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Primer Design of Sumatran Striped Rabbit (*Nesolagus netscheri* Schlegel, 1880) using Primer-BLAST and AliView Program

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Abstract: The Sumatran striped rabbit (Nesolagus netscheri) lacks specific primers to amplify the chytochrome oxidase 1 (CO1) gene and the chytochrome b (cytb) gene, at present. Therefore, it is important to design primers to amplify the CO1 gene and cytb gene in N. netscheri. The aim of this study is to compare the primer design methods used, namely Primer-BLAST and AliView programs, to design specific primers for the chytochrome oxidase 1 (CO1) and chytochrome b (cytb) genes in N. netscheri. This research was conducted using the descriptive method with molecular observation. In this study, CO1 gene primers, namely [(forward: 5' TGTATGATATGGGGGAGGGC 3'), (reverse: 5' TGGTCCGTCCTTATTACAGCG 3')] and cytochrome b (cytb) gene primers, namely [(forward: CCAGCTCCATCCAATATCTC, (reverse: 5' GTTAGGGTTAGAAGGTCTGC 3')] and showed that primer design using the AliView program produced specific primers in the genus Nesolagus. The conclusion of this study is that primers designed using the AliView program are more specific than those designed using Primer-BLAST.

Keywords: AliView, primer-BLAST, primer design, sumatran striped rabbit

Introduction

The Sumatran striped rabbit (*Nesolagus netscheri*) was first discovered by E. netscher who was a high-ranking Dutch East Indies official who served and lived in Padang Panjang in 1880. The specimen was preserved in alcohol and given to the National Museum of Nature in the Netherlands (Museum Leyden) where it was described by Schlegel in 1880 (Schlegel, 1880). *N. netscheri* is one of the endemic animals of Sumatra Island that is protected under the Regulation of the Minister of Environment and Forestry of the Republic of Indonesia P.20/MENLHK/SETJEN/KUM.1/6/2018 (Setiawan *et al.*, 2018; Setiawan *et al.*, 2019). *N. netscheri* is listed as a Data Deficient species in IUCN (2018) due to a lack of data on which to base estimates of population size, density and distribution (McCarthy *et al.*, 2019). The Sumatran striped rabbit (*N. netscheri*) and the Annam striped rabbit (*N. timminsi*) are highly morphologically similar. Both species have hair with a striped pattern of yellowish ash that becomes rusty brown (Schlegel, 1880; Setiawan *et al.*, 2018). There are still not many studies conducted for both species, especially *N. netscheri*.

Molecular studies of *N. netscheri* have been conducted, including Surridge *et al.* (1999) who identified *N. netscheri* and *N. timminsi* based on the 12s rRNA gene, Matthee *et al.* (2004) on the molecular matrix of species in the Leporidae family, Aurora (2023) on DNA bar coding characteristics of *N. netscheri* based on the Cytochrome Oxidase 1 (CO1) gene for identification, and Priyono *et al.* (2025) made mtDNA and phylogenetic analysis of *N. netscheri*. Based on the search of previous

studies, there has been no research on the specific primer design of *N. netscheri*. Specific and precise primers are important for good amplification results. The success of DNA amplification depends on the accuracy of the primers used (Diss, 2003). Primer binding specificity is determined by several considerations, namely primer length, melting temperature and G/C base content (Ye *et al.*, 2012).

Some methods that can be used in primer design are using Primer-BLAST, NCBI and AliView programs. Primer-BLAST incorporates a global alignment mechanism and is designed to be highly sensitive in detecting potential amplification targets. Primer-BLAST can check the specificity of pre-existing primers with or without templates (Ye *et al.*, 2012). AliView is an alignment viewer and editor designed to meet the requirements of next-generation sequencing-era phylogenetic data sets (Larsson, 2014). In addition to sequence alignment, AliView also has the function of finding degenerate primers in selected semiconserved regions (target genes). The primers are presented as a sequential list sorted by the number of degenerate positions, self-binding value, and melting temperature (Larsson, 2014). Based on this background, it is illustrated that it is necessary to conduct primer design research for this *N. netscheri* species by comparing primer design methods with Primer-BLAST and AliView programs to obtain more specific primers.

