BIODIVERSITAS
Volume 24, Number 1, January 2023
Pages: 241-249

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240129

DNA primer design for sex identification of Sumatran tiger body samples

IKRIMA ASRORI, DJONG HON TJONG, WILSON NOVARINO, MANSYURDIN, SYAIFULLAH, DEWI IMELDA ROESMA♥

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas. Jl. Raya Unand, Limau Manis, Padang 25163, West Sumatra, Indonesia. Tel./Fax.: +62-751-71671, ♥email: dewiroesma@sci.unand.ac.id

Manuscript received: 15 November 2022. Revision accepted: 4 January 2023.

Abstract. *Asrori I, Tjong DH, Novarino W, Mansyurdin, Syaifullah, Roesma DI. 2023. DNA primer design for sex identification of Sumatran tiger body samples. Biodiversitas 24: 241-249.* Many reports of cases of illegal trade in animal body parts have resulted in more and more samples of animal body parts being seized. Seized sample from illegal trade needs to be identified with the help of molecular methods to ensure the profile of the seized samples including the determination of their sex. At the molecular level, amelogenin gene amplifications are used to determine the sex of mammals. Previous studies using primers for amelogenin gene amplification found that amelogenin X (AMELX) and amelogenin Y (AMELY) bands in male samples were difficult to distinguish due to very small differences, 20 base pairs (bp). The difficulty of distinguishing these bands resulted in errors in detecting male and female individual samples. Therefore, it was to design a more specific primer as a way to avoid this error. The purpose of this study was to design a DNA primer for the sex identification of the Sumatran tiger (*Panthera tigris sumatrae* Pocock, 1929). The research was carried out using descriptive methods and molecular observation of the AMELX and AMELY Sumatran tiger sequences. The primer design results in this study were 100% able to identify the sex of the Sumatran tiger sample. The present primer design (F= 5' TCGGTTAACAATTCCCTGGGC'3 and R= 5'AGGCCAAATAGGAGTGTGCT'3) is more specific than the primers previously reported.

Keywords: Design primer, gen amelogenin, intron, *Panthera tigris sumatrae*, specific primer

INTRODUCTION

The Sumatran tiger is one of nine tiger subspecies (Seidensticker et al. 1999). There used to be three tiger subspecies in Indonesia, but, the Javan tiger (*Panthera tigris sondaica* Temminck, 1844) and the Balinese tiger (*Panthera tigris balica* Schwarz, 1912), became extinct in the 1940s and 1980s (Xue et al. 2015). The Sumatran tiger (*Panthera tigris sumatrae* Pocock, 1929) is an endangered species on the IUCN (International Union for Conservation of Nature) Red List. Although there are already international laws and regional regulations on tiger parts trade, poaching, and illegal activities, many cases continue to this day (Goodrich et al. 2015).

The increasing demand for Sumatran tiger body parts and cases of habitat destruction were the main causes of the decline in tiger numbers during the twentieth century. Therefore, monitoring the population of this species is very important. Sex ratio information is important to provide information for animal conservation and management (Joshi et al. 2019). The sex ratio is important to obtain information on the estimated number of males and females caught in illegal hunting, and to estimate the Sumatran tiger population in ecological studies (Colorado et al. 2012).

Identification of the sex of an adult Sumatran tiger can be determined by direct observation. However, often the results of confiscations from illegal trade are only body parts in the form of nails, flesh, skin, hair, bones, and other biological materials (Kamarcharya et al. 2018). Therefore, it is necessary to carry out a comprehensive examination to ascertain the profile of the confiscated samples, which are thought to be part of the Sumatran tiger. DNA molecular marker is one of the methods to identify separate from the animal's bodies. Previous research using molecular methods to identify body part samples, among others, was reported by Ashrifurrahman et al. (2019) that there is a specific site on the 23rd sequence nucleotide base of the COI gene, and then Ashrifurrahman et al. (2022) reported that the COI gene can be used as a marker to identify Sumatran tiger samples. Molecular sex identification can be done by amplifying the amelogenin gene (Gokulakrishnan et al. 2012; Farahvash et al. 2016; Dutta et al. 2017). The amelogenin gene is used for molecular sex identification in mammals. Electrophoresis results from the amplification of the amelogenin gene, indicated by two bands in the male sample, and one band in the female sample (Ahmad et al. 2021; Lucas et al. 2022).

Sex identification of the Sumatran tiger based on the amelogenin gene using primers from Pilgrim et al. (2005) has also been reported by Asrori et al. (2022). Asrori et al. (2022) reported that the results of the sex identification of the Sumatran tiger based on Pilgrim et al. (2005) showed that the amelogenin gene amplification bands on the X and Y chromosomes in male samples were difficult to distinguish. the difficulty to distinguish AMELX and AMELY bands due to the difference in the length of the

DNA produced only 20 bp (214 bp and 194 bp). The problem of distinguishing these bands results in errors when detecting male and female individual samples. So, it is necessary to design a more specific primer as a way to avoid this error.

The factors that must be considered in designing primers are primer length, melting temperature (Tm), primer annealing temperature (Ta), number of GC bases, and specificity or uniqueness (Ye et al. 2012). Reports on AMELX and AMELY sequences in several mammalian species indicate that there are differences in the sequences of the AMELX and AMELY introns (Pilgrim et al. 2005; Das et al. 2019; Kawasaki et al. 2020; Lohay 2020; Ahmad et al. 2021). It can be concluded that the sequence of the amelogenin Y intron gene in each animal species is different. Therefore, it was possible to design specific primers for the sex identification of the Sumatran tiger.

