

**SEX DETERMINATION OF RACING PIGEONS (*Columba livia*)
MOLECULARLY BASED ON BLOOD SAMPLES**

Undergraduate Thesis

**Written By
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**DEPARTMENT OF BIOLOGY
FACULTY OF MATHEMATICS AND NATURAL SCIENCES
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ABSTRACT

SEX DETERMINATION OF RACING PIGEONS (*Columba livia*) MOLECULARLY BASED ON BLOOD SAMPLES

By

Laila Salwa Azzahra

Pigeons (*Columba livia*) belong to the Family Columbidae with most of their activities flying. Nowadays, pigeons have very diverse ecological, economic, and aesthetic values. Pigeons are monomorphic, having similar characteristics between male and female individuals, especially at a young stage. This is the main challenge for pigeon breeders in selecting male and female individuals. Blood samples are a source of genetic material that can be used in determining 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 female sex chromosomes in female individuals have heterozygous chromosomes (ZW) and male individuals have homologous sex chromosomes (ZZ). Blood samples were taken in two locations, East Lampung and Kota Metro. This study aims to confirm the determination of pigeon sex at a young age molecularly using the Polymerase Chain Reaction (PCR) technique. This research was carried out under the Lampung Disease Investigation Center and carried out at the Biotechnology Laboratory, Lampung Disease Investigation Center through stages, DNA extraction, DNA amplification, electrophoresis and visualization. Of eleven pigeon blood samples, it was confirmed that 7 female and 4 male individuals, with 1 error in the gender estimate by the pigeon owner

Keywords: blood, CHD gene, *Columba livia*, PCR, sex determination

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Written By

Laila Salwa Azzahra

Undergraduate Thesis

**In partial fulfillment of the requirements for the degree on
BACHELOR OF SCIENCE**

At

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**FACULTY OF MATHEMATICS AND NATURAL SCIENCES
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PIGEON (*COLUMBA LIVIA*)
MOLECULARLY BASED ON BLOOD
SAMPLES**

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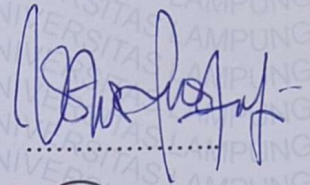
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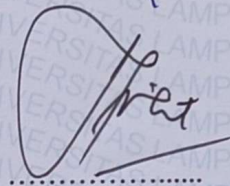
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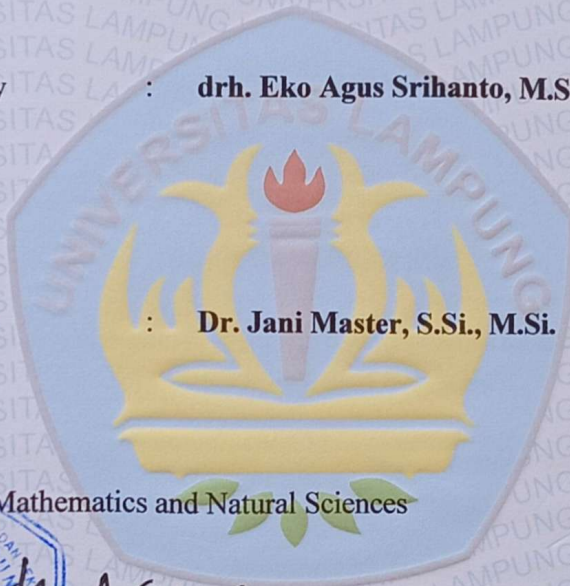
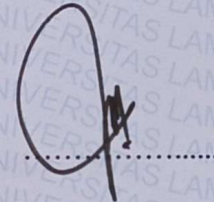
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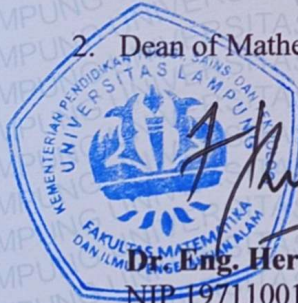
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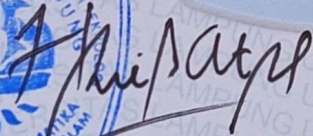
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Hereby I declare that my undergraduate thesis title:

“Sex Determination of Racing Pigeon (*Columba livia*) Molecularly Based on Blood Samples”

In truly and entirely my own work, created in accordance with the applicable norms and academic ethics. Furthermore, I do not object to the use of some or all of the data from this undergraduate thesis by faculty members and/or the study program for publication purposes, provided that my name is mentioned.

If it is later proven that my statement is untrue, I am willing to accept academic sanctions, including the revocation of my bachelor's degree, as well as legal consequences.

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BIODATA



The author was born in Rajabasa Lama on June 19, 2003 and is the first of three children of Mr. Imawanto and Mrs. Titin Murdaningsih

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Testing Center (BPKIL) with the title White Spot Syndrome Virus (WSSV) Detection in Post Larva Vannamei Shrimp (*Litopenaeus vannamei*) with the Conventional Polymerase Chain Reaction (PCR) Method at the Serang Fish and Environmental Health Testing Center (BPKIL). The author has also carried out a Community Internship Course for 40 days in July – August 2024 in Sribhawono, Bandar Sribhawono, East Lampung. The author has also attended the National Seminar Conservation III as a speaker with the title “Sex Determination of Racing Pigeon (*Columba livia*) Molecularly Based on Blood Samples”, as well as attending the Workshop on “primary preparation” organized by the Lampung Disease Investigation Center. Scientific article on Sex Determination of Racing Pigeon (*Columba livia*) Molecularly Based on Blood Samples is in ongoing process to be published in Journal of Agricultural Sciences (Agrosci). In addition to participating in academic activities, the researcher has been a member of the Communication, Information and Public Relations (KOMINHUM) division of the Biology Student Association (HIMBIO), on year 2022, a member of the Social and Community Service Service (SPM) BEM FMIPA Unila. In addition, I have contributed to the Natural Resources Conservation Week (PKSDA) as a Member of the Public Relations Division.

PRAYER

Bismillahirrahmanirrahim

By expressing gratitude to Allah SWT, I dedicate my small work to my parents,
my beloved father Imawanto and my beloved mother Titin Murdaningsih who
have given all the affection, and always prayed, gave encouragement and
delivered to this place

Mr. and Mrs. Supervisor who have patiently educated me to make me a better
person

Hopefully my work may benefit others. Ameen.

MOTTO

"Surely with that hardship comes more ease. So once you have fulfilled (your duty) strive (in devotion). Turning to your Lord alone with hope."

(QS. Al-Insyirah: 6-8)

"Whatever your destiny is, it will find its way to find you"

Ali bin Abi Talib

"Live your life better. Because Tomorrow I think you can be a better version of you, no matter what. I think you're doing fine. Then you can be fine. Thumbs up!"

