Turk. J. Vet. Anim. Sci.
2007; 31(6): 371-374


Research Article

# Sex Determination by CHDW and CHDZ Genes of Avian Sex Chromosomes in *Nymphicus hollandicus*

Harun CERİT*, Kozet AVANUS

Department of Animal Breeding and Genetics, Faculty of Veterinary Medicine, İstanbul University, İstanbul - TURKEY

Received: 12.04.2006

**Abstract:** The aim of this study was sex determination in *Nymphicus hollandicus* without giving it any harm and obtaining accurate results by DNA analysis. CHD genes are preserved within avian Z and W sex chromosomes. The intron regions of the CHDW and CHDZ genes vary between male (ZZ) and female (ZW) individuals. The method used in this study was based on this difference. DNA was extracted from feathers instead of blood. The intron regions of CHDW and CHDZ genes were amplified by sex specific primers (P2 and P8). PCR products were screened by agarose gel electrophoresis. Individuals showing double (ZW) and single (ZZ) bands were identified as females and males, respectively.

**Key Words:** *Nymphicus hollandicus*, CHD1W, CHD1Z, avian, sex identification

## *Nymphicus hollandicus*'ta Kanatlı Cinsiyet Kromozomu Genlerinden CHDW ve CHDZ ile Cinsiyet Tayini

**Özet:** Bu çalışmanın amacı, *Nymphicus hollandicus*'lara (sultan papağanı) fiziksel bir zarar vermeden DNA analiz metoduyla cinsiyet saptaması yapmaktır. CHD genleri kanatlı Z ve W cinsiyet kromozomları arasında korunmuştur. CHD geninin intron bölgesi erkek (ZZ) ve dişi (ZW) bireyler arasında farklılık göstermektedir. Çalışmada kullanılan metotta bu farklılık esas alınmıştır. DNA kan yerine tüyden izole edilmiştir. PCR uygulaması CHDW ve CHDZ genlerinin intron bölgesini çoğaltan cinsiyete özel primerler kullanılarak (P2 ve P8) gerçekleştirilmiştir. PCR ürünleri agaroz jel elektroforezi ile görüntülenmiştir. Sonuç olarak, çift bant gösterenlerin dişi, tek bant gösterenlerin erkek olduğu saptanmıştır.

**Anahtar Sözcükler:** *Nymphicus hollandicus*, CHD1W, CHD1Z, kanatlı, cinsiyet tayini

## Introduction

People have always been attracted by parrots because of their ability to imitate any type of sound and because of their nice colourful feathers. Parrots are the most expensive birds in the pet market. Knowledgeable customers want to know the sex of their future or present parrot. Parrot salesmen generally respond unthinkingly to the customer's question about parrots' sex in an unscientific way. The material we used in our study was a Cockatoo (parrot) species, *Nymphicus hollandicus*, which belongs to the class Aves, order Psittaciformes, and family Cacatuidae (1).

Generally determination of the sex of birds is quite difficult before puberty but in monomorphic species it is difficult even after puberty. Some avian species–such as chickens, nestling turkeys, ducks, geese, owls, and parrots–are difficult to sex morphologically; for most of these species it is impossible even in adult ages. This difficulty causes a big problem for evolutional studies, wildlife conservation, and parrot breeders and owners (2,3).

Male and female birds must be kept in the same cage for reproduction. Because breeders are not sure of the birds' sex in cages, they cannot obtain any newborns from monomorphic birds just by keeping two of them in the same cage. First of all they need to be sure about the sex of birds they breed and put them together in the same cage. Because of their lack of success in breeding, mostly

*E-mail: hcerit@istanbul.edu.tr

they choose to import newborns illegally. Since males have the ability to talk and sing harmonically, this creates differences in the sale prices and care costs between male and female parrots. Illegal parrot imports and the time spent for the reproduction process cause very significant financial losses. In our study *Nymphicus hollandicus* was chosen because it is cheaper than other parrots and the pet shop owners were more understanding during the gathering of feathers from the birds. Additionally, *Nymphicus hollandicus* is one of the most preferred pets in and around İstanbul.

