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Primer design of the CO1 gene (Cytochrome Oxidase-1) for Sumatran elephant (*Elephas maximus sumatranus*) for rapid detection using real-time PCR method

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Abstract. *Sari MT, Susilowati A, Aritonang SB, Astirin OP, Etikawati N, Saamia V. 2024. Primer design of the CO1 gene (Cytochrome Oxidase-1) for Sumatran elephant* (Elephas maximus sumatranus) *for rapid detection using real-time PCR method. Biodiversitas 25: 3840-3849.* The population of Sumatran elephants is classified as endangered according to the IUCN due to conflicts between humans and elephants, resulting in many elephants being killed by humans to obtain ivory to be sold. The traded ivory is often processed into other ornaments. A rapid forensic examination with real-time PCR using the molecular approach of the CO1 gene (Cytochrome Oxidase-1) is necessary to determine the original species of the processed elephant ivory. This research aims to design and optimize a primer for identifying Sumatran elephants (*Elephas maximus sumatranus*). The CO1 gene primer for Sumatran elephants was designed using the Primer3Plus and IDT websites. The primers were used to amplify and identify the species of Sumatran elephants from blood, feces, and urine samples. Blood and feces samples were collected from Sumatran elephants at Taru Jurug Zoo Surakarta, while feces and urine samples were taken from Sumatran elephants at Ragunan Zoo Jakarta. DNA from blood and urine samples was extracted using the TIANamp Genomic DNA Kit, and DNA from feces samples was extracted using the TIANamp Stool DNA Kit. The purity and concentration of the obtained DNA extracts were measured. The designed primers were synthesized by Macrogen (Korea), and primer optimization was performed with a PCR gradient. The identification process of Sumatran elephant DNA extract samples was conducted by amplification using real-time PCR quantification with the standard curve method. The results of this research include the COI-270 primer with the sequences 3'-TTAGGTCAACCAGGCTCTCTTC-5' and 5'-AGGATATACGGTCCAACCAGTG-3', capable of amplifying the target 270 bp and having a single peak at the melt curve temperature of 79°C. The identification protocol with specific COI-270 primer can be used in forensic examinations to identify samples originating from Sumatran elephants.

Keywords: Cytochrome Oxidase-1, DNA, primer design, real time PCR, Sumatran elephant

INTRODUCTION

Poaching is one of illegal trade practice that involves the killing or capturing of wild animals, such as wildcats (like tigers, leopards, and lions), rhinos, elephants, pangolins and sea turtle for their valuable body parts. This trade violates national and international laws, such as the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). In 1989, CITES placed African elephants (*Loxodonta africana*) under Appendix I, which includes species threatened with extinction. This means the commercial trade of ivory is prohibited. However, some populations have been placed under Appendix II (with certain restrictions) to allow controlled trade in specific countries. Illegal trade in wild animals includes the process of transporting, torturing, killing, sending, transferring, storing, and receiving animals for exploitation purposes. Poaching of elephants is widespread in Africa and Asia, leading to the killing of many elephants by poachers to obtain elephant ivory (Ewart et al. 2020). The high demand for elephant ivory, with prices reaching up to 1800 dollars per kilogram, even in Indonesia, has become a contributing factor to illegal wildlife hunting and trade (Guntur and Sabar 2019). According to Williams et al. (2020), in the IUCN Red List Threatened Species, Sumatran elephants are classified as critically endangered species. As of 2017, their population was only 1,694-2,038 individuals spread across seven provinces in Indonesia (KLHK 2020). The traded elephant ivory is processed into sculptures, ornaments, and traditional medicines, making morphological-based macroscopic identification difficult for investigative purposes (Linacre 2021).

Processed elephant ivory that is trafficked needs to be investigated for legal purposes. Molecular approaches, such as DNA barcoding using the CO1 gene located in mitochondria, are employed for the taxonomic identification of organisms through amplification and analysis of short, standardized, and previously identified DNA sequences (Ewart et al. 2020). The CO1 gene has a combination of conserved sequences and polymorphisms, making it suitable for DNA barcoding to identify animal species (Rodrigues et al. 2017). This identification is carried out to check the authenticity of elephant ivory because there are also many processed imitation ivory involving other wild animals

(Linacre 2021). The condition of forensic samples found has often experienced DNA genome fragmentation or what is commonly referred to as DNA degradation due to environmental influences (Sahajpal et al. 2021). Mitochondrial DNA (mtDNA) is highly polymorphic, with thousands of copies inside each cell. Therefore, its amplification and sequencing could be a powerful tool in species identification, even in old or degraded samples (Mayada et al. 2020). In forensic cases, the identification process needs to be rapid and accurate, prompting the use of real-time PCR methods for elephant species identification. This method amplifies the CO1 gene of Sumatran elephants, providing melt curve graphs that differentiate nucleotide variations among species. Melt curve shape and melt peak (Tm) are potentially indicative of sequence variation among PCR products. Differences in DNA melt curves, stem from the effects nucleotide sequence chemistry has on melt peak intensity and curve shape. The success of the real-time PCR process is, in part, determined by using a specific primer. The design of a specific primer is a crucial initial step in obtaining primer sequences specific to the CO1 gene of Sumatran elephants. Primer design was performed using several programs, including Primer3Plus and IDT (Integrated DNA Technologies®), with Primer3Plus being widely used for genomic/mitochondrial DNA applications due to its accurate results. Research on identifying Sumatran elephant species using real-time PCR in forensic examinations has not been conducted before. Therefore, this study aims to obtain specific and sensitive primers for amplifying the CO1 gene in Sumatran elephants and determine the optimal primer conditions in the real-time PCR process. This research will develop a protocol to identify Sumatran elephant forensic cases.

