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Identification and Genetic Diversity of *Phalanger* sp. Origin Papua Based on 12S rRNA Gene Sequence

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Abstract

Taxonomically *Phalanger* sp. or cuscus is a member of the Phalangeridae family, this animal has a distribution that is widely found in eastern Indonesia, has endemic status, its existence is legally protected because it is often hunted and consumed for food by the surrounding community. The study of molecular biology is one of the efforts in the management of cuscus conservation in Indonesia, namely by completing the database and molecular information in the Gene Bank so that later it is hoped that it can be used to trace kinship relationships. The aim of the study was to identify *Phalanger* sp. from Papua molecularly using COX1, ND4L, and 12S rRNA sequences. The methodology used is DNA isolated from blood samples of *Phalanger* sp. as template DNA in the amplification process by PCR. This study used 12S rRNA primers. The results showed that 12S rRNA could be used to identify *Phalanger* sp. while the COX1 and ND4L sequences have not been successfully used to identify *Phalanger* sp.

Keywords: genetic diversity; *Phalanger* sp; 12S rRNA gene.

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1. Introduction

Papua is the province with the highest biodiversity in Indonesia, predicted to account for around 30%-50% [1]. It is estimated that there are approximately 25,000 species of woody plants, 164 species of mammals, 329 species of amphibians and reptiles, 650 species of birds, and 1,200 species of marine fish [1]. Papua as one of the islands that supports the high biodiversity in Indonesia, not only has a high species richness, but also has a high level of species endemicity when compared to other islands. In Papua there are approximately 200 species of land mammals and 154 species of which are known to form large populations that include both endemic and introduced species [2].

At the present time, although Papua is home to high biodiversity, it also has serious problems, especially in poor social and economic development planning. This condition has worsened since the central government granted Special Autonomy status to Papua in 2001. Rapid and often unplanned development has increased habitat destruction in Papua, which has a direct negative impact on the declining level of biodiversity in the region. In fact, this biodiversity is increasingly threatened by various economic activities such as the conversion of primary forest land into oil palm plantations, industrial forest plantations and mining [3].

Changes in forest areas into cultivation, agriculture, industry, settlements, shops, roads, and abandoned areas into alang-alang fields, result in the reduction or extinction of certain species populations. Some endemic and rare species may be extinct in their natural habitat before their names are identified and their potential is known. The main cause of the poor condition of Indonesia's biological diversity is because over-harvesting of biological resources is not balanced with sustainable-based conservation efforts. One example of endangered biological wealth in the forest area of Papua is *Phalanger* sp. This animal has a fairly wide range of distribution, starting from eastern Indonesia, Papua New Guinea, Cape York to Queensland in Australia [4]. Furthermore, it is stated that there are 11 types of cuscus in New Guinea and several surrounding islands, but in detail the behavior, distribution, and species are not known with certainty [5, 4].

According to [2] stated that of the 11 species of cuscus found in New Guinea, five species of which have been protected by the Indonesian government through the Decree of the Minister of Agriculture No. 247/KPTS/UM/4/1979, namely *Phalanger orientalis* (eastern cuscus), *Phalanger maculatus* (ordinary spotted cuscus), *Phalanger gymnotis* (gray cuscus), *Phalanger vestitus* (silk hair cuscus), and *Phalanger rufoniger* (black spotted cuscus). In fact, most of the Phalangeridae family is legally protected through the Wild Animal Hunting Regulation no. 226/1931, Law No. 5/1990, Law No. 7/1999, has also been included in the IUCN list in the endangered species category, according to CITES has also been classified in Appendix II [6]. In addition, the Government has also made efforts to conserve cuscus both *in-situ* and *ex-situ.*

Referring to the passage of time, it turns out that in reality until now the government has not been able to stop the illegal hunting and trade of wild animals. If this condition is not immediately carried out prevention and conservation measures, it will add to the list of animals that have become extinct.

A genetic approach is very important in animal conservation efforts. Within a population, genetic variation is

very significant for the short-term survival of a species. Electrophoresis or DNA sequencing can be used to determine unique genetic variations within a particular population. Genetic data make an important contribution to taxonomy. Characterization in traditional taxonomy is always based on morphological data which may be due to local adaptation or genetic flexibility. In this case genetics can help clarify relationships and guide conservation efforts toward distinct distributions or unique taxa. Genetic principles can guide the selection of broodstock, control the breeding structure, monitor the overall genetic variation, and the loss of genetic variation in conservation areas.

Based on several sources of accurate information, it is known that in the recent period a number of studies have proven that mitochondrial DNA (mtDNA) can be used as a maternal marker [7.8]. This can happen because it is supported by several advantages possessed by mtDNA which are generally not found in nuclear DNA. Some of the advantages referred to are the size of mtDNA about 16.5 kb smaller than nuclear DNA, a very fast evolutionary rate, derived from the maternal line and can be obtained from samples that have undergone degradation [9, 10, 11].

