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P2/P8 primer PCR effective in sex determination in Domestic birds

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Abstract
In avian species, sex determination is based on ZW-ZZ chromosomes. Males are homogametic have two Z chromosomes (ZZ), while females are heterogametic and have a Z and a W chromosome (ZW). The PCR has been successfully used for the detection of the sex in birds. In the current study, an effort to compare the accuracy of sex determination using primers P2/P8 & P2/NP/MP, targeting the Chromo-Helicase DNA binding (CHD) gene, in Domestic birds of known gender using PCR technique. A total of seventy-four sexed samples were identified by the P2/P8 PCR. A total of 72 valid results were obtained while two samples failed to provide an amplification. The female (ZW) showed two bands (350 & 365 bp approximately) separated from each other by a mere 15 bp, while, males (ZZ) showed a single band at 350 bp. Out of the seventy-two results all seventy-two samples were correctly identified by the test. The overall accuracy of the test stood at 97.29% (72/74).
In the P2/NP/MP PCR, the female (ZW) showed two bands (330 & 270 bp) separated from each other by a 60 bp, while, males (ZZ) showed a single band at 270 bp. A total of 74 valid results were obtained. Out of the 74 samples sexed using P2/NP/MP PCR test, all 74 samples provided an amplification. However, out of the seventy-four samples, seven samples provided erroneous results. The overall accuracy of the test stood at 90.54%.
Thus, the P2/P8 primer was more accurate in the sex estimation in domestic birds, whereas, the P2/MP/NP PCR has comparatively less accurate in detecting the sex in domestic birds. Although, it is worth mentioning that the P2/MP/NP PCR was 100% accurate in the estimation of sex in Khaki campbell duck (*Anas platyrhynchos domesticus*), Guinea fowl (*Numida meleagris*), Turkey (*Meleagris gallopavo*) and Japanese quail (*Coturnix japonica*). Depending on targeted species the P2/P8 PCR can be used to identify birds with precision.

Keywords: Poultry, P2/P8, P2/NP/MP, CHDI, sex determination, molecular sex determination

Introduction
Many bird species exhibit sexual monomorphism, meaning that males and females have similar physical traits, including colouration. It can be challenging for even experienced ornithologists to determine the sex of an individual bird accurately. Males and females are monomorphic in colour for at least 60% of passerine species (Griffiths *et al.*, 1998) [1]. The sex of nestling birds can be even more difficult than adults, as young birds do not typically show morphological differences between males and females shortly after hatching (Price & Birch, 1996). In most cases, the differences between males and females become apparent a few weeks after hatching. This lack of distinguishable physical differences between males and females in adult and juvenile birds can make it challenging to gather accurate data on sex ratios in broods.
In avian species, sex determination is based on the presence of a Z or W sex chromosome. Males are homogametic and have two Z chromosomes (ZZ), while females are heterogametic and have a Z and a W chromosome (ZW) (Solari, 1994) [15].
In most birds, the W chromosome is a degraded Z chromosome and contains most of the repetitive heterochromatic DNA. However, in ratites, like emus and ostriches, the W chromosome is similar in size and gene to the Z chromosome. The mechanism of avian sex determination has been a mystery for many years. Many studies have concentrated on developing effective molecular techniques for sex identification, and these molecular methods are becoming more popular in research and conservation efforts for various bird species.

