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Application of Two Complementary Molecular Sexing Methods for East Asian Bird Species

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ABSTRACT

Numerous avian species are sexually monomorphic, especially nestlings, posing difficulties in determining their sex by morphological traits alone. Despite the difficulties, sex identification of birds is an essential part of *ex situ* conservation breeding programs for endangered species, and the sex of individuals is an integral component of information required for research concerning ecology, behavior, genetics, and conservation biology. Therefore, molecular sexing methods are gaining increasing attention as an aid in study and conservation of many bird species. An amplification refractory mutation system (ARMS) method using the sequence differences between *CHD1Z* and *CHD1W* was applied for sex identification of 29 East Asian bird species to test the applicability of the method for a wide range of wild bird species. The sex of 25 bird species was successfully identified by the ARMS method using the P2/NP/MP primer set, and the sex of four additional species was identified by another primer set P2/P8. The methods were applied to sex 124 individual samples that were preliminarily sexed by only morphological characteristics of the birds. Of the specimens tested, 16 (12.9%) individuals showed different gender from the preliminary data. This showed that sex identification of monomorphic avian species by external morphology alone has a high risk of misidentification. Therefore, alternative molecular sexing methods are recommended in the sexing process where monomorphic individuals, damaged carcasses or parts of carcasses are involved.

Key words: Chromo-Helicase-DNA binding (*CHD*) gene, sex identification, birds.

INTRODUCTION

In many birds, individuals can not be reliably sexed in their earlier life stage due to sexual monomorphism in their external morphological characters (Dubiec and Zagalska-Neubauer, 2006). Nestlings, in particular, pose greater difficulties in determining their sex based solely on their morphological traits because their external characters and other signs are not yet fully developed and it is also practically impossible to discern their sexual organs through laparoscopy.

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Despite the difficulties, sex identification of birds is an essential part of *ex situ* conservation breeding programs for endangered species and it is essential information in various fields of research such as ecology, behavior, genetics, and conservation biology of avian species. Up to now, there have been various approaches being used for sex identification other than molecular techniques for monomorphic birds including avian laparoscopy, biochemical analysis, and cytogenetic analysis (Dubiec and Zagalska-Neubauer, 2006; Richner, 1989). However, these approaches are usually time-consuming or invasive to individuals.

Sexual monomorphism is not the only obstacle in sexual identification of birds. Ecological or genetic studies utilizing damaged carcasses suffer from difficulties in distinguishing the sex of individuals. One of the fields that has to deal with such difficulty are studies concerning vehicle mortality which have been gaining increasing interest among researchers recently (Choi and Park, 2006). The external character and internal organs of road killed specimens are usually partially decomposed or are critically damaged preventing the identification of sex by morphology alone. Other cases that require sex identification through non-morphological clues are genetic studies using DNA from non-invasive genetic samples (e.g., feathers, feces, egg shells) or body parts found in the field. Under the situations where only a small part of an individual is available for sex identification, molecular techniques are the only option for sexing (Hattori et al., 2003; Robertson et al., 1999). Accordingly, a number of recent studies focused on developing efficient molecular methods for identification of sex, and molecular sexing methods are gaining increasing attention as an aid in research and conservation of many bird species (Dubiec and Zagalska-Neubauer, 2006; Cerit and Avanus, 2007).