MATERIALS AND METHODS

Sample collection

The sample in this study consisted of ten blood samples from individuals of the Sumatran tiger (*P. t. sumaterae*) whose sex was known (four males and six females). So that there is no doubt in the application for sex identification of seized samples in the future. The use of blood samples aims to speed up the isolation process and get better results because blood samples have a higher concentration of DNA. Blood samples were collected by veterinarians at Sumatran tiger individuals who were rehabilitated at the Dharmasraya Sumatran Tiger Rehabilitation Center (PR-HSD).

Procedures

DNA isolation and Polymerase Chain Reaction (PCR)

DNA isolation was carried out from Sumatran tiger blood following the Invitrogen DNA Isolation KIT protocol. The results of DNA isolation were stored at -20°C for further analysis. The results of the DNA isolation were then electrophoresed on a 1.2% agarose gel. The PCR process targets the amelogenin gene sequence based on Pilgrim et al. (2005) (F 5' CGAGGTAATTTTTCT GTTTACT 3'; R 5' GAAACTGAGTCAGAGAGGC 3'. The temperature PCR process refers to Pilgrim et al. (2005) and is modified by Asrori et al. (2022). The PCR reaction with a total volume of 25 µL, by mixing 12.5 µL of GoTaq green supermix, 9.5 µL of nuclease free water, 1 µL forward primer, 1 µL reverse primer, and 1 µL DNA isolation. Predenaturation was carried out at 95°C for 3 minutes, then denaturation was carried out at 95°C for 30 seconds, and after that annealing at 48°C for 60 seconds and 72°C for 25 seconds. The final extension was carried out at 72°C for 5 minutes. The amplification process with this PCR machine runs for 35 cycles. The results of the PCR will be visualized and analyzed by 3% agarose gel electrophoresis.

Gel extraction and sequencing

AMELX and AMELY DNA were extracted from male Sumatran tiger samples using gel extraction. The QIAquick Gel Extraction Kit protocol was followed for gel extraction. The extraction results were visualized on a 3% agarose gel. After the AMELX and AMELY DNA were separated, the AMELX and AMELY nucleotide base sequencing was carried out.

Sequencing

DNA amplification products (DNA amplicon) from the PCR process were sent to Firstbase Company Singapore to be purified and used as samples in nucleotide sequencing (DNA sequencing) reactions.

Primer design

The primer design was carried out using PrimerBlast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), with reference to Ye et al. (2012). The primer design was based on an analysis of the Sumatran tiger amelogenin gene sequence. Then, also one pair of primers based on the alignment results of the Sumatran tiger amelogenin sequences. After that, the most qualified primer was selected.

Specificity and efficiency test of design results with PCR primer

Specificity and efficiency test of primers designed by PCR using DNA isolates from nine Sumatran tiger blood samples (Table 1). The PCR reaction was performed with the composition of Supermix Bioline 12.5 µL, Nuclease free water 3.5 µL, forward primer 3 µL, reverse primer 3 µL, and DNA isolation results in 5 µL. Predenaturation was carried out at a temperature of 95°C for 15 minutes, then denaturation was carried out at a temperature of 94°C for 30 seconds. After that, annealing was carried out at a temperature that was in accordance with the results of temperature optimization using gradient PCR for 60 seconds and elongation at 72°C for 60 seconds. The last cycle, the final extension, was carried out at 72°C for 10 minutes. The amplification process with this PCR machine runs for 45 cycles. The results of the PCR will be visualized and analyzed by 3% agarose gel electrophoresis.

Table 1. Sumatran tiger blood samples

Sample code	Sex
HS01	Male
HS02	Male
HS03	Male
HS04	Male
HS05	Female
HS06	Female
HS07	Female
HS08	Female
HS09	Female

Data analysis

The data from the sequences were contiguous using the SeqMan software, and the contiguous sequences were aligned with the comparison sequences from GenBank (https://www.ncbi.nlm.nih.gov/) using the BioEdit program. Variations in nucleotide bases between Sumatran tiger amelogenin gene sequences and comparison sequences indicate that the amelogenin gene can be used as a specific molecular marker.

RESULTS AND DISCUSSION

Polymerase Chain Reaction (PCR)

The results of amplification of the amelogenin gene in Sumatran tiger nuclear DNA using forward primer 5' CGAGGTAATTTTTCTGTTTACT 3' and reverse primer 5' GAAACTGAGTCAGAGAGGC 3' (Pilgrim et al. 2005) with an annealing temperature of 48°C are shown in Figure 1. Two DNA bands for male samples measuring approximately 190 bp and 210 bp and one DNA band for female samples measuring approximately 210 bp, were identified using a 50 bp DNA ladder comparator (Figure 1).

Sumatran tiger amelogenin gene sequence variations with comparative sequences

Before designing a specific primer, it is necessary to know the sequence of the Sumatran tiger amelogenin gene variation. The results of the alignment of the Sumatran tiger amelogenin gene sequences, with the comparison amelogenin gene sequences are shown in Figure 2. The alignment results showed that there were many differences (variations) in the nucleotide base sequences for the Sumatran tiger AMELX and AMELY sequences with 18 comparison sequences from different families (Figure 2). The alignment of the amelogenin gene sequences in nine Sumatran tiger samples had many similarities (Figure 2).

Primer design

The Sumatran tiger sample AMELY sequence with code HS01Y represents all Sumatran tiger AMELY samples. The designed primer is expected to amplify about 150 bp of the Sumatran tiger AMELY sequence. The results of the primer design using PrimerBlast on the NCBI site obtained 10 primer candidates for AMELY and also one pair of primers based on the alignment results of the Sumatran tiger amelogenin sequences (Table 2). The eleven primer candidates were then re-selected according to the criteria for good primers for the PCR process (Wang 2016). Based on the primer candidate results from PrimerBlast, one primer pair, primer pair 11 (F = 5' TCGGTTAACAATTCCCTGGGC 3'; R = 5' AGGCCAAATAGGAGTGTGCT 3') was selected that met the characteristics of a good primer for the PCR process, referring to Wang's (2016) report.