As a result, molecular sexing approaches are gaining more attention as a valuable tool in this field (Cerit & Avanus, 2007; Dubiec & Zagalska-Neubauer, 2006) [2-3]. A PCR-based approach using an Amplification Refractory Mutation System (ARMS) that relied on differences in the CHD1Z and CHD1W sequences to determine the sex of Falconiformes more effectively (Ito *et al*., 2003) [6]. This method complemented the technique established by (Griffiths *et al.*, 1998) [1]. (Ito *et al.*, 2003) [6] Used two sets of primers for sex identification in Falconiformes P2/NP and P2/P8. The P2/NP primer set was used for the method based on intronic length variation between CHD1W and CHD1Z. This primer set was designed to amplify a fragment that spans the CHD1 intron, resulting in different banding patterns between males and females. On the other hand, the P2/P8 primer set was used to amplify the CHD1 region and identify nucleotide differences between CHD1W and CHD1Z. However, the P8 primer site was found to be less conserved than the NP primer site, leading to weak amplification of the CHD1Z region in some species. The ARMS method is based on the principle that point mutations can potentially impact a primer binding site of the CHD1Z homology target sequence. In such cases, an inner primer may be unable to amplify the CHD1Z gene due to nucleotide differences but can still amplify the CHD1W gene. However, after conducting a pairwise sequence comparison, it was observed that there were no sequence differences at the ARMS site. As a result, the primer set (P2/NP/MP) examined in this study produced two bands regardless of the gender tested, suggesting that this primer set was unsuitable for identifying the sex of some species. In the current study, an effort to compare the efficiency of sex determination using primers P2/P8 & P2/NP/MP, targeting the Chromo- Helicase DNA binding (CHD) gene, in Domestic birds of known gender using PCR technique.

Material and Methods

A total of seventy-four samples were collected from various sources for the sex identification. A total of fifty tissue samples of chicken were obtained from local chicken vendors. The sex of the sacrificed bird was identified by the observation of gonads and noted. The tissue was transported on ice and stored at -20 °C until it processing. Also, blood samples from Khaki Campbell duck (*Anas platyrhynchos domesticus*) (n=6), guinea fowl ((*Numida meleagris*) (n=10), turkey (*Meleagris gallopavo*) (n=4) and quails (*Coturnix japonica*) (n=4) were collected from Poultry Research and Training Centre, under Maharashtra Animal & Fishery Sciences University, Seminary Hills, Nagpur.

The DNA was isolated from the blood and tissue of the birds using DNeasy Blood and Tissue Kit (Mfg. Qiagen Pvt Ltd., USA) following the standard protocol. The DNA was eluted in 170 µl Nuclease Free Water (NFW) (Mfg. Ambion). The DNA was stored at -20C until further use.

Gradient PCR for P2/P8 and P2/NP/MP Primers

In order to determine the exact annealing temperature, a gradient PCR was set up employing P2/P8 (P2: 5'-TCTGCATCGCTAAATCCTTT-3'; P8: 5'-CTCCCAAGGA TGAGRAAYTG3') and P2/NP/MP (MP: 5'-AGTCACTAT CAGATCCGGAA-3'; NP: 5'-GAGAAACTGTGCAAAAC AG-3') primers (Table 1 and 2). For primer pair P2/P8 the annealing temperature range of 48 °C to 50 °C was utilised while for the P2/NP/MP PCR a range from 48 °C to 53 °C was used. The amplicons were subjected to 3% agarose gel electrophoresis in case of P2/P8 PCR while 2% for P2/NP/MP PCR.

PCR for screening bird samples

Based on the results of the gradient PCR, the samples were screened for the amplification of the CHD1Z and CHD1W genes using P2/P8 and P2/NP/MP Primers. The samples were screened thrice and the records were maintained for the estimation of accuracy of both the approaches.

Results

Gradient PCR for the standardization of P2/P8 and P2/NP/MP amplification

The gel electrophoresis followed by gel documentation clearly exhibited distinct well separated bands true to the gametotype at 49 °C (Fig. 1). A four-sex replicate reaction was carried out at annealing temperatures from 48 °C to 50 °C (Table 1). The gel electrophoresis followed by gel documentation clearly exhibited distinct well separated bands true to the gametotype at 48 °C (Fig. 2). Hence the temperature was utilized for the screening protocols carried out subsequently.

