The Journal of Agricultural Sciences - Sri Lanka
Vol. 14, No. 3, September 2019. Pp 201-206
http://doi.org/10.4038/jas.v14i3.8603

Comparison of DNA Isolation and Dominant and Co-dominant Molecular Markers to Reveal the Genetic Sex of *Gallus domesticus* (Domestic Chickens)

W.K.R. Nirmali[1,2], L. Warnakula[1], R. Cooray[1],N.S. Hapuarachchi[1] and M.P.S. Magamage[2*]

Received:31st January 2019/ Accepted: 30th May 2019

ABSTRACT

Purpose: *The quality of DNA and reliability of molecular markers are crucial for the success of Polymerase Chain Reaction (PCR) based genetic sex determination. This study was aimed at investigating the optimum conditions for isolation of DNA from chicken blood and the reproducibility of dominant and co-dominant sex markers to be validated as a tool for successful sexing in avian research.*

Research Method: *Efficacy of six different extraction procedures including manual and solution based commercial purification kit were evaluated with different combinations of initial blood, lysis buffer and protein denaturant in relation to the DNA yield and purity. Three primer sets namely CHD1, HUR 0423 and HUR 0424 were evaluated by PCR.*

Findings: *The study results showed that 10µl of initial blood volume yields a significantly high DNA yield with high purity. Dominant marker HUR0424 showed to be a reliable marker system for the genetic sexing of domestic chickens over co-dominant markers.*

Research Limitation: *For the accuracy of the results, protocols had to be followed at the same time and using same sample to avoid any errors.*

Originality/ Value: *PCR based sexing is considered, the most accurate and inexpensive method and hence validation of the method is important for success of future avian research.*

Keywords: *Co-dominant markers, Dominant markers, Gallus domesticus, Genetic sexing, Protocol optimization*

INTRODUCTION

Avianblood comprised of erythrocytes, leukocytes and thrombocytes. Unlike mammalian blood, its red blood cells and thrombocytes are nucleated, making it a rich source of DNA (Maxwell, 1993). Therefore, DNA isolation from avian blood may result high yields of DNA even with small amounts of initial blood, making it a major problem of clogging in column-based purification methods *(*Honkatukia *et al.,* 2010*)*. If the sample volume is reduced to counteract this problem, it may cause yield reductions and impure end products. Also, high levels of proteins, minerals and fatty acids in avianblood plasma forms jelly like compounds during DNA isolations making it more complicated. Therefore, optimisation of extraction process is vital in avian researches to obtain PCR grade DNA for downstream applications.

Z and W are the two sex chromosomes found in birds where females are heterogametic (ZW) and males are homogametic (Griffiths and Tiwari, 1996; Griffiths and Korn, 1997;

[1] *Section of Genetics, Institute for Research and Development, Colombo, Sri Lanka.*
[2*] *Laboratory of Reproductive Biology and Biotechnology, Department of Livestock production, Sabaragamuwa University of Sri Lanka, PO Box 02, Belihuloya. Sri Lanka.*
manjula.magamage@fulbrightmail.org
https://orcid.org/0000-0003-1227-3607

Griffiths *et al.,* 1998; ZZ; Ellegren, 2001). The accurate sex determination of the chickens is important in the embryonic research and the sex reversal trials based on chicken model and also economically crucial in the layer industry. Application of the polymerase chain reaction (PCR) to identification of genetic sexes of birds is ideal because it requires only a small sample, such as a drop of blood or a single plucked feather, for DNA extraction, minimising trauma to individual birds (Itoh, 2001). The PCR based sex identification is therefore, considered to be the most accurate and inexpensive method over most of the available sex determining methods (Dhanasekaran *et al.*, 2016).