Comparison of design result primers with previously reported primers

The results of the PrimerBlast test showed that the designed primer could detect the AMELY sequence in the cat (*Felis catus* Linnaeus, 1758) with one nucleotide base difference in the forward primer. While the results of the Pilgrim primer test using PrimerBlast showed that the Pilgrim primer could detect AMELY sequences in cats and several other species. Based on Table 3 and Table 4, it is known that the primer design result is more specific than Pilgrim's primer.

Primer test

The primer test of the design results on the Sumatran tiger sample showed only one band in the male sample. While the female sample did not show a band because it did not have AMELY. Based on Figure 3, it is known that the primer designed successfully amplified the AMELY sequence in all-male Sumatran tiger samples. The designed primer targets the PCR product at a size of 141 bp. The primer amplifies the Y chromosome amelogenin gene sequence starting from the sequence 2034-2195 bp, referring to the AMELY sequence from the lion (NC_056697.1) (Figure 4).

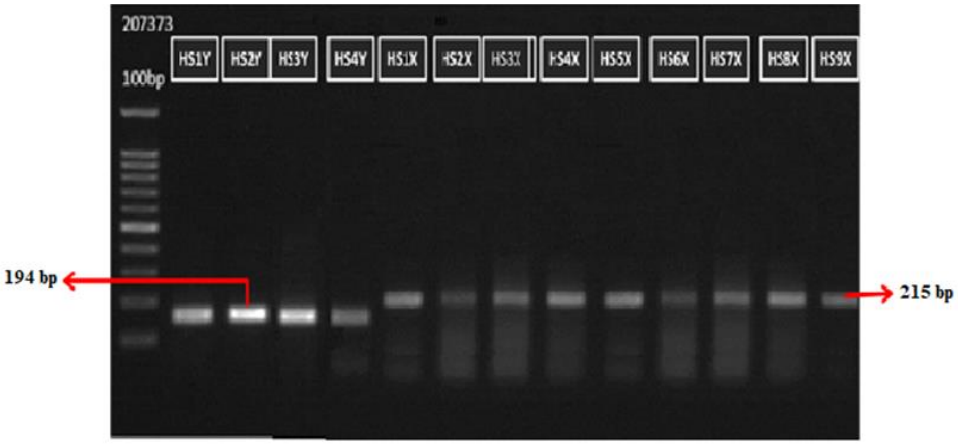

Figure 1. Visualization of DNA purification results in Sumatran tiger samples to be sequenced. L= Ladder 50 bp. Note: HS01-HS04 = Male individual, HS05-HS09 = Female individual