Claude Clawmark

"I'm not afraid of wounds and pain. Whatever happens, I won't be afraid. Going to find my dreams. Let's be the first rabbit."

JKT48

"If you're not a good shot today, don't worry. There are other ways to be useful"

Sasha Novikov/Sova

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There are still many shortcomings in writing this study. Therefore, constructive suggestions and criticism are still very much needed in writing scientific papers in the future.

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I. INTRODUCTION

1.1 Background

Indonesia is one of the countries that has a high bird diversity. In 2018 the diversity of birds was recorded at 1,771 species and in 2019 it became 1,777 species (Latumahina *et al.*, 2020). Various species of birds are spread throughout Indonesia ranging from Sulawesi, Maluku, Nusa Tenggara Timur, Java, and Sumatra. One of the birds found in Indonesia is from the Columbidae including *Streptopelia chinensis* (tekukur), *Geopelia striata* (Javanese perkutut), *Treron griseicauda* (punai pengantin), *Treron vernans* (punai gading), and *Streptopelia bitorquata* (Javanese deduruk), *Columba livia* (pigeon) (Eprilurahman *et al.*, 2018).

Playing a role in the socio-economic aspect, pigeons are used in bird racing competitions and consumed. Generally, pigeons that are included in bird racing competitions are male. This is the main challenge for pigeon breeders in selecting superior male and female individuals, in order to produce superior offspring as well. Disastra (2021) shows that there is a mistake of bird sellers in determining the sex (sexing) of the Columbidae morphologically. Gender determination is an effort to breed, increase maintenance, population development, and increase conservation efforts for pigeons (Morinha *et al.*, 2012). According to Fitriana (2014), the sexing is generally carried out based on morphological characteristics, such as body size and feather color. This technique has obstacles as birds are monomorphic, the morphological characteristics of individual males and females are difficult to distinguish, especially at the young or early age (Fitriana *et al.*, 2022). Morphologically, in male pigeons, the head looks

rough, the leg and neck bones are large, and the toes are long. As for the female pigeon, the head is flat, 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 require long costs and time and low accuracy (Fitriana *et al.*, 2022). DNA analysis using the Polymerase Chain Reaction (PCR) technique is an alternative that can be done because the technique is easy to use, fast, accurate and requires only a small number of DNA samples. The samples used in the analysis can be derived from bird feathers or blood (Wulansari *et al.*, 2013).

A sexing method in birds with DNA has been developed based on the detection of intron size differences in the Helicase DNA-binding Protein (CHD) on Z and W chromosomes. The CHD gene is the first gene to be proposed as a valid marker for sex differentiation in different types of birds. The CHD gene has fewer differences in size and nucleotide sequence between the CHD-1Z and CHD-1W introns (El Islami *et al.*, 2021). The sex chromosomes of birds are different from mammals, on birds the sex of the female individual has heterozygous (ZW) chromosomes and male individuals homozygous (ZZ). Amplification of the CHD gene segment in male birds results in only one amplicon fragment of the Z chromosome, while in female birds it produces two fragments of the Z and W chromosomes that have different band lengths due to the difference in the length of the amplified introns. Sex identification using molecular methods can be applied to young monomorphic birds, by targeting sex chromosomes directly (Purwaningrum *et al.*, 2019).

Yimtragool and Changtor (2022) stated that DNA samples can use blood and feathers (calamus). Blood and feather sampling is an invasive technique, by direct contact with the individual bird. Trimbo *et al.* (2009) show the sexing

on birds using non-invasive samples such as eggshell waste that can contaminate DNA material with other proteins that are not needed. According to Harvey *et al.* (2006) the most common method of sampling to obtain genetic material in sexing is to take blood samples.

1.2 Research Objective

This study aims to confirm the determination of the sex of pigeons (*Columba livia*) using blood samples using the Polymerase Chain Reaction (PCR) technique.

1.3 Theoretical Framework

Pigeons have the ability to recognize locations and fly long distances and act as pollinators, and seed dispersers. Over time, pigeons were used for consumption, communication and competition. Pigeon racing is a race that relies on the ability to fly fast to a certain destination point using male pigeons.

Sexing in birds plays an important role in the selection of superior individuals. Pigeons have monomorphic properties, which makes it difficult to determine sex based on morphological observations, especially in young individuals. Sexing in monomorphic birds can be done by laparoscopy, vent sexing, steroid sexing and karyotyping. Molecularly, sexing can be conducted by the Polymerase Chain Reaction (PCR) technique, which is carried out based on differences in the combination of male and female sex chromosomes. The sex chromosomes of male birds are composed of homogametes (ZZ), while in female individuals they are heterogamous (ZW).

The Chromo-Helicase-DNA-binding Protein (CHD) gene is one of the genes on the sex chromosome that can be used in molecular birds sexing. In male

individuals have two CHD-Z genes that will produce amplification into one sequence, while female individuals have CHD-W and CHD-Z genes that will produce amplification into two sequences during the PCR process. A study to determine the sex of birds based on blood samples based on the CHD gene using a pair of primers 2550F and 2718R with PCR techniques was carried out. This research is expected to provide accurate information related to the sex of monomorphic birds at a young age.

II. LITERATURE REVIEW

2.1 Pigeon (*Columba livia*)

2.1.1 General Characteristics and Classification

Birds are warm-blooded animals (homeotherms) that can regulate their body temperature according to the environment around where they live, and have feathers that cover their bodies (derived from the epidermis). Most of the feathers are found on the wings, and as the bird ages, its feathers will be lighter, wider, stronger and denser. The bristles are arranged at the ends of the bristles in such a way that they are waterproof and warm the body from cold temperatures. The bird's front limbs are modified into wings and the hind limbs are used for walking, swimming and perching. In birds, the mouth is modified to be a beak with the lower jaw having no teeth, and has air sacs and has four heart chambers (Hidayat, 2022).

According to Aji *et al.* (2015) pigeons found in Indonesia are a local birds, estimated to come from a species of wild pigeon (*Columba livia*) from Europe that has been domesticated for a long time. Pigeons are known by the local name of burung dara, merpati and in Tidore they are called virgins furuh (Hamid *et al.*, 2016). Domestic pigeons in general can be used as racing pigeons, consumption, ornamental pigeons (aesthetic) and mail pigeons (Aji *et al.*, 2015). Other abilities that pigeons have, including recognizing their mates,

owners, or trainers remotely, with high flight speeds. Pigeons are often used in competitions as racing pigeons (Hamid *et al.*, 2016). Pigeons have small heads with the shapes like perkutut bird, flat, and round types. The shape of the pigeon's wings has a wider and harder surface, with the body shape divided into a banana heart shape and a ship-like shape (Kadri *et al.*, 2016). The beak type of pigeon is short and pointed, used to peck at food such as seeds. The type of pigeon's tail is round and the legs of the pigeon are used to perch on trees (Figure 1) (Kahby *et al.*, 2023).

