HAYATI
Journal of Biosciences

Vol. 30 No. 5, September 2023 816-824
DOI:10.4308/hjb.30.5.816-824
ISSN: 1978-3019
EISSN: 2086-4094

Development of DNA Extraction Method for Forensics Studies of Preserved Hair and Skin Samples from Sumatran Tiger (*Panthera tigris sumatrae*, Pocock 1929)

Noviar Andayani[1*], Andi Eko Maryanto[1,2], Muhammad Naufal Nur[1]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia*
[2]*Wildlife Biology and Sustainable Landscape Research Group, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia*

ARTICLE INFO

Article history:
Received March 12, 2023
Received in revised form April 20, 2023
Accepted May 2, 2023

KEYWORDS:
DNA extraction,
species identification,
Sumatran tiger,
wildlife forensics,
wildlife trades

ABSTRACT

Poaching and illegal wildlife trade present severe threats to the Sumatran tiger. The high demand for tiger body parts leads to a high number of imitations in illegal markets, complicating the morphological identification of any confiscation cases. Accurate identification is essential in legal due process, given that the national protection law only regulates Indonesia's native species. Identification using molecular approaches may overcome the problem. However, most illegally traded tiger body parts have been preserved for an extended period of time, reducing the quantity and quality of the recovered DNA. This study aimed to develop a fast and effective method to recover DNA from preserved forensic samples. The methods had been tested with several museum samples of arsenic-treated hairs and a tiger skin piece obtained from the National Research and Innovation Agency (BRIN, formerly LIPI), tiger hairs obtained from Conservation of Natural Resources Agency (BKSDA) of Bengkulu Province, and a confiscated tiger skin sample from BKSDA Aceh. The DNA was extracted using ion-exchange, salting out, and protease-based methods. The results showed that the protease-based extraction outperformed the others to yield applicable DNA isolates for PCR-based species identification by *Cyt b* and *ND2* mtDNA partial genes from preserved samples. However, further works are still needed to recover sufficient DNA yields for sex identification.

1. Introduction

As the only remaining tiger population in Indonesia, the Sumatran tigers suffer from threats such as poaching and illegal wildlife trade (Wibisono and Pusparini 2010). The high demand for tiger body parts has been inducing the rise of imitations, leading to bias in evaluating poaching impact and hinders law enforcement on wildlife trafficking due to difficulties in the identification process (Nyhus and Tilson 2004). Molecular-based species identification is considered promising to overcome the morphological approach limitations in terms of species identification, including tiger (Karmacharya *et al.* 2018; Lopez-Oceja *et al.* 2016; Vipin *et al.* 2016). However, most illegally traded tiger body parts have been preserved for an extended period of time, reducing DNA quantity and quality for an effective identification process.

The identification process is an essential stage, given only the Sumatran tiger is protected by the national law among other tiger subspecies. Thus, recovering a sufficient amount of DNA from wildlife forensic samples is key and developing DNA recovery protocols for wildlife forensic casework remains needed. This study aimed to develop a simple, fast, and relatively cost-efficient method to enable species identification from preserved hair and skin samples of Sumatran tiger and evaluate the recovered DNA using tiger-specific mtDNA markers that have been developed (Luo *et al.* 2004; Wetton *et al.* 2004). This study also examined genome enrichment-method to obtain a higher yield and integrity of recovered DNA for further wildlife forensic applications.

* Corresponding Author
E-mail Address: andayani@ui.ac.id

2. Materials and Methods

2.1. Samples collection

Biological samples (museum specimen, hairs, and skins) from 17 tigers were collected to assess the extraction methods. The museum samples obtained from Museum Zoologicum Bogoriense, Center of Biological Research, Indonesian Institute of Sciences (LIPI). The museum specimens were arsenic-treated hairs and skins with various preservation ages ranging from year 1911 to 1956. Besides museum specimens, we used confiscated samples of hairs and skins, consist of unpreserved hairs (four months) obtained from a dead female tiger found in Selingsingan, Bengkulu Province, provided by the Conservation of Natural Resource Office (BKSDA) of Bengkulu Province, and hairs from a piece of confiscated tiger skin (two years) provided by BKSDA Aceh. The tiger skin was confiscated in South Aceh Regency in 2018, originated from a male-predicted individual. Sample access were under the permission (SK.84/KSDAE/SET/KSA.2/3/2020) issued by the Directorate of Conservation of Natural Resources, the Indonesian Ministry of Environment and Forestry. We also used positive control of tiger blood obtained from two tigers belong to Taman Safari Indonesia, Bogor, West Java.

2.2. DNA Extraction and Genomic DNA Enrichment

Prior to the extraction process, samples were decontaminated from debris and exogenous contaminating DNA by immersion in 0.5% of bleach. We used 10 hair shafts (cut into ±0.5 cm each) and a piece of skin (cut into 0.5 cm × 0.5 cm) for each reaction. We examine three DNA extraction methods, [1] the ion exchange-based method using Chelex® 100 resin [Bio-Rad], [2] protease-based method using KAPA Express Extract Kit [KAPA Biosystems], and [3] salting out-based method as described by Sambrook and Russel (2001). The ion-exchange-based extraction was performed by incubating the sample in 200 µL 10% Chelex resin (75°C 25 mins; 95°C 20 mins). The lysate was centrifuged to precipitate the resin and the supernatant was transferred to a new fresh tube. The protease-based extraction was performed following the manufacturer protocol (incubation at 75°C 10 mins; 95°C 5 mins) in a 100 µL lysis buffer comprising 88 µL nuclease-free water, 10 µL 10× KAPA Express Extract Buffer, and 2 µL KAPA Express Extract Enzyme (1 U/µL). Salting out-based method was performed by following the protocol described by Sambrook and Russel (2001) with DTT addition into the lysis buffer resulting in 1 mM final concentration, increased SDS concentration to 0.5%, and increased proteinase K volume to 20 µL. The lysis step was performed overnight, and the pelleted-DNA was resuspended in 50 µL TE buffer (low EDTA). The salting-out method also carried out for positive control DNA extraction. All the extracts were quantified using a nano volume spectrophotometer and stored at -20°C until further analysis.