MATERIALS AND METHODS

Sample collection

Blood and feces samples were collected from 2 Sumatran elephants at the Taru Jurug Zoo Surakarta (TSTJ), Central Java, Indonesia. Feces and urine samples were obtained from Sumatran elephants at the Ragunan Zoo in Jakarta (TMR) with permission from SK Dirjen KSDAE Number SK174/KSDA/SET.3/K54.2/9/2022 (Table 1). Research involving taking blood samples from animals is permitted under an ethical clearance letter No.4426/C.1/KEPK-FKUMS/VIII/2022. The samples were taken to the National Police Criminal Investigation Agency Forensic Laboratory Center, Sentul, Bogor, based on a permit from the BKSDA Jakarta with SATS-DN (Domestic Plant and Wildlife Transport Letter) Number S.401/K.13/TU/TSL/09/2022. Blood was collected in 3 mL from the posterior auricular vein and stored in EDTA vacuum container tubes. Feces were collected in 100 g and stored in conical tubes. Urine was collected in 25 mL and stored in conical tubes. The collected blood, feces, and urine samples were then stored at -80°C to preserve DNA quality and remain usable for an extended period.

DNA extraction

DNA extraction was carried out using a spin column following the TIANAMP Genomic DNA Kit protocol (TIANGEN®) and the TIANAMP Stool DNA Kit (TIANGEN). A total of 200 µL of blood sample was placed in a 1.5 mL tube, and 1700 µL of urine sample was placed in a 1.5 mL tube. Subsequently, the samples were centrifuged at a speed of 12,000 rpm for 3 minutes. DNA extraction was performed on the blood and urine samples following the protocol specified in the TIANamp Genomic DNA Kit. For the feces sample, 200 mg were finely ground into powder using a mortar with the addition of liquid nitrogen. The powdered feces were then placed in a 1.5-mL tube, and DNA extraction was performed according to the TIANamp Stool DNA Kit protocol.

Primer design and in silico primer specificity analysis

The primer design was conducted by downloading the CO1 gene sequence of Asian elephants (*Elephas maximus*) from NCBI with the accession number NC_005129.2 as a reference sequence in FASTA format. The CO1 gene sequence of the Asian elephant is 1551 bp. In designing primers, we follow the general requirements. General requirements in designing primers are that the primer length is commonly 18-24 bp. Furthermore, the optimal Tm value used in the Real-time PCR process is between 59-68°C. Real-time PCR's ideal amplicon product size ranges from 80-150 bp. Repeated nucleotide repetitions should be avoided because they can cause mispriming. To avoid mispriming, the maximum nucleotide repetition is only four nucleotides. Finally, there is the GC Clamp, which is the maximum value of AG 5 based on the end of the 5' chain on the primer. The ideal GC Clamp is 1 to 2, which will bind the primer strongly to the DNA template so that the primer will be specific to the target DNA template (Thornton and Basu 2017). Primers were designed using the Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and IDT websites (https://sg.idtdna.com/PrimerQuest/Home/Index), followed by secondary structure analysis using the Oligo Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html), IDT, and NCBI BLAST websites.

The best primers obtained (Table 2) were then analyzed in silico to test their specificity and the level of similarity of the PCR product sequences with the nucleotide database available in GenBank NCBI. The analysis was done by aligning the primer sequences using Clustal W and BLAST nucleotide similarity on the NCBI website (Sihotang et al. 2021).

Primer optimization

The primer optimization was carried out using the PCR gradient method with the Proflex PCR System Thermoscientific. The amplification process was performed with a total volume of 25 µL, comprising 12.5 µL of 2xTaq PCR Master Mix (with dye) from TIANGEN, 1 µL each 5 µM of forward and reverse primer, 40-50 ng/µL of DNA sample, and Nuclease Free Water (NFW). Amplification optimization was conducted in this study, and the PCR conditions obtained were as follows: pre-denaturation at

98℃ for 2 minutes, 35 cycles of denaturation at 98℃ for 5 seconds, annealing at 60℃ for 45 seconds, extension at 60℃, and post-extension at 72℃ for 10 minutes. The best optimum primer includes annealing temperature and primer concentration, which will then be used for DNA template amplification using Real-time PCR.

Electrophoresis of PCR products

PCR product electrophoresis was performed to validate the specificity of the COI-270 primer. The electrophoresis process involved creating an agarose gel with a concentration of 1.5% and a gel thickness of 0.25 cm. A total of 0.375 g of agarose powder was mixed with 25 mL of 1X TBE buffer, then homogenized on a hotplate magnetic stirrer at 200℃ for 10 minutes. The gel was stained with a fluorescent dye, specifically 1.5 µL of GelRed 10000x. Electrophoresis was carried out using a 100-bp DNA ladder since the target PCR product of the COI-270 primer was 270 bp. The electrophoresis ran for 60 minutes with an electric current of 70 volts.