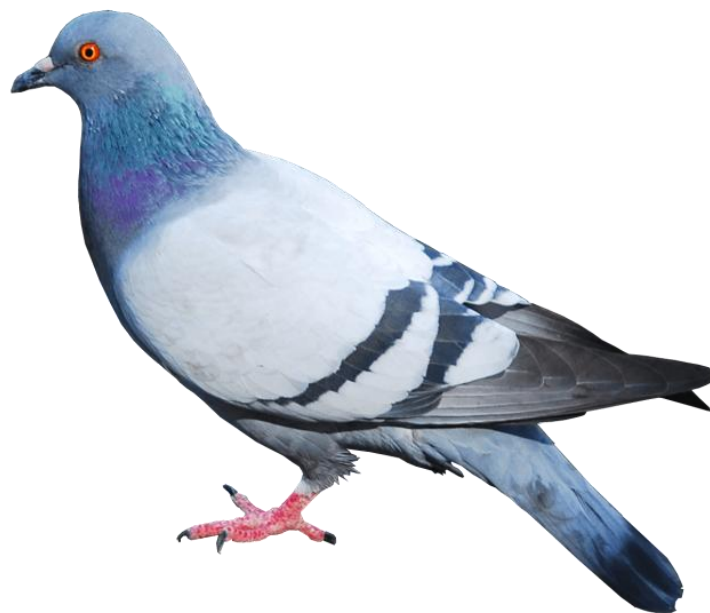


Figure 1. Pigeon (*Columba livia*) (Source: Fapet IPB, 2023)

Columbidae consists of 344 species, 50 genus scattered throughout the world, except for thirteen extinct species. *Columba*, a genus of the Columbidae consisting of about 35 species. This genus was first introduced by Carl Linneus in 1758 in the paper *Systema Naturae* (Sirhandi *et al.*, 2021). Domestic pigeons are known as rock pigeons in some countries due to their life perched on the edge of stone buildings (Chitty, 2018).

The classification of pigeons based on the *Handbook of the Birds of the World* cited by Chitty (2018) is as follows:

Kingdom : Animalia
 Phylum : Chordata
 Class : Aves
 Order : Columbiformes
 Family : Columbidae
 Genus : *Columba*
 Species : *Columba livia*

2.1.2 Morphology of Aves Blood

Blood is a component in the circulatory system that plays a role in circulating various essential compounds needed by the body. Blood cells are divided into red blood cells (erythrocytes), white blood cells (leukocytes), and blood platelets (thrombocyte). In the Aves Class, erythrocytes have nuclei (Rousdy and Linda, 2018) (Figure 2) and are larger in size than mammalian erythrocytes and smaller compared to reptilian erythrocytes (Kinanti *et al.*, 2017). The size of erythrocytes in aves varies depending on the species, with a general range ranging from $10.7 \times 6.1 \mu\text{m}$ to $15.8 \times 10.2 \mu\text{m}$. Mature erythrocytes are elliptical in shape with the cell nucleus located in the middle. Chromatin inside the nucleus tends to clump together and become denser as the cell ages. The lifespan of erythrocytes in aves is shorter, ranging from 28-45 days (Utami, 2013).

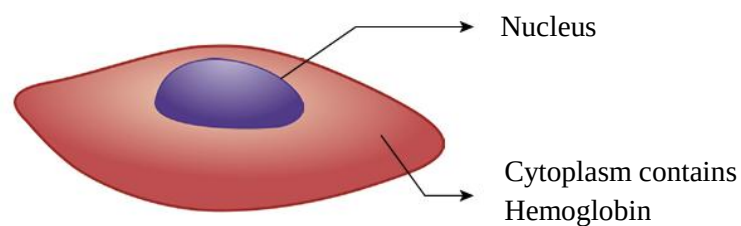


Figure 2. Erythrocytes in aves (Source: Scanes and Dridi, 2021).

2.1.3 Habitat and Behavior

In general, pigeons live in tropical and subtropical areas, and are found in a variety of landscapes adjacent to settlements. Pigeons can adjust adaptively and can survive in different environments including forests, parks, and cities (Elvanda, 2023). In its natural habitat, pigeons nest and perch on rock banks. Pigeons are monogamous and live in groups, forming nests made of plant material and feathers. In young pigeons (squab) or already able to fly at the age of 5 weeks, they are given 'crop milk' by their mother. Crop milk is produced from the layer of the plant that is shed, vomited, and given to the squab. While adult pigeons usually eat seeds and small insects as a source of protein (Chitty, 2018).

2.1.4 Ecological Status

One of the threats faced by pigeons is exploitation by humans. Pigeons are not categorized as endangered animals, as they have a high rate of reproduction and optimal adaptability. Various factors such as habitat destruction, encroachment by humans, climate change, need to be a concern for pigeons' needs for access to clean water, food, safe places to nest and care for their young (AAAC Wildlife Removal, 2022).

Based on their diversity and conservation status, pigeons are categorized as "least concern" or at low risk by the International Union for Conservation of Nature (IUCN) because their population is stable (IUCN, 2016). Pigeon conservation efforts are focused on protecting habitats. This includes cultivating protected areas, planting trees, and restoring degraded habitats, as well as public education in increasing awareness of the importance of pigeons (AAAC Wildlife Removal, 2022).

2.2 Sex Chromosomes In Birds

The sex chromosomes in birds are made up of Z and W that develop differently. In female individuals, they are heterogamous (heterogamous ZW) by carrying copies of the Z and W chromosomes while in male individuals they are homogamous (homogamet ZZ) and carry two copies of the Z chromosome (Fitriana *et al.*, 2022). The size of the Z chromosome is larger because it has largely been conserved, in contrast to the smaller W chromosome because it has lost most of its genes through evolution (De Silva *et al.*, 2023). Female individuals have different sequences of DNA nucleotides, which are specific to the W chromosome and can be used as a bird sex identifier. This plays a role in identification using DNA-based techniques in determining the sex of birds (Griffiths, 2000).

The Chromodomain helicase DNA binding (CHD) marker gene is a gene found on the W and Z chromosomes in the aves group. Fridolf-sson and Ellegren (1999), have developed a method for determining DNA sex based on the detection of differences in the size of the Chromodomain helicase DNA binding (CHD) gene intron on the Z chromosome and W chromosomes. Female individuals are characterized by displaying one (CHD1W) or two fragments (CHD1W) and (CHD1Z), while male individuals display only one fragment (CHD1Z) whose size is clearly different in size from the CHD1W fragment specific to females (Figure 3) (Sulandart and Zein, 2012).

