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Primer Optimization as Genetic Marker Primer Based on Cytochrome B Gene on Sumatran Elephant (*Elephas maximus sumatranus*) Non-Invasive Samples

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Abstract

Sumatran elephant is declared as critically endangered by IUCN since 2011. In supporting conservation efforts on sumatran elephant in its natural habitat, molecular data collection of their population needs to be done. Genetic source material is mostly obtained in its natural habitat is its dung/feces. Study on cytochrome B gene as genetic marker from sumatran elephant fecal samples can be done to get additional genetic data as a form of conservation effort. Primer optimization as genetic marker based on cytochrome B gene has been carried out as an early study on sumatran elephant genetics. Primer optimization program based on cytochrome B genetic marker from sumatran elephant fecal samples was under Higher Education Applied Research funded by The Ministry of Research, Technology, and Higher Education. DNA was isolated from fecal samples of captive sumatran elephants in Elephant Training Center, Way Kambas National Park. Primer optimization was done via amplification by increasing certain temperature. Specificity test was done to ensure that the primer only recognized sumatran elephant DNA. The best result was obtained with the annealing temperature of 52^oC .

Keywords: Sumatran Elephant, Way Kambas National Park, Primer Optimization, Genetic Marker, Cytochrome B Gene.

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1. Introduction

Sumatran elephant (*Elephas maximus sumatranus*) is an endemic mammal in Sumatera from Nanggroe Aceh Darussalam, North Sumatera, Riau, Jambi, Bengkulu, South Sumatera, and Lampung. Its existence has been categorized as critically endangered since 2011 by the International Union for Conservation of Nature (IUCN) [1]. Based on Urgent Rescue Action Plan on Sumatran Elephant, the decline of sumatran elephant individual from 2011-2017 has reached 700, with the remaining amount ranging between 1,694-2,038, confirming its critical status [2]. Besides habitat fragmentation, illegal hunting, and human-wildlife conflict, the challenge faced in its conservation is inbreeding for captive sumatran elephants. Its low population size causes the increasing of inbreeding probability which leaves concern towards the genetic drift in their population, thus affecting the individual viability. Study on sumatran elephant kinship pattern has been studied, especially related to phylogeny of asian elephants in Indonesia, Nepal, India, and african elephants. Sumatran elephant is related to asian elephant with varying degree of relatedness [3]. Genetic variation based on mitochondrial DNA between sumatran elephants in Lampung, South Sumatera, and Bengkulu is considered low [4]. Data on genetic diversity can be used as a base in identifying the priority for sumatran elephant conservation efforts.

Molecular DNA technology is the solution for updating information and supporting sustainable population. Genes in mitochondrial DNA (mtDNA) can be used for breaking genetic differentiation pattern at different phylogenetic scales. Certain segments of mtDNA are tagged according to higher sequence evolution rates which are

generally used to study the degree of genetic differences between populations and for historical reconstruction of distribution patterns [5]. Genetic information in mtDNA can also be used to evaluate the viability of a population. Studies have shown the utilization of mtDNA for animal species identification.

In the last decades, molecular genetics has played an important role in helping, clarifying, and determining the identification of species, especially on closely related species, cryptic species. Molecular DNA approach to taxonomy has been used and there is a faster and larger access protocol available nowadays [6]. Molecular DNA technique is known as a tool for species identification through the sequence of DNA barcodes sequences from mitochondrial DNA [7]. The CO1 gene is known to have low variation in species (intraspecies), but has high interspecies variations [8]. Amplification of CO1 gene fragments using standard DNA barcode primers developed by Ivanova *et al.* (2006) [9] has been widely used for the identification of mammals, while the sequence of cytochrome B gene fragments has not been widely used. Optimization of the cytochrome B primer PCR program as one of the genetic markers for the DNA of the captive sumatran elephants.

2. Materials and Methods

Two fresh fecal samples were obtained from two captive sumatran elephants of Elephant Training Center, Way Kambas National Park. This work is part of research under Higher Education Applied Research funded by The Ministry of Research, Technology, and Higher Education [10]. Materials include QIAamp Fast DNA Stool Mini Kit (Qiagen; cat no. 51604), forward and reverse primer for sumatran elephant cytochrome B gene, MYTaq™ HS Red Mix (Bioline; cat no. BIO 25043), absolute ethanol (Merck; cat. no. K51871183942), agarose, 100 bp DNA ladder, SYBR® safe DNA gel stain (Invitrogen; cat. no. S33102) dan TBE 1x (Invitrogen; cat. no. 15581-044). DNA Amplification was done using T100 Thermocycler (Biorad).

Table 1. Sequence of primers used

Primer	Sequence	Product	Position
CytbR1F	5'-TCACCCCCTGTTTAAAATCATC -3	639	14169-14190
CytbR1R	5'-TGAAGTGAGACCTAGTGGGTTGT -3'		14765-14787
CytbR2F	5'-CGGTAGATAAAGCAACCTTAAACC-3'	601	14654-14677
CytbR2R	5'-GCGATTGGTAGGAAAGCTAGAAT-3'		15232-15254

Primer used for DNA amplification is specific for sumatran elephant with cytochrome B gene as its target (Table 1). Primers were designed using Primer3 (bioinfo.ut.ee) with the reference to *Elephas maximus* acc. No AB002412. The design was based on parameters, such as primer length which determines the specificity of the primer itself. The optimal length ranges between 18-24 bases. GC content was also considered as well [11];[16]. Palindromic sequences are not allowed to be used in the primer as it will avoid annealing the target sequence and will fold back on itself [12];[15].