COX1, 12S rRNA, and ND4L genes are encoding genes to encode certain enzymes that play a role in the process of cellular respiration in the mitochondria. According to [12], states that the COX1 gene can be used as a genetic marker to distinguish Tarsier species from Sumatra and Sulawesi. Likewise, according to [13], because the ND4L gene sequence has diversity in its nucleotides, it can be used as a genetic marker for *Acipenseriformes.*

One of the efforts to maintain the existence and protection from the threat of deforestation which is a serious problem for the survival of the cuscus, it is necessary to study this animal in more detail, especially at the genome level using molecular methods. It is important to do this because through this molecular method, the characteristics and information about cuscus can be known precisely so that conservation actions can be carried out optimally. According to [14], scientific studies on the characteristics of the genomes of endemic animals are still limited, so it is necessary to conduct related research that can contribute more accurate information.

The aim of the study was to identify *Phalanger* sp. from Papua molecularly using COX1, ND4L, and 12S rRNA sequences. The expected benefit of this research is to help preserve *Phalanger* sp. both in-situ and ex-situ, the return of *Phalanger* sp. to their habitat (ex-situ conservation results or wild catches), and is expected to reveal kinship relationships between the Phalangeridae family.

2. Method

2.1 Field research

The location of cuscus blood sampling in the Arfak Mountains, Manokwari, West Papua, was carried out for 5 months. The number of cuscus used was 2 male and in healthy condition (Figure 1). Location for cuscus blood analysis by isolating its DNA using PCR at BIOTROP's Laboratory in Bogor, West Java.

A. first sample B. second sample

Figure 1: Two samples of cuscus from the Arfak Mountains, Manokwari, West Papua.

Some of the equipment used for this research are micropipette (Gilson) with a size of 200 to 1,000 *µl*, micropipette (Nichipet) with a size of 20 to 100 *µl*, 2 to 20 *µl* and 1 to 10 *µl*, pipette tips (blue, yellow, white), disposable 3 *ml* syringe, water bath (Eyla, Uni Thermo Shaker NTS 3000), centrifuge (5804R), freezer, 1 PCR machine, 1 electrophoresis device, microwave, 1 agar printing device (well printing plate and comb), balance, magnetic stirer , vortex mixer, spin down, measuring cup, gloves, erlenmeyer tube, and UV transluminator.

2.2 Data sampling

Blood collection was carried out using 3 *ml of disposable syringe* on the lateral tail vein or the sapenic vein of the leg. The blood is then put into a tube that already contains 1 *mg* of anti-coagulant (heparin), and is labeled. Then the tube was shaken briefly and then stored in a cool box.

2.3 Data analysis

DNA isolation

Total DNA isolation from blood samples using Qiagen's QIAamp® DNA Blood kit (containing AL buffer, ATL buffer, AW1 buffer, AW2 buffer, AE buffer) [12] and proteinase K used to lyse membranes in blood cells and also degrade enzymes DNAse and other proteins to avoid DNA degradation, especially mitochondrial DNA in the sample solution. The isolation process begins with homogenizing the sample by centrifuging at a speed of 8,000 rpm for one minute at room temperature. The resulting supernatant was transferred to a new 1.5 *ml* microcentrifuge tube. The next step was the addition of 200 *µl* of absolute ethanol, then vortexed for 15 seconds.

All solutions and precipitates are taken with a micropipette and transferred in a column (there is a filter and a collection tube is installed underneath). After that, the solution was centrifuged again at 8000 rpm for one minute. The solution filtered in the collection tube was discarded. 500 *µl* of buffer AW1 was added to the column, then the solution was centrifuged again at 8,000 rpm for one minute. The solution filtered in the collection tube was discarded as in the previous step. 500 *µl* of AW2 buffer was added to the column, after which the solution was centrifuged again at 8,000 rpm for one minute. The solution filtered in the collection

tube was discarded, then centrifuged at 10,000 rpm, at room temperature for five minutes, and the solution filtered in the collection tube was discarded.

After that, the column (filter) was transferred to a 1.5 *ml* microcentrifuge tube, then 50 *µl* of AE buffer was added to the column. The solution contained in the column was left at room temperature for five minutes. The tube containing the column was then centrifuged at 10,000 rpm for one minute. This process is then repeated once again, starting with the addition of 50 *µl* of buffer AE. The filtered solution below is the result of DNA isolation. The isolated DNA was stored in a freezer at -20°C. The DNA isolation results were checked or identified using 1% agarose gel. In this study, the primary design (Table 1) was based on data from the mitochondrial genome sequence of the closest relative of *Phalanger*, namely *Tricosurus* using the online primer 3 program of online.

