence of W chromosome triggers female development (Hori *et al*., 2000; O'Neil *et al*., 2000) while *DMRT1* on Z chromosome supports the opinion that the number of Z chromosomes regulates sex (Nanda *et al*., 2000).

The gene on the female-specific W chromosome was only recently identified, and sexing methods had developed so far do not work across species. What is needed is a method that is universal for sex identification. The first gene on W chromosome, *CHD1*, can be used for sex identification in a wide range of species. However, the *CHD1* gene is present not only on W chromosome but also on Z chromosome. Sex identification with *CHD1* needs a way to distinguish between these two genes. Thus, an evolutionally conserved sequence such as a functional gene present only on W chromosome is required. However, no such sequence has yet been identified. The genes or sequences including *CHD1* found on W chromosome until now, such as *ATP5A1* (ATP synthase α-subunit; Fridolfsson *et al*., 1998), *Wpkci* (W-linked gene for the altered form of protein kinase C-interacting protein; Hori *et al*., 2000; O'Neill *et al*., 2000), *spindlin* (Itoh *et al*., 2001a) and EE0.6 related sequences (Itoh *et al*., 2001b) have their homologous copies on the Z chromosome. Therefore, identification of sex with these genes or sequences poses the same problem as *CHD1*.

Identification of sex with these genes is done with any of three methods; with restriction enzyme (Griffiths and Tiwari, 1995; Griffiths *et al*., 1996), with SSCP (Ellegren, 1996; Cortés *et al*., 1999), and based on intronic length polymorphism between two homologous copies (Ellegren, 1996; Griffiths *et al*., 1998; Kahn *et al*., 1998; Fridolfsson and Ellegren, 1999). The last method is the simplest and most rapid.

In this study, we used a method based on intronic length polymorphism between *CHD1W* and *CHD1Z* for sex identification of Falconiformes. P2/P8 primer pair detected a weak band in some species, especially in kestrel and falcon, although P2/NP primer pair detected strong signal band in all species (data not shown). This result suggested that P8 primer site is less conserved rather than NP primer site, and that the P2/NP primer pair may be able to identify the sex in more species. This method was successful for 2 species of Falconidae, but not successful for 6 species of Accipitridae (Fig. 1). The differences in size between *CHD1W* and *CHD1Z* are 2-8 bp in Accipitridea, against 20 bp in Falconidae. Indeed, it was reported that the merlin (*F. columbarius*) and hobby (*F. subbuteo*) have PCR products that differ by only one base (Nesje and Røed, 2000). In consideration of the separation ability of agarose gel and length of amplified product (about 400 bp), it is probably impossible to determine sex in Accipitridae by this method with agarose gel. It is difficult to sex these species, except for mountain

hawk-eagle, without gel for sequence analysis, which is laborious and time-consuming.

We performed identification of sex with ARMS, which is also referred to as allele-specific PCR (ASPCR) (Ugozzoli and Wallace, 1991), mismatch amplification mutation assay (MAMA) (Cha *et al*., 1992), and PCR amplification of specific alleles (PASA) (Dutton and Sommer, 1991). ARMS is a technique based on PCR, and can accomplish allele-specific amplification by using a 3'-termial mismatch primer; it has been used for detection of genetic disease (Newton *et al*., 1989) and for genome mapping (Smith *et al*., 1996, 1997). ARMS has several advantages. First, it requires only PCR, which is easily and rapidly performed. Second, if there is not a restriction enzyme-recognizing site, it can distinguish between two homologous copies. In the present study, identification of sex with ARMS was successful in all species tested (Fig. 3). Because it amplifies not only *CHD1W* by NP/MP primer pair but also simultaneously *CHD1Z* by P2/NP primer pair, false negative problems are avoided at the same time. Indeed, since there are about 100 bp of difference in size between *CHD1W* and *CHD1Z*, these fragments can be clearly separated by electrophoresis on agarose gel. ARMS using three primers is useful for sex identification in Falconiformes, especially in species that cannot be sexed by the method based on intronic length polymorphism. In this study, as the point mutation between *CHD1W* and *CHD1Z* exists in both the Accipitridae and Falconidae, it is thought that this point mutation is conserved throughout Falconiformes, suggesting that identification of sex by ARMS could be applied to all species of Falconiformes, including the merlin and hobby. Therefore, this result provides information useful in any future conservation plan for rare raptors.

Our results showed that the alternative PCR approach, based on intronic length polymorphism and a few sequence differences between *CHD1W* and *CHD1Z*, provides a simple, useful and rapid technique for sex identification, since both methods require only PCR and agarose gel electrophoresis. If there is a conserved point mutation in species that can not be sexed by a method based on intronic length polymorphism, these alternative sexing methods may be universally applicable over a wide range of species in the future.

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