DNA Extraction

DNA extraction was conducted using QIAamp Fast DNA Stool Mini Kit. It started with inserting 200 µl of sample to a 1.5 microtube containing 1000 µl inhibitex buffer. Suspension was homogenized for 1 minute and centrifuged at 14,000 rpm for 1 minute. Amount of 600 µl of the centrifuged solution was added into another 1.5 microtube containing 25 µl of proteinase K and 600 µl AL buffer. The suspension was homogenized for 15 seconds and incubated for 10 minutes at 70°C. Then, 600 µl absolute alcohol was added to the suspension and homogenized for 15 seconds. The suspension is then transferred to the spin column and centrifuged at 14,000 rpm for 1 minute. This procedure is repeated until there's no suspension left in the tube. After that, 500 µl AW1 buffer is inserted into the spin column and centrifuged for 1 minute at 14,000 rpm. Collection tube is replaced with the new one. Of 500 µl AW2 buffer is inserted and centrifuged at 14.000 rpm for 1 minute. Collection tube is replaced with 1.5 ml microtube. Then, 100 µl ATE buffer is inserted. The suspension is incubated at room temperature for 1 minute and centrifuged at 14.000 rpm for 1 minute.

DNA Amplification

Optimization was done to find the best annealing temperature suitable for the primer. Annealing temperature can be obtained from the melting temperature (Tm) using the formula: 2 (A+T) + 4 (G+C) [13]. Reaction mix total volume in the PCR tube was 20 µl, consisting 10 µl MYTaq™ HS Red Mix, 0.8 µl of each forward and reverse primer with the concentration of 20 pmol, and 3 µl DNA template. Thermocycler is set to the program as follows: predenaturation at 95°C for 5 minutes; denaturation at 95°C for 20 seconds; annealing at 51°C; 52°C;53°C;54°C; 55°C; 56°C for 45 seconds; extension at 72°C for 1 minute for 35 cycles; post extensionat 72°C for 7 minutes.

Electrophoresis was done on 1.5% agarose gel dissolved in TBE buffer 1x solution and stained by SYBR® safe DNA gel stain. The gel was visualized in UV transilluminator and documented. Amplification result analysis is done by comparing the position of the DNA band with the marker. Specificity test as a form of verification by

amplifying other species (sumatran rhinoceros, cow, pig, and buffalo) using the designed primer.

3. Results and Discussion

DNA amplification of fecal samples of captive sumatran elephants, Elephant Training Center, Way Kambas National Park was conducted after the extraction has finished in order to obtain copied DNA. Primer used for DNA amplification was designed specifically for cytochrome B gene with the size of 1137 bp. To amplify the whole gene, overlapping primer design was made, consisting of two regions (Figure 1). In designing primer, targeted gene was examined.

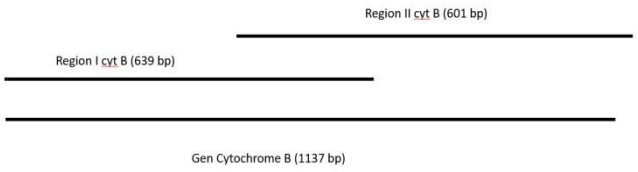

Figure 1. Primer design scheme used for amplifying the whole gene of cytochrome B

Optimization was done to find out the best amplification program by changing the annealing temperature gradually. It can be determined from the lowest melting temperature of the primers used. The optimization was carried out at 51-56^{o}C. Single band DNA at certain size, respectively 639 bp (region I) and 601 bp (region II) was obtained from the amplification program (Figure 2). The best result was obtained at the annealing temperature of 52^{o}C.

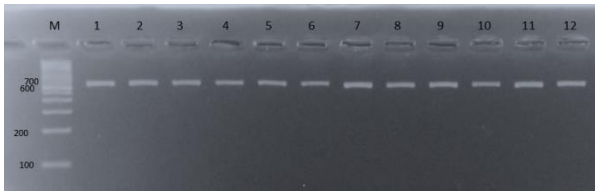

Figure 2. Electrophoresis result of the primer optimization process of Cytochrome B Region I (1-6) and Region II (7-12). (Note: M: marker; 1: 51^{o}C; 2: 52^{o}C; 3: 53^{o}C; 4: 54^{o}C; 5: 55^{o}C; 6: 56^{o}C)

Primer designed and used for species detection must be specific for sumatran elephants. Specificity test is done to find out if a certain designed primer can detect other species that are not intended [14]. Primer is said to be specific if it can only detect a certain target species. Specificity test was done to compare the DNA amplification result among ruminant species (Figure 3).

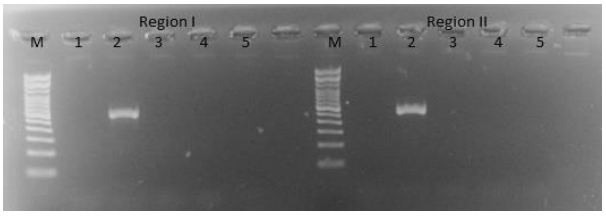

Figure 3. Cytochrome B (Region I and II) primer specificity test. (Note: M: Marker, 1: Cow, 2:Sumatran elephant, 3: Sumatran rhinoceros, 4: Pig, 5: Buffallo)

DNA amplification showed positive results for captive sumatran elephants' fecal samples (Table 2). Amplicon produced are 639 bp in region I and 601 in region II. Other species were not detected by using the designed primer.

Table 2. Specificity test result among other species

Samples	Result	
	Region I	Region II
Cow	-	-
Sumatran elephant	+	+
Sumatran rhino	-	-
Pig	-	-
Buffallo	-	-

4. Conclusion

Both primers used in this research (CytBR1 and CytBR2) are able to amplify the cytochrome B gene of sumatran elephant. Sequencing is needed to be done as a form of validation on the primers made for detection.

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