The Z and W sex chromosomes evolved differently in birds from the mammalian X and Y chromosomes (4). In birds females are heterogametic and carry a copy of Z and W but males are homogametic and carry 2 copies of the Z sex chromosome (5). The most critical question in sex identification of birds is how the 2 types of sex chromosomes play a role. It is not clear yet if female characteristics are developed by female specific W chromosomes or male characteristics are defined by the dose of chromosome Z. Even though it is female specific, the W chromosome has a similar structure to the mammalian male Y chromosome, except for its poorer gene structure, smaller size, and richness in heterochromatin and repeat sequences. There are 2 genes defined on the W chromosome. These are chromo helicase DNA binding protein (CHD1W) and ATP synthesis $\alpha$-sub unit (ATP5A1W). Both genes are located in the non-recombined part of the W chromosome and their similar homologues (CHD1Z and ATP5A1Z) exist in the Z chromosome (6-9).

With the invention of the polymerase chain reaction (PCR) by Kary Mullis in the 1980s a new revolution began in genetic science. It became possible to amplify target DNA sequences. The PCR procedure requires a template DNA that can be obtained from a drop of blood or a couple of feathers of the bird. Before the DNA analysis method was developed birds' sex was determined by laparoscopic or karyotyping methods. However, during the application of these methods the birds were harmed or even killed (10-12). The PCR method is fast and reliable. The DNA specification method can be applied by using the blood or the feathers as materials for template DNA. In order to avoid giving pain to the birds and reducing the risk of biological contamination chest feathers were used for the DNA isolation.

## Materials and Methods

Feathers from 41 *Nymphicus hollandicus* were used as material. The materials were collected from pet shops in and around İstanbul. Five or six chest feathers were collected carefully from each bird and transported to the lab in sterile bags.

Extraction of DNA from the apex of feathers was as follows: after the addition of 180 µl of lysis buffer (100 ml of 1 M Tris, 200 ml of 0.5 M EDTA, 2 ml of 5 M NaCl, 100 ml of 10% SDS), 25 µl of 100 mg/ml DTT (dithiothreitol) and 20 µl of 10 mg/ml proteinase K were added to the samples of 25 mg (3-4 calamuses). The mixture was incubated at 50 ºC, until the calamuses completely dissolved (3-5 h). After the addition of 400 µl of phenol (Tris-HCl pH 8.0), the mixture was left for 30 min. It was then centrifuged at 13,000 rpm for 3-5 min and the lower phase was discarded. After the addition of 400 µl of chloroform isoamyl alcohol (24:1) it was left for 10 min. This was followed by centrifuging at 13,000 rpm for 3-5 min and the lower phase was discarded. Pure ethanol and 40 µl of sodium acetate were added and the mixture was left for 45 min at -20 ºC. Then it was centrifuged at 13,000 rpm for 30 min at 4 ºC and washed with 10% ethanol and centrifuged at 13,000 rpm for 10 min at 4 ºC. DNA was regained with 100 µl of TBE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). To define the purity level of the DNA samples the optical densities were measured at 260 and 280 nm wavelengths in spectrophotometers (13-17).

For the PCR reaction P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3') primers were used. Amplification was performed in 25 µl total volume; 1.5 µl of 10× PCR buffer (Sigma), 1.5 µl of 25 mM $MgCl_2$, 100 ng of each primer, 200 µM of each dNTP, 0.5 U Taq DNA Polymerase, and 250 ng of DNA sample. The conditions for PCR amplification were a denaturing step at 94 ºC for 1 min 30 s, 35 cycles of 95 ºC for 30 s, 52 ºC for 30 s, and 72 ºC for 30 s, and final elongation at 72 ºC for 5 min. PCR products were visualized on 5% agarose gel stained with ethidium bromide (3,17-21).

## Results

The method used for DNA extraction is quite easy, fast, and practical. The OD rates were measured to find out the purity of DNA templates. OD rates of 41 samples were 1.7458 on average.

We tried to visualize PCR products in 2% and 3% agarose gels, consecutively. Because the 2 intronic regions on Z and W chromosomes have similar sizes they appeared overlapped with accumulation of ethidium bromide (EtBr) in 2% and 3% agarose gels. Consequently, it is not possible to obtain the female bands from 2% and 3% agarose gel. Since we could not distinguish and see clearly the double bands derived from the females we moved the PCR products into 5% agarose gel and were able to visualize and differentiate DNA bands easily. However, we distinguished 2 different band patterns easily in 5% agarose gel, where males and females showed single and double bands, respectively (Figure).

