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SPECIFIC DIFFERENCES IN AMELX AND AMELY GENES FROM SUMATRAN TIGERS, *PANTHERA TIGRIS SUMATRAE* (FELIDAE), FOR MOLECULAR SEX IDENTIFICATION

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Specific Differences in AMELX and AMELY Genes from Sumatran Tigers, *Panthera tigris sumatrae* (Felidae), for Molecular Sex Identification. Asrori, I., Tjong, D. H., Novarino, W. & Roesma, D. I. — Sex determination by DNA-based molecular techniques in Sumatran tigers needs to be investigated and developed for forensic and population genetic purposes. The amelogenin gene is a marker commonly used for sex determination. In some species, the difference between the AMELX and AMELY sequences has been reported to be in the intron region. However, the difference between the AMELX and AMELY sequences in the Sumatran tiger is unknown. Therefore, it is necessary to investigate the sequence differences in introns between AMELX and AMELY Sumatran tigers to determine the specific differences between male and female samples. This study aimed to analyse the sequence of nucleotide bases in the Sumatran tiger amelogenin gene introns based on the nucleotide base sequences in the amelogenin gene introns. The method in this research is descriptive, with a molecular observation of the AMELX and AMELY Sumatran tiger sequences. The amplified samples were sequenced, and it was found that the lengths of the AMELX and AMELY Sumatran tiger sequences were 215 bp and 194 bp, respectively, with a 21 bp deletion in the AMELY sequence.
Key words: tiger, DNA sexing, intron, sequence.

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Introduction

The Sumatran tiger (*Panthera tigris sumatrae* Pocock, 1929) requires appropriate conservation efforts as a protected wildlife species. Habitat studies and population estimates are indispensable for decision-making on the protection of these animals. In addition, genetic conservation efforts are no less important for the Sumatran tiger. Information on population structure, genetic diversity, and the proportion of male and female individuals in the population can be obtained through a genetic approach (Yamazaki et al., 2011). Sex determination of wildlife populations, particularly Sumatran tigers, is an effective technique for evaluating population structure, managing conservation, and maintaining population dynamics (Eggert et al., 2003; Shaw et al., 2003). A genetic approach using non-invasive samples is very helpful in studying this umbrella species (Smith, 2012). These non-invasive samples can be in the form of hair, dung, and bones obtained without the need to meet directly with wild animals, especially Sumatran tigers (Statham, 2007).

Sex identification using DNA-based molecular techniques in wildlife needs to be studied and developed for forensics (Gupta et al., 2006) and population genetics (Smith, 2012). Sex identification using DNA extracted from flesh, blood, hair, bones, nails, and even feces is an important analysis in forensic science. In some mammals such as primates (Bradley et al., 2001; Ensminger and Hoffman, 2002), cattle (Ennis and Gallagher, 1994; Chen et al., 1999; Weikard et al., 2006), and other species of the family Bovidae including sheep, goats, red deer, sika deer, bison, gaur, European bison, buffalo and anoa (Yamauchi et al., 2000; Pfeiffer and Brenig, 2005; Weikard et al., 2006), as well as in horses (Fukushima et al., 1999), Japanese black bear (Yamamoto et al., 2002), domestic cat, bobcat, puma (Pilgrim et al., 2005), and Sumatran tiger (Asrori et al., 2022), sex determination is based on amelogenin gene (AMEL) located on the sex chromosomes (AMELX and AMELY).

A simple and accurate method of sex determination with DNA data can be widely applied in humans and animals. Based on the study of the differences between AMELX and AMELY that have been reported, it is known that the intron sequences of the amelogenin Y gene in each animal species may have differences. Therefore, analysis of the amelogenin gene sequence can be an alternative to increase the accuracy of sex identification (Santos et al., 1998).

The sex identification of Sumatran tiger samples (Asrori et al., 2022) showed that the X chromosome and the Y chromosome of the Sumatran tiger carried a copy of the amelogenin gene. However, the AMELX and AMELY gene amplification bands in male samples were difficult to distinguish. Difficulties are because the difference in molecular weight of AMELX and AMELY is very small, affecting the resulting band's size (Pilgrim et al., 2005; Pfeiffer and Brenig, 2005). The difficulty of distinguishing these bands results in errors when detecting male and female individual samples (Asrori et al., 2022). Therefore, this study was conducted to analyze the differences in amelogenin gene sequences on the X and Y chromosomes of the Sumatran tiger used for sex identification to get faster and more accurate results.