Sequencing of PCR products

Sequencing of PCR products was performed to validate the sequence of COI gene of Sumatran elephant (*E. maximus sumatranus*). Sequencing is done by sending a PCR product sample to 1st Base DNA Sequencing (Apical Scientific Laboratory) at Selangor, Malaysia. The PCR product DNA were then sequenced using single pass Sanger sequencing with the ABI 3730 Genetic Analyzer (Applied Biosystems). The sequence that has been obtained are then analyzed by BLAST using the NCBI online website for identify similarities between sample sequences and sequences in the GenBank database.

In vitro primer specificity and sensitivity analysis used Real Time PCR

To determine the primer specificity in this study, used samples from other species, namely bovine and human, were used as negative controls. The real-time PCR method utilized was the Quantification Standard Curve from the handbook of Real time PCR, aiming to determine primer sensitivity through a standard curve created with eight levels of serial dilution. Real-time PCR was carried out with a total volume of 20 µL, consisting of 10 µL of PowerUp SYBR Green Master Mix, 1 µL each of 10 µM forward and reverse primer, a DNA sample with a concentration of 8-10 ng/µL, and nuclease-free water (NFW) to reach the total volume. Running was carried out using the real-time PCR 7500 Fast Applied Biosystem machine. The real-time PCR amplification conditions used were UDG (Uracil DNA Glycosylase) activation at 50℃ for 2 minutes, dual-lock DNA polymerase (pre-denaturation) at 98℃ for 2 minutes, 40 cycles of denaturation at 98℃ for 5 seconds, and annealing/extension at 60℃ for 50 seconds (Kitpipit et al. 2016). After the amplification process, a melt curve analysis was performed at 95℃ for 15 seconds and 60℃ for 1 minute. Real-time PCR analysis was performed by reading the Ct (Cycle threshold) value and analyzing the peak of the melt curve graph that had been created. A single specific peak melt curve indicates the success of the Sumatran elephant (*E. maximus sumatranus*) CO1 gene amplification process using the designed primers.

RESULTS AND DISCUSSION

In silico primer specificity analysis

In silico primer specificity analysis was conducted to analyze secondary structure criteria, including hairpin, self-dimer, and cross-dimer formations (Table 3).

Attachment position of primer 270 with Asian elephant COI sequence

Figure 1 shows the attachment position of the COI 270 primer: the forward primer will start to attach at the 121st base sequence, and the reverse primer will attach at the 390th base sequence on the Asian elephant COI gene sequence. In silico primer specificity analysis was also conducted using NCBI BLAST to determine the primer binding specificity with the target (Table 4).

The in silico analysis of the forward primer 270 specificity in Table 4 has results of species other than *Laelapidae* sp. and *Solo* sp., which are included in the invertebrate group and are very different from the *Elephas maximus* species, so the results are still acceptable. However, in vitro testing is still needed to strengthen the results obtained. The analysis of the reverse primer 270 has resulted in the form of the *Elephas maximus* and *Loxodonta africana* species. This indicates that the reverse primer 270 is less specific for the *Elephas maximus* species because it can detect the *Loxodonta africana* species, which is an African elephant. Based on the research that has been done, primer 270 has NCBI BLAST results that match, according to Narita et al. (2012).

Table 1. ID codes for Sumatran elephant samples

Sample ID code	Sample type	Sample origin
G1	Blood	TSTJ
G3	Blood	TSTJ
A6	Faeces	TSTJ
B7	Faeces	TSTJ
P1	Faeces	TMR
R1	Faeces	TMR
UP	Urine	TMR

Note: TSTJ: Taru Jurug Zoo Surakarta, TMR: Ragunan Zoo in Jakarta

Table 2. Primer sequence

ID primer	Forward sequence	Reverse sequence	Product size
Primer COI-270	TTAGGTCAACCAGGCTCTCTTC	AGGATATACGGTCCAACCAGTG	270 bp

Visualization of PCR product electrophoresis

The PCR product electrophoregram was visualized under a UV transilluminator with wavelengths of 254 nm and 312 nm (Figure 2). Single and thick DNA bands were formed, consistent with the target 270 bp in the *E. maximus sumatranus* samples in wells 1 to 3. In the non-target human sample (M) in Well 4, a thin DNA band was formed at the 100 bp target. In the non-target bovine sample (SB) in Wells 5 and 6, no DNA bands were formed. Finally, the NTC sample in Well 7 did not show any DNA bands. In Figure 2, in wells 1, 2 and 3, two bands or faint bands can happen due to incorrect sample preparation, low protein concentration, insufficient electrophoresis conditions, or problems with gel or buffer.

Sequence similarity

The obtained sequences of amplicon COI-270 primer were analyzed using NCBI BLAST, and the similarity (Table 5) between the PCR product and the *E. maximus* species was determined.