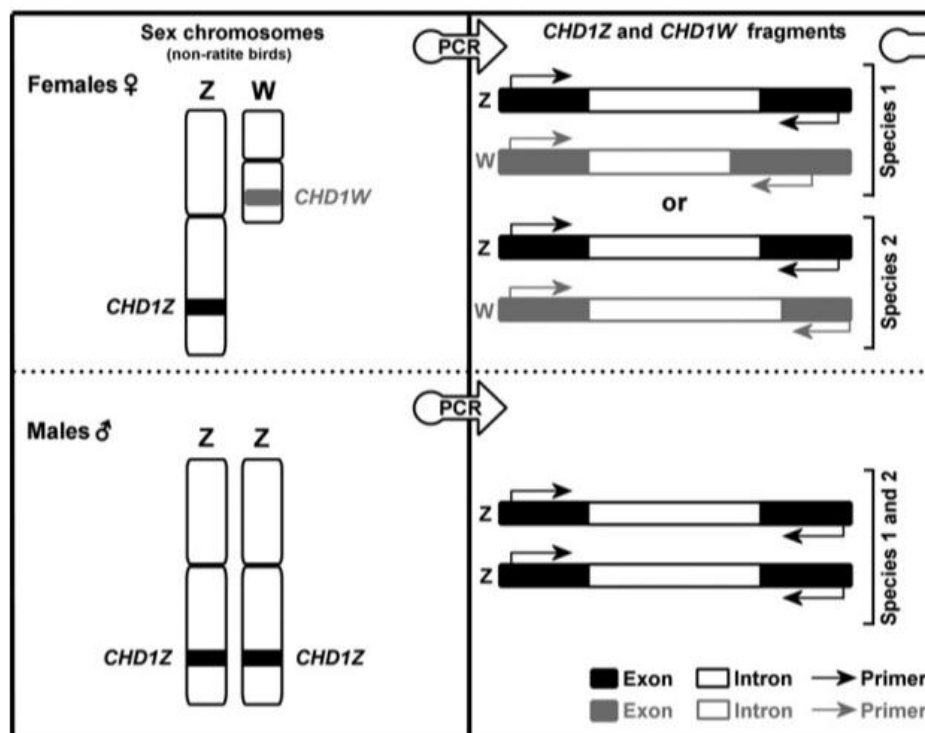


Figure 3. Introns of W Chromosome and Z Chromosomes in aves (Source: Morinha *et al.*, 2012)

The use of the poultry W chromosome Chromo-helicase-DNA-binding Protein gene (CHD-W) which is similar to the Y chromosome in mammals, was first discovered by Griffiths and Tiwari (1993).

2.3 Sex Determination Techniques

The sexing method in monomorphic birds can be done in various ways such as morphological characteristics, laparoscopy, vent sexing, steroid sexing and karyotyping (Fitriana *et al.*, 2022).

1. Laparoscopy

The Laparoscopic method is performed by cutting the small lateral part of the left side parallel to the bird's posterior ribs so that the physical characteristics of the reproductive organs can be seen on a laparoscopic or otoscope device (De Silva *et al.*, 2023). The weakness of the laparoscopic

method requires anesthesia which can risk the reproductive organs and cause death in individual birds (Nattalia, 2018).

2. Karyotyping

Karyotyping is a method that is carried out by differentiating the size of the Z and W chromosomes in birds (Nattalia, 2018). In general, the W chromosome is smaller than the Z chromosome. The karyotyping method is carried out by isolating chromosomes and karyotypes from blood and hair samples. According to De Silva *et al.* (2023), weaknesses of karyotyping methods, it takes a long time to obtain cell culture and requires trained and experienced skills.

3. Vent Sexing

According to Susilawati (2014), vent sexing is a method to determine sex based on its cloacae morphology. In this method, the presence or absence of a phallus in the male reproductive organs or the presence of the clitoris in female individuals can be determined by pressing the cloaca (De Silva *et al.*, 2023). The vent sexing method gets accurate results up to 95%. The application of the vent sexing method is difficult to do, as it requires an experienced and trained expert in differentiating small protrusions in the cloaca called the phallus (Natalia, 2018) (Figure 4).

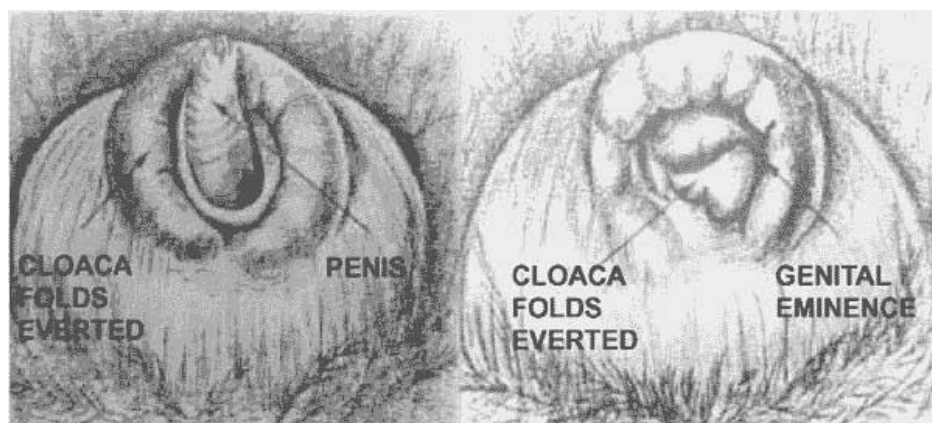


Figure 4. Protrusion at the top of the male individual cloaca (left) and the absence of a protrusion at the top of the female individual cloaca (right) (Food and agriculture organization, 2002).

4. Sexing steroids in feces

The steroid sexing method is based on the ratio of estrogen and testosterone (E/T) in bird droppings in determining the sex of the bird. This method is an alternative to non-invasive techniques with a sex determination accuracy of more than 70%, although there are seasonal variations that can affect hormones during the mating season in certain bird species (De Silva *et al*, 2023). Blank *et al.* (2020) stated that in male individuals, fecal androgen levels during the approach period were higher than during copulation, while fecal estrogen concentrations were higher during the approach and copulation periods in female individuals.

2.4 Polymerase Chain Reaction (PCR)

Rapid and accurate gender determination can be done using the Polymerase chain reaction (PCR) method. The invention of the PCR technique was invented by Kary Mullis in 1983. The PCR technique is a molecular technology that allows target DNA to be multiplied in vitro using enzymatic reactions (Mullis, 1990). In determining the sex of birds molecularly, PCR techniques consist of three stages, namely DNA extraction, DNA amplification and electrophoresis.