Material and Methods

The method used in this research is descriptive method with molecular observation. The primary test of primary results was carried out by visualizing the amplification results of the CO1 gene and the cytochrome b (cytb) gene.

Tissue sampling

Sampling of muscle tissue (meat) from one individual of *Nesolagus netscheri* that has been collected at the Genetics and Biomolecular Laboratory, FMIPA, Andalas University on June 22, 2022. For molecular data purposes, the collection results were stored in 96% PA solution in a sterile 1.5 microtube. Then stored in a refrigerator with a temperature of 4℃.

DNA isolation and visualization of isolation results

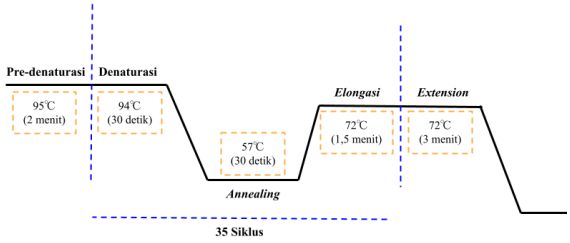
DNA isolation is a process to separate DNA molecules from most other components, such as proteins, lipids, and RNA (Sambrook and Russel, 2001; Lodish *et al.*, 2000). DNA isolation is done by following the GeneAll Exgene Genomic DNA micro protocol. Then the isolation results will be electrophoresed with 1.2% agarose gel and visualized using gel documentation.

Primer design

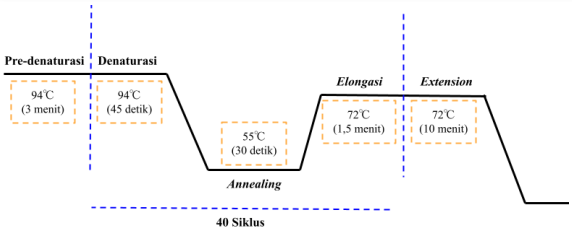
Primer design of CO1 gene of *Nesolagus netscheri* was done by referring to the complete gene CO1 of *Nesolagus timminsi* (NC_063946.1) in GenBank, NCBI. Primers were designed using Primer-BLAST, NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Then the primer design of the cytochrome b gene of *Nesolagus netscheri* was carried out with reference to the complete cytochrome b gene sequence of *Nesolagus timminsi* (NC_063946.1) in GenBank, NCBI. Both methods provided several primers which were then selected based on the criteria of ideal primers for amplification.

DNA amplification and visualization of amplification results

DNA amplification is the process of multiplying copies of a target DNA segment to obtain the number of copies needed for analysis (Brown, 2006). DNA amplification of *N. netscheri* refers to Ruedas *et al.* (2017) with modified annealing temperature. The DNA amplification steps of CO1 gene and cytochrome b (cytb) gene are presented in Figure 1 below:



(a)



(b)

Figure 1. Illustration of *N. netscheri* DNA amplification cycle (a) CO1 gene amplification cycle, (b) cytochrome b (cytb) gene amplification cycle

DNA amplification of CO1 gene and cytochrome b (cytb) gene was carried out with primers that have been designed using Primer-BLAST and AliView. The amplification results and visualization of the amplification results will prove that the primers that have been designed successfully bind to the target gene or not.

Results and Discussion

Based on the research conducted, the following results were obtained :

DNA isolation and visualization of isolation results

The isolation process of *N. netscheri* DNA was successfully carried out using the GeneAll Exgene Genomic DNA micro protocol. Visualization results of DNA isolation from muscle tissue (meat) samples of one individual of *N. netscheri* showed thick and bright DNA bands (Figure 2). These results were obtained after electrophoresis on a 1.2% agarose gel and visualized with DNA BIOSTEP.

