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Sex Determination on Racing Pigeons (*Columba livia*) Molecularly Using Blood Samples

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Abstract.
Background. Pigeons (*Columba livia*) belong to the Columbidae. Pigeons possess diverse ecological, economic, and aesthetic values, as they are monomorphic, exhibiting similar characteristics between male and female individuals, especially at a young age.
Aims. In collaboration with the Lampung Disease Investigation Center, a study has been done to confirm the sex determination of pigeons at a young age by the PCR technique. Blood samples were taken in two locations, East Lampung and Kota Metro. Molecular análisis was carried out at the Biotechnology Laboratory, Lampung Disease Investigation Center.
Methods. The technique includes DNA extraction, DNA amplification, electrophoresis and visualization.
Result. It poses a significant challenge for pigeon breeders in sex determination. Blood is a source of genetic material that can be used to determine the sex of birds. The Chromo-Helicase-DNA-binding Protein (CHD) gene is one of the genes used as a marker to molecularly differentiate the sex of birds. The sex chromosomes in female individuals have heterozygous chromosomes (ZW) and male individuals have homozygous sex chromosomes (ZZ).
Conclusion. Of eleven pigeon blood samples, there were seven female and four male individuals, and there was one mistake in the gender prediction by the pigeon owner.

Keywords: blood, CHD gene, PCR technique, pigeon, sex determination

INTRODUCTION

Various types of birds are spread throughout Indonesia, ranging from Sulawesi, Maluku, East Nusa Tenggara, Java, and Sumatra, including the Columbidae, including *Streptopelia chinensis* (tekukur), *Geopelia striata* (perkutut jawa), *Treron griseicauda* (punai pengantin), *Treron vernans* (punai gading), and *Streptopelia bitorquata* (dederuk jawa/puter), *Columba livia* (pigeon, merpati) (Eprilurahman *et al*., 2018). According to Elvanda (2023), pigeons belong to the Family Columbidae, with most of their activity being flying. Ecologically, pigeons play a role in the dispersal and pollination of various plants (Syamsia, 2022). In terms of aesthetic value, pigeons are recognized as pets and cultivated birds. In the socio-economic context, pigeons are utilized in bird racing competitions and are also consumed as food. Generally, pigeons that are included in bird racing competitions are male. Preliminary research by Disastra (2021) showed that there was a mistake of bird breeders in determining the sex on Columbidae morphologically. According to Fitrian (2014), sex determination is generally carried out based on morphological characteristics, such as body size and coat color. It has obstacles, as the morphological characteristics of individual males and females are difficult to distinguish, especially at an early age (Fitriana *et al*., 2022).

Morphologically, the male pigeon has a head that appears rough, with large leg and neck bones, and long toes. The female pigeon has a flat head, the leg and neck bones look small, and the toes are short (Kadri *et al.*, 2016). Sexing in monomorphic birds can be done by various methods, including laparoscopy, karyotyping, vent sexing, and steroid sexing. The disadvantages of these methods include high costs and time, as well as low accuracy (Fitriana *et al*., 2022). DNA analysis using the Polymerase Chain Reaction (PCR) technique can be done as it is easy to apply, fast, accurate and requires only a small number of DNA samples.

The female bird sex chromosomes are heterozygous (ZW) and male individuals are homozygous (ZZ). A method of determining sex in birds with DNA has been developed based on the detection of intron size differences in the Helicase DNA-binding Protein (CHD) Chromodomain Helicase DNA-binding Protein (CHD) gene on Z and W chromosomes. The CHD gene is the first gene proposed as a valid marker for sex differentiation in various bird species. The CHD gene has fewer differences in size and nucleotide sequence between the CHD-1Z and CHD-1W introns (El Islami *et al*., 2021).

Yimtragool and Changtor (2022) stated that DNA samples can use blood and feathers (calamus). According to Harvey *et al.* (2006) the most common method of sampling to obtain genetic material in determining sex is to take a blood sample. This study aims to confirm the sex determination of pigeons (*Columba livia*) using blood samples and the PCR technique.