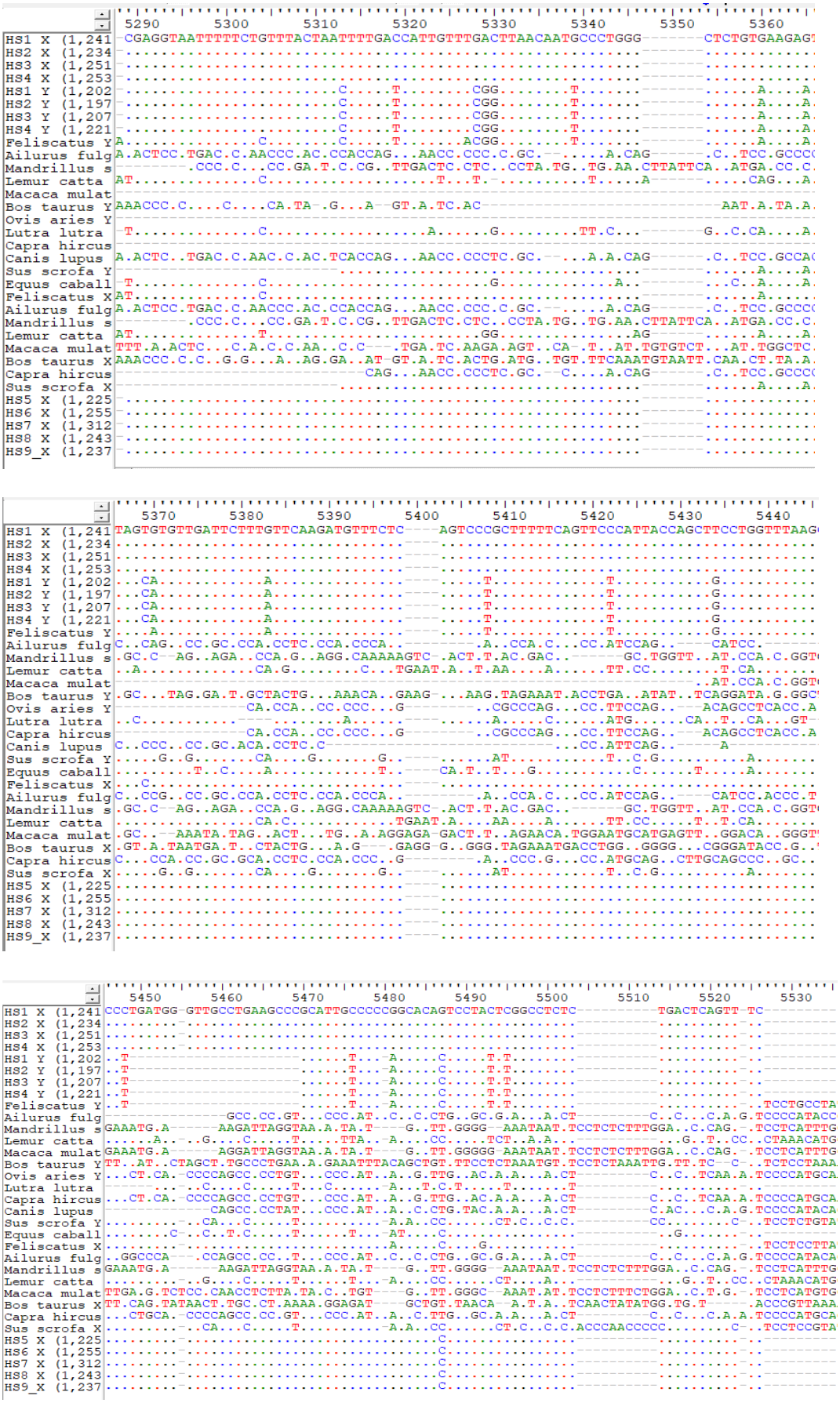

Figure 2. Results of alignment of Sumatran tiger amelogenin gene sequences with comparison sequences. Note: HS: Sumatran tiger

Table 2. Primer candidates based on primer blast for AMELY Sumatran tiger

	Sequence 5'-3'	Length	Tm	GC%	Self 3' Complementary
	Primer Pair 1				
Forward	CGGTTAACAATTCCCTGGGCT	21	60.62	52.38	1
Reverse	CCAGGCAGCTGGTAATGAGA	20	59.46	55	0
	Product Length :101				
	Primer Pair 2				
Forward	TCATTACCAGCTGCCTGGTTT	21	59.92	47.62	3
Reverse	GAAACTGAGTCAGAGAGGCCA	21	59.38	52.38	2
	Product Length : 72				
	Primer Pair 3				
Forward	GGTTAACAATTCCCTGGGCTC	21	58.9	52.38	2
Reverse	TGCAGGCTTAAACCAGGCAG	20	60.9	55	2
	Product Length : 112				
	Primer Pair 4				
Forward	ACAATTCCCTGGGCTCTGTAAA	22	59.62	45.45	2
Reverse	AGAGGCCAAATAGGAGTGTGC	21	60.07	52.38	2
	Product Length : 136				
	Primer Pair 5				
Forward	GTTAACAATTCCCTGGGCTCTG	22	59.24	50	3
Reverse	GCCAAATAGGAGTGTGCTGG	20	58.9	55	0
	Product Length : 136				
	Primer Pair 6				
Forward	CAATTCCCTGGGCTCTGTAAA	21	57.91	47.63	2
Reverse	AACCAGGCAGCTGGTAATGAG	21	60.34	52.38	1
	Product Length : 96				
	Primer Pair 7				
Forward	GTTAACAATTCCCTGGGCTCT	21	57.91	47.62	1
Reverse	GAGGCCAAATAGGAGTGTGC	20	58.62	55	2
	Product Length : 139				
	Primer Pair 8				
Forward	AGTTCTCATTACCAGCTGCCTG	22	60.36	50	3
Reverse	ACTGAGTCAGAGAGGCCAAAT	21	58.74	47.62	2
	Product Length :74				
	Primer Pair 9				
Forward	CCTGGGCTCTGTAAAGAATAGC	23	60.12	47.83	0
Reverse	AAACCAGGCAGCTGGTAATG	20	58.45	50	0
	Product Length : 91				
	Primer Pair 10				
Forward	TTCGGTTAACAATTCCCTGGGC	22	61.14	50	3
Reverse	GTGTGCTGGGACAATGCAG	19	59.42	57.89	2
	Product Length :129				
	Primer Pair 11				
Forward	TCGGTTAACAATTCCCTGGGC	21	60.61	52.38	3
Reverse	AGGCCAAATAGGAGTGTGCT	20	59	50	1
	Product Length : 141				

Table 3. Comparison of the design primer blast primer with Pilgrim's primer

Sequences detected by	
Design result primer	**Pilgrim's primer**
AMELY *P.t sumatrae*	AMELY *F. catus*
AMELY *F. catus*	AMELY *Bos taurus*
	AMELX *Bos taurus*
	AMELY *Lutra lutra*
	AMELX *Lutra lutra*
	AMELY *Meles meles*
	AMELY *Equus caballus*
	AMELX *Equus caballus*
	Kromosom *Pyrochroa serraticornis*

Table 4. Report on the application of Pilgrim primers for amplification of the amelogenin gene in Felidae

Author	Species tested
Pilgrim et al. (2005)	*F. catus* (domestic cat)
	Lynx (Bobcat)
	Bobcat (Bobcat)
	Cougar (Puma)
Pandhee et al. (2016)	*F. catus* (domestic cat),
	F. catus (jungle cat),
	Pardofelis temminckii (Asean golden cat)
	Panthera tigris (tiger)
	Prionailurus viverrinus (fishing cat)
Kamarcharya et al. (2018)	*P.t tigris* (benggalan tiger)
Asrori et al. (2022)	*P.t sumatrae* (sumatran tiger)

