Sex Determination of Superorder *Neognathae* (class *Aves*) by Molecular Genetics Methods

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Abstract

The aim of this study was optimization molecular genetic method for sex determination of superorder *Neognathae* from class *Aves.* The basis of the molecular-genetic methods was the amplification of a chromo-helicase DNA binding 1 (CHD) gene region, which is located in both sex chromozomes Z and W. Genomic DNA was isolated from whole blood and feathers by using commercial column kit QIAamp DNA Mini kit. The intron regions of CHDW and CHDZ genes were amplified by sex specific primers P2 and P8. The PCR method used in this study was based on two differences between CHDW and CHDZ genes. The one of these differences is restriction site for endonuclease *Hae*III located only in CHDZ and the second is the lenght polymorphism between CHDW and CHDZ where for the males was detected one band and for the females were detected two bands in 3.5 % agarose gel. These molecular-genetics methods were successfully used for sex determination in 36 species from superorder *Neognathae*.

Keywords: CHD gene, PCR method, sex determination, superorder *Neognathea*

1. Introduction

Sex determination of same species of superorder *Neognathea* is not clearly identified because they do not exhibit any external sexual dimorphism between males and females. But the problem with sex determination is present in many species of young birds from class *Aves*. The traditional techniques (laparotomy, cloacal examination or cytogenetic analyse) used for sex determination of monomorphic birds are time-consuming or requires well-trained stuff. In recent years some DNA-based tests were design for sex determination in many birds of superorder *Neognathea* that represent the most numerous superorder of class *Aves*. The most widely used molecular methods for sexing birds are based on detection of differences between conserved CHD (chromo-helicase-DNA-binding) genes located on the sex chromosomes Z and W of all non-ratite birds [1-3]. One of these differences is the size variations in introns between the the CHD gene on the Z- and W-sex chromosomes [3-5] in many species of birds from superorder *Neognathae* especialy in order *Anseriformes.* The base of this method is amplification of specific CHD1Z and CHDW1 gene fragments and the further separation in agarose or polyacrylamide gel. The PCR product from males shows a single band whereas that from females shows two bands. The second difference is the present of any recognized sites for some restriction endonucleases such as *Mae*II , *Hae*III, *Dde*I [6, 7] *Bsh*NI [8] in the CHD1Z gene fragment or the present of restriction site for *Asp*700I enzyme [3] in the CHD1W gene fragment. This difference of the CHDZ1 and CHDW1 gene is detected by a polymerase chain reaction–restricted fragment length polymorphism (PCR-RFLP) method. For using of these both specific differences for detection the CHDZ1 and CHDW1 genes were designed a sets of

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oligonucleotides such as 1237/1272H [9], 2550F/2718R [10] or P2/P3 and P2/P8 [11].
The aim of our study was using of the two differences of CHDZ and CHDW gene (variation of intron size and/or the presence of restricion sites) in sex determination of 36 species of birds from superorder *Neognathae* by using PCR and PCR-RFLP method.