Chromosome W is female specific and Z is its homologous chromosome. Any DNA sequence that is present only on the W chromosome and is widely conserved among species would be a target of PCR based sex identification (Itoh, 2001). Therefore, W specific targets have been widely studied and several W specific dominant targets have been proposed for the identification of avian sex. Chromodomain Helicase DNA (CHD) binding protein loci which is preserved within both avian Z and W chromosomes has been another target for the sex determination of birds including domestic chickens. The size of the CHD gene is slightly different in Z and W chromosomes due to a variance in the intron regions (Griffiths *et al.,* 1998), making it an ideal co-dominant marker to distinguish homozygotes from heterozygotes. Even though many chromosomal targets have been identified for early sex determination of birds, reproducibility of those markers is still problematic. Therefore, finding a reliable marker system for genetic sexing of birds is crucial in avian research as it can save resources, time and money of the researchers.

Therefore, the aim of this research was to investigate the optimum conditions of DNA isolation from chicken whole blood and to compare the reproducibility of two previously described genetic sex determination marker systems (dominant and co-dominant) for the early sex determination of domestic chickens.

MATERIALS AND METHODS

Animal Use Statement

All animal experiments were conducted under the guidance and approval of institutional animal care and use committee recommendations of Sabaragamuwa University of Sri Lanka (Approval Number: ERC/A/06/2017/01).

Experimental design for DNA extractions

Five different extraction procedures including conventional methods such as Sodium Dodecyl Sulphate (SDS) and CetylTrimethyl Ammonium Bromide (CTAB) extraction methods and solution based commercial purification kit (QIAGEN Flexi Gene® DNA Purification Kit) were evaluated (Table 01) (Sambork, 2001; Qiagen, 2010). Column based kits were excluded as previous studies have reported clogging issues. Efficacy of procedures was assessed with different combinations of initial blood, lysis buffer and protein denaturant in relation to the DNA yield and purity.

Table 01: Experimental data of DNA extraction procedures.

Procedure	Initial Blood Volume (µl)	Type of Lysis Buffer	Protein Denaturant	Reference
1	100	FG1 buffer	Proteinase K	(Maxwell *et al.*, 1987)
2	50	FG1 buffer	Proteinase K	
3	10	FG1 buffer	Proteinase K	
4	10	SDS	Cloroform/ Iso amyl	(Valdez *et al.,* 2010)
5	10	CTAB	Cloroform/Iso amyl	(Valdez *et al.,* 2010)

Samples collection, DNA isolation and quantitation

200µl of blood from shaver brown day old chicks was collected into 2ml EDTA tubes, from the brachial vein, using sterile 31gauge needles and were stored at -20°C. DNA extraction was performed in triplicates as given in Table 1. DNA concentration, 260/280 and 260/230 purity ratios were measured by Nano Drop UV/ Visible spectrophotometer. The integrity of samples was checked by 1% agarose gel stained with Diamond fluorescent dye.

Comparison of genetic sexing markers by PCR

DNA of 20 randomly selected day old non-sexed chicks, extracted by the procedure 3 discussed above was used for the PCR assay. Two known samples from mature male and female were used as reference samples. Three primer sets namely CHD1, HUR0423 and HUR0424 (Maxwell *et al.*, 1987; Valdez *et al.,* 2010) were used for the PCR amplification (Table 02).

PCR amplification was performed in 20µl final volume containing 10ngµl^{-1} of genomic DNA, 10pM of forward and reverse primer,1X FIREPol® Colourless Master Mix (Solis BioDyne) and PCR grade water (Solis BioDyne). Initial denaturation of 94°C for 3 min followed by 40cycles at 94°C for 20 seconds, 54°C for 30 sec and 72°C for 1 min, and a final extension at 72°C for 7 min was used for CHD1. Initial denaturation of 95°C for 5 min followed by 35 cycles at 94°C for 30 sec, 47°C (HUR0423)/ 54°C (HUR0424) for 30 sec and 72°C for 30 sec, and a final incubation at 72°C for 7 min were used with HUR0423 and HUR0424 primers. PCR amplicons were visualised by 1.5% agarose gels stained with Diamond fluorescent dye. Data were analysed using ANOVA bythe Statistical Analysis System (SAS) 9.0.