The chromosomal sex determination system of bird is different from that of mammals. In birds, males have two identical sex chromosomes (ZZ), whereas females have heterogametic chromosomes (ZW). The reverse is true for mammals in which males have heterogametic chromosomes (XY), and females are homogametic (XX). In mammals, sex-specific sequences of *Sry*, *ZFY/ZFX*, or amelogenine genes play an important role in identifying sex by molecular techniques (Bryja and Konečný, 2003; Williams et al., 2004; Yamamoto et al., 2002). For determination of sex in birds, various experimental methods using sex-specific genetic markers such as *CHD1W/CHD1Z* (Ellegren, 1996; Griffiths et al., 1998; Fridolfsson and Ellegren, 1999) and EE0.6 (Itoh et al., 2001), and *Wpkci* (Hori et al., 2000; O'Neill et al., 2000) are detected by PCR or Southern blot analysis. Griffiths et al. (1998) reported that 27 species of birds could be sexed successfully using one set of primers, P2/P8 by changing PCR conditions (annealing temperature and $MgCl_2$ concentration). More recently, Ito et al. (2003) reported an amplification refractory mutation system (ARMS)-based sexing method using the sequence differences between *CHD1Z* and *CHD1W*, and showed that sex of Falconiformes could be efficiently determined by the alternative PCR-based approach to complement the method by Griffiths et al. (1998). Additionally, several researchers have described assigning the sex of monomorphic birds using PCR-RFLP *Nla*III, Intron PCR assay and CSL (crane sex-linked DNA) that are utilized in conservation projects such as breeding programs and field research (Duan and Fuerst, 2001; Bermúdez-Humarán et al., 2002). Molecular sexing enables researchers to identify sex using only a feather or drop of blood (Sacchi et al., 2004), which is often the only available samples for endangered bird species. In other cases of endangered species, bird feces have been used for DNA extraction and sex identification (Robertson et al., 1999).

In the present study, we extended the approach developed by Ito et al. (2003), which had been applied only to Falconiformes. We tested the applicability of the method to a wider range of bird species occurring in Korea and in East Asia; 36 species including 15 natural monuments species designated by the Korea Cultural Heritage Administration and 13 endangered species designated by the Korea Ministry of Environment. We elucidated why the method by Ito et al. (2003) is not applicable to some species and showed an alternative approach would be useful for molecular sexing of a wide variety of bird species (Griffiths et al., 1998).

Table 1. Species and number of samples analyzed in this study.

Order	Family	Scientific name	Common name	Sample size	
				Male	Female
Procellariiformes	Procellariidae	*Calonectris leucomelas**	Streaked shearwater	1	0
Ciconiformes	Ardeidae	*Ardea cinerea*	Grey heron	2	3
		Egretta alba	Great egret	1	1
		Egretta garzetta	Little egret	1	2
		Nycticorax nycticorax	Black-crowned night heron	3	2
	Threskiornithidae	*Platalea minor**	Black faced spoonbill	1	0
Anseriformes	Anatidae	*Anser albifrons*	White-fronted Goose	5	7
		Anas formosa	Baikal Teal	11	5
		Anas poecilorhyncha	Spot-billed duct	1	2
Falconiformes	Accipitridae	*Haliaeetus albicilla*	White tailed sea eagle	1	1
		Haliaeetus pelagicus	Stella's sea eagle	1	1
		Aegypius monachus	Cinereous vulture	12	15
		Accipiter soloensis	Chinese sparrowhawk	1	2
		Accipiter nisus	Eurasian sparrowhawk	2	2
		Accipiter gentilis	Northern goshawk	2	2
		Buteo buteo	Common buzzard	2	3
	Falconidae	*Falco tinnunculus*	Common kestrel	3	6
Galliformes	Phasianidae	*Phasianus colchicus*	Ring-necked Pheasant	4	2
Gruiformes	Gruidae	*Grus monacha*	Hooded carne	1	1
		*Grus japonensis**	Red crowned crane	0	1
		*Grus vipio**	White napped crane	1	0
Charadriiformes	Scolopacidae	*Scolopax rusticola**	Eurasian Woodcock	0	3
Columbiformes	Columbidae	*Streptopelia orientalis*	Rufous turtle dove	3	1
Strigiformes	Strigidae	*Otus scops*	Eurasian scops owl	1	4
		Otus lempiji	Collared scops owl	1	5
		Bubo bubo	Eurasian eagle owl	7	10
		Strix aluco	Tawny Owl	1	1
		*Strix uralensis**	Ural Owl	0	2
		Ninox scutulata	Brown Hawk Owl	3	3
Caprimulgiformes	Caprimulgidae	*Caprimulgus indicus*	Jungle nightjar	1	1
Piciformes	Picidae	*Picus canus**	Grey headed woodpecker	0	2
Passeriformes	Turdidae	*Zoothera dauma*	White's Thrush	2	2
	Sylviidae	*Acrocephalus orientalis*	Oriental Great Reed Warbler	3	2
	Paridae	*Parus major*	Great Tit	6	2
	Emberizidae	*Emberiza chrysophrys*	Yellow-browed Bunting	1	3
		Emberiza elegans	Yellow-throated Bunting	5	4

* Samples from only one sex were available for these species.