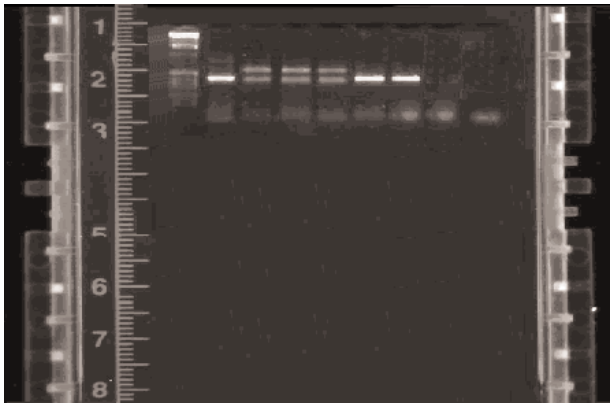


Figure. Amplified by using P2 and P8 primers PCR products show banding patterns for female and male N. hollandicus in 5% agarose gel. (From left to right: pUC19 marker, a male, 3 female and 2 male individual band patterns).

According to our results 23 males and 18 females were determined out of 41 specimens of *Nymphicus hollandicus*. Because 3 females had laid eggs and 2 males had mated with females they were used for verification in this study.

## Discussion

In this study DNA was isolated from bird (*Nymphicus hollandicus*) feathers, because feather sample collection gives less pain to the bird than blood sample collection. Additionally its low cost and reduced risk of contamination and preventing the breakage of DNA with requirement of less tube transfer make this method preferable.

Purity of DNA was measured by spectrophotometer and the results were similar to those of previous studies using different methods (14,16,22-24). In the present study the OD rates were over 1.6 and therefore this extraction method can be used in gene cloning, and RFPL and DNA analyses (21).

The method in this study was based on avian CHD genes (CHDW and CHDZ). Introns, which are the regions that do not code the genetic cipher, are less preserved compared to exons and their length varies among genes. Intron regions of CHD genes are located in the middle of 2 conserved regions that primers bind. The lengths of them differ between CHD-W and CHD-Z genes, making sex identification possible. Conserved exonic and length varied intronic regions were amplified by PCR primers (P2 and P8) following the primer annealing. That is why when the PCR products are screened by agarose gel males showed a single (CHD-Z) band, and females clearly showed an additional (CHD-W) band (3). The electrophoresis results were similar to the results reported by Griffiths et al. (2,25).

In this study a fast, reliable, correct, and cheap procedure was applied so that DNA could be extracted and sex could be identified in *Nymphicus hollandicus*. DNA was extracted from chest feathers instead of from blood without harming the animals or endangering their lives and so this method respects animal welfare and is suitable for sex determination in *Nymphicus hollandicus*.

In conclusion, the DNA extraction method used in this research has primacy because of its easy process, requirement of a short time to complete, and its low cost. In this study the sex identification based on P2 and P8 primers is a reliable and age independent method that needs a small amount of DNA and is applicable in both adults and nestlings (26). Therefore CHD gene amplification is a convenient, safe, and simple technique for sexing *Nymphicus hollandicus*. As Garcia-Moreno and Mindel (27) reported, primers used in sex identification can definitely help to illuminate phylogenetical studies. This method will be useful for studies and conservation programmes of rare birds like parrots. Moreover, the primers used for sex identification can be used in some other species like ducks, geese, pheasants, and falcons.

## Acknowledgements

This study was supported by the Research Fund of İstanbul University (Project number BYP-677/26042005 and UDP-787/23062006).