2.4.1 DNA Extraction

DNA extraction is the process of separating DNA from other cellular components such as proteins, carbohydrates, and fats (Hutami *et al.*, 2018). The main stages of DNA extraction consist of four main stages including cell division (lysis), DNA binding (binding), DNA washing (wash), and elution using reagents/buffers that have been provided by the Extraction Kit manufacturer (Ariyanti and Sianturi, 2019). The stage of cell breakdown aims to destroy the cell membrane so that the inside of the cell can come out (Hutami *et al.*, 2018). The DNA

binding stage occurs due to the presence of a silica membrane in a minispin column tube. The basic principle of silica membranes in the spin column is that DNA binds to silica in the spin column with the help of chaotropic salts with high concentrations (Ariyanti and Sianturi, 2019). The washing stage aims to separate DNA from other macromolecules such as proteins, small portions of RNA, lipids and polysaccharides. The last stage is DNA purification aimed at removing residues from substances used at the stage of DNA lysis and separation (Hutami *et al.*, 2018). DNA material is elusive from the silica membrane with water or low-concentration buffer salts (Ariyanti and Sianturi, 2019).

2.4.2 DNA Amplification

The DNA amplification stage is a stage to increase the amount of DNA obtained after the extraction stage. The PCR method aims to produce specific DNA fragments with a predetermined length and sequence from a small piece of complex printed DNA, based on enzymatic amplification of the DNA fragment using two complementary primary oligonucleotides (Yuniarti and Sua, 2021).

The PCR process involves several stages, namely pre-denaturation of the template DNA, denaturation of the template DNA, primary attachment to the template (annealing), primary extension and stabilization (post extension). The stage of denaturalization of the DNA of the template to the extension is a repetitive stage, in each cycle there is a duplication of the amount of DNA (Aminah *et al.*, 2019). According to Lorenz *et al.* (2014) The PCR process generally takes place in three stages, denaturation stage, annealing stage, and extension stage (Figure 5).

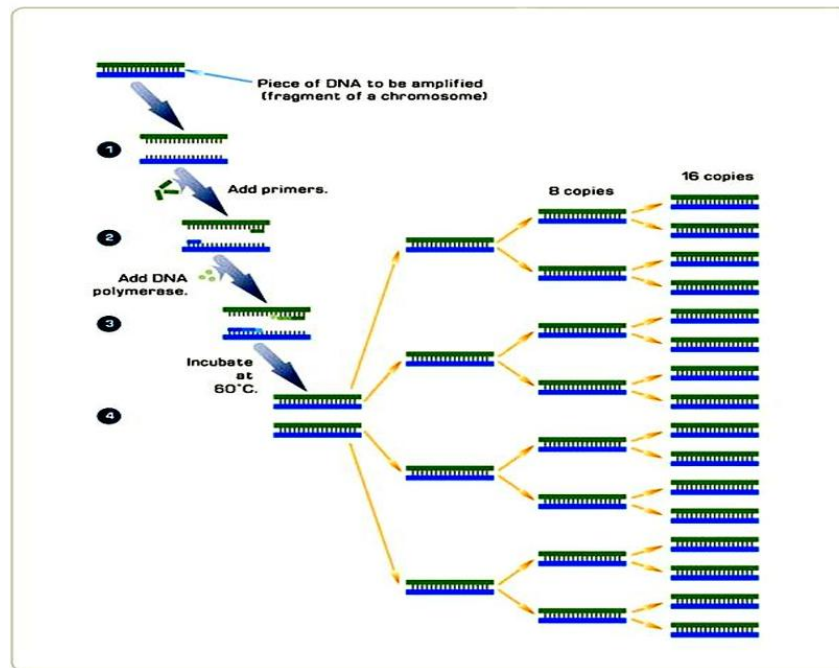


Figure 5. PCR one-cycle scheme (Yusuf, 2010).

Denaturation is the process of separating double-stranded DNA into a single DNA strand by heating it to a temperature of 94°C. Annealing is a primary attachment process. The enzyme Taq polymerase can initiate the formation of a new DNA strand if there is a DNA strand attached to the target DNA strand that has been separated. Extension is the process of DNA lengthening. After the primer attaches to the target DNA strand, the DNA polymerase enzyme will lengthen and form new DNA from the combination of the primer, molded DNA and nucleotide (Riana, 2013).

Primers are single-stranded oligonucleotide molecular sequences consisting of about 30 bases that play an important role in the PCR process (Salsabila *et al.*, 2021). Generally, the primer design used in PCR consists of a pair of primers known as forward primers and reverse primers (Yuniarti and Sua, 2021). According to Fitriana *et al.* (2023), there are four primers that have been developed in determining the sex of the aves group, namely P2/P8 (Griffiths *et al.*, 1998),

1237L/1272H (Kahn *et al.*, 1998), 2550F/2718R (Fridolfsson and Ellegren, 1999), and CHD1F/CHD1R (Lee *et al.*, 2010).

The PCR method is known to be able to determine the sex of birds precisely and has high sensitivity (Fitriana *et al.*, 2022). According to Suriyaphol *et al.* (2014) in determining the sex of birds will be flexible using dried blood spots (DBS) on paper discs without a long genome DNA extraction process. A4 photocopy opaque paper can be used in blood sampling.

2.4.3 Electrophoresis

Electrophoresis is a method to separate DNA fragments based on their size (molecular weight) and physical structure using an electric field (Saiallagan *et al.*, 2022). The working principle of electrophoresis is based on the movement of negatively charged particles (anions) such as DNA will move towards the positive pole, while positively charged particles (cations) will move towards the negative pole. The smaller the size of the molecule, the faster the migration rate, because the gel matrix contains a complex network of pores so that the particles can move through the matrix. The size of DNA fragments can be estimated by comparing the migration speed with the DNA ladder molecules whose size is known (Nugraha *et al.*, 2014).

DNA visualization was then carried out using exposure to ultraviolet light after first adding an ethidium bromide solution to the gel in its manufacture (Nugraha *et al.*, 2014). To avoid the harm caused by ethidium bromide, SYBR safes are used as a substitute. SYBR safe dye makes DNA fluorescent under UV light or blue light (Natasya, 2019).

2.5 DNA Qualitative and Quantitative Test

The success of DNA isolation was qualitatively analyzed to show the presence or absence of DNA using 1% agarose gel electrophoresis in a 1X TAE solution. The results of DNA isolation show that DNA can be isolated well, if a clear and bright band appears on the subsection. The thickness and light of the band qualitatively indicates a high concentration of DNA isolation results, while a thin band indicates a low concentration of DNA produced (Hidayat *et al.*, 2016) (Figure 6).