RESULTS

Comparison of extraction methods

Using of 100µl of initial blood for the extraction of DNA from the commercial DNA purification kit resulted a clump of DNA which did not dissolve properly in the re-suspension buffer. 10µl of blood therefore, showed to be the ideal initial volume for avian DNA extractions (Table 03).

Statistical analysis of extraction procedures in related to the DNA yield and purity by ANOVA showed a significant difference of average DNA yield between the 5 extraction procedures (p value <0.0001). According to Duncan grouping, Procedure 3 yielded the best DNA concentration followed by procedures 2 and 5 respectively. The least yield was recorded in procedure1followed by 4. The 260/280 ratios were statistically significant among the procedures (p value is 0.0019). Methods 5, 4, 3 and 2 showed similar highest ratios while 1 showed the lowest ratio. The 260/230 ratios were also relatively, statistically significant among the procedures (p value is 0.0009). Method 5 showed the highest value while others showed similar low ratios.

Comparison of dominant and co-dominant markers

Co-dominant marker CHD1 resulted 2 bands at 310bp and 271bp positions in chicken female samples and one band at 271bp in chicken male samples (Figure 01), but the results were not reproducible in all cases replicated.

Table 02: Primer details

Primer	Sequence (5'-3')	Annealing Temperature
CHD1	Forward:CAAGGATGAGAAACTGTGCAAAACAG Reverse:CAAGGATGAGAAACTGTGCAAAACAG	54°C
HUR 0423	Forward:GAGCTGATTGCTTCAGAGGG Reverse:TCTGGTTCCAATTCAATGTCA	47°C
HUR 0424	Forward:GGTCGGGGAGAGGAATAAAA Reverse:GCACCACAGGCTTACGCTAT	54°C

Table 03: DNA yields and purity values

Procedure	Average DNA yield ± SD(ngµl^{-1})	260/280± SD	260/230± SD
1	4.6 ± 1.652	1.23± 0.329	0.57± 0.542
2	397.1± 101.025	1.68± 0.075	1.04± 0.170
3	1226.4± 61.37	1.87± 1.867	2.25± 0.019
4	51.33± 17.69	1.87±0.127	0.56± 0.385
5	301.87± 12.58	1.94± 0.028	5.98± 2.495

Dominant marker HUR0423 failed to give a clear amplification in PCR. Dominant marker HUR0424 resulted a single band for female and no bands for male samples. Results were reproducible and reliable among samples being investigated (Figure 02).

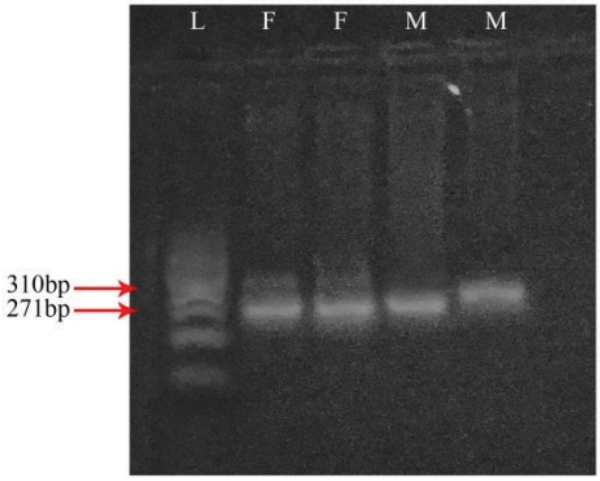

Figure 01: PCR amplification of CHD1 locus of Chicken DNA. L: 100bp DNA size ladder, F: Female sample and M: Male sample