MATERIALS AND METHODS

Genomic DNA was extracted from the tissue and blood samples deposited at the Conservation Genome Resource Bank for Korean Wildlife (CGRB; www.cgrb.org) using the DNeasy Tissue Kit (Qiagen) and phenol-chloroform extraction method (Sambrook et al., 1989). A sum of 193 specimens from 36 species used in this study is described in **Table 1**.

Of the 193 specimens, 182 samples represent both sexes from 29 species, but samples of only one sex were available for the remaining 7 species (11 specimens).

We initially used 65 samples from 36 species of known sex, verified by external morphology and anatomical examination to test the molecular sexing method. We further examined additional 126 individual tissue samples deposited in CGRB whose sex were unclear due to monomorphic morphology and heavily spoiled condition of carcasses. These additional samples had only preliminary sex identification data based on obscure external morphology.

A primer set of P2 (5'-TCTGCATCGCTAAATCCTTT-3')/NP (5'-GAGAAACTGTGCAAAACAG-3')/MP (5'-AGTCACTATCAGATCCGGAA-3') was utilized in this study (Ito et al., 2003; and Griffiths et al., 1998). The primer set can only detect female-specific *CHD1W* gene by including 3'-terminal mismatch primer MP. Alternatively, another primer set of P2 and P8 (5'-CTCCCAAGGATGAGRAAYTG-3') was used for bird species whose sex can not be determined by the former primer set (Griffiths et al., 1998). The latter primer set was designed to detect the intronic length difference between *CHD1W* and *CHD1Z* in many birds.

The final concentrations of 20 μl PCR mixture were as follows: 2.5 mM each dNTP, 0.5 mM of each primer, 1 unit of *i*-star *Taq* polymerase (iNtRON Biotechnology, South Korea), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 ng of genomic DNA as template. An initial denaturing step at 94℃ for 4 min was followed by 35 cycles at 94℃ for 30 sec, 48 ~ 51℃ for 30 sec, 72℃ for 40 sec, and a final run at 72℃ for 4 min completed the program. PCR products were separated by 2% agarose gel electrophoresis in 0.5 × TBE for 40-50 min at 100 V and visualized by staining with ethidium bromide.

To investigate the molecular explanation to the failure of P2/NP/MP primer set to differentiate some species of Anatidea (*Anser albifrons, Anas formosa, Anas poecilorhyncha*) and Phasianidae (*Phasianus colchicus*), the fragment of *CHD1Z* was amplified by a primer pair, NP and P2 and purified with the Zymoclean Gel DNA recovery kit (Zymo research, USA). The purified DNA was sequenced in both directions using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., USA), followed by detection on an ABI 3730 XL instrument. Primers used for the sequencing were the same as those used for the amplification.