Screening of the sexed samples using P2/P8 primer pairs

A total of seventy-four sexed samples were identified by the P2/P8 PCR (Table 2). A total of 72 valid results were obtained while two samples failed to provide an amplification (Fig. 3). The female (ZW) showed two bands (350 & 365 bp approximately) separated from each other by a mere 15 bp, while, males (ZZ) showed a single band at 350 bp.

The same seventy-four sexed samples were sex typed by the P2/NP/MP PCR. The female (ZW) showed two bands (330 & 270 bp) separated from each other by a 60 bp, while, males (ZZ) showed a single band at 270 bp. A total of 74 valid results were obtained (Fig. 4).

The samples were tested thrice to ensure reliability of the results and the results so obtained have been summarized in table 3.

Accuracy of the P2/P8 PCR test

Out of the 74 samples sexed using P2/P8 PCR test, 72 valid results were obtained. Out of the seventy-two results all seventy-two samples were correctly identified by the test. The overall accuracy of the test stood at 97.29%. While, out of the 74 samples sexed using P2/NP/MP PCR test, all 74 samples provided an amplification. However, out of the seventy-four samples, seven samples provided erroneous results. The overall accuracy of the test stood at 90.54%.

Discussion

Various molecular methods have been described in the sex determination of avian species. A vast diversity in birds limits the generalization of the protocols available for the accurate sex detection in birds. In the recent years, protocols given by Griffith *et al*., and Ito *et al*., have been used by various workers with varying amount of success in detection of sex in various taxa of birds. In the current study, Khaki Campbell duck (*Anas platyrhynchos domesticus*), Chicken (*Gallus gallus domesticus*), Guinea fowl (*Numidam eleagris*), Turkey (*Meleagris gallopavo*) and Japanese quail (*Coturnix japonica*) were used as models for the comparison of the efficiency of the above two primer pairs in identification of the sex in varied taxa of domestic birds.

The PCR reaction is universally employed for the diagnosis of

disease and molecular biology. The technique has been successfully used for the detection of the sex in birds (Ogawa *et al*., 1997; Griffith *et al*., 1998; Ito *et al*., 2003; Han *et al*., 2009; Zhang, 2009) [9, 1, 6, 4, 14]. There are certain factors that can influence the efficiency of the PCR reaction *viz*. the concentration of $MgCl_2$, purification methods for primers, annealing temperature, etc. Though the annealing temperature for the P2/P8 primer has been described by many to be in the range of 48 °C-50 °C, an effort to standardize the same was made in the lab. Local variables like the concentration of $MgCl_2$ in the master mix, synthesis methods employed in the synthesis of the primers, etc. can influence the outcome of the test in the lab (Huang *et al*., 2003) [5]. Hence it was thought prudent to first optimize the reaction using the master mix. A gradient PCR provides for the flexibility in the annealing temperature (Ishii & Fukui, 2001) [7] and was utilized to standardize the annealing temperature of the PCR to 49 °C. Similarly, for the P2/MP/NP primer sets, gradient PCR followed by gel electrophoresis indicated 48 °C as optimum annealing temperature for PCR reaction.

Molecular sex identification of birds utilized the PCR technique to target the CHD region located on the Z and W chromosomes in birds. In case of the P2/P8 approach, the heterogametic females produce two fragments measuring 343 and 364 bp were produced, while, a single 334 bp fragment was generated from male birds. The amplification produced varies from species to species and the difference in between the Z and W fragment may be as less as 15 base pairs in some species of the birds. For the effective detection of the fragments amplified by P2/P8 primer pairs, the gel electrophoresis employed using ethidium bromide is crucial. There are reports of use of 3% to 5% agarose gel for the effective separation of the amplicon fragments. In the current study, 3% gel was utilized with 80V voltage for 90 minutes. The gel percentage is crucial for the effective separation and correct allotment of sex identification to birds. The small difference in the male and female birds limits the success of the P2/P8 primer pairs in sex detection of birds. However, the accuracy and universal application makes it best candidate for the sex identification in a wide variety of birds.