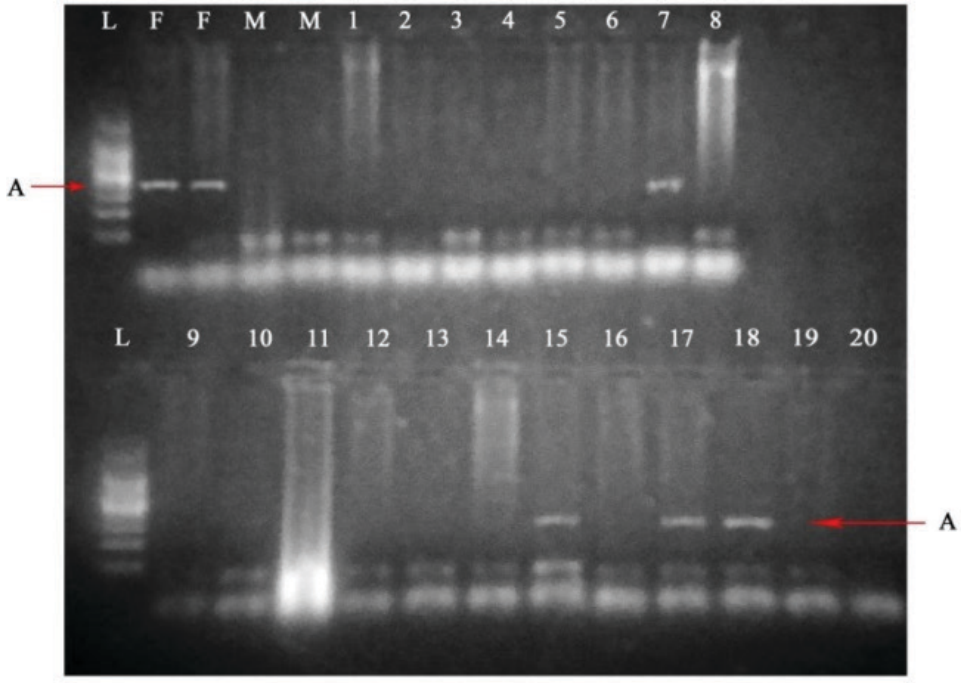

Figure 02: PCR amplification of HUR0424 locus of Chicken DNA. L: 100bp DNA size ladder, F: Female Samples and M: Male Samples, 1-20: Amplicons for 20 random samples.

DISCUSSION

The occurrence of DNA clump which couldn't be dissolved when using a higher initial blood volume can be supported by the previous evidence by Honkatukia *et al.,* (2010), for the extraction of large amount of DNA by a small volume of blood making the clump since there is a large number of nucleated cells in avian blood. According to the DNA extraction results, the best protein denaturation method for DNA extraction using avian blood is the enzymatic method which involves protease K enzyme and yielded the highest DNA yields. However, the manual method, chloroform isoamyl alcohol method can also be efficient with an efficient lysis buffer although it involved several human errors comparatively. Out of the lysis buffers considered, the CTAB buffer yielded the best yield compared to SDS buffer. According to previous literature, the SDS buffer was used in DNA extraction from the blood following an RBC lysis step in the protocol (Qamar *et al.,* 2016). Accordingly, it can be suggested that the CTAB buffer was efficient in the lysis of both RBCs and WBCs yielding, a higher DNA yield and SDS buffer was efficient in lysis of only WBCs but not RBCs reducing the DNA yield.

According to the results of the purity ratios, the low 260/280 ratios from procedure 1 could be because of high protein content in larger volume of initial blood which could not be properly removed from the medium during protein denaturation while the other procedures could manage the removal of protein using both enzymatic and manual methods when the initial blood volume is lower. The result of highest 260/230 ratio from the procedure 5 and similar low ratios in other procedures could be due to the presence of organic contaminants in the extracted DNA samples of all procedures except for CTAB method which suggests that the best removal of the organic contaminants can be obtained by using CTAB buffer.

When considering the results of the PCR amplification, even though clear bands were obtained from the molecular markers except HUR0423 to differentiate the sex of chicken samples, based on the reproducibility of results, it could be concluded that Dominant marker HUR 0424 to be the best marker in genetic sexing of chicken.