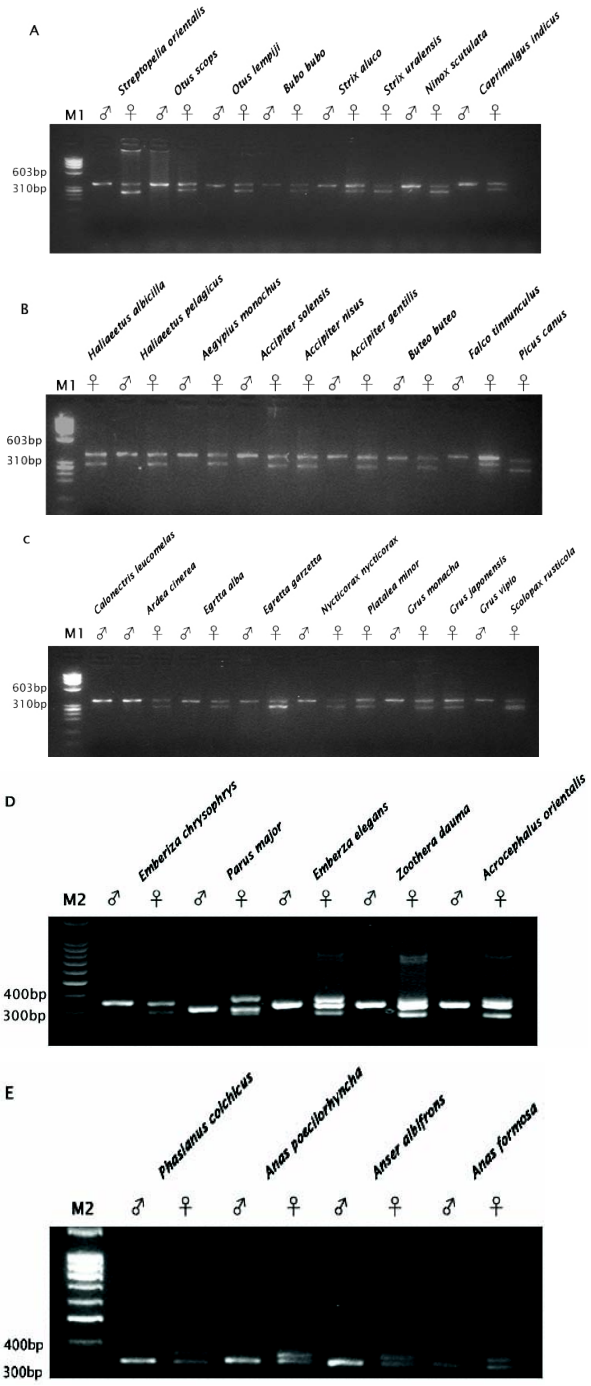

Figure 1. Polymerase chain reaction–based sex identification using P2/NP/MP (A–D) and P2/P8 (E) primer sets. P2/NP/MP primer set (Ito et al., 2003) was used in 32 bird species and P2/P8 primer set (Griffiths et al., 1998) was used in four species where P2/NP/MP primer set failed to identify sex. Males have a single band whereas females have double or triple bands. The known sex of individuals is indicated above the gel. Seven species were tested with samples from only one sex (**Table 1**). M1, Molecular size marker (Ø 174/*Hae*III Promega, USA); M2, Molecular size marker (100 bp ladder; Promega, USA).

RESULTS

As shown in Figure 1, the PCR method using a primer set of P2/NP/MP was able to correctly identify the sex of 25 species across seven different orders successfully (single band in males, and double or triple bands in females; Fig. 1A–D). The band patterns of seven species with only single sex samples were also consistent with the known sex of the samples, but the results need further verification of the methods with samples of the other sex. However, both sexes of four species, *P. colchicus A. albifrons, A. formosa* and *A. poecilorhyncha* produced double bands, and the sizes of the bands were identical in males and females. Sequence analyses (GeneBank Accession nos EU196234-EU196237) were performed to elucidate the reason for amplification of two bands in the four species above. The result showed that, at the complementary base of 3'-terminal ARMS primer site, both *CH1DW* and *CHD1Z* genes of the four species had thymine, whereas other species had cytosine in *CHD1Z* and thymine in *CHD1W* (Fig. 2). To determine the sex of the four species, another primer set, P2/P8, applied to a wide range of bird species, was utilized (Griffiiths et al., 1998) produced a single band for males and double bands for females (Fig. 1E).

Thus, the band patterns of all 65 samples from 36 species were in agreement with the patterns expected from the known sex of the samples. The sex of 126 additional samples of individual birds with only preliminary sex data was determined by the alternative methods. Of the 126 samples tested, the results of 110 (87.3%) concurred with the preliminary identification of morphological traits, but 16 (12.7%) did not agree with the preliminary identification (data not shown).