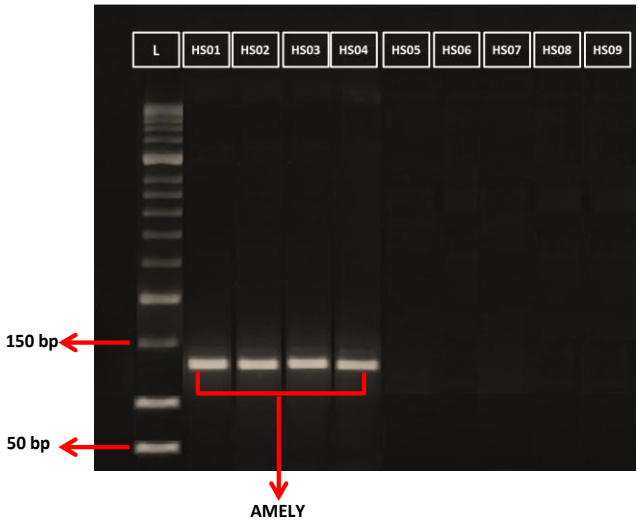

Figure 3. Visualization of the amelogenin gene amplification of the Sumatran tiger using the design primer. Note: HS01-HS04 = Male individual, HS05-HS09 = Female individual

Discussion

Sumatran tiger amelogenin gene amplification results based on Pilgrim et al. (2005) primer is in line with the reports of Pilgrim et al. (2005) that the identification of male domestic cat samples was characterized by the visualization of two DNA bands (AMELX= 195 bp; AMELY= 214 bp), and one DNA band for female (AMELX= 214 bp) at 214 bp. The difference of domestic cats has a difference of 20 nucleotide bases.

Based on the sequence of amplification results, the primer was designed to identify the sex of the Sumatran tiger. Before designing the primer, alignment was carried out to determine the variation and similarity of the amelogenin gene sequence of the Sumatran tiger with other mammals. This same or similar sequence of bases is called a conserved sequence. According to Huber et al. (2019), a large number of conserved sequences can be used as universal PCR primers. While, designing specific primers according to Hisao and Ayana (2016) must have unique or varied sequences, sequences that are different from other DNA sequences. Sequence variations between Sumatran tigers and comparison sequences indicate that primers can be designed to identify the sex of Sumatran tiger samples. Alignment results did not show specific sequences for AMELX and Sumatran tiger AMELY that could be amplified simultaneously (other than in the Pilgrim primer attachment area). So in this study, a primer was designed that would only amplify Sumatran tiger AMELY.

Primer design was carried out using PrimerBlast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al. 2012). Because it will only amplify AMELY, the visualization of the amplification results showing bands for male samples and no bands for female samples. The primer pair chosen in this study was primer pair 11. Referring to Wang's (2016) report, the results of BLAST analysis, primer length, GC percentage, melting temperature, and dimer primer analysis of 11 primer pairs were the best of all suggested primer pairs. The primer lengths selected in this study were 21 bp and 20 bp. A good primer length according to Wang (2016), is 17 bp-28 bp. In line with the report, the study by Chuang et al. (2013) reported that a good primer length for PCR was 16-28 bases. Wu et al. (2004) also reported that in addition to the primer length, it is also necessary to pay attention to the difference between the forward and reverse primer lengths.

Another characteristic that needs to be considered in the selection of primers for PCR is the percentage of GC base in the primer. The percentage of GC base is an important characteristic of primers because it affects the adhesion strength of a primer (Chuang et al. 2013; Wang 2016; Banaganapalli et al. 2019). Primer pair 11 has a GC base percentage of 52.38% and 50%, respectively. The percentage of good GC base according to Wang (2016) is 40-60%.

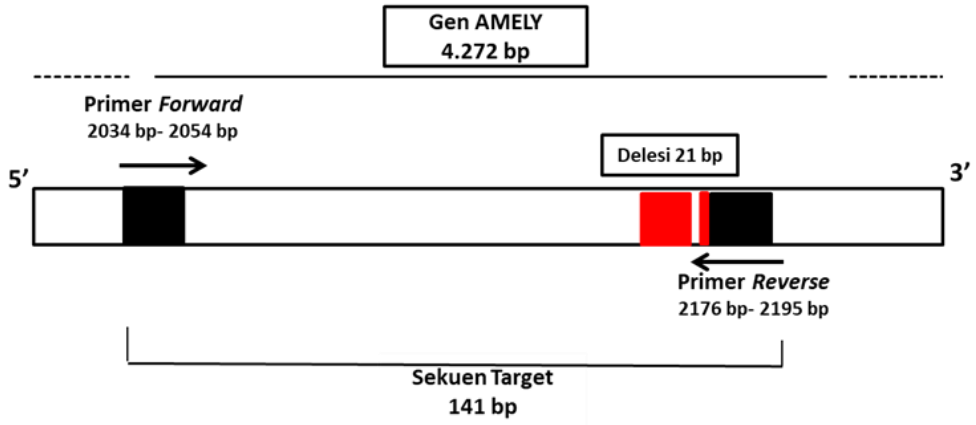

Figure 4. Illustration of the location of the primer attachment of the design result (*black*), on the Sumatran tiger AMELY sequence based on the AMELY lion sequence reference (NC_056697.1) (*white*)

Melting temperature (Tm) is also a characteristic that needs to be considered in the selection of primers. Based on Table 2, it is known that the melting temperatures of primer pair 11 are 60.61 C and 59°C. Melting temperature is one of the most important things to consider in the selection of PCR primers (Chuang et al. 2013). According to Wang (2016), the melting temperature of a good primer pair for PCR is 50°C-62°C with a maximum difference in melting temperature between forward and reverse primers a maximum of 3-5°C. Melting temperature is related to the percentage of GC of a primer. Melting temperature and GC percentage are primer characteristics that influence each other (Kumar et al. 2014; Victoriano et al. 2022).

Another characteristic that needs to be considered in primer design is the possibility of dimer primers occurring. Based on analysis using DINAMelt software, it is known that all primer pairs form dimers. Thus, primer pair 11 was chosen, which had the least number of primer dimers and fulfilled other good primer characters (Figure 5). To minimize the possibility of false dimers and priming, the PCR process is generally carried out at high temperatures (>50°C) (Mona et al. 2021).