The primers P2/MP/NP were designed by Ito *et al*., 2003 [6] primer NP was designed to replace primer P8 described by Griffith *et al*., 1998 [1] owing to the polymorphism exhibited by a diverse class of birds. The primer NP is located 15 bp towards the 3' end of the Z and W chromosome. The primer NP annealing site is supposed to be more conserved and thus allowing the sex determination a wide variety of birds. To further ease the detection of the sex, a primer MP was designed to targeting the CHD-W midway between NP and P2 site, causing an amplification of approximately 260 - 280 bp in the domestic bird species. The gel electrophoresis required 2% gel and due to the 50-80 bp difference in the male and female birds, the primer pairs could be utilized with ease in the detection of sex. However, the Lee *et al*., 2008 [8] observed that due to point mutations in the CHD1Z the P2/NP/MP method, which is based on the ARMS, yielded two bands irrespective of the sex in Anatidea (*A. albifrons*, *A. formosa*, and *A. poecilorhyncha*) and one species of Phasianidae (*P. colchicus*). Thus, there could be species variation due to the point mutations which can be further studied using sequencing technique. Thus, P2/MP/NP is a technique that can be used with ease but is prone to errors owing to intrinsic DNA characters like point mutations.

In the current study, P2/P8 PCR was able to detect the sex in 72 out of the 74 samples, it failed to amplify two samples consistently, in order to confirm that the DNA is present, universal primer as described by Verma and Singh, 2003 [13] were utilized to amplify a 440 bp fragment, indicating the presence of DNA. The failure of P2/P8 in certain individuals can be attributed to the P8 site which can be prone to point mutations at the annealing site resulting into a complete amplification failure. Rest of the samples confirmed the sex identity of the birds as per the validated identification rendered based in the examination of gonads and physical appearance. The P2/P8 primers have been regarded as universal primer for the detection of sex in birds owing to the accurate and highly efficient nature of the PCR.

The P2/MP/NP PCR was able to amplify fragments from all the seventy-four samples. The test proved to be erroneous in determining the sex of seven samples. It is worth noting that all the seven samples were of chicken origin and the P2/MP/NP PCR fared well with rest of the four species with hundred percent accuracy. The misidentification males as females were seen in six instances in the current study. The possible reason for the misidentification could be as communicated by Lee *et al*., 2008 [8]. However, in one instance, the female was identified as male, wherein the failure could be attributed to the failure of MP primer to anneal at the CHD1W gene leading to the amplification of only one fragment of approximately 330 bp corresponding to the homogametic males (ZZ). The overall accuracy of the P2/MP/NP PCR was found to be 90.54%, however, the accuracy was 100% (n=24) in samples other than chicken samples. The work of Purwaningrum *et al.*, 2019 [11] pointed out to the 100% accuracy of P2/MP/NP PCR in detection of sex in captive birds (n=52) belonging to 16 species. No bird belonging to Phasianidae family was included in the study.