Sensitivity analysis of COI-270 primer - Real-time PCR

The sensitivity analysis of the COI-270 primer using real-time PCR was generated in a standard curve graph (Figure 3) with eight serial dilution levels. The COI-270 primer demonstrated the ability to amplify the lowest DNA quantity, 0.000704 ng/µL at CT 33.2

Specificity analysis of COI-270 primer: Real-Time PCR

The specificity of primer B using real-time PCR is shown by the Ct value in Table 6. The specificity of primer B with a target of 270 bp in elephant samples has been successfully amplified with a Ct value ranging from 18.0 to 23.5 with different quantities of DNA. The greater the amount of target DNA, the smaller the Ct value will be, such as in the UP elephant sample, which has a DNA quantity of 8.240 ng/µL with a Ct value of 18.4. The less the amount of target DNA, the greater the Ct value will be, such as in sample B7, which has a DNA quantity of 0.261 ng/µL with a Ct value of 23.5. According to Tamara et al. 2021, the Ct value range states that a Ct value of less than 29 is strongly positive with a large amount of DNA target, 30-37 is moderately positive with a moderate amount of DNA target, and 38-40 is weakly positive with a small amount of DNA target, and there is a possibility of contamination. These results are reinforced by the melt curve graph in Figure 4, which shows that the melt curve graph has successfully amplified the Sumatran elephant sample specifically, as indicated by the formation of a single peak melt curve at a temperature of 79°C.

Table 3. Primer secondary structure

Primer COI-270	Hairpin (kcal/mol)	Self-dimer (kcal/mol)	Cross dimer (kcal/mol)
Reverse	-1.04	-2.10	-2.18
Forward	-0.97	-2.70	

```
Reverse.C   ------------------------------------------------------------ 0
Elephas     ATGTTTGCTAACCGCTGACTATATTCAACAAACCACAAAGATATTGGAACACTGTATCTA 60
Forward     ------------------------------------------------------------ 0

Reverse.C   ------------------------------------------------------------ 0
Elephas     CTATTTGGTGCTTGAGCTGGTATAGTAGGGACTGCTTTTAGTATTCTAATTCGGGCAGAA 120
Forward     ------------------------------------------------------------ 0

Reverse.C   ------------------------------------------------------------ 0
Elephas     TTAGGTCAACCAGGCTCTCTTCTTGGAGACGACCAAATCTATAATGTCATTGTCACAGCA 180
Forward     TTAGGTCAACCAGGCTCTCTTC---------------------------------------- 22

Reverse.C   ------------------------------------------------------------ 0
Elephas     CACGCCTTTGTAATAATCTTCTTTATAGTTATGCCAATCATAATTGGAGGCTTTGGAAAC 240
Forward     ------------------------------------------------------------ 22

Reverse.C   ------------------------------------------------------------ 0
Elephas     TGGTTAATTCCACTTATAATTGGAGCACCTGATATAGCTTTTCCTCGAATAAACAATATA 300
Forward     ------------------------------------------------------------ 22

Reverse.C   ------------------------------------------------------------ 0
Elephas     AGTTTCTGACTACTGCCTCCGTCTTTCCTACTACTTTTGGCATCCTCCATAGTAGAAGCT 360
Forward     ------------------------------------------------------------ 22

Reverse.C   --------CACTGGTTGGACCGTATATCCT---------------------------- 22
Elephas     GGAACAGGCACTGGTTGGACCGTATATCCTCCTCTGGCAGGAAACTTAGCCCATGCAGGA 420
Forward     ------------------------------------------------------------ 22
```

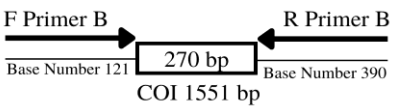

Figure 1. The attachment position of the COI 270 primer

Table 4. NCBI BLAST primer analysis

Sequence	Description	Max score	Total score	Query cover	E value	Percentage of identity
Primer 270 Forward	*Laelapidae* sp. cytochrome oxidase subunit 1(CO1) gene	44.1	44.1	100%	0.090	100%
	Elephas maximus cytochrome oxidase subunit 1(CO1) gene	44.1	44.1	100%	0.090	100%
	Solo sp. partial CO1 gene	44.1	44.1	100%	0.090	100%
Primer 270 Reverse	*Elephas maximus* cytochrome oxidase subunit 1 (CO1) gene	44.1	44.1	100%	0.090	100%
	Loxodonta africana cytochrome oxidase subunit 1(CO1) gene	44.1	44.1	100%	0.090	100%

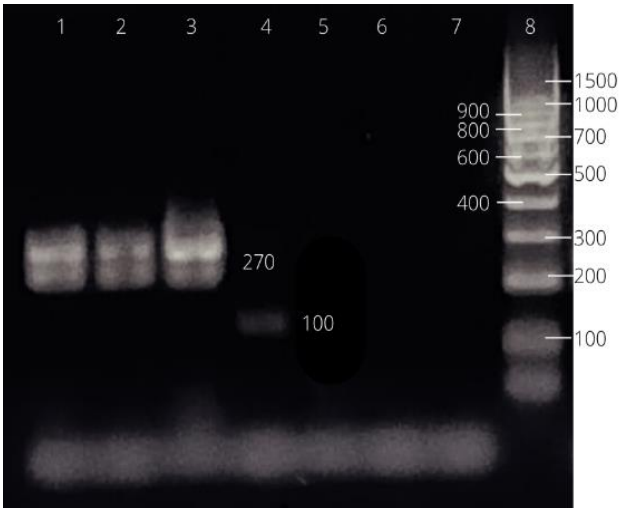

Figure 2. Visualization of PCR Product by Electrophoresis. Wells Order: 1: *Elephas maximus sumatranus*, 2: *Elephas maximus sumatranus*, 3: Positive control (Sumatran elephant), 4: Non-target human sample (M), 5: non-target bovine sample (SB), 6: non-target bovine sample (SB), 7: No Template Control (NTC) sample, 8: DNA ladder 100 bp