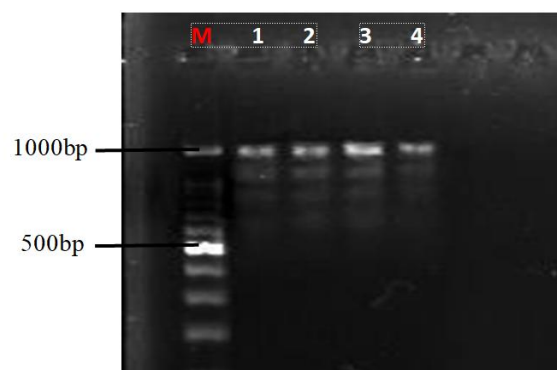


Figure 6. Results of qualitative tests to determine the sex of Black-Capped Lory (*Lorius Lory*) (Ramadhanail, 2019).

Quantitatively, the analysis of the concentration and purity of DNA isolation can be known using a spectrophotometer. The principle of the spectrophotometer is the absorption of ultraviolet light radiation by nucleotides and proteins in the solution, due to the presence of purine bases and pyrimidines. The maximum absorption of UV radiation by DNA is achieved with a wavelength of 260 nm, while the maximum absorption of proteins at a wavelength of 280 nm. The results of DNA isolation are said to be pure if the ratio value ranges from 1.8–2.0 (Hidayat *et al.*, 2016). If the ratio of A260 values divided by A280 is less than 1.8, it indicates the presence of contaminants caused by proteins or phenols in the insulation results. If the ratio is more than 2.0, it is possible to cause RNA contamination (Perwitasari *et al.*, 2020).

III. RESEARCH METHODS

3.1 Time and Location

The research "Sex Determination of Racing Pigeon (*Columba livia*) Molecularly Based on Blood Samples" was carried out in February – April 2025 and under the program of drh. Eko Agus Srihanto, M.Sc. The Lampung Disease Investigation Center in collaboration with Dra. Elly Lestari Rustiati, M.Sc. Blood sampling was carried out in two main locations, in East Lampung (Labuhan Ratu Induk, Rajabasa Lama Induk, Labuhan Ratu 4, Labuhan Ratu), (Braja Asri, Way Jepara), (Raman Daya 4, Raman Utara) and Kota Metro (Rejomulyo, South Metro). Molecular analysis was carried out at the Biotechnology Laboratory, Lampung Disease Investigation Center.

3.2 Tools and Materials

The tool used in sampling is a sterile needle. Tools used in molecular analysis include micropipette and tip, 1.5 ml microtube and rack, spin column, collection tubes, Class II biosafety cabinet (BSC), Qubit Fluorometer, vortex, centrifuge, water bath, spin down, 0.2 ml PCR tube, thermal cycler, microwave, agar mold and comb, electrophoresis tool set, and PC or computer.

The materials used in sampling are A4 opaque paper and plastic clips. The materials used in molecular analysis are blood samples, DNA isolation kits from Invitrogen, PureLink™ Viral RNA/DNA Mini Kits catalog number 12280050 (consisting of lysis buffer, proteinase K, Wash Buffer, and RNase-

free Water), absolute alcohol, DNA marker Invitrogen TrackIt™ 100 bp DNA Ladder catalog number 104488058, agar dye SYBR safe DNA gel stain catalog number S33110, agarose gel powder catalog number 75510019, and TAE buffer solution (Tris Acetate EDTA), Qubit™ dsDNA BR Assay kit catalog numbers Q32850 (consisting of Qubit™ dsDNA BR buffer and Qubit™ reagent), amplification kit from Bioline, namely MyTaq™ HS Red Mix catalog number BIO-25047, one pair of primers i.e. 2550F and 2718R, and nuclease-free water.

3.3 Implementation

3.3.1 Sampling Location

Blood sampling of pigeons was carried out in two main locations, East Lampung (Labuhan Ratu Induk, Rajabasa Lama Induk, Labuhan Ratu 4, Labuhan Ratu), (Braja Asri, Way Jepara), (Raman Daya 4, Raman Utara) and Kota Metro (Rejomulyo, Metro Selatan) based on information from breeders regarding the existence of young pigeons. The retrieval process is carried out randomly.

Sampling of Columbiformes birds was carried out using the two-handed handling-restraint method according to references from Yulianti and Arief (2020). This method is done by holding together a pair of pigeon legs, then the other hand grabs the body and wings. The bird's body is pressed towards the handler with the neck positioned flanked by the arms and body, and the head is directed towards the handler's back. The other hand fixes a pair of animal legs with one finger (usually the index finger) between the animal's legs to lock the leg so that it does not come off. Blood sampling is carried out by spreading the wings of young pigeons. The part where the blood will be drawn is wiped using an alcohol swab. Sterile needles are inserted into the venous veins, blood is collected and dripped on

5x5 mm² A4 opaque paper (Asawakarn *et al.*, 2018). The blood samples are stored in different plastic clips and labeled including the sample number and sampling location. Storage is carried out in existing refrigerators in East Lampung and Kota Metro. Blood samples were taken and stored at the Lampung Disease Investigation Center Biotechnology Laboratory for further analysis.

3.3.2 Sample Preparation

Preparations are made on blood samples that have been taken by collecting them on opaque paper and stored in plastic clips. Blood samples on opaque paper were removed in plastic clips using tweezers and inserted into a 1.5 mL microtube containing 1 mL of PBS buffer (Figure 7). Homogenization of the sample is carried out by vortex for 20-30 seconds to ensure that the blood that has been absorbed on the opaque paper can mix evenly with the PBS buffer. The tubes are labeled with markers so that each sample can be clearly identified and not confused with each other. The information that must be included includes the place, date, and sampling method. The sample is stored in a refrigerator.

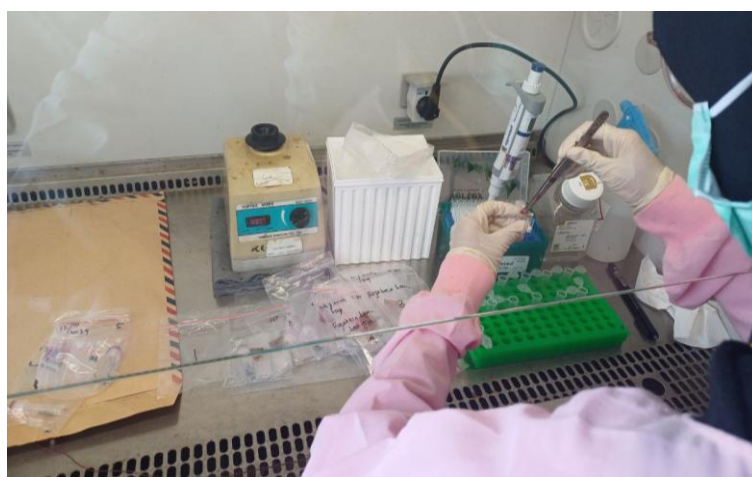


Figure 7. Implementation of pigeon blood sample preparation at the Biotechnology Laboratory of the Lampung Disease Investigation Center.