DISCUSSION

Recent accelerated rates of environment deterioration due to human activities have caused natural habitat losses and decline of wildlife populations. As a consequence of this, the concerns about conservation for wildlife and their habitat have gradually increased in South Korea and this has leaded to building conservation projects and efforts such as reintroduction projects of Asiatic black bear and goral, rehabilitation center for oriental white stork, and conservation genome resource bank. Conservation of endangered wildlife species requires fundamental knowledge of ecology and biology of the species, and identification of species, sex and individuals by molecular genetic techniques are increasingly being utilized for this purpose (Deyoung and Honeycutt, 2005). Sex identification using molecular techniques is one of the better

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M. migrans          CHD1Z ACTGACTT N(144) GTAGCTTTGAACTACCTATTCTGAAATTCCAGATCAGCTTT
M. migrans          CHD1W GACC.AC. N(147) ..............T..A......................
A. formosa          CHD1Z ....G... N(104) ....T........G.T.......................
A. poecilorhyncha   CHD1Z ....G... N(106) ....T........G.T.......................
A. albifrons        CHD1Z ........ N(106) ....T.C......GTT.......................
P. colchicus        CHD1Z T...T... N(108) ....T......T.-.T.G.....................

                                                                MP 3'-AAGGCCTAGACTATCACTGA-5'
M. migrans          CHD1Z AATGGAAGTGAAGGGAGACGCAGTAGGAGCAGAAGATACTCTGGATCAGATAGTGACT
M. migrans          CHD1W ..............A.....................T........T.........
A. formosa          CHD1Z ..............A.....................T........T.........
A. poecilorhyncha   CHD1Z ..............A.....................T........T.........
A. albifrons        CHD1Z ..............A.....................T........T.........
P. colchicus        CHD1Z ..............A.....T.................T........T.........

M. migrans          CHD1Z CCATCTCAGAAAGAAAACGGCCTAAAAAACGTGGAAGACCACGAACTATTCCTC
M. migrans          CHD1W ...................A..A............C...................
A. formosa          CHD1Z .....A.G...........T..A.....G.........................
A. poecilorhyncha   CHD1Z .....A.G...........T..A.....G.........................
A. albifrons        CHD1Z .....A.G...........T..A.....G.........................
P. colchicus        CHD1Z .....A.............A.....G.....G.....T.............
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Figure 2. Comparison of partial *CHD1Z* and *CHD1W* genes in four species. The nucleotide sequences of partial *CHD1Z* and *CHD1W* genes were compared in four species where sex identification was unsuccessful with the P2/MP/NP primer set. The poorly conserved region was eliminated and represented by N. Numbers in parenthesis indicate the length (bases) of this region. Nucleotide sequences of *CHD1Z* and *CHD1W* genes of *M. migrans* were from NCBI (AB096141 and AB096142).

examples of the practical applications of genetic techniques for conservation and research on wildlife species.

Several techniques have been developed for sex identification of bird species using molecular methods. Among them, one introduced by Griffiths et al. (1998) has been the most frequently used. It is a simple method, based on the intronic length polymorphism between two homologous *CHD* genes on Z and W chromosomes. Amplification of the *CHD1W* and *CHD1Z* genes produces two bands with sizes different enough to be distinguished on an agarose gel. However, in certain species, including Falconiformes and Strigiformes, Griffiths' approach needs time consuming and cumbersome steps such as restriction enzyme digestion and acrylamide gel electophoresis. The size difference between *CHD1Z* and *CHD1W* differ between species, 2-8 bp in Accipitridea and 20 bp in Falconidae (Ito et al., 2003). Nesje and Røed (2000) reported that the PCR product length difference between merlin (*F. columbarius*) and hobby (*F. subbuteo*) was only by one base. To complement this problem, an amplification refractory mutation system (ARMS) method using the sequence differences between *CHD1Z* and *CHD1W* was developed by Ito et al. (2003). So far, the method has been applied to Falconiformes only (Ito et al., 2003).