Melt curve - Real time PCR analysis

Analysis of the melt curve of COI-270 primer on *E. maximus* sample showed a specific single peak at 270 bp at a temperature of 79°C (Figure 5.A). The non-target bovine sample (SB) did not show any peaks, indicating no amplification occurred (Figure 5.B). The non-target human sample (M) exhibited a peak at 77.4°C (Figure 5.C) with a CT value of 39.4, indicating the formation of a melt curve peak due to the designed primer for the CO1 gene of Sumatran elephants amplifying human DNA in the final cycles of real-time PCR. The No Template Control (NTC) sample did not show any peaks, indicating that the COI-270 primer did not undergo primer dimerization (Figure 5.D).

Discussion

This research has been conducted, and the COI-270 primer with a product length of 270 bp has been designed, demonstrating the capability to specifically and accurately amplify *E. maximus sumatranus* DNA using real-time PCR. Analysis of the secondary structure specificity of the COI-270 primer showed the presence of a hairpin, self-dimer, and cross-dimer. A secondary structure or hairpin is formed by intramolecular interaction within the nucleotide of primer. Delta G (▲G) is the energy that breaks the secondary structure. Usually, it is effective when the energy is less than -9 kcal/mol. Primer COI-270 has ▲G -0,97 to -2,70 kcal/mol in any structures. A number of ▲G indicates that the actual secondary structure will not form at all (Rianti et al. 2021). In this study, an in-silico primer analysis was performed using NCBI BLAST to avoid nonspecific binding. The specificity analysis by NCBI BLAST for the COI-270 primer showed sequence binding of both the forward and reverse primers to species other than the target CO1 gene of *Elephas maximus*. These different species bind to the COI-270 forward primer and belong to the invertebrate group, which is distantly related to *Elephas maximus*. Therefore, the results of the NCBI BLAST analysis are deemed acceptable. The NCBI BLAST results for the reverse primer COI-270 in silico showed binding to *Loxodonta africana*, which is closely related to the target CO1 gene of *Elephas maximus*, indicating that the reverse primer COI-270 sequence is less specific. If a primer has many nonspecific binding results in the NCBI BLAST specificity test, indicating binding to species outside the target species, then that primer candidate is considered less specific (Kumar and Chordia 2015). To strengthen the accuracy of in silico analysis results, it is necessary to conduct specificity tests and DNA sequencing of the COI-270 primer in vitro in the laboratory.

Table 5. Similarity sequences of COI-270 primer

Primer	Sequence similarity	Query cover	Percentage of identity
Forward	*Elephas maximus* CO1 gene	95%	100%
Reverse	*Elephas maximus* CO1 gene	96%	98.3%

Table 6. CT Values of COI-270 primer

Sample ID	CT value	Target DNA quantity (ng/µL)
A6	22.0	0.797
B7	23.5	0.286
R1	20.1	2.714
P1	19.7	3.594
UP	18.4	8.240
Non-target control sample; Bovine DNA (SB)	Undetermined	-
Non-target control sample; Human DNA (M)	39.4	0
WK (positive control sample; Sumatran elephant DNA)	18.0	10.753
NTC (No Template Control) sample	Undetermined	-

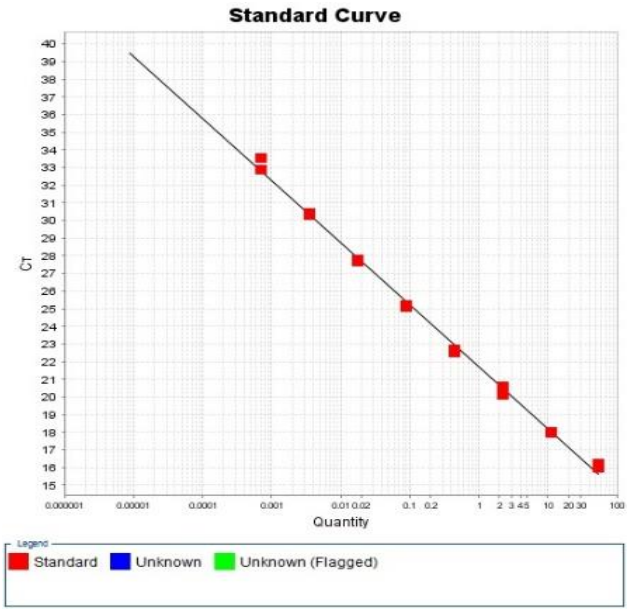

Figure 3. Standard curve of COI-270 primer

The specificity for the COI-270 primer in this study were strengthened by performing DNA sequencing on *E. maximus sumatranus*. Sequencing was carried out using the dideoxytermination Sanger sequencing method, which produces nucleotide sequences from DNA fragments. This method utilizes DNA polymerase enzymes purified to synthesize DNA chains of varying lengths. The main material used in the Sanger method is ddNTPs (dideoxynucleotide triphosphates), which function in the sequencing process involves separating DNA fragments based on size using capillary electrophoresis with an electric current that draws molecules through a gel substrate in capillary form, resulting in different DNA fragments (Gummadi and Kandula 2020). The obtained sequences were analyzed using NCBI BLAST, revealing a similarity (Table 6) between the PCR product and the *Elephas maximus* species.