Dimer analysis is required to ensure that the primers do not hybridize with the DNA template sequence. The occurrence of dimers will have a negative impact on the PCR results (Meagher et al. 2018; Jansson and Hedman 2019). Cross-dimer occurs when the forward and reverse primers stick together. Meanwhile, self-dimer is formed when the forward primer attaches to another forward primer, or the reverse primer attaches to another reverse primer (Garafutdinov et al. 2020). The percentage of GC base in a primer also influences the formation of primer dimers. Because it is made up of three hydrogen bonds, the bond between bases G and C is very strong. This makes the nucleotide bases in primers with GC bases easier to bind. To reduce the occurrence of dimer primers, primers with the smallest G-C bonds were chosen (Assal and Lin 2021).

According to Garafutdinov et al. (2020), the accumulation of dimer primers in PCR can be reduced or eliminated. The possibility of dimer primer formation can be suppressed by adding a sequence of nucleotide bases at the 5' end (inactive side) of the primer. Other studies (Brodin et al. 2013; Mubarak et al. 2020) also suggested optimization by increasing the temperature of the annealing process to reduce the rate of dimer primer formation. In addition, based on the reports of Pandhee et al. (2016), Kamarcharya et al. (2018) and Asrori et al. (2022), primers designed by Pilgrim can also amplify the amelogenin gene of other species in the Felidae family.

The amelogenin gene is a gene encoding the amelogenin protein that functions as the main matrix of tooth enamel (Chowdhury et al. 2018). The amelogenin gene found in most mammals is located on the X and Y chromosomes (Fábián et al. 2017). Due to the diversity between AMELX and AMELY, this gene has been used as a marker to check sex in mammals, including humans (Chowdhury et al. 2018; Dash et al. 2020; Ahmad et al. 2021; Lucas et al. 2022). The amelogenin gene has been used to identify sex in several species in the family Felidae (Lynx, Bobcat, Domestic cat, and Puma) (Pilgrim et al. 2005). Pandhee et al. (2016) reported that the primer designed by Pilgrim et al. (2005) was successful in amplifying the amelogenin gene in five species of the Felidae family, namely *F. catus* (Persian cat), *F. catus* (forest cat), *Pardofelis temminckii* (Vigors & Horsfield, 1827) (Asian golden cat), *P. tigris* (tiger), and *Prionailurus viverrinus* (Bennett, 1833) (mangrove cat). Kamarcharya et al. (2018) also reported that these primers can amplify the amelogenin gene in the Bengal tiger (*P. t tigris*). The three studies used primers designed by Pilgrim with a PCR product length range of 214 bp for AMELX and 194 bp for AMELY. Specific primer design experiments for the mammalian amelogenin gene have previously been reported by Erwanto et al. (2012) who designed specific primers for pork identification. Specific primers were designed with a fragment length of 300 bp. However, the PCR test found cross-amplification reactions with other species (chickens, goats, and cattle).

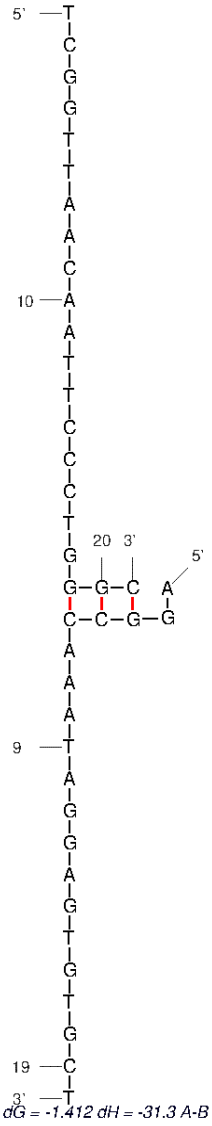

Figure 5. Dimer structure in the design primer, primer pair no 11

The primer is designed to amplify AMELY which is only present in males. Interpreting results show bands for male samples and no bands for female samples. The alignment results show that the amelogenin gene sequences of the Sumatran tiger and the amelogenin gene sequence data that have been reported in the EMBL and GenBank databases have variations in nucleotide bases, so that specific sequences can be determined to identify the Sumatran tiger. Thus, the results of the primary design, 100% can be used to determine the sex of a sample of Sumatran tiger body parts. The method used in this study can be used on various types of samples (bone, meat, skin, nails, teeth, etc.), including old samples. According to Hofreiter et al. (2001), animal specimens that have been stored for hundreds of years can still be identified, and their DNA can be sequenced. However, according to Schweitzer et al. (2008), hypothesized that DNA analysis is not useful for very ancient (tens of thousands of years) specimens.

In conclusion, primer for sex identification samples of Sumatran tiger body parts has been successfully designed and can be used to quickly sex identification samples of Sumatran tiger body parts. The sequences of primer forward and primer reverse in this study were F= 5' TCGGTTAACAATTCCCTGGGC'3 and R= 5'AGGCCAAATAGGAGTGTGCT'3.

ACKNOWLEDGEMENTS

We thank the Directorate General of Learning and Student Affairs, Indonesia, who provided a student research grant (No. 033/E5/PG.02.00/2022). Our gratitude goes to Ir. Catrini Pratihari Kubonbody, M. Arch. The Executive Director of the Arsari Djojohadikusumo Foundation (YAD) and Dr. Erly Sukrismanto as the Head of the Natural Resources Conservation Center (KSDA) of West Sumatra, Indonesia, who has received assistance in the form of blood and samples of the Sumatran tiger's body parts that provided the preserves used in this study.

