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Sex determination by CHD (Chromo helicase DNA binding) gene in local rock pigeons (*Columba livia*) from Lahore, Pakistan

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Abstract
Visually identification of sex is difficult in Class Aves because birds are monomorphic to identify sex on the basis of phenotypic characteristics was impossible. The Birds grown-up females seem indistinguishable to the males in half of the world. This issue can hinder by both human-helped reproducing of winged animals and transformative examinations. DNA-based sex distinguishing proof gives an answer. Researchers depict a test in light of CHD (chromo-helicase-DNA) gene present on the avian sex chromosomes of all bird species with the conceivable special case of the ratites. The CHD-W is arranged on the W chromosome; it is for females. On the Z chromosome CHD-Z, is found, it occurs in both sexes (female, ZW; male, ZZ). In this study, sex of Rock Pigeons (*Columba livia*) was identified. Three birds were obtained from local market. DNA was extracted from the tissue samples. DNA was quantified by Nano Drop. Gene was amplified on a PCR using sex specific primers. Amplicons were analysed on agarosegel. It intensified homologous segments of the two genes and includes intron's lengths which normally vary. When PCR products were revealed on gel electrophoresis single Z-band was obtained which indicates that one sample was male birds and two were female having double bands. It was the first study in Pakistan to utilize CHD gene for sex identification in rock pigeon which proved the validity of the genetic marker for this species. This study can pave a way to build effective conservation programs and successful breeding strategies.

Keywords: Rock pigeons, CHD gene, DNA, molecular sexing, CHD marker, sex identification

Introduction
On the basis of different phenotypic characteristics, sex of birds had been identified. Different methods used for gender identification includes behavioural inspection, pre brooding pop presence, morpho-metric attribute differences, gonads inspection by laparotomy or laparoscopy and sex chromosome analysis. Only during the breeding season first two methods could be applied and the study of morphometric characteristic might be arguable. In the adult birds the evaluation of gonads might be difficult during the breeding and the nesting season because of their small body size [1]. Sex identification from external morphological traits was difficult at the time of pairing. If determination of sex in birds was well definite then better conservation program would be attained [2]. This was impossible to determine sex on the bases of phenotypic traits because birds are monomorphic [3]. In diploid species, genes are present on the sex chromosomes that are responsible for sex determination. In mammals SRY gene is located on Y chromosome that is responsible for maleness. Sex determination pattern in mammals and birds is opposite. In birds, females are heterogametic (ZW) and males are homogametic (ZZ) [4]. During evolutionary process, some genes on W chromosomes were lost and size become smaller while Z chromosome is larger and highly conserved [5]. By molecular techniques these problems had been solved using karyotyping and intensifying the sex chromosome gene CHD that is found on both sexes [6]. By PCR amplification technique, sex genes give different fragments size that identify gender in birds. Females presenting two bands of different sizes, W chromosome is small and males presenting a single band corresponding to the two Z chromosomes of equivalent size. For sex determination (CHD) gene is used as marker in bird's species [7]. For the separation of PCR products, it does not require restriction enzyme and is therefore simple, cheap and quicker. Molecular diagnostic method of sex identification in pigeon's uses CHD1 gene, which is located on both W and Z chromosomes. Isolation of DNA from pigeon's tissue.

PCR intensification of the CHD quality with single arrangement of primers and agarose gel electrophoresis was used to mask PCR products. Individuals shows double (ZW) bands for females and single (ZZ) band for males. The purpose of this study was gender identification. This study can pave a way to build successful breeding strategies and effective conservation programs

Materials and Methods

Three rock pigeon (*Columba livia)* were obtained from local market of Lahore. The birds were identified on the basis of their physiological and morphological features. Tissue sample (100 mg) was taken from the sternum of the rock pigeon. Sample was taken in autoclaved petri plates, and crushed with sterile blade into fine pieces. Crushed sample was and washed with chilled wash buffer (1mM EDTA, 10 mM Tris, Cl (1000 uL). DNA was extracted by organic method, following the manufacturer's protocol [8]. At -20 °C isolated DNA was stored. DNA was quantified by measuring absorbing at 260 and 280 nm using Nano Drop 2000c. Gene was amplified on a PCR using sex specific primers. 2550 F (5′-GTTACTGATTCGTCTACGAGA-3′) and 2718R (5′-ATTGAAATGATCCAGTGCTTG-3′) as describe by [9]. The PCR conditions were optimized for the amplification of CHD gene by changing the concentration of deoxynucleotide triphosphate (dNTPs), magnesium chloride (MgCl2) and Taq polymerase. Annealing temperature was also adjusted to achieve amplified CHD gene. The PCR recipe and conditions applied in experimental process to get amplified products are listed in the table 1.

Table 1: PCR Composition

Sr. No	Reagents (working concentration)	Quantity	Reagent Concentration
1.	DNA Sample	1µL	50ng/µL
2.	PCR Buffer with ammonium sulphate	2.5µL	1X
3.	MgCl2	2.5µL	2.5mM/µL
4.	DNTPs	2.5µL	2.5mM/µL
5.	Taq Polymerase	0.5µL	2.5U/µL
6.	Forward primer	1µL	0.8pmoles
7.	Reverse primer	1µL	0.8pmoles
8.	Deionized water	14µL	
	Total volume	25µL	

The following are the optimized cycling conditions of PCR for amplification of CHD gene. The cyclic conditions for PCR were as per the following: an initial denaturation step at 95°C for 4 min followed by 30 cycles of final denaturation at (94°C, 30 sec), annealing (52°C, 30 sec), Extension (72°C, 60 sec) and finial DNA extension step at 72°C for 10 min.