The results show a Query Cover value of 95% and 96%, representing the percentage of the sequence that matches its counterpart. These results indicate a high sequence similarity between the PCR product and the *E. maximus* species. The Persent Identity values of 98.3% and 100% indicate DNA sequence similarity between the PCR product and *E. maximus* species. A Persent Identity value of 98%-100% suggests that they originate from the same species. Genomes of the same species commonly show genome-aggregate average nucleotide identity >95% and if score <95% means include of other species (Rodriguez et al. 2024).

In this study, the target PCR product used is 270 bp, the shorter PCR product produces more pronounced melt curve differences than longer nucleotide (Galuszynski and Alastair 2020). This study used Real-time PCR since it is more time-efficient, reduces the risk of contamination, and is more cost-effective than species identification through DNA sequencing (Vuong et al. 2016). The method amplifies DNA using a specific primer targeting the desired DNA, and a fluorescent dye, such as SYBR Green, is used to determine the success of specific PCR fragment detection. This study used SYBR Green fluorescent dye, which fluoresces when bound to double-stranded DNA and is detectable at wavelengths of 498 nm and 522 nm. Fluorescent signals are recorded in each real-time PCR cycle, providing direct visualization of the abundance of the amplified target DNA products during the real-time PCR reaction.

The real-time PCR process conducted in this study used the Quantification Standard Curve method by creating a standard curve as a reference to determine the sensitivity value of the primer. The sensitivity results were determined based on the standard curve graph with eight serial dilution levels (Figure 3), showing an efficiency value of 92.6%, a slope value of -3.5, and an R2 value greater than 0.996. These values were considered positive and satisfactory, because the ideal slope of a standard curve is -3.32 which indicates 100% Real-Time PCR efficiency, although a range from -3.1 (110%) to -3,58 (90%) and a standard curve should demonstrate strong linear fits with R2 values ranging from 0.980 to 1.00 (Bivins et al. 2021). The sensitivity test was conducted to determine the lowest quantity of target DNA detectable in real-time PCR using the designed primer (Agustiningsih 2021). The COI-270 primer demonstrated sensitivity, amplifying the lowest quantity of target DNA at the 8th dilution level with a DNA quantity of 0.000704 ng/µL and a CT value of 33.2. This indicates that the COI-270 primer is sensitive and specific to the CO1 gene of *E. maximus sumatranus*.

The specificity test of real-time PCR was conducted to determinethe specificity of the designed primer to the CO1 gene of *E. maximus sumatranus*. Specificity testing is a

critical parameter that determines the success of the target gene amplification process. Primer specificity can be further confirmed by verifying the identity of the formed sequences through gel electrophoresis and DNA sequencing (Broeders et al. 2014). In this study, sample negative control was used from cows and humans. The use of negative control samples derived from human DNA for research purposes is carried out by humans and prone to human DNA contamination. So, a specificity test is required to ensure that the COI-270 primer is specific to the *E. maximus sumatranus* target and does not amplify human DNA. The use of negative control samples from *Bos taurus* is because the *Bos taurus* species belongs to a class of large mammals whose body parts are often processed into artificial tusks in forensic cases, thus requiring a specificity test to ensure that the COI-270 primer does not amplify DNA frombovine.

Next, the specificity test of the COI-270 primer was demonstrated with amplification plot graphs (Figure 4) and melt curve graphs (Figure 5). The amplification plot graph shows the reactions occurring in each cycle in real-time PCR, allowing the detection of the first occurrence of PCR product amplification because the fluorescence signal crosses the threshold line automatically (Jalali et al. 2017). The cycle when the fluorescence signal is detected crossing the threshold line is called the cycle threshold (CT) value (Coyle et al. 2021). The CT value is related to the quantity of target DNA (Ferguson et al. 2014). The lower the quantity of DNA target, the lower the CT value; conversely, the higher the quantity of DNA target, the higher the CT value (Shah et al. 2021). The melt curve graph in real-time PCR is formed due to a decrease in fluorescence signal, indicating that double-stranded DNA (dsDNA) molecules have melted into two single-stranded DNA (ssDNA) molecules, and the fluorescent dye loses its ability to produce fluorescence signals on the graph, marked by a descending melt curve (Zhang et al. 2020). The temperature on the melt curve graph will produce a single peak when half of the target dsDNA molecules have denatured and the other half still remain in the ssDNA form (Ruijter et al. 2019). In the real-time PCR process, the specificity test with CT and melt curve values can be used to identify of animal species (Malewski et al. 2021).