3.3.3 DNA Extraction

DNA extraction is carried out through 4 main stages, lysis, binding, washing, and elution, by following the protocol from invitrogen, PureLink™ Viral RNA/DNA Mini Kits catalog number 12280050, in a Class II biosafety cabinet based on the available built-in protocol (Figure 8). DNA extraction was carried out using the silica base extraction method. The process of entering samples and buffer solutions into the microtube is laid out on the microtube rack using a micropipette, while the centrifugation stage is carried out by a centrifuge.

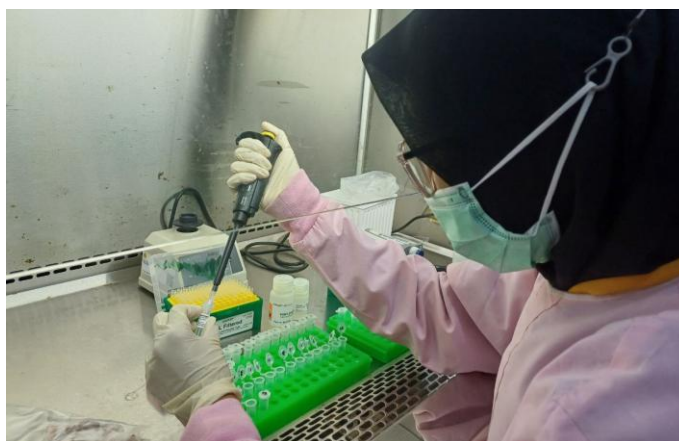


Figure 8. Implementation of DNA extraction of pigeon blood samples at the Biotechnology Laboratory of the Lampung Disease Investigation Center.

The lysis stage is carried out by inserting a blood sample of 200 μ l into a microtube containing 200 μ l of lysis buffer and 25 μ l of proteinase K. Homogenization of the suspension uses a vortex and is incubated at 56°C for 15 minutes.

The binding stage is carried out by involving a spin column in which there is a silica-based membrane by adding 250 μ l of absolute alcohol to the suspension, did vortex and incubated at room temperature for 5 minutes. The suspension is moved on a spin column and centrifuge at

a speed of 8000 g (10000 rpm) in a temperature of 4-8°C for 1 minute. The collection tubes are replaced with new ones after the centrifugation is completed.

The washing or precipitation stage is carried out by adding 500 µl of wash buffer into the spin column. Suspension of the centrifuge at a speed of 8000 g (10000 rpm) in 4-8°C for 1 minute. The collection tube was replaced with a new one and added with a 500 µl wash buffer into the spin column. The suspension is centrifuged at a speed of 8000 g (10000 rpm) in a temperature of 4-8°C for 1 minute. The collection tube was replaced with a new one without adding anything and returned to centrifuge at a speed of 8000 g (10000 rpm) at a temperature of 4-8°C for 1 minute. The collection tube was replaced with a 1.5 ml recovery tube.

The elution stage is carried out by adding 50 µl of RNase Free Water (NFW) and incubating at room temperature for 1 minute. Suspension is centrifuged at 12000 rpm at 4-8°C for 1 minute. The spin column is removed and labeled on the tube. Storage of extracted DNA can be done in a freezer with a temperature of -20°C so that the DNA is not damaged.

3.3.4 DNA Concentration Measurement

DNA concentration measurement is carried out with a Qubit fluorometer device which aims to check the quantity of DNA extracted (Figure 9). The reagents used include 199 µL Qubit™ dsDNA BR Buffer, and 1 µL Qubit™ reagent. Both reagents are put into a 0.5 ml tube. The extracted DNA was added as much as 10 µL to the 190 µL test reagent resulting in a final volume of 200 µL. The suspension was slowly homogenized by repeatedly sucking and removing the liquid using a micropipette so as not to form bubbles or

liquids attached to the tube walls. The mixture is incubated for 2 minutes at room temperature and placed in a place not exposed to light. The DNA sample is read using a Qubit fluorometer on a program that corresponds to the size expressed in ng/ μ L.



Figure 9. Implementation of DNA concentration measurement from extraction at the Biotechnology Laboratory of the Lampung Disease Investigation Center.

3.3.5 DNA Amplification

DNA amplification is carried out through Polymerase Chain Reaction (PCR) using a thermal cycler. The DNA amplification stage is carried out through three stages, namely making a master mix, adding templates, and running PCR. The process of making this master mix is carried out in a PCR workstation (Figure 10) by mixing four reagents, namely 12.5 μ l MyTaqTM HS Red Mix, 1 μ L Forward Primer with a concentration of 20 pmol, and 1 μ L Reverse Primer with a concentration of 20 pmol, as well as 5.5 μ L Nuclease-Free Water. The three reagents were put into a 0.2 ml PCR tube. The primer used is a primer that is specific to the CHD gene (Table 1). The reagents are homogenized in the PCR tube using spin down to ensure that the reagent is mixed with the rest of the reagent and that no reagent sticks to the wall of the tube.



Figure 10. Implementation of the master mix at the Biotechnology Laboratory of the Lampung Disease Investigation Center.

Table 1. Arrangement of the primary sequence of the CHD gene in pigeons (Fridolfsson and Ellegren, 1999).

Primery	Primary Sequence
2550F	5'- GTTACTGATTCGTCTACGAGA -3'
2718R	5'- ATTGAAATGATCCAGTGCTTG -3'

Template addition is done in a different room, specifically where the template is added (Figure 11), which is done by adding 5 μ l of DNA template to the prepared master mix. Running PCR is performed by setting the amplification profiles for the 2550F and 2718R primers on the thermal cycler machine with the following cycles: 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) (Figure 12).

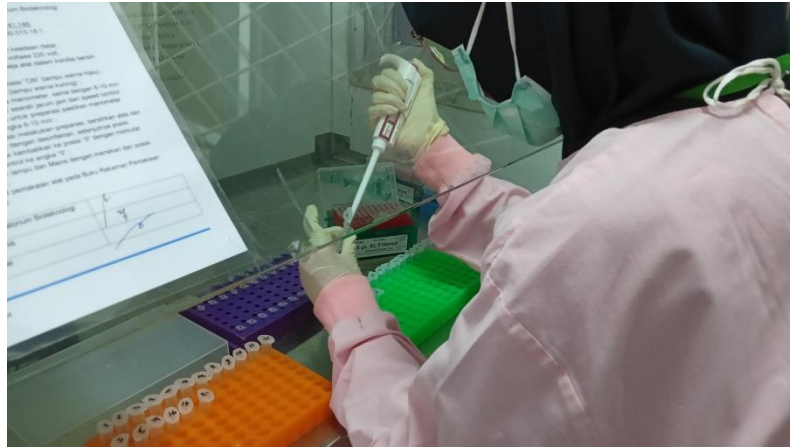


Figure 11. Implementation of addition templates in the Laboratory Biotechnology of the Lampung Disease Investigation Center.