CONCLUSION

DNA extraction from lesser volume of chicken whole blood yields more DNA with high purity. Protein denaturation was efficient when using both enzymatic as well as Chloroform isoamyl alcohol methods but the efficacy of lysis buffer determines DNA yield and the purity. The Dominant marker showed to be a reliable marker system for the genetic sexing of domestic chickens over co-dominant markers.

Data Availability Statement

The data generated during the current study are available in the manuscript.

ACKNOWLEDGEMENT

We acknowledge the Institute for Research and Development, Battaramulla, Sri Lanka for partially funding this research.

REFERENCES

Dhanasekaran, S., Raj, G., Vignesh, A., Selvan, S., Prakash, B., Perumal, P., Arivudainambi, S. andBabu, T. (2016). Gender identification in Chicken (Gallus gallus) by PCR using whole blood and dried blood spot on filter paper as template: without prior DNA isolation. bioRxiv. 046888. DOI: https://doi.org/10.1101/046888

Ellegren, H. (2001). Hens, Cocks and avian sex determination. EMBO reports. 2(3): 192-196. DOI: https://doi.org/10.1093/embo-reports/kve050

Griffiths, R., Double, M., Orr, K. and Dawson, R. (1998). A DNA test to sex most birds. *Molecular Ecology*. 7(8): 1071-1075. DOI: https://doi.org/10.1046/j.1365-294x.1998.00389.x

Griffiths, R. andKorn, R. (1997). A CHD1 gene is Z chromosome linked in the chicken Gallus domesticus. Gene. 197(1-2): 225-229. DOI: https://doi.org/10.1016/s0378-1119(97)00266-7

Griffiths, R. andTiwari, B. (1996). Sex of the last wild Spix's macaw. *Nature*. 375(6531): 454-455. DOI: https://doi.org/10.1038/375454a0

Honkatukia, M., Soderback, P. and Kulmala, J. (2010). Isolation of genomic DNA from chicken blood using QuickPick™ gDNA kit, Technical Note: *Molecular Biology*. BN Products & Services. http://www.bionobile.com/literature/TN_gDNA_chicken_blood.pdf (29.09.2017)

Itoh, Y. (2001). Identification of the sex of a wide range of Carinatae birds by PCR using primer sets selected from chicken EE0.6 and its related sequences. *Journal of Heredity*. 92(4): 315-321. DOI: https://doi.org/10.1093/jhered/92.4.315

Maxwell, B.L., McDonnell, D.P., Conneely, O.M., Schulzm T.Z., Greene, G.L. and O'Malley, B.W. (1987). Structural organization and regulation of the chicken estrogen receptor. *Molecu*l*ar Endocrinology*. 1(1): 25-35. DOI: https://doi.org/10.1210/mend-1-1-25

Maxwell, M. (1993) Avian blood leucocyte responses to stress. *Worlds Poultry Science Journal*. 49(1): 34-43. DOI: https://doi.org/10.1079/wps19930004

Qamar, W., Khan, M.R. andAraf[ah], A. (2016). Optimization of conditions to extract high quality DNA for PCR analysis from whole blood using SDS-proteinase K method. *Saudi Journal of Biological Sciences*. DOI: http://doi.org/10.1016/j.sjbs.2016.09.016.

QIAGEN. (2010). QIAGEN FlexiGene® DNA Handbook, Qiagen Handbook. Available at:https://www.qiagen.com/fr/resources/resourcedetail?id=6b147421-7846-411e-9993-bb01563b807e&lang=en. (23.09.2017)

Sambork, J., Fritsch, E.F. and Manutis, T. (2001). *Molecular cloning: A laboratory manual*. 2nd Edition. Volume 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. DOI: https://doi.org/10.1002/abio.370050118

Valdez, M.B., Mizutani, M., Kinoshita, K., Fujiwara, A., Yazawa, H., Shimada, K., Namikawa, T. and Yamagata, T. (2010). Differential development of sex-related characters of chicken from the GSP and PNP/DO inbred lines after left ovariectomy. *Journal of Reproductive Development*. 56(1): 154-161. DOI: https://doi.org/10.1262/jrd.09-156s