The results of the specificity test for the COI-270 primer indicate that samples with the target CO1 gene of *E. maximus sumatranus* were amplified with CT values ranging from 18.4 to 23.5, consistent with the positive control sample of *E. maximus sumatranus* with a CT value of 18.0 (Table 6). This signifies that the CT values are positive, indicating the presence of target DNA corresponding to the COI-270 primer. The CT value range is considered positive when it falls between 11 and 20, indicating that the sample has a large quantity of target DNA, while a CT value range of 21-30 suggests a moderate quantity of target DNA (Shah et al. 2021). Furthermore, the melt curve graph in the specificity test for the COI-270 primer (Figure 5) showed a single peak at a temperature of 79°C, indicating specific binding between the COI-270 primer and the target DNA of *E. maximus sumatranus*. This result is reinforced by the gel electrophoresis visualization (Figure 2) in wells 1, 2, and 3, showing a bright single DNA band at 270 bp corresponding to the target. This indicates specific amplification between the COI-270 primer and the target DNA, forming the appropriate amplicon.

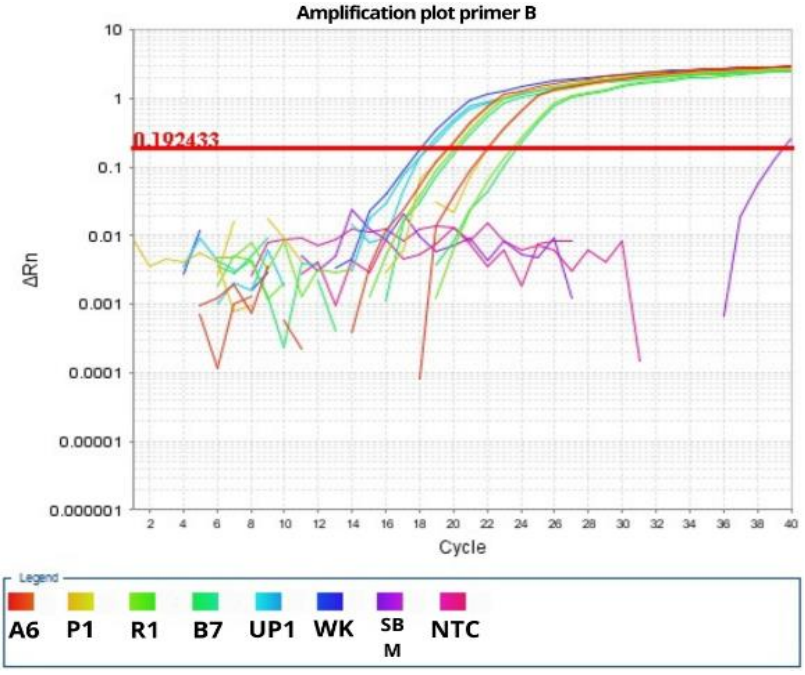

Figure 4. Amplification plot graph of COI-270 primer. The designed primers successfully amplified the COI gene specifically in Sumatran elephants from faecal and urine samples and did not amplify COI in humans and bovine. A6-B7: Sumatran elephant feces DNA sample, P1: Sumatran elephant urine DNA sample, WK: positive control DNA sample (Sumatran elephant), SB: Non-target control sample (Bovine DNA), M: Non-target control sample (Human DNA), NTC: No Template Control