Material and Methods

Sample

The sample in this study consisted of ten blood samples from individuals of the Sumatran tiger (*Panthera tigris sumaterae*) whose sex was known (four males and six females). 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. For that reason, there is no doubt about the application for sex identification of confiscated samples in the future. Blood samples were collected from Sumatran tiger individuals who were rehabilitated at the Dharmasraya Sumatran Tiger Rehabilitation Center (PRHSD).

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 DNA isolation results were then electrophoresed using 1.2 % agarose gel. The PCR process targets the amelogenin gene sequence based on the forward 5' CGAGGTAATTTTTCTGTTTACT 3' primer and the reverse 5' GAAACTGAGTCAGAGAGGC 3' primer (Pilgrim et al., 2005). The temperature PCR process refers to Pilgrim et al. (2005) and is modified by Asrori et al. (2022). The PCR reaction was carried out with a total mixture 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 1µl DNA isolation. First, predenaturation was carried out at 95 ˚C for 3 minutes, and 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

Gel extraction was carried out to separate AMELX and AMELY DNA from male samples of Sumatran tigers. Gel extraction was carried out according to the QIAquick Gel Extraction Kit protocol. The extraction results were visualized on 3 % agarose gel. After the AMELX and AMELY DNA were separated, the AMELX and AMELY nucleotide base sequencing was carried out.

Fig. 1. Visualization of amelogenin gene amplification results in Sumatran tigers using 3 % agarose gel. L = Ladder 50 bp (base pair). NCBI accession number HS1 = LC770322, HS2 = LC770323, HS3 = LC770324, HS4 = LC770325, HS5 = LC770326, HS6 = LC770327, HS7 = LC770328, HS8 = LC770329, HS9 = LC770330.

Sequence Analysis

Sequencing data analysis was carried out using several molecular software. First, SeqMan (Swindell and Plasterer, 1997) was used to contiguous forward and reverse sequences of AMELX and AMELY samples of Sumatran tiger samples. Then, sequence alignment was performed using Bioedit software (Hall, 1999).

Results

Amelogenin Gene Amplification

The results of amplifying the amelogenin gene in Sumatran tiger are shown in fig. 1. The DNA band for male samples is about 190 bp and 210 bp, while the female sample has one DNA band of about 210 bp, which is known using a 50 bp DNA ladder comparison.

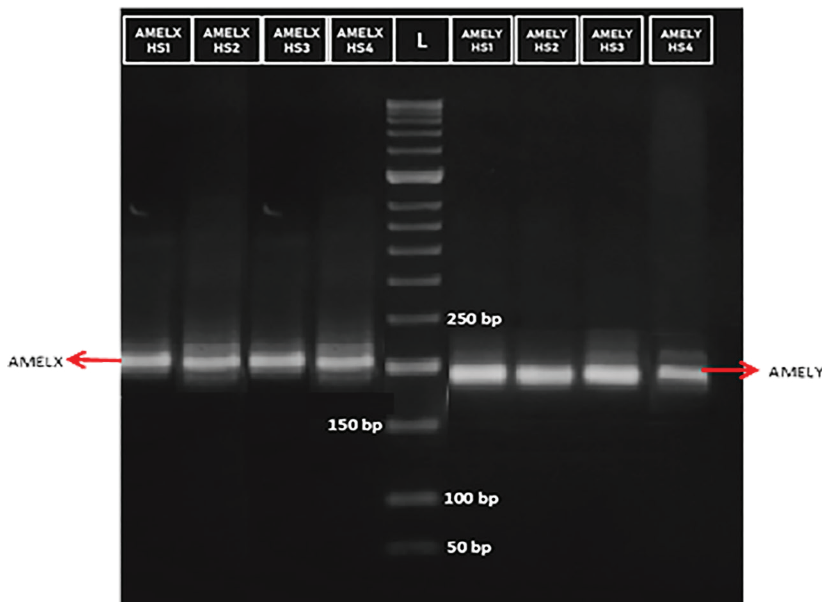

Fig. 2. Visualization of AMELX and AMELY gel extraction results on Sumatran tiger samples using 3 % agarose gel. L = Ladder 50 bp (base pair).