Table 1: Specific oligonucleotide primers for amplification of COX1, ND4L, and 12S rRNA genes.

Primary			
Target	F dan R	Base order	PCR product (bp)
COX1	COX1F (21 nt)	5' GTGGTTATGAGGTTGGCTTGA 3'	1633
	COX1R (21 nt)	5' GCTCTTTCAGCCATTTTACCC 3'	
ND4L	ND4LF (20 nt)	5' TCCTTATTCTCCCCGGATTT 3'	843
	ND4LR (20 nt)	5' TAGGGGGTTCAATTCCTTCC 3'	
12S rRNA	12S rRNAF (20 nt)	5'CAGTGAGAATGCCCTCAAAA3'	879
	12S rRNAR (20 nt)	5'CTCCAAGTGCACCTTCCAGT3'	

Annotation: F= *forward*; R= *reverse*

Amplification of 12S rRNA gene

The results of DNA isolation were used for templates in the amplification process by the PCR method. The PCR reaction mixture for one reaction was 40 *µl* consisting of 20 *µl* KAPA, 2 *µl* for each primer 1 and 2 with a concentration for each primer of 10 *pmol*, 1 *µl* of total DNA, and to fill the volume of one reaction ddH2O was added with volume of 17 *µl* to reach a concentration of 40 *µl*. The PCR conditions for amplification of the COX1 and ND4L genes were as follows: initial denaturation for 5 minutes at 94°C, followed by denaturation at 94°C for 30 seconds, 54°C for 45 seconds, 72°C for 1.5 minute; amplification reaction for 35 cycles and then ended with the addition (extension) for 5 minutes at a temperature of 72 ° C. As for the 12S rRNA gene with PCR conditions as follows: initial denaturation for 5 minutes at a temperature of 94 ° C followed by denaturation at a temperature of 94° C for 1 minute 30 seconds, at a temperature of 48.7° C for 45 seconds for annealing, at a temperature of 72° C for 1 minute for elongation; amplification was carried out for 35 cycles and then terminated for 5 minutes at 72°C.

Electrophoresis of 12S rRNA gene PCR results

The electrophoresis process with agarose gel was carried out to see the results of DNA isolation by PCR. The

first thing to do in this process is to make a gel. The gel concentration used for electrophoresis of PCR results was 1%, consisting of 0.25 *grams* of agarose dissolved in 25 *ml* of TBE 0.5X or 0.5 *grams* of agarose in 50 *ml* of TBE 0.5X heated in a microwave until boiling. After that, 2 μl of Good View as dye was added to the liquid gel for a 25 *ml* gel volume and 4 μl for a 50 *ml* gel volume. The agarose solution is awaited until it reaches a temperature of about 55°C, then the agarose solution is poured into the agar plate, then the well-printing comb is installed. After the gel hardened, the plate containing the agarose gel was placed in an electrophoresis tank containing TBE 0.5X buffer solution (attempted to match the buffer used to make agarose gel). Samples from DNA isolation and PCR as much as 3 μl were taken with a micropipette then mixed with 1 μl of Glycerin Bromphenol Blue (GBB) and put in agarose gel wells. Electrophoresis was carried out with an electric current of 80 volts and waited for the process to complete about 30 minutes. The DNA molecule fragments were observed with the help of a UV transluminator ($\boldsymbol{\lambda}$ = 260 *nm*), while the length of the amplified DNA could be identified/identified with 100 *basepair* (bp) DNA markers.

3. Results and discussion

In accordance with the purpose of DNA isolation is to separate DNA from other materials such as proteins, fats, and carbohydrates. This DNA isolation process refers to the main principle that in isolation there will be destruction (lysis), extraction or separation of DNA from solid materials such as cellulose and protein, and DNA purification. The totality of DNA isolation from blood samples 1 and 2 has been successfully carried out which is then continued with DNA quality checking. The DNA quality checking process was carried out using 1% gel electrophoresis and the results were quite satisfactory because the quality of the DNA obtained was intact (Figure 2). Intact DNA characters were indicated by the absence of electrophoretic DNA smears. This is important because in the PCR process, DNA that is still intact will provide relatively more accurate results.

Figure 2: Electrophoresis of DNA isolation.