METHODS

Blood sampling was carried out by carefully piercing sterile needles in the wing veins of young pigeons. The blood is collected on 5x5 mm² of A4 opaque paper until it is absorbed and stored in plastic clips. The blood samples were prepared using PBS buffers and were homogenized. DNA extraction is performed using the PureLinkTM Viral RNA/DNA Mini Kits protocol, which involves lysis, binding, washing, and elution steps. DNA concentration measurements are performed using a Qubit fluorometer. The next stage of the DNA template is added into a microtube that has been filled with the master mix reagent. The PCR mixture is homogenized, fed into the Thermal Cycler. Amplification was carried out with the PCR program, Cycle 1 (1x): 95°C for 5 minutes, Cycle 2 (35x) stage 1: 94°C for 30 seconds, stage 2: 50°C for 45 seconds, stage 3: 72°C for 1 minute, Cycle 3 (1x): 72°C for 5 minutes, Cycle 4 (1x): 12°C (Fitriana *et al*., 2023). The results were separated using 1% agarose gel in 100 mL of TAE 1X solution and SYBR® safe DNA gel stain. The gel containing the PCR product was then electrophoresed using electrophoresis with a voltage of 100 volts for 35 minutes. The presence of PCR products is visualized under bluelight and documented using a PC-connected camera tool via the EOS Utility app.

DISCUSSION

The pigeon blood samples were obtained from pigeon breeders in East Lampung and Kota Metro from February to March 2025. Blood samples were taken from young pigeons with an age ranging from 2-3 weeks. Information about the pigeons sampled was collected through direct interviews with breeders for predicting sex (Table 1).

Table 1. Pigeon blood samples in East Lampung and Kota Metro

No.	Location	Sample Code	Sample Type	Sampling Date	Sex prediction	Bird Age (weeks)	Owner Name
1.	Labuhan Ratu Induk, Labuhan Ratu, East Lampung (5° 6'50.38"S, 105°40'25.22"E)	1	Blood	February 28, 2025	♀	3	Iskandar
2.	Rajabasa Lama Induk, Labuhan Ratu, East Lampung (5° 6'22.50"S, 105°39'4.80"E)	2	Blood	March 1 2025	♂	3	Haikal Sydemham
3.	Braja Asri, Way Jepara, East Lampung (5° 9'25.34"S, 105°42'26.83"E)	3	Blood	March 12, 2025	♀	3	Rahmad Mulyadi
		4	Blood		♀	3	
4.	Labuhan Ratu 4, Labuhan Ratu, East Lampung (5° 9'47.90"S, 105°36'59.73"E)	5	Blood	March 13, 2025	♀/♂	2	Tri Nugroho
5.	Mulyojati 16A, West Metro, Kota Metro (5° 8'47.28"S, 105°17'16.68"E)	6	Blood	March 14, 2025	♀	2	Mufit
6.	Rejomulyo 26 Plains, South Metro, Kota Metro (5° 9'32.75"S, 105°17'4.04"E)	8	Blood	March 14, 2025	♂	2	Alfaro
		9	Blood		♂	2	
7.	Raman Daya 4, North Raman, East Lampung (4°58′10.55"S, 105°27′41.77"E)	7	Blood	March 15, 2025	♀/♂	2	Rohmad
		10	Blood		♀/♂	2	
		11	Blood		♀/♂	2	

Information : The sign (♂) indicates a male individual, (♀) indicates a female individual, **(♀/♂)** showing doubts about the predicted sex

DNA extraction utilizes the silica-based extraction method, which employs silica columns to separate DNA from other components. As Pratiwi and Widodo (2020) stated, DNA quantity testing using a qubit fluorometer was carried out to determine the DNA concentration of extracted samples based on the fluorescent dye principle, which binds to double-stranded DNA (dsDNA) (Table 2).

Table 2. Results of DNA Extraction Test: Quantity of Pigeon Blood Samples with Tools Qubit fluorometer.

No.	Sample Code	DNA concentration (ng/µl)
1.	D1	7,88
2.	D2	1,61
3.	D3	1,44
4.	D4	2,56
5.	D5	9,84
6.	D6	4,20
7.	D7	10,1
8.	D8	0,964
9.	D9	12,4
10	D10	27,6
11.	D11	22,6

The quantity of DNA extraction results was measured using a Thermo Scientific Nanodrop spectrophotometer to determine the concentration and purity of the extracted DNA with wavelengths of 230 nm, 260 nm, and 280 nm. The level of DNA purity is good if the ratio value of 260/280 nm obtained is between 1.8-2.0 (Setiani *et al*., 2021) and the concentration is above 100 ng/µL (Hikmatyar *et al.*, 2015). If the purity value of DNA obtained is less than 1.8 ng/µl, it is known that there has been contamination of the extraction results in the form of phenols, proteins and carbohydrates, while if the purity value of DNA obtained is more than 2.0 ng/µl, the DNA extraction results are contaminated by the buffer carried during extraction (Mustafa *et al*., 2016).