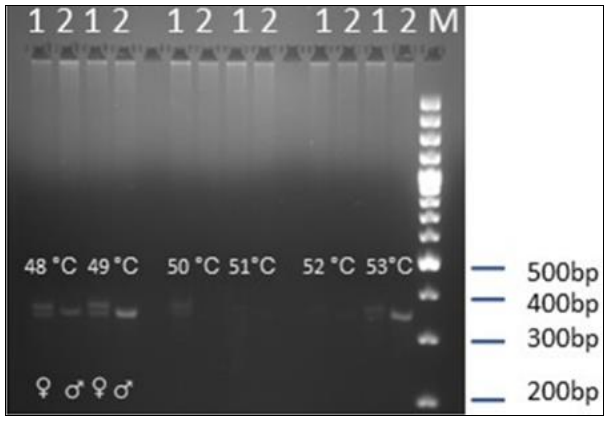

Fig 1: Gradient PCR for standardization of P2/P8 primer pairs

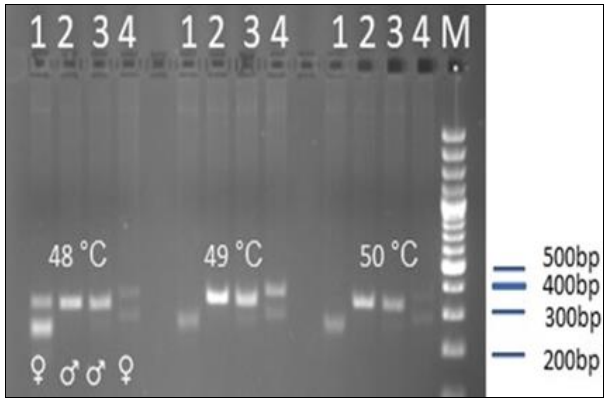

Fig 2: Gradient PCR for standardization of P2/MP/NP primer pairs

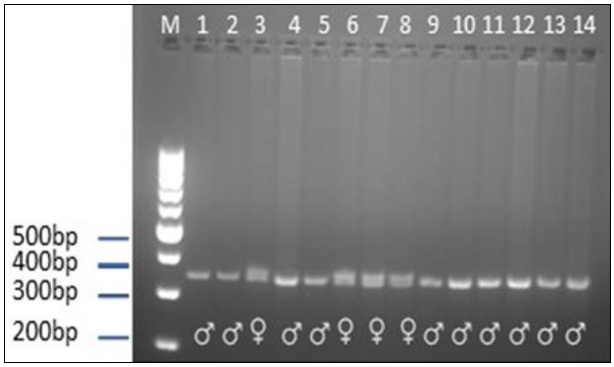

Fig 3: PCR using P2/P8 primer pairs for the sex determination of birds

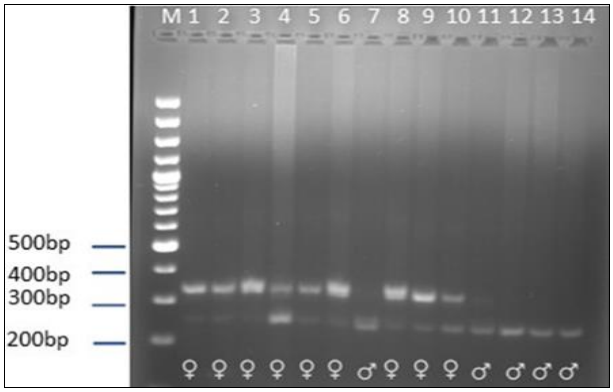

Fig 4: Gradient PCR for standardization of P2/MP/NP primer pairs

Table 1: Composition of PCR reaction utilised in the study for P2/P8 and P2/NP/MP Primers.

Component	**P2/P8 PCR (Griffiths *et al.*,1998)** [1]	**P2/MP/NP PCR (Ito *et al.*, 2003)** [6]
Master Mix	5 µL	5 µL
P2 Primer	1 µL	1 µL
P8 Primer / NP Primer	1 µL	0.5 µL
MP Primer	-	0.5 µL
Nuclease Free Water	1 µL	1 µL
DNA Template	2 µL	2 µL

Table 2: PCR cycling conditions utilised in the study for P2/P8 and P2/NP/MP Primers.