Notation 1: cuscus blood sample 1

2: cuscus blood sample 2

The isolated total DNA was used as a template for amplification of COX1, ND4L, and 12S rRNA genes using PCR. The PCR results were analyzed by agarose gel electrophoresis method, and visualization of the PCR results could be seen under UV light. The process of amplification of COX1, ND4L, and 12S rRNA genes was

carried out using COX1, ND4L, and 12S rRNA primers, which then produced PCR products of 1633, 843, and 879 *base pairs* (bp). PCR products were detected by being migrated to 1.5% agarose gel using 1xTBE buffer in a Submarine Electrophoresis device (Hoefer, USA). Observations were made with the help of ultraviolet light (l = 260 nm) after the gel was stained with DNA staining (1st Base). DNA marker with a size of 100 bp (1st Base) or Marker is used as an indicator of the length of the nucleotide base. The PCR products of these genes are presented in Figures 3 and 4. The PCR results were then used for sequencing. The purpose of sequencing is to analyze the nucleotide sequence of the sample. Based on the electrophoresis (Figures 3 and 4), it is known that only the 3rd column contains bands, namely the 12S rRNA gene. In both samples 1 and 2, precisely in the 3rd column, the 12S rRNA gene was well amplified, while in columns 1 and 2 (COX1 and ND4L genes) there were no bands or bands in both samples. This indicates that the COX1 and ND4L genes are not well amplified. The cause of the unamplification of the two genes was because the primers used were not specific or had a low level of similarity.

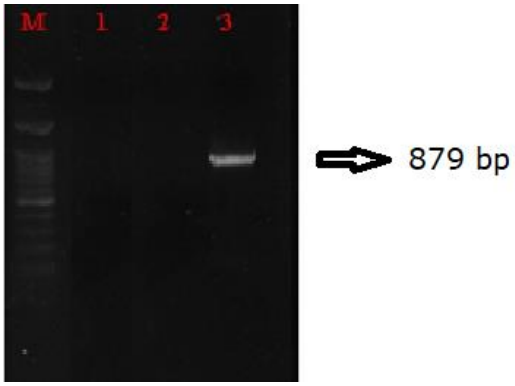

Figure 3: Electrophoresis of COX1, ND4L, and 12S rRNA gene amplification of sample 1 on 1.5% agarose gel.

Notation

M : marker DNA Ladder 100 bp

1 : COX1 gene

2 : ND4L gene

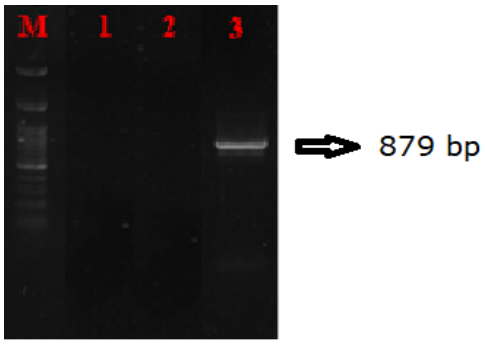

Figure 4: Electrophoresis of COX1, ND4L, and 12S rRNA gene amplification of sample 2 on 1.5% agarose gel.

Notation

M : marker DNA Ladder 100 bp

1 : COX1 gene

2 : ND4L gene

Primer is a series of nucleotides that will be used as the initiation of DNA replication. Selective selection of oligonucleotide primers plays an important role in DNA amplification using PCR, oligohybridization, and DNA sequencing. Meanwhile, a factor that is quite important in analyzing DNA is the process of properly designing primers [15](Abd-Elsalam, 2003). The primer that will be selected to attach to the target sequence must meet several criteria such as primer length, guanine-cytosine concentration, attachment temperature, melting temperature, 5' end stability and 3' end specificity [15](Abd-Elsalam, 2003). In designing primers, sufficient detailed data or information is needed regarding the genes of the *Phalanger* sp. species, because for species that have never been analyzed molecularly, it is necessary to design primers using their closest relatives who are still in the same family. This can be an obstacle in designing specific genes for *Phalanger* sp. In this study, 2 types of primers were used, namely general primers (12S rRNA gene) and specific primers (COX1 and ND4L genes). Referring to the results of this study that was successfully amplified and well sequenced was a gene with a common primer (12S rRNA). The results of the 12S rRNA gene sequencing were obtained in the form of an electrophorogram. The occurrence of color differences at the peaks of the electrophorogram indicates that there are different types of bases. One color of the peak of the electrophorogram is a representation of one base that has different intensities with different notations. The notation A is for adenine bases, C is for cytosine bases, G is for guanine bases and T is for thymine bases, while the N notation has the meaning of the peaks being unclear due to overlapping of several peaks at one position or too low peaks resulting from nucleotides. This N notation can be corrected and replaced manually with the appropriate notation. After the results of the sequencing were copied and put into the blast at www.ncbi.nlm.nih.gov, the species with the sequences appeared, including *Phalanger* sp.

3.4 Conclusion

Based on the results of the analysis and discussion, it can be concluded that the 12S rRNA sequence can be used to identify *Phalanger* sp., while the COX1 and ND4L sequences have not been successfully used to identify *Phalanger* sp. Thus the identification of *Phalanger* sp. using the 12S rRNA sequence will facilitate and strengthen the identification of cuscus not only morphologically but also molecularly.

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