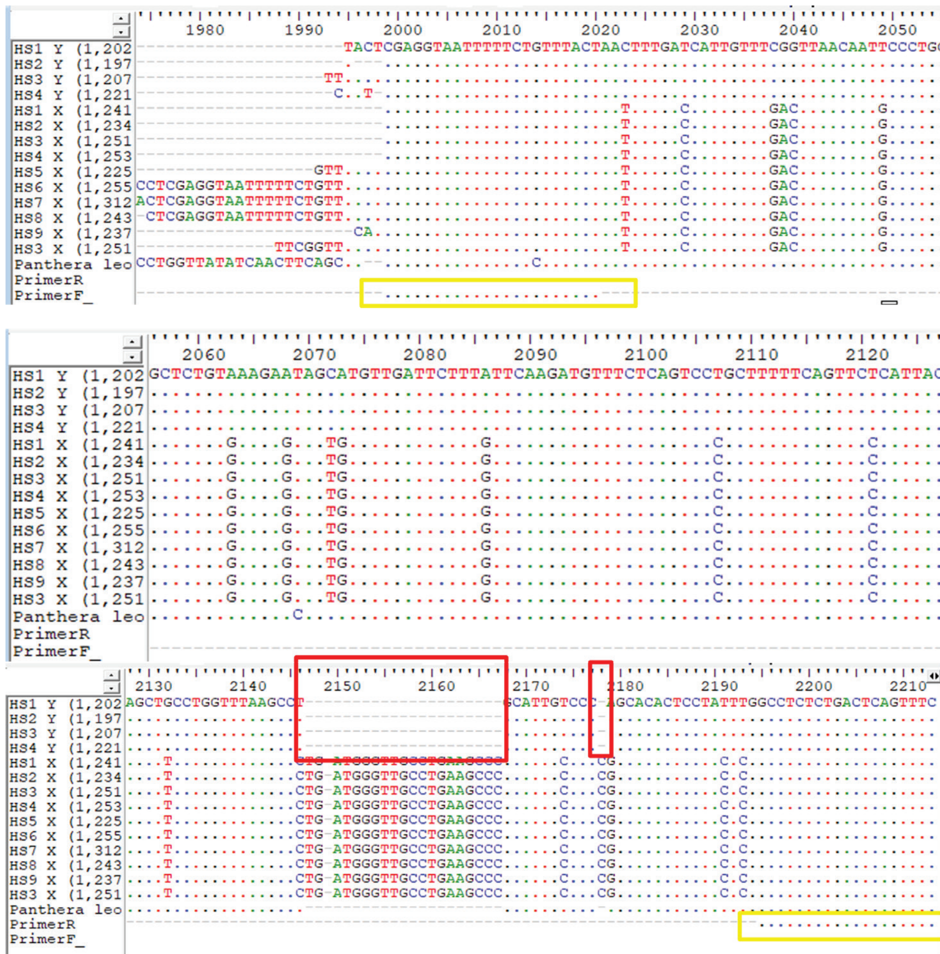

Fig. 3. Results of alignment of Sumatran tiger amelogenin gene sequences with reference sequences of AMELY lion and Pilgrim et al. (2005) using the BioEdit program. Description: red box = Primary attachment, yellow box = Deletion in AMELY

The results of AMELX and AMELY sequencing from our samples are reported to NCBI and can be accessed with accession numbers LC770322, LC770323, LC770324, LC770325, LC770326, LC770327, LC770329, LC770330.

Gel Extraction

Gel extraction was carried out on AMELX and AMELY amplified samples of male Sumatran tigers. The results of gel extraction on Sumatran tiger samples are shown in fig. 2. Extraction of gel to separate DNA from agarose gel based on the QIAquick Gel Extraction Kit protocol was successfully carried out. The results of the gel extraction were then sequenced.

Sequence Analysis

Nine AMELX sequences and four Sumatran tiger AMELY sequences that have been edited are then aligned to determine the length of the target sequence, determine the deletion that occurs in the AMELY sequence and estimate the position of the deletion in the Sumatran tiger AMELY. The position of the Sumatran tiger AMELY deletion was determined by referring to the reference sequences of other species. The choice of the sequences is because there is no report on the complete sequence of the amelogenin gene or the Sumatran tiger Y chromosome in GenBank. So, the whole lion Y chromosome sequence with acces-

sion number NC_056697.1 was chosen for the reference sequence, which has the closest kinship with the Sumatran tiger. Based on the alignment results, it is known that the length of the AMELX and AMELY sequences of the Sumatran tiger amplified using Pilgrim et al. (2005) were 215 bp and 194 bp with a 21 bp deletion in the AMELY sequence (fig. 3).