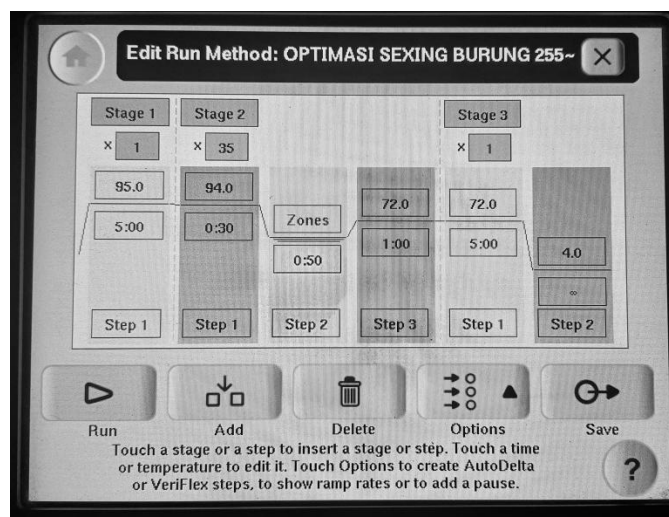


Figure 12. Amplification program on the thermal cycler engine screen

3.3.6 Electrophoresis

Electrophoresis is carried out by draining the DNA in the agarose gel well in a container with an electric current (Figure 13). Agarose 1% gel is made by dissolving 1 g of agarose powder and 100 mL of 1X TAE solution into an erlenmeyer flask. The solution is homogenized and heated using the microwave for 3 minutes. SYBR® safe DNA

gel stain is added as much as 12 μ l. Agarose is poured into a gel mold that has been combed. Agarose is waiting to harden and ready to use.

So that the solid ones are put into a chamber that has contained 1x TAE buffer solution until the agarose gel is submerged. DNA markers are inserted as much as 6 μ l into a separate well as a reference for the size of DNA molecules obtained from the amplicon results. The power supply is connected with an electrophoresis chamber. The electrophoresis process is carried out for 35 minutes with a voltage of 100 V and a strong current of 300 A.

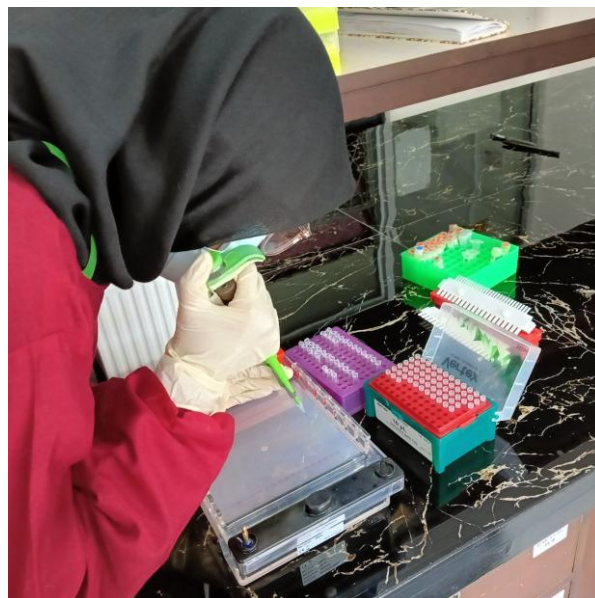


Figure 13. Implementation of electrophoresis in the laboratory Biotechnology of the Lampung Disease Investigation Center

3.3.7 Visualization

The results of the electrophoresis were visualized under blue light and documented using a PC-connected camera tool via the EOS Utility app. Observations were made by observing the presence of DNA bands present in agarose gel.

3.3.8 Data Analysis

Data analysis was carried out descriptively based on the assumption that bands or bands were formed on agarose gel indicating male and female individuals. Assuming the test results in male individuals produce 1 band with a molecular size of 600-650 bp and assuming the test results in female individuals produce 2 bands with molecular sizes of 600-650 bp and 400-450 bp.

3.4 Research Procedure Flow Chart

Pigeons (*Columba livia*), are monomorphic, which have almost similar morphological characters between male and female individuals, so it is difficult to determine the sex of pigeons, especially in young birds. The study of the determination of the sex of racing pigeons molecularly using blood was carried out based on (Figure 14).

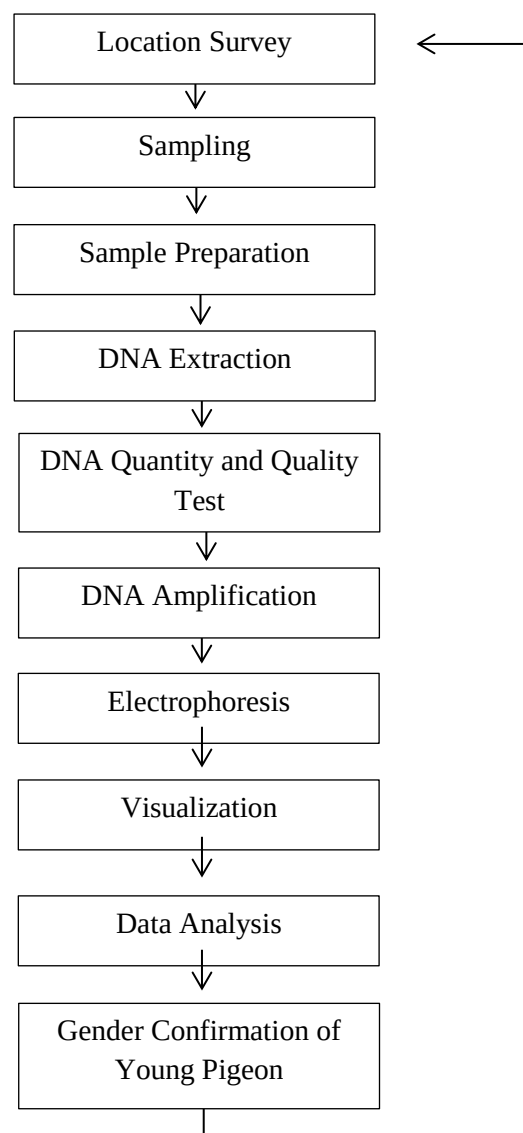


Figure 14. Flow chart of the implementation of research on determining the sex of racing pigeons (*Columba livia*) molecularly using blood.

V. CONCLUSIONS AND SUGGESTIONS

5.1 Conclusion

The conclusion obtained from the study "Sex Determination of Racing Pigeons (*Columba livia*) Molecularly Based on Blood Samples" is that from the eleven pigeon blood samples can confirm that 7 female individuals and 4 male individuals and there is 1 error in the gender estimate by the pigeon owner.

5.2 Suggestion

Bird sexing can be further studied on other species.

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