2. Materials and methods

In this study were collected blood samples or samples of feathers from 36 species of birds from superorder *Neognathae* (order *Galliformes* – 3 species, order *Anseriformes* – 4 species, order *Passeriformes* – 3 species, order *Strigiformes* – 1 species, order *Falconiformes* – 1 species, order *Psittaciformes* – 24 species). Genomic DNA was isolated by using commercial column kit QIAamp DNA Mini kit (Qiagen).
PCR: The intron regions of CHDW and CHDZ genes were amplified by primer set P2 and P8 decribed by Griffiths et al. [11]. The reaction mixture in the total volume 25 µl containing 10-50 ng DNA, 1 U Taq polymerase (Fermentas), 1 x PCR buffer $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 200 µM dNTP, 0,8 pM of each primer. The PCR reaction was optimized in the gradient thermocycler C1000^{TM} (Biorad, USA). The following amplification parameters were applied: 95^{o}C for 3 minutes followed by 30 cycles: 95°C for 15 seconds, 50°C for 25 seconds, 72°C for 35 seconds. The reaction was completed by the final extension: 72°C for 5 minutes. The PCR products of all species were separated on 3,5 % agarose gel (Serva) containing GelRed dye (Biotium) at 180 V in 1 x sodium borate buffer for 30 minutes and the gel was analyzed by UV transilluminator and photographed with an documentation system Olympus C 7070.
RFLP: The PCR products were digested by restriction endonuclease FastDigest *HaeIII* (Fermentas). The digestion was performed with 10 ul of PCR product mixed with 1 µl of the restriction enzyme, 2 µl of 10 x FastDigest buffer in total volume 25 µl. The run conditions were 10 minutes at 37 ^{o}C. Digested fragments were visualized by electrophoresis on 2 % agarose gel (Serva) containing GelRed dye (Biotium) at 180 V in 1 x sodium borate buffer for 15 minutes and the gel was analyzed by UV transilluminator and photographed with a documentation system Olympus C 7070.

3. Results and discussion

In our study we analyzed 36 species of birds from superorder Neognathae for sex determination by using PCR and PCR-RFLP. We analysed samples of male and female of each species for verification of sex determination by molecular-genetics method. Sex determination by PCR amplification or PCR-RFLP analysis of the CHD gene was successful in all 85 samples from 36 species of superorder *Neognathae* used in this study:
Meleagris gallopavo var. domesticus, Gallus gallus domesticus, Anser anser domesticus, Anas platyrhynchos domesticus, Phasianus colchicus, Branta ruficollis, Cygnus olor, Melopsittacus undulatus, Myiopsitta monachus, Psittacus erithacus, Poicephalus senegalus, Cyanoliseus patagonus, Pyrrhura perlata, Pyrrhura rupicola sandiae, Amazona aestiva, Amazona barbadensis, Amazona autumnalis, Aratinga jendaya, Aratinga finschi, Aratinga solstitialis, Ara ararauna, Ara nobilis, Ara severa, Psittacula krameri, Alisterus scapularis, Pioneites melanocephala, Pionus menstruus, Platycercus eximius, Nymphicus hollandicus, Eolophus roseicapillus, Cacatua alba, Corvus monedula, Corvus corax, Passer domesticus, Tyto alba, Buteo jamaicensis

PCR: The PCR reactions were performed in the gradient thermocycler C1000^{TM} (Biorad) by using primers P2 and P8. The size variations in intron region of amplified fragment of CHDZ and CHDW gene were detected in 3.5% agarose gel. The size of specific fragments of CHDZ and CHDW genes amplified with primers P8 and P2 were not uniform for all species. The PCR product from males shows a single band (approximately 378 bp for chromosome Z) whereas that from females shows two bands (approximately 378 bp for chromosome Z and longer approximately 390 bp for chromosome W). The presence of the size different fragments for CHDZ and CHDW gene were not detected for species of orders *Galliformes*, *Falconiformes* and *Strigiformes* (Figure1). For these orders we have to used the PCR-RFLP methods.

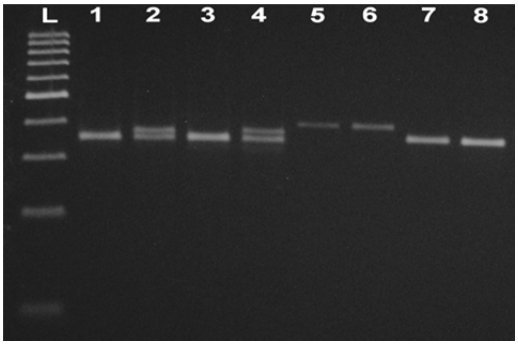

Figure 1. Representatively results of PCR analysis for identification of the size different fragments of CHDZ and CHDW genes on 3.5 % agarose gel.