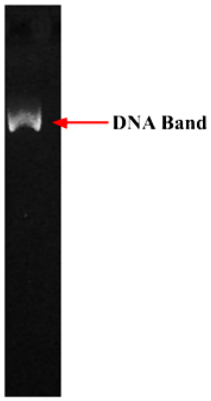


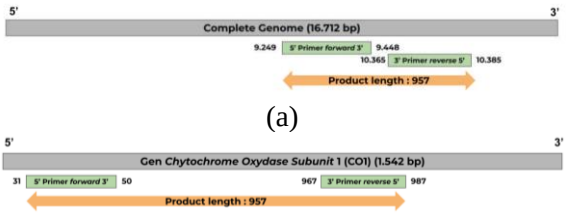
Figure 2. DNA isolation results of *Nesolagus netscheri*

Primer Design

DNA isolation was declared successful because it produced thick and bright DNA bands. Thick and bright bands indicate that the isolated DNA has a high concentration of DNA and good quality and purity. This is in accordance with Karp *et al.* (1998) which states that a thick and bright band indicates that the DNA concentration of the resulting isolate is high, while a thin band indicates that the DNA concentration of the resulting isolate is low.

CO1 gene primer design using primer-BLAST

Primer design of CO1 gene of *Nesolagus netscheri* was done by referring to the complete sequence of CO1 gene of *Nesolagus timminsi* (NC_063946.1) in GenBank, NCBI. Based on research that has been done using Primer-BLAST, NCBI, 10 pairs of primers were obtained that can amplify the CO1 gene in *N. netscheri*. However, primer selection is still needed based on ideal primer characteristics for successful amplification of more specific genes. The success of DNA amplification depends on the accuracy of the primers used (Diss, 2003).



(a)

(b)

Figure 3. Illustration of primer attachment of CO1 gene from primer design with Primer-BLAST (a) Insertion in the complete genome of *Nesolagus timminsi*, (b) Insertion in the complete CO1 gene of *Nesolagus timminsi*.

The primer pair selected in this study is primer pair 10 with forward primer (5' TGTATGATATGGGGGAGGGC 3') and reverse primer (5' TGGTCCGTCCTTATTACAGCG 3') because this primer has the best ideal primer criteria. Primer 10 has a primer length of 20 bp forward and 21 bp reverse. This result is in line with Wang's report (2016) which states that a good primer has a primer length of 17 bp - 28 bp. In the complete genome of *N. timminsi*, the forward primer attaches to the base position to 9,249 - 9,448 and the reverse primer attaches to the base

position to 10,365 - 10,385 (Figure 3a). In the complete gene CO1 *N. timminsi* forward primer attached to the base position to 31 - 50 and reverse primer attached to the base position to 967 - 987 (Figure 3b). Based on this attachment, the length of the CO1 gene primer design product in *N. netscheri* is 957 bp. Referring to Hillis *et al.* (1996) which states that mitochondrial DNA and nuclear genes have good product lengths ranging from 500 bp -1500 bp (depending on the targeted gene). Therefore, these two forward and reverse primers can be used to amplify the CO1 gene in *N. netscheri*.

It has a melting temperature of 59.86℃on the forward primer and 59.86℃ on the reverse primer. Melting temperature is one of the important things to consider in primer selection (Rychilik *et al.*, 1990). According to Chuang *et al.* (2013) and Wang (2016) the melting temperature of a good primer pair for PCR is 50℃ - 62℃. This primer also has a GC percentage of 52.38% - 55% which is greater than the percentage of AT bases. This result is in line with what Wang (2016) reported that a good GC percentage is 40% - 60%. In another report, Deffenbach *et al.* (1993) suggested that the ideal GC base concentration for primers is 45% - 55%.

Primer design of cytochrome b gene using aliview program

Primer design for the cytochrome b gene of *Nesolagus netscheri* was carried out by referring to the complete cytochrome b gene sequence of *Nesolagus timminsi* (NC_063946.1) in GenBank, NCBI. Based on research that has been done using the AliView program (Larsson, 2014), several primers were obtained that can amplify the cytochrome b gene (cytb) in *N. netscheri.* The number of primer candidates generated from the AliView program is more than the primer candidates generated by Primer-BLAST, which is 500 - 1,000 primer candidates. Then primer selection was carried out based on ideal primer characteristics for more specific amplification success. The success of DNA amplification depends on the accuracy of the primers used (Diss, 2003).