Table 1. Sumatran tiger blood samples

No	Sample Code	Sex
1	HS01	Male
2	HS02	Male
3	HS03	Male
4	HS04	Male
5	HS05	Female
6	HS06	Female
7	HS07	Female
8	HS08	Female
9	HS09	Female

Discussion

The analysis of the sequencing results was carried out in several stages. Beginning with unification of the sequence (contig) using the SeqMan application (Swindell and Plasterer, 1997). The results of the sequence analysis are in accordance with the report of Pilgrim et al. (2005), that identification of male domestic cat samples was marked by visualization of two DNA bands at 194 bp and 214 bp (AMELX and AMELY), and one female DNA band (AMELX) at 214 bp. So it is known that the domestic cat AMELX and AMELY sequences have a difference of 20 nucleotide bases.

Based on the alignment results, it is known that the lengths of the AMELX and AMELY Sumatran tiger sequences were amplified using Pilgrim et al. (2005) were 215 bp and 194 bp with a 21 bp deletion in the AMELY sequence. These results indicate that there are differences between the amelogenin gene sequences of Sumatran tigers and domestic cats. Pilgrim et al. (2005) reported that PCR products using the same primer in domestic cats were 214 bp for AMELX and 194 bp for AMELY, with a 20 nucleotide base deletion in AMELY. This result is supported by the report of Pandhee et al. (2016) that the length of the PCR product in the fishing cat (*Prionailurus viverrinus*), Asian golden cat (*Pardofelis temminckii*), and jungle cat (*Felis chaus*) produced PCR product sizes with different lengths even though they used the same primer.

AMELY deletion studies in several mammalian species have previously been reported. For example, Pfeiffer and Brenig (2005) reported that the AMELY sequences of red deer (*Cervus elaphus*) and goat had 55 bp deletions compared to AMELX. Fontanesi et al. (2008) also reported that the AMELY sequence in three intron regions of the porcine amelogenin (*Sus scrofa*) gene had a deletion of 9–10 bp compared to the AMELX sequence. In addition, Pilgrim et al. (2005) also reported that there was a 20 bp deletion in the AMELY intron region when compared to the X chromosome copy (AMELX) in a domestic cat (*Felis catus*). Analysis of the differences in the AMELX and AMELY sequences showed that the differences in the AMELX and AMELY sequences for several species occurred due to deletions in the AMELY intron region. This difference is a marker of sex identification in animals, especially mammals. However, there are no reports on the amelogenin gene sequences of other tiger subspecies. Therefore, this method cannot be used to differentiate between tiger subspecies. So that further research is needed to obtain sequence data from other tiger subspecies.

Conclusion

The sequence of nucleotide bases in the introns of the Sumatran tiger amelogenin gene has 21 base differences in AMELX and AMELY. The difference between AMELX and AMELY is due to a deletion in AMELY.

References

Asrori, I., Tjong, D. H., Roesma, D. I., Novarino, W. & Mansyurdin, S. 2022. DNA Sexing Sumatran tiger (*Panthera tigris sumatrae*) based on amelogenin gene. *World Journal of Andvanced Research and Review*, **14** (03), 190–194. https://wjarr.com/sites/default/files/WJARR-2022-0482.pdf

Bradley, B. J., Karen, E. C. & Linda V. 2001. Accurate DNA-based sex identification of apes using non-invasive samples. *Conservation Genetics*, 2, 179–181. https://doi.org/10.1023/A:1011847528045

Chen, C. M., Hu, C. L., Wang, C. H., Hung, C. M., Wu, H. K., Choo, K. B. & Cheng, W. T. 1999. Gender determination in single bovine blastomeres by polymerase chain reaction amplification of sex-specific polymorphic fragments in the amelogenin gene. *Molecular Reproduction and Development*, 54, 209–214. https://doi.org/10.1002/(SICI)1098-2795(199911)54:3<209::AID-MRD1>3.0.CO;2-6

Eggert, L. S, Eggert, J. A. & Woodruff, D. S. 2003. Estimating population sizes for elusive animals: the forest elephants of Kakum National Park, Ghana. *Molecular Ecology*, 12, 1389–1402. https://doi.org/10.1046/j.1365-294X.2003.01822.x

Ennis, S. & Gallagher, T. F. 1994. A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. *Animal Genetics*, 25, 425–427. https://doi.org/10.1111/j.1365-2052.1994.tb00533.x

Ensminger, A. L. & Hoffman, S. M. 2002. Sex identification assay useful in great apes is not diagnostic in a range of other primate species. *American Journal of Primatology*, 56, 129–134.