## References


1. Alderton, D. (1992): Evcil Kuş Bakımı, İnkılap Yayınevi. İstanbul. 1992; 78-79.
2. Griffiths, R., Double, M.C., Orr, K., Dawson, R.G.J.: A DNA test to sex most birds. Mol. Ecol., 1998; 7: 1071-1075.
3. Griffiths, R., Tiwari, B.: Sex of the last wild Spix's macaw. Nature, 1995; 375: 454.
4. Ellegren, H., Carmichael, A.: Multiple and independent cessation of recombination between avian sex chromosomes. Genetics, 2001; 158: 325-331.
5. Handley, L.J., Ceplitis, H., Ellegren, H.: Evolutionary strata on the chicken Z chromosome: implications for sex chromosome evolution. Genetics, 2004; 167: 367-376.
6. Ellegren, H.: Hens, cocks and avian sex determination. A quest for genes on Z or W? Embo Rep., 2001; 2: 192-196.
7. Fridolfsson, A.K., Ellegren, H.: Molecular evolution of the avian CHD1 genes on the Z and W sex chromosomes. Genetics, 2000; 155: 1903-1912.
8. Griffiths, R., Korn, R.M.: A CHD1 gene is Z chromosome linked in the chicken *Gallus domesticus*. Gene, 1997; 197: 225-229.
9. Griffiths, R., Tiwari, B.: Avian CHD genes and their use in methods for sex identification in birds. Isis Innovation, Oxford. 1996.
10. Heinsohn, R., Legge, S., Barry, S.: Extreme bias in sex allocation in *Eclectus* parrots. Proc. Biol. Sci., 1997; 264: 1325-1329.
11. Saino, N., Ellegren, H., Møller, A.P.: No evidence for adjustment of sex allocation in relation to paternal ornamentation and paternity in barn swallows. Mol. Ecol., 1999; 8: 399-406.
12. Trewick, S.A.: On the skewed sex ratio of the Kakapo *Strigops habroptilus:* sexual and natural selection in opposition? Ibis, 1996; 139: 652-663.
13. Sefc, K.M., Payne, R.B., Sorenson, M.D.: Microsatellite amplification from museum feather samples: effects of fragment size and template concentration on genotyping errors. The Auk, 2003; 120: 982-989.
14. Leeton, P., Christidis, L., Westerman, M.: Feathers from museum bird skins: a good source of DNA for phylogenetic studies. The Condor, 1993; 95: 465-466.
15. Malagó, W.Jr., Franco, H.M., Matheucci, E.Jr., Medaglia, A., Henrique-Silva, A.F.: Large scale sex typing of ostriches using DNA extracted from feathers. BMC Biotechnol., 2002; 2: 19.
16. Pfeiffer, I., Völkel, I., Täubert, H., Brenig, B.: Forensic DNA-typing of dog hair: DNA-extraction and PCR amplification. Forensic Sci. Int., 2004; 141: 149-151.
17. Shephard, J.M., Catterall, C.P., Hughes, J.M.: Discrimination of sex in the white-bellied sea-eagle *Haliaeetus leucogaster* using genetic and morphometric techniques. Emu, 2004; 104: 83-87.
18. Arnold, K.E., Orr, K.J., Griffiths, R.: Primary sex ratios in birds: problems with molecular sex identification of undeveloped eggs. Mol. Ecol., 2003; 12: 3451-3458.
19. Cerit, H.: Determination of the allele frequency of some genomic loci in a Holstein cattle population and its importance in individual identification. Turk. J. Vet. Anim. Sci., 2003; 27: 81-91. (article in Turkish with an abstract in English).
20. Cerit, H., Altınel, A., Elmaz, Ö., Avanus, K.: Polymorphism evaluation of various genomic loci in the Kıvırcık sheep breed of Turkey. Turk. J. Vet. Anim. Sci., 2004; 28: 415-425.
21. Sambrook, J., Frisch, F.F., Maniation, T.: Spectrophotometric Determination of the Amount of DNA or RNA Appendix E: Commonly used Techniques in Molecular Cloning: Molecular Cloning: 3A Laboratory Manual. 2nd edn., Cold Spring Harbor Laboratory Press, New York, 1989.
22. Moazami-Goudarzi, K., Laloë, D., Furet, J.P., Grosclaude, F.: Analysis of genetic relationships between 10 cattle breeds with 17 microsatellites. Anim. Genet., 1997; 28: 338-345.
23. Gustincich, S., Manfioletti, G., Del Sal, G., Schneider, C., Carninci, P.: A fast method for high-quality genomic DNA extraction from whole human blood. Biotechniques, 1991; 11: 298-301.
24. Miller, S.A., Dykes, D.D., Polesky, H.F.: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res., 1988; 16: 1215.
25. Griffiths, R., Daan, S., Dijkstra, C.: Sex identification in birds using two CHD genes. Proc.: Biol. Sci., 1996; 263: 1251-1256.
26. South, J.M., Wright, T.F.: Nestlings sex ratios in the Yellow Naped Amazon: no evidence for adaptive modification. The Condor, 2002; 104: 437-440.
27. Garcia-Moreno, J., Mindell, D.P.: Rooting a phylogeny with homologous genes on opposite sex chromosomes (gametologs): a case study using avian CHD. Mol. Biol. Evol., 2000; 17: 1826-1832.