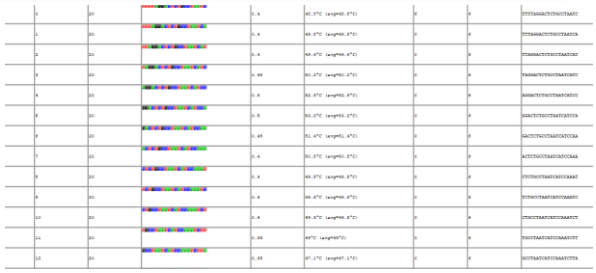
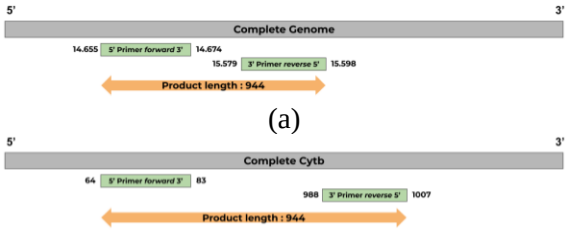


Figure 4. Some of the primers generated from primary generation from the AliView program



(a)

(b)

Figure 5. Illustration of primer attachment of cytochrome b (cytb) gene from primer design with AliView program (a) Attachment in complete genome of *Nesolagus timminsi*, (b) Attachment in complete cytochrome b (cytb) gene of *Nesolagus timminsi*.

The forward primer selected is (5' CCAGCTCCATCCAATATCTC 3') and the reverse primer selected is (5' GTTAGGGTTAGAAGGTCTGC 3') which is the result of primer selection based on the ideal primer requirements (Figure 4). In the complete genome of *N. timminsi*, the forward primer attaches to base position 14,655-14,674 and the reverse primer attaches to base position 15,579-15,598 (Figure 5a). In the complete gene cytb *N. timminsi* forward primer attached to the base position to 64 - 83 and reverse primer attached to the base position to 988 - 1007 (Figure 5b). Based on this attachment, the length of the cytochrome b (cytb) gene primer design product in *N. netscheri* was 944 bp. Referring to Hillis *et al.* (1996) which states that in phylogenetic studies using mitochondrial DNA and nuclear genes have a good product length ranging from 500 bp -1500 bp (depending on the targeted gene). Therefore, these two forward and reverse primers can be used to amplify the cytochrome b (cytb) gene in *N. netscheri*.

These primers consist of a forward primer and a reverse primer that function to ensure efficient and specific amplification at the 5' and 3' ends so as to obtain a well-amplified target gene. Both primers (forward and reverse) have

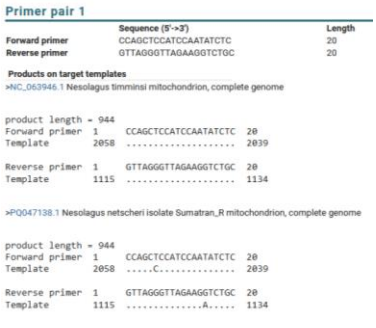
the same length, which is 20 bp. According to Wang's (2016) report, a good primer has a primer length of 17 bp - 28 bp. Referring to Koressaar & Remm (2007) which states that both primers are recommended to have almost the same length to maintain efficiency in the amplification process because a significant difference in length between the forward and reverse primers can cause unbalanced amplification.

The forward and reverse primers have melting temperatures of 55℃ and 55.17℃ respectively. Melting temperature is one of the important things to consider in primer selection (Rychilik *et al.*, 1990). According to Chuang *et al.* (2013) and Wang (2016) the melting temperature of a good primer pair for PCR is 50℃ - 62℃. Then the forward and reverse primers have a GC base percentage of 50%. The percentage of GC bases is in line with that reported by Wang (2016) which states that a good GC percentage is 40% - 60%. In another report, Deffenbach *et al.* (1993) suggested that the ideal GC base concentration for primers is 45% - 55%.