The amplification of blood samples was carried out by electrophoresis on eleven amplicons. The amplification process aims to increase the amount of DNA in specific segments according to the primers used, namely the CHD, 2550F, and 2718R gene primers. Visualization of PCR products showed positive results on all samples (Figure 2). The positive results consisted of four male individuals and seven female individuals (Table 2).

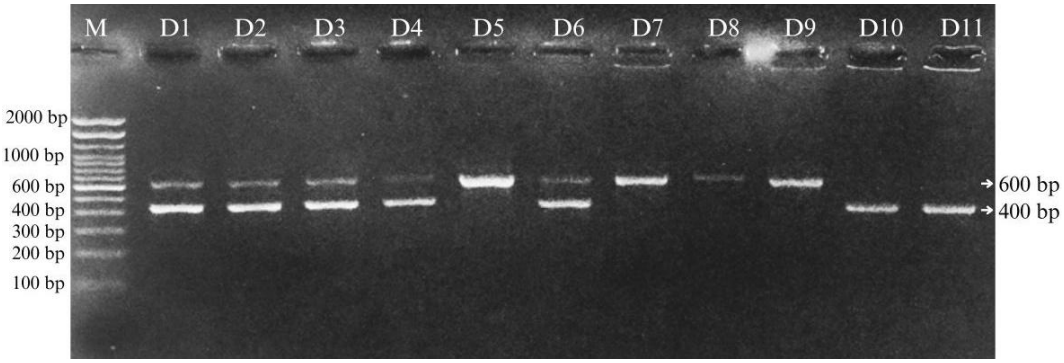

Figure 2. Visualization of pigeon DNA PCR products using blood samples using 2550F and 2718R primers.

Table 2. Results of sex determination in pigeon blood samples

No.	Sample Code	Sex	
		♂	♀
1.	D1		+
2.	D2		+
3.	D3		+
4.	D4		+
5.	D5	+	
6.	D6		+
7.	D7	+	
8.	D8	+	
9.	D9	+	
10	D10		+
11.	D11		+
	Sum	4	7

Information

(+) : A positive sign indicates that there is a band luminescence in the male (♂) or female (♀) on agarose gel.

The success of the amplification results was demonstrated by the appearance of one band in male individuals and two bands in female individuals on 1% agarose gel. The positive results consisted of two male individuals and four female individuals. The luminescence of the DNA bands seen in agarose gel is formed due to the interaction between positive ions in the SYBR® safe DNA gel stain and negative ions in the DNA molecule (Virnarenata, 2019). As referenced by Fridolfsson and Ellegren (1999), primers of 2550F and 2718R in male individual birds will produce 1 band measuring 600-650 bp, while female individuals will produce 2 bands measuring 600-650 bp and 400-450 bp.

Of the eleven samples, 4 samples showed male individuals and 7 female individuals. Based on the predicted sex in pigeons of the D2 was male individuals. However, after testing

for molecular sexing techniques showed female individuals. This shows that from the eleven samples there is one miss predicted by the bird owner.

The DNA amplification process of the DNA isolated template with pigeon blood samples produced clear bands in samples D1, D2, D3, D5, D7 and D9, while the D8 sample had faint bands. Dim bands in the D3 sample can be caused by inhibitors in PCR reactions such as hemoglobin and immunoglobulins. Hemoglobin can interfere with the activity of DNA polymerase enzymes during the PCR process, while Immunoglobulin G (IgG) interferes with genomic DNA binding, thereby inhibiting the attachment of both primary and DNA polymerase (Sidstedt *et al*., 2018; Pratomo *et al*., 2021). Meanwhile, in samples D10 and D11, only 1 clear band was produced indicating amplified CHD1-W fragments and 1 other band was dim, indicating a CHD1-Z fragment that was not fully amplified. This event is known as allelic dropout (ADO), which is the failure of the amplification process of an allele in a PCR reaction (Stevens *et al*., 2017).

CONCLUSION

Of the eleven pigeon blood samples, it can be confirmed that there are 7 female and 4 male individuals and there is 1 mistaken by morphology characteristics.

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