In the present study, the ARMS method by Ito et al. (2003) was utilized to determine the sex of 193 individuals of 36 East Asian species to test the applicability of the method in a wide range of bird species. The results showed that of the 29 species tested with samples of both sexes, the method was able to successfully determine the sex of 25 species which belong to 7 different orders: Ciconiformes, Falconiformes, Gruiformes, Columbiformes, Strigiformes, Caprimul-giformes, and Passeriformes (**Fig. 1 A–D**). This method was able to identify sex of specimens without the additional steps required in Griffiths' approach which didn't work particularly well for Strigiformes. It has been reported by Cheng et al. (2006) that black faced spoonbill (*Platalea minor*), one of the rarest species in the world, listed as 'critically endangered' on the IUCN red list, could not be sexed with Griffiths' method without extra steps because there was no large size difference between the bands. Although only one female of the species was tested in this experiment, our result showed two obvious bands for the female, and the sex of all four other species in the same Ciconiforme order could be identified with the ARMS method (**Fig. 1C**), *Platalea minor* is also expected to be easily sexed using the same method. Clearly, these results prove that the ARMS method is more convenient and time-saving than Griffiths' approach when applied to Strigiformes and Ciconiformes.

In three species of Anatidea (*A. albifrons, A. formosa, and A. poecilorhyncha*) and one species of Phasianidae (*P. colchicus*), the attempt to identify sex by ARMS method failed because two apparent bands of same sizes were observed on 2% agarose gel in both genders. On the basis of the principle of ARMS method, there would be a possibility that point mutations affect a primer binding site of *CHD1Z* homology of this target sequence where an inner primer can't amplify *CHD1Z* gene but can amplify *CHD1W* gene because of the nucleotide differences. The result of sequence pairwise comparison showed that there was no sequence difference at the ARMS site (**Fig. 2**). Therefore, the primer set examined in this study amplified two bands irrespective of the both genders tested, which indicates that the primer set, P2/NP/MP was not suitable to identify sex of the four species. An alternative primer set, P2/P8 was applied to solve this problem (Griffiths et al., 1998). With the alternative primer set, a single band in males and double bands in females were observed in the four species (**Fig. 1E**). These cases imply that when ARMS is applied to certain species for the first time, the results must be verified with sex determination data obtained by alternative methods (anatomical or morphological methods, other molecular approaches etc.) are essential for positive confirmation.

We performed molecular sex identification of 53 samples of *Otus scops* and *Otus lempiji*, designated a natural monuments species (No. 324) by the Korea Cultural Heritage Administration. The samples were obtained from road killed individuals and identification was performed by using the ARMS method, verified in this study, for the two species to see if sexual bias exists in the vehicle mortality rates of certain species. The preliminary results (unpublished, Choi et al.) suggest that there are indeed sexual biases in

some species mortality rates (data not shown). This emphasizes the utility of molecular sexing methods to answer certain ecological inquiries using body parts as the source of genetic samples. This is a case that well reveals the importance and a need of a molecular sexing method of unknown sex of birds.

Using the alternative molecular sexing methods verified in this study, we tested the accuracy of sex determination by morphological and anatomical characters. Using specimens stored in the CGRB which had preliminary sex information available was subjected to the test. Of the 126 specimens tested, results of 110 (87.3%) samples coincided with the result of morphological and anatomical sex identification but 16 (12.7%) individuals revealed a different gender than recorded in the tissue bank data. This illustrates the high risk of misidentification of sex for monomorphic avian species by external morphology alone. Therefore, molecular sexing methods are recommended in the sexing process where monomorphic individuals, damaged carcasses or parts of carcasses are involved to provide more reliable and complementary data. The method verified in this study has already been applied to the sex identification of pheasants (*Phasianus colchicus*) in a wildlife forensic investigation case of poaching (An et al., 2007).

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