Primer BLAST Analysis

Based on the BLAST results of the primers, it can be seen that the CO1 gene primer is not species-specific for *N. netscheri* because it also amplifies other species (Figure 6a). This primer can also amplify other species, such as *Nesolagus timminsi*, *Ochotona alpina*, and *Hippidion saldiasi*. BLAST results of the cytochrome b gene primers also showed that these primers are not specific to *N. netscheri*. This primer can also be used for *N. timminsi* (Figure 6b). This means that these primers are specific to the Nesolagus genus. The creation of specific primers requires sufficient sequence to ensure the primers do not bind to non-target sites. This is particularly important in mammals, as gene sequence similarity between species can lead to unwanted amplification (Dieffenbach *et al.* 1993). The use of genomic databases to collect many target sequences from different mammalian species helps in the identification of conservative regions and avoiding non-specific amplification (Roux, 2009). Thus, primer generation using the AliView program resulted in more specific primers than using Primer-BLAST.

(a)

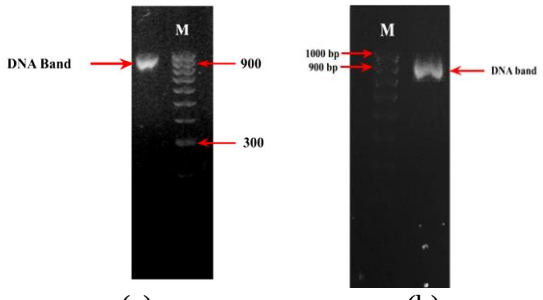


(b)

Figure 6. Results of BLAST analysis of primers in NCBI (a) Blast of CO1 gene primers from Primer-BLAST (b) Blast primer of cytochrome b (cytb) gene from AliView program.

Primer Test with DNA Amplification

Visualization of amplification results obtained after electrophoresis on 2% agarose gel and visualized using DNA BIOSTEP. The amplification results were compared with a 100 bp DNA Ladder or marker (M) to estimate the length of the resulting amplification results. The CO1 gene amplification results are estimated to have a DNA fragment size of about ± 900 bp (Figure 7a).



(a) (b)

Figure 7. Visualization of amplification results (a) CO1 gene amplification results of primer design from Primer-BLAST (b) cytochrome b (cytb) gene amplification results from AliView program.

The length of the resulting DNA fragment is estimated to be part of the CO1 gene fragment of *N. netscheri*. Then the amplification of the cytochrome b gene is estimated to have a DNA fragment size of about ± 900 bp (Figure 7b). The length of the resulting DNA fragment is estimated to be part of the cytochrome b (cytb) gene fragment of *N. netscheri*. Based on GenBank data, NCBI with accession number NC_063946.1, it is known that *N. timminsi* has a complete CO1 gene length of 1,542 bp and a complete cytochrome b (cytb) gene length of 1,140 bp. Amplification of the CO1 gene and the cytochrome b (cytb) gene of *N. netscheri* was successfully carried out with a base length of about ± 900 bp. Sambrook and Russel (2001) stated that the success of DNA amplification is influenced by several factors, namely DNA template, PCR buffer, primers, $MgCl_2$, polymerase enzyme, time, temperature and number of cycles used in the PCR process. Thus, it can be concluded that the two primers designed were able to amplify the CO1 gene and the cytochrome b (cytb) gene in *N. netscheri*.

Conclusions

Based on the research that has been done, it can be concluded that: Primer design of Cytochrome Oxidase Subunit 1 (CO1) gene using Primer-BLAST has been successfully carried out with primer results, namely forward (5' TGTATGATATGGGGGAGGGC 3') and reverse (5' TGGTCCGTCCTTATTACAGCG 3'). Cytochrome b (cytb) gene primer design using the AliView program has been successfully carried out with primer results, namely forward (5' CCAGCTCCATCCAATATCTC 3') and reverse (5' GTTAGGGTTAGAAGGTCTGC 3'). Primer design using the AliView program produced more primer candidates and more information (melting temperature, %GC, and primer dimers) which facilitated the primer selection process compared to Primer-BLAST. Primer design using the AliView program produces primers that are more specific than Primer-BLAST.

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