Condition	**P2/P8 (Griffiths *et al.*,1998)** [1]	**P2/MP/NP (Ito *et al.*, 2003)** [6]
Pre-Denaturation	94 °C, 5min	95 °C, 5min
Denaturation	95 °C, 30sec	94 °C, 30sec
Annealing	49 °C, 30sec	48 °C, 30sec
Extension	72 °C, 30sec	72 °C, 30sec
Final Extension	72 °C, 5min	72 °C, 7min
Cycles	40	40

Table 3: Sex determination of birds based on P2/P8 and P2/NP/MP PCR along with the sexing results based on the gonadal observation/ sexing

Sr. No	**Sample code**	**P2/P8**	**P2/MP/NP**	**Established SEX ID**
1	Du 1	Male	Male	Male
2	Du2	Male	Male	Male
3	Du3	Female	Female	Female
4	Du4	Male	Male	Male
5	Du5	Female	Female	Female
6	Du 6	Female	Female	Female
7	Tu 1	Female	Female	Female
8	Tu 2	Male	Male	Male
9	Tu 3	Male	Male	Male
10	Tu 4	Female	Female	Female
11	GF1	Female	Female	Female
12	GF 2	Female	Female	Female
13	GF 3	Male	Male	Male
14	GF 4	Male	Male	Male
15	GF 5	Female	Female	Female
16	GF 6	Male	Male	Male
17	GF 7	Female	Female	Female
18	GF 8	Male	Male	Male
19	GF 9	Male	Male	Male
20	GF 10	Male	Male	Male
21	Qu 1	Female	Female	Female
22	Qu 2	Male	Male	Male
23	Qu 3	Male	Male	Male
24	Qu 4	Female	Female	Female
25	Ch 1	Male	Female	Male
26	Ch 2	Male	Female	Male
27	Ch3	Female	Female	Female
28	Ch4	Male	Female	Male
29	Ch5	Male	Female	Male
30	Ch6	Female	Female	Female

31	Ch7	Female	Male	Female
32	Ch8	Female	Female	Female
33	Ch9	Male	Female	Male
34	Ch10	Male	Female	Male
35	Ch11	Male	Male	Male
36	Ch12	Male	Male	Male
37	Ch 13	Male	Male	Male
38	Ch14	Male	Male	Male
39	Ch15	Male	Male	Male
40	Ch16	Male	Male	Male
41	Ch17	Female	Female	Female
42	Ch18	Male	Male	Male
43	Ch19	Female	Female	Female
44	Ch20	Male	Male	Male
45	Ch 21	Male	Male	Male
46	Ch22	Male	Male	Male
47	Ch23	Female	Female	Female
48	Ch24	Unidentified	Male	Male
49	Ch25	Male	Male	Male
50	Ch26	Male	Male	Male
51	Ch27	Female	Female	Female
52	Ch28	Male	Male	Male
53	Ch29	Female	Female	Female
54	Ch30	Male	Male	Male
55	Ch31	Male	Male	Male
56	Ch32	Male	Male	Male
57	Ch33	Female	Female	Female
58	Ch34	Female	Female	Female
59	Ch35	Female	Female	Female
60	Ch36	Male	Male	Male
61	Ch37	Male	Male	Male
62	Ch38	Female	Female	Female
63	Ch39	Female	Female	Female
64	Ch40	Male	Male	Male
65	Ch41	Male	Male	Male
66	Ch42	Female	Female	Female
67	Ch43	Male	Male	Male
68	Ch44	Male	Male	Male
69	Ch45	Unidentified	Female	Female
70	Ch46	Male	Male	Male
71	Ch47	Male	Male	Male
72	Ch48	Female	Female	Female
73	Ch49	Male	Male	Male
74	Ch50	Female	Female	Female

Conclusion

Thus, the P2/P8 primer was more accurate in the sex estimation in birds, whereas, the P2/MP/NP PCR has comparatively less efficient in detecting the sex of birds. It is worth mentioning that the P2/MP/NP PCR was 100% accurate in the estimation of sex in Khaki Campbell duck (*Anas platyrhynchos domesticus*), Guinea fowl (*Numida meleagris*), Turkey (*Meleagris gallopavo*) and Japanese quail (*Coturnix japonica*). The primer failed in chicken primarily due to the drawbacks discussed previously. Depending on targeted species the P2/P8 PCR can be used to identify birds with precision.

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