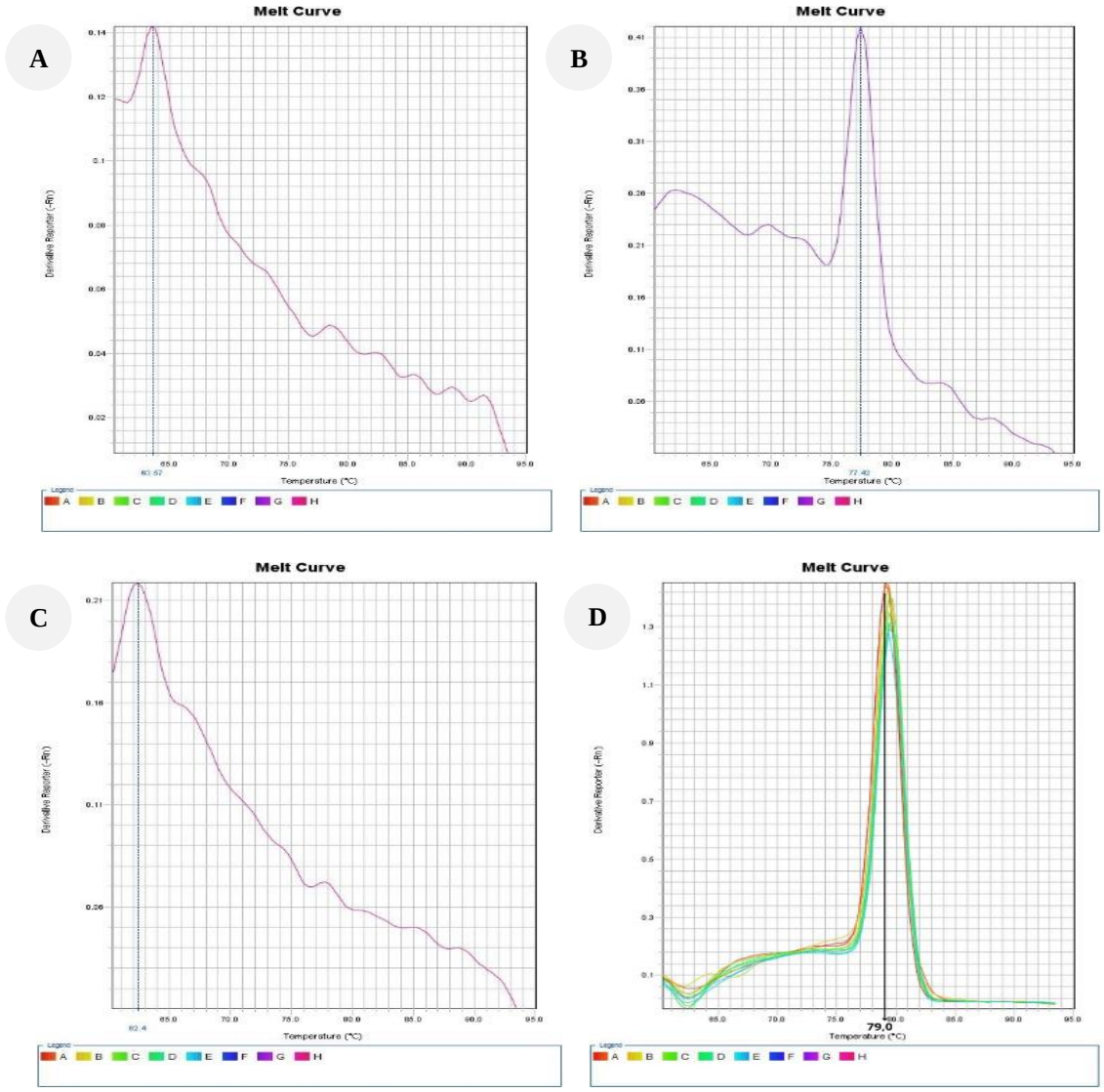

Figure 5. Melt curve graphs. A. Melt curve graph of Elephas *maximus sumatranus* sample; B. Melt curve graph of non-target bovine sample (SB); C. Melt curve graph of non-target human sample (M); D. Melt curve graph of NTC (no template control) sample

The specificity test for the COI-270 primer for negative samples from bovine did not yield a CT value and was marked as undetermined (Table 6), indicating the absence of target DNA corresponding to the primer. Thus, no amplification occurred in the real-time PCR reaction, and no fluorescence signal crossed the threshold line. This is consistent with the melt curve graph (Figure 5.B), which showed no peak formation. The absence of a peak is due to the lack of amplification, confirming the absence of amplicons in the real-time PCR reaction, as visualized in the electrophoresis (Figure 2) in wells 5 and 6, where no DNA bands were formed, indicating the absence of amplicons.

The specificity test for the COI-270 primer for human negative samples yielded a CT value of 39.4 with a target DNA quantity of 0 (Table 6). Although the CT value is high, it is acceptable because it approaches the end cycle in the real-time PCR process, which is 40 cycles. According to Onwuamah et al. (2021), real-time PCR results for negative samples with CT values above 38 is showing negative. For each run to be valid, the negative control should have a CT value between 35-40 and the positive control should have a CT<35. The melt curve graph for the non-target human sample (M) showed a peak at a temperature of 77.4°C (Figure 5.C). This peak temperature is lower than the peak temperature of the *E. maximus sumatranus* target DNA, which is 79°C, indicating the formation of a non-specific product in the late cycles of real-time PCR. This non-specific product can be considered negative. According to Ruiz-Villalba et al. (2017), a peak in the melt curve with a lower temperature than the amplification temperature of the desired target DNA represents denatured, non-specific DNA sequences that are no longer fluorescent. To reinforce the real-time PCR results, gel electrophoresis visualization of the PCR product (Figure 2) in well 4 showed the formation of a thin single DNA band at 100 bp, indicating the absence of a specific amplification between the COI-270 primer and the non-

target human sample, as evidenced by the lack of amplicon formation.

The specificity test for the COI-270 primer on the No Template Control (NTC) sample yielded an undetermined CT value (Table 6), indicating that the COI-270 primer did not form primer dimers, as evidenced by the absence of amplification. The undetermined CT value also signifies that the real-time PCR reaction process was not contaminated. This result aligns with the melt curve graph (Figure 5.D), which shows no peak formation. The absence of a peak is attributed to the lack of amplification, confirming the absence of PCR products in the real-time PCR reaction. This result is further supported by gel electrophoresis visualization (Figure 2) in well 7, where no DNA bands were formed, indicating the absence of amplicons in the real-time PCR reaction. It is important to include NTC in the reaction because NTC samples serve to ensure that the tested samples are free from contamination and that the obtained real-time PCR results are valid and reliable (Smith 2021).

In conclusion, it can be stated that the COI-270 primer is specific and sensitive, making it optimal for the identification testing of *E. maximus sumatranus* using real-time PCR.

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