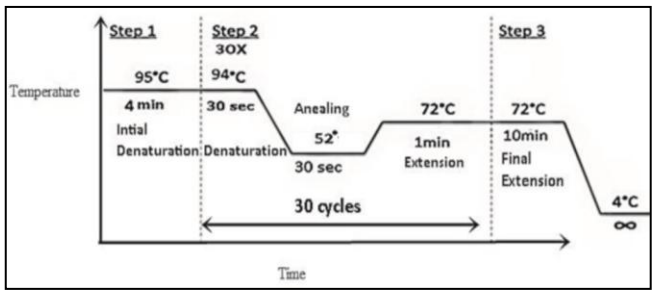

Fig 1: PCR Profile

The PCR products were run on 2% agarose gel for 30 min at 110 V by electrophoresis in TAE buffer and staining the gel with ethidium bromide. Bands were visualized through UV light in Gel Doc.

Results

All samples were successfully intensified. Distinction between the lengths of introns on conserved chromo-helicase-DNA binding protein (CHD1) gene was used for sex identification. PCR products on gel electrophoresis revealed the presence of a single band for male and double bands for female. Male have only one band of same size (ZZ) while female have two bands of different sizes (ZW). Z chromosome fragment was present in both male and female. Two samples were female birds and one was male bird. In all birds, females are heterogametic (ZW) while males are homogametic (ZZ).

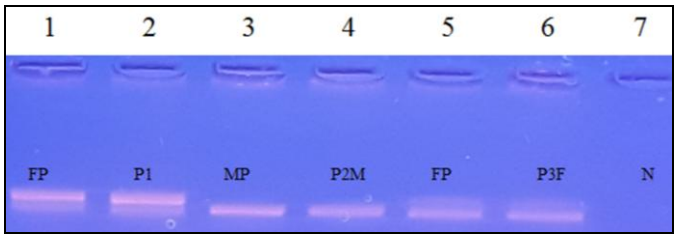

Fig 2: Gene amplification of sample

Lane 1: female positive control; lane 2: pigeon female; lane 3: male positive control; lane 4: pigeon male; lane 5: female positive control; lane 6; pigeon female; lane 7: Negative control

Discussion

Different methods have been described for Sex determination of Avian including steroid sexing, laparoscopy, vent sexing, feather sexing or karyotyping for monomorphic birds species [10]. In specific species feather sexing is possible in which an auto sex character has been introduced by crossing [10, 11]. Molecular sexing technique use PCR to produced gene fragments for gender identification. Chromo helicase DNA binding protein gene (CHD) is present on sex chromosome that have different fragments size for males and females [11, 12, 13]. DNA should provide a versatile way to separate female and male birds. Unfortunately, the selection of genetic markers is difficult. As Z sex chromosome is present in both genders while W sex chromosome occurs only in the female (ZW). CHD gene is highly conserved coding region used for DNA sexing technique using single set of PCR primers 2550F /2718R to identify sex throughout the class Aves, with the exception of ratites. The present study used the strategy to validate the utilization of CHD gene for sex identification of rock pigeon. For this purpose, DNA was extract from tissue samples. PCR amplification of the CHD gene with single set of primers 2550F/2718R was done. On gel electrophoresis a single Z-band was obtained which indicates that one sample was male birds and two were female having double bands. Our results was similar to previously published articles [14, 15] by using same primers. A single Z band indicates male birds and female having double bands. Some species could be tested with Z43B marker, Different sizes of Z and W amplicons were obtained, making it possible to distinguish between males (ZZ) and females (ZW). Z43B marker would be useful for obtaining sex-typing data for species of birds. [16]. this showed similarity with our report. Alternative primer

pairs have also been described for sex identification in birds. It was reported that several primer pairs other than 2550F/2718R have been applied for sex identification in birds of Iran. P2/P3 is another CHD1 specific primer that has been used for sex identification in birds of prey. It amplified 109 bp fragments in both female and male birds that could not distinguish between females and males on the agarose gel. Therefore, an additional step of restriction enzyme digestion of the PCR products was performed to determine the sex of the birds [17]**.** This is contrary with our findings. P8/P2 primer was not successful, when performing genetic sex-typing. An error is associated with the occurrence of Z polymorphism, Z-polymorphism leads to some males possessing two differently sized (Z) alleles. It is common to assume that when two different sized amplicons are observed, it indicates the individual is female, whereas observation of a single amplicon indicates a male, and because of these assumptions, unrecognized Z-polymorphism leads to the incorrect classification of true males as females [18]**.** But our finding is not compared with this. It was the first study in Pakistan to utilize CHD gene for sex identification in rock pigeon which proved the validity of the genetic marker for this species. It was also confirmed that the present strategy is effective for sex determination of rock pigeon. This study can pave a way to build successful breeding strategies and effective conservation programs.

Conclusion

For sex identification in Rock pigeons, we determined the applicability of 2550F/2718R primer pair. CHD gene is amplified by 2550F/2718R primer pair revealed distinction between the lengths of introns in both sexes, enabling exact sex identification.

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