REFERENCES

Ahmad A, Rahat MA, Wahab A, Subhanuddin, Shah M, Rasool A, Israr M. 2021. PCR-based sex determination in Cattle using gene specific markers. Pak J Biochem Biotechnol 2 (2): 134-139. DOI: 10.52700/pjbb.v2i2.93.

Ashrifurrahman, Fadhlullah A, Ericca C, Simamora S, Novarino W, Roesma DI. 2019. Characteristic of CO1 gene in "Bonita", one of the female tiger *Panthera tigris sumatrae* in East Sumatra. World J Adv Res Rev 4 (2): 043-048. DOI: 10.30574/wjarr.2019.4.2.0055.

Ashrifurrahman, Simamora S, Ritonga R, Novarino W, Tjong DH, Rizaldi, Syaifullah, Roesma DI. 2022. Sumatran tiger identification and phylogenetic analysis basedon the CO1 gene: Molecular forensic application. Biodiversitas 23 (4): 1788-1794. DOI: 10.13057/biodiv/d230410.

Asrori I, Tjong DH, Roesma DI, Novarino W, Syaifullah, Mansyurdin. 2022. DNA Sexing Sumatran tiger (*Panthera tigris sumatrae*) based on amelogenin gene. World J Adv Res Rev 14 (3): 190-194. DOI: 10.52700/pjbb.v2i2.93.

Assal N, Lin M. 2021. PCR procedures to amplify GC-rich DNA sequences of *Mycobacterium bovis*. J Microbiol Methods 181: 106121. DOI: 10.1016/j.mimet.2020.106121.

Banaganapalli B. 2019. In silico PCR. In: Shaik N, Hakeem K, Banaganapalli B, Elango R (eds.). Essentials of Bioinformatics, Volume I. Springer, Cham. DOI: 10.1007/978-3-030-02634-9_16.

Brodin J, Krishnamoorthy M, Athreya G, Fischer W, Hraber P, Gleasner C, Green L, Korber B, Leitner T. 2013. A multiple-*alignment* based primer design algorithm for genetically highly variable DNA targets. BMC Bioinform 14: 255-283. DOI: 10.1186/1471-2105-14-255.

Chowdhury RM, Singhvi A, Bagul N, Bhatia S, Singh G, Goswami S. 2018. Sex determination by amplification of amelogenin gene from dental pulp tissue by Polymerase Chain Reaction. Indian J Dent Res 29: 470-476. DOI: 10.4103/ijdr.IJDR_274_17.

Chuang LY, Cheng YH, Yang CH. 2013. Spesific primer design for the Polymerase Chain Reaction. Biotechhnol Lett 35 (10): 1541-1549. DOI: 10.1007/s10529-013-1249-8.

Colorado FAG, Ne Matta C, Sánchez A. 2012. Sex-determination systems and their evolution: Mammals. Acta Biol Colomb 17 (1): 3 - 18.

Das PP, Krishnan G, Doley J, Bhattacharya D, Deb SM, Chakravarty P, Das PJ. 2019. Establishing gene Amelogenin as sex-specific marker in yak by genomic approach. J Genet 98: 7. DOI: 10.1007/s12041-019-1061-x

Dash HR, Rawat N, Das S. 2020. Alternatives to amelogenin markers for sex determination in humans and their forensic relevance. Mol Biol Rep 47: 2347-2360. DOI: 10.1007/s11033-020-05268-y.

Dutta P, Bhosale S, Singh R, Gubrellay P, Patil J, Sehdev B, Bhagat S, Bansal T. 2017. Amelogenin gene - The pioneer in gender determination from forensic dental samples. J Clin Diagn Res 11 (2): ZC56-ZC59. DOI: 10.7860/JCDR/2017/22183.9407.

Erwanto Y, Sugiyono A, Rohman, Abidin MZ, Ariyani D. 2012. Identifikasi daging babi menggunakan metode Pcr-Rflp gen *Cytochrome* B dan PCR primer spesifik gen Amelogenin. Agritech 32 (4): 370-377. DOI: 10.22146/agritech.9579.

Fábián R, Kovács A, Stéger V, Frank K, Egerszegi I, Oláh J, Bodó S. 2017. X and Y chromosome specific variants of the amelogenin gene allow non-invasive sex diagnosis for the detection of pseudohermaphrodite goats. Acta Vet Hung 65 (4): 500-504. DOI: 10.1556/004.2017.047.

Farahvash T, Torshizi RV, Masoudi AA, Rezaei HR, Tavallaei M. 2016. AMELX and AMELY Structure and application for sex determination of Iranian maral deer (*Cervus elaphus maral*). Iran J Appl Anim Sci 6 (4): 963-968.

Garafutdinov RR, Aizilya A, Galimova, Assol R, Sakhabutdinova. 2020. The influence of quality of primers on the formation of primer dimers in PCR. Nucleos Nucleot Nucl Acids 39 (9): 1251-1269. DOI: 10.1080/15257770.2020.1803354.

Gokulakrishnan P, Kumar R, Sharma B, Mendiratta S, Sharm D, Malav O. 2012. Sex determination of cattle meat by polymerase chain reaction amplification of the Amelogenin (AMELX/AMELY) gene. Vet World 5: 526-529. DOI: 10.5455/vetworld.2012.526-529.

Goodrich J, Lynam A, Miquelle D, Wibisono H, Kawanishi K, Pattanavibool A, Htun S, Tempa T, Karki J, Jhala Y, Karanth U. 2015. *Panthera tigris*. The IUCN Red List of Threatened Species e.T15955A50659951. https://www.iucnredlist.org

Hisao Y, Ayana I. 2016. Human Blastocystis subtyping with subtype-specific primers developed from unique sequences of the SSU rRNAgene. Parasitol Intl 65 (6 Pt B): 785-791. DOI: 10.1016/j.parint.2016.03.00.