Fontanesi, L., Scotti, E. & Russo, V. 2008. Differences of the Porcine Amelogenin X and Y Chromosome Genes (AMELX and AMELY) and Their Application for Sex Determination in Pigs. *Molecular Reproduction and Development*, 75, 1662–1668. https://doi.org/10.1002/mrd.20903

Fukushima, Y., Mukoyama, H., Sato, F., Hasegawa, T., Ishida, N. & Muramatsu. S. 1999. Sex determination of equine somatic and germ cells by PCR amplification based on the sequence polymorphism of X and Y-chromosomal amelogenin genes. *Animal Science Journal (Japan)*, 70, J6–J10. https://doi.org/10.1292/jvms.62.1109

Gupta, S. K., Thangaraj, K. & Singh, L. 2006. A simple and inexpensive molecular method for sexing and identification of the forensic samples of elephant origin. *Journal of Forensic Sciences*, 51, 805–807. https://doi.org/10.1111/j.1556-4029.2006.00154.x

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 Journal of Veterinary Medicine*, **46** (1), 41–47. https://he01.tci-thaijo.org/index.php/tjvm/article/view/49782

Pfeiffer, I. & Brenig, B. 2005. X- and Y-chromosome specific variants of the amelogenin gene allow sex determination in sheep (*Ovis aries*) and European red deer (*Cervus elaphus*). *BMC Genetics*, 6, 16. https://doi.org/10.1186/1471-2156-6-16

Pilgrim, K. L, Mckelvey, K. S., Riddle, A. E. & Schwartz, M. K. 2005. Felid sex identification based on non-invasive genetic samples. *Molecular Ecology Notes*, **5** (1), 60–61. https://doi.org/10.1111/j.1471-8286.2004.00831.x

Santos, F. R. 1998. Reliability of DNA-based sex tests. *Nature Genetics*, 18, 103. https://doi.org/10.1038/ng0298-103

Shaw, C. N., Wilson, P. J. & White, B. N. 2003. A reliable molecular method of gender determination for mammals. *Journal of Mammalogy*, 84, 123-128. https://doi.org/10.1644/1545-1542 (2003)084<0123:ARM-MOG>2.0.CO;2

Smith, O. 2012. *Population Genetics and Structure of the Sumatran tiger*. PhD Thesis. Imperial College London.

Statham, M. J., Peter, D. Turner, & Catherine, O. R. 2007. Molecular Sex Identification of Five Mustelid Species. *Zoological Studies*, **46** (5), 600–608. http://zoolstud.sinica.edu.tw/Journals/46.5/600.pdf

Weikard, R., Pitra, C. & Kühn, C. 2006. Amelogenin cross-amplification in the family Bovidae and its application for sex determination. *Molecular Reproduction and Development*, 73, 1333–1337. https://doi.org/10.1002/mrd.20554

Yamamoto, K., Tsubota, T., Komatsu, T., Katayama, A., Murase, T., Kita, I., & Kudo, T. 2002. Sex identification of Japanese black bear, Ursus thibetanus japonicus, by PCR based on amelogenin gene. *The Journal of Veterinary Medical Science*, 64, 505–508. https://doi.org/10.1292/jvms.64.505

Yamauchi, K., Hamasaki, S., Miyazaki, K., Kikusui, T., Takeuchi, Y. & Mori, Y. 2000. Sex determination based on fecal DNA analysis of the amelogenin gene in sika deer (*Cervus nippon*). *The Journal of Veterinary Medical Science*, 62, 669–671. https://doi.org/10.1292/jvms.62.669

Yamazaki, S., Motoi, Y., Nagai, K., Ishinazaki, T., Asani, M. & Suzuki, M. 2011. Sex determination of Sika deer (*Cervus nippon yesoensis*) using nested PCR from feces collected in the field. *The Journal of Veterinary Medical Science*, **73** (12), 1611–1616. https://doi.org/10.1292/jvms.11-0235

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