***L** - 100 bp ladder (Fermentas), **1** – Branta ruficollis* (♂), ***2** – Branta ruficollis* (♀), ***3** – Anser anser domesticus* (♂), ***4** – Anser anser domesticus* (♀), ***5** – Gallus gallus domesticus* (♂), ***6** – Gallus gallus domesticus* (♀), ***7** – Buteo jamaicensis* (♂), ***8** – Buteo jamaicensis* (♀),

PCR-RFLP: The digestion of PCR products with restriction endonuclease *Hae*III differentiated the sex in species of all orders with the exception of order *Anseriformes.* The *Hae*III digestion of PCR products for CHDZ gene produced two fragments and for CHDW gene three fragments (Figure 2). We detected in all species of birds from order Anseriformes only one fragment with the size equal to the size of PCR product. The reason is that the species from order Anseriformes have not the recognizing site for restriction endonuclease *Hae*III. The absence of restriction site for *Hae*III for this order disallow to use PCR-RFLP method for sex determination.

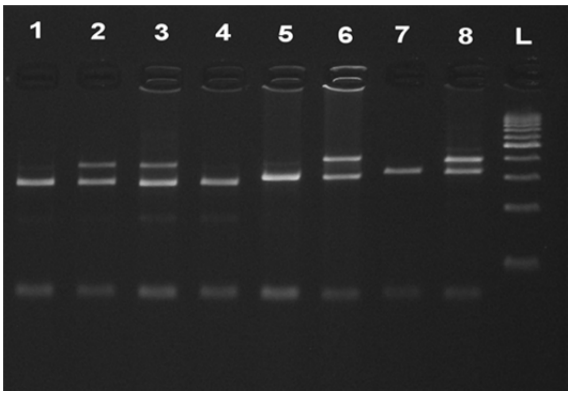

Figure 2. Representatively results of PCR-RFLP analysis by restriction enzyme *Hae*III on 2 % agarose gel.

***1** – Gallus gallus domesticus* (♂), ***2** – Gallus gallus domesticus* (♀), ***3** – Meleagris gallopavo var. domesticus* (♀), ***4** – Meleagris gallopavo var. domesticus* (♂), ***5** – Melopsittacus undulatus* (♂), ***6** – Melopsittacus undulatus* (♀), ***7** – Buteo jamaicensis* (♂), ***8** – Buteo jamaicensis* (♀), ***L** - 100 bp ladder (Fermentas).*

The primer set P8 and P2 designed by Griffiths et al. [11] used in this study gave good results for sex determination in all species of orders *Galliformes*, *Anseriformes*, *Strigiformes*, *Passeriformes*, *Falconiformes*, *Psittaciformes*. This set of primers P8 and P2 allowed the using of combination of two molecular techniques such as PCR amplification and restriction fragment lenght polymorphsim. Vučićević et al. [12] used for sex determination by molecular-genetis methods the other primer set 2550F/2718R designed by Fridolfsson and Ellegreen [10]. The 2550F/2718R primers may in some species produce only 1 fragment both in males and females [10]. This results from a preferential amplification of the shorter gene copy from the W chromosome, which in turn results in no detectable product from the Z chromosome. However, in such cases the birds can be easily sexed on the basis of the difference in size of both amplified fragments. The single fragment in males and females was found in the *Anatidae, Gruidae, Scolopacidae, Falconidae* and *Accipiteridae* [13]. The advantage of primer set P8 and P2 is utilization of two differences (different intron size and the presence of restricion site for restriction endonucleases) of specific fragment CHDZ and CHDW gene. On basis combination of this facts we can determine of sex in all 36 species of superorder *Neognathae* by using one primer set.

4. Conclusions

In our study we used reliable, inexpensive, fast and simple molecular-genetics methods for sex determination in 36 species of birds from superorder *Neognathae*. The combination with isolation of genomic DNA from feathers is the using of this methods optimal for determining sex in young birds or birds without sexual dimorphism.

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