Hofreiter M, Jaenicke V, Serre D, von Haeseler A, Pääbo S. 2001. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. Nucleic Acids Res 29 (23): 4793-9. DOI: 10.1093/nar/29.23.4793.

Huber P, Cornejo FM, Ferrera I, Sánchez P, Logares R, Metz S, Balagué V, Acinas SG, Gasol JM, Unrein F. 2019. Primer design for an accurate view of picocyanobacterial community structure by using high-throughput sequencing. Microbial Ecol 85 (7): E02659-18. DOI: 10.1128/AEM.02659-18.

Jansson L, Hedmana J. 2019. Challenging the proposed causes of the PCR plateau phase. Biomol Detect Quantif 17: 2214-7535. DOI: 10.1016/j.bdq.2019.100082.

Joshi DB, De R, Goyal SP. 2019. Utility and applicability of a universal set of primers in identifying the sex of south and Southeast Asian Mammals. Zool Stud 58: e19. DOI: 10.6620/ZS.2019.58-19.

Kamarcharya D, Sherchan AM, Dulal S, Manandhar P, Manandhar S, Joshi J, Bhattarai S, Bhatta TR, Awasthi N, Sharma AN, Bista M, Silwal NR, Pokharel P, Lamichhane RR, Sharma N, Llewellyn B, Wultsch C, Kelly MJ, Gour D, Waits L, Hero JM, Hughes J. 2018. Species, sex and geo-location identification of seized tiger (*Panthera*

tigris tigris) parts in Nepal—A molecular forensic approach. PLoS ONE: 1-16. DOI: 10.1371/journal.pone.0201639.

Kawasaki K, Mikami M, Goto M. 2020. The evolution of unusually small Amelogenin genes in cetaceans; pseudogenization, X-Y gene conversion, and feeding strategy. J Mol Evol 88: 122-135. DOI: 10.1007/s00239-019-09917-0.

Kumar A, Kaur J, Kumar A, Kaur J. 2014. Primer based approach for PCR amplification of high GC content gene: *Mycobacterium* gene as a model. Mol Biol Intl 2014: 937308. DOI: 10.1155/2014/937308.

Lohay GG. 2020. An accurate molecular method to sex savannah elephants using PCR amplification of Amelogenin gene. Pachyderm 61: 90-96. DOI: 10.1101/856419.

Lucas CG, Spate AM, Samuel MS. 2022. A novel swine sex-linked marker and its application across different mammalian species. Transgenic Res 29: 395-407. DOI: 10.1007/s11248-020-00204-z.

Meagher RJ, Priye A, Light YK, Huang C, Wang E. 2018. Impact of primer dimers and self-amplifying hairpins on reverse transcription loop-mediated isothermal amplification detection of viral RNA. Royal Soc Chem 143: 1924-1933. DOI: 10.1039/c7an01897e.

Mona S. 2021. Basic concepts of primer designing: A minireview. Intl J Latest Trends Eng Technol 17 (4): 010-012. DOI: 10.21172/1.174.03.

Mubarak SM, Al-Koofee DAF, Radhi OA, Ismael JM, Al-Zubaidi ZF. 2020. An optimization and common troubleshooting solving in Polymerase Chain Reaction technique. Sys Rev Pharm 11 (2): 427 436. DOI: 10.5530/srp.2020.2.63.

Pandhee S, Phavaphutanon J, Sirinarumitr K, Laopiem S, Sirinarumitr T. 2016. Evaluation of Amelogenin and Zinc-finger Loci for sex identification in captive felids. Thai J Vet Med l46 (1): 41-47.

Pilgrim KL, Mckelvey KS, Riddle AE, Schwartz MK. 2005. Felid sex identification based on noninvasive genetic samples. Mol Ecol Notes 5 (1): 60-61. DOI: 10.1111/j.1471-8286.2004.00831.x.

Schweitzer MH, Avci R, Collier T, Goodwin MB. 2008. Microscopic, chemical and molecular methods for examining fossil preservation Méthodes d'études microscopiques, chimiques et moléculaires pour l'examen de la conservation des fossiles. Comptes Rendus Palevol 7: 159-184. DOI: 10.1016/j.crpv.2008.02.005.

Seidensticker J, Christie S, Jackson P. 1999. Introducing the tiger. In: Seidensticker J, Christie S, Jackson P (eds). Riding The Tiger: Tiger Conservation in Human Dominated Landscape. Univ Press Cambridge, UK.

Victoriano CM, Megan EP, Nicole AM, Seegmiller A, Simmons S, Schmitz JE, Haselton FR, Adams NM. 2022. Direct PCR with the CDC 2019 SARS-CoV-2 assay: Optimization for limited-resource settings. Sci Rep 12: 11756. DOI: 10.1038/s41598-022-15356-7.

Wang C. 2016. Primer Design. Centre for Medical Parasitology University of Copenhagen. Health Science, Denmark.

Wu JS, Lee C, Wu CC, Shiue YL. 2004. Primer design using genetic algorithm. Bioinformatics 20 (11): 1710-1. DOI: 10.1093/bioinformatics/bth147.

Xue HR, Yamaguchi N, Driscoll CA, Han Y, Bar-Gal GK, Zhuang Y, Mazak JH, Macdonald DW, O'Brien SJ, Luo S-J. 2015. Genetic ancestry of the extinct Javan and Bali Tigers. J Hered 2015: 247-257. DOI: 10.1093/jhered/esv002.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: A tool to design target-specific primers for Polymerase Chain Reaction. BMC Bioinform 13: 134-144. DOI: 10.1186/1471-2105-13-134.