Genomic DNA enrichment was performed with multiple displacement amplification (MDA) method using REPLI-g Mini Kit [QIAGEN]. Genome enrichment was carried out following the manufacturer's protocol. Enrichment success was confirmed by running the MDA products on 0.8% agarose gel.

2.3. Molecular Species and Sex Identification

We qualitatively evaluated each method's performance if the extracts are effective for species and sex identification by PCR. Two different tiger-specific primers were used, C9366F/T9882R (Luo *et al.* 2004) targeting 571 bp area of *ND2* gene and Tig117F/Tig231R (Wetton *et al.* 2004) targeting 156 bp area *Cyt b* gene. For *Cyt b*, we tailed each forward and reverse primer with M13 vector sequences of (5'-TGTAAAACGACGGCCAGT) and (5'-CAGGAAACAGCTATGAC) respectively, resulting a 35 bp longer amplicon (191 bp). The M13-tailed primers were chosen due to brighter PCR bands on agarose gel during optimization process compared to the original pairs. For sex identification, duplexed PCR reaction of ZFX-PF/ZFX-PR and DBY-PF/DBY-PR was performed (Sugimoto *et al.* 2006).

PCR reaction comprised of 12.5 µL 2× TopTaq™ PCR Master Mix Kit [QIAGEN], 0.5 µM each forward and reverse primer or 0.2 µM each for duplex reaction, additional 0.5 mM $MgCl_2$ [Thermo Scientific] for duplex reaction, 3 µL template DNA, and RNase-free water [QIAGEN] up to 25 µL final volume. PCR cycles followed (95°C 3 mins; 35× of 9 5°C 30s, Ta 30s, 72°C 30'; 72°C 5 mins) on SimpliAmp™ thermal cycler [Applied Biosystem], with Ta comprised of 46°C, 53°C, and 56°C for *Cyt b, ND2*, and duplexed ZFX/DBY respectively. PCR products were assessed on 1.5% (species identification) and 2% (sex identification) agarose gel stained with GreenSafe [NZYTech] and visualized under trans-UV illuminator.

2.4. Data Analysis

Successful PCR products proceeded for DNA sequencing by Macrogen, Inc. (South Korea and Singapore). The retrieved sequences were trimmed for ambiguous base-removal using DNA Baser Assembler version 5.15 [Visual Bioinformatics]. The trimmed sequences and primers were mapped to the Sumatran tiger mitochondrial reference sequence (JF357969.1) to verify primers and product specificity using Geneious Prime version 2021.0.1 [Biomatters, Ltd.]. The sequences were analyzed to find Sumatran tiger-specific nucleotide variation by performing multiple sequence alignment with other tiger subspecies sequences (Table 1) using ClustalW algorithm on BioEdit version 7.2.5. Felis catus mitochondrial sequence (U20753.1) was used for nucleotide numbering reference, following Luo *et al.* (2004). Phylogenetic analysis was performed to determine the species relationship to Sumatran tiger using Bayesian inference on BEAST2 version 2.6.3. Prior to running the phylogenetic analysis, tthe sequences were run on jModelTest to obtain the best substitution model and the MCMC was set to 10 million times with data retrieval every 10 thousand chains.

3. Results

3.1. Concentration and Purity of DNA Extracted from Hair and Skin Samples

DNA concentrations from all samples are listed in Table 2 and summarized into plots in Figure 1.

Table 1. GenBank species references were involved in phylogenetic analysis

Scientific name	Common name	GenBank accession number	
		Cyt b	*ND2*
Bos javanicus	Javan cattle	7901819	NC_012706.1
Capra hircus	Goat	1485868	MK341077.1
Cervus unicolor	Sambar deer	AF423201.1	NC_031835.1
Muntiacus muntjak	Deer	AF042717.1	NC_004563.1
Neofelis diardi	Sunda clouded leopard	HM748845.1	-
Neofelis nebulosa	Asian clouded leopard	-	KU133958.1
Panthera pardus	Leopard	6262383	NC_010641.1
Panthera tigris altaica	Siberian tiger	AF053026.1	HM185182.2
Panthera tigris amoyensis	South China tiger	NC_014770.1	NC_014770.1
Panthera tigris corbetti	Indochinese tiger	AF053049.1	JF357971.1
Panthera tigris sumatrae	Sumatran tiger	AF053040.1	JF357969.1
Panthera tigris tigris	Bengal tiger	AF053018.1	JF357968.1

Table 2. DNA concentration and purity yielded from preserved hair and skin samples of Sumatran tiger extracted using three different methods.

Source (individual)	Sample type (total specimen)	Method	Averaged concentration per method (ng/µL)	Averaged purity per method
BKSDA Aceh	Skin (1)	Chelex	115.48	1.44
		KAPA	244.83	1.23
(n = 1)		*Salting out*	26.25	1.97
	Hair (1)	Chelex	25.58	1.01
		KAPA	23.48	0.92
		Salting out	24.73	1.94
BKSDA Bengkulu	Hair (1)	Chelex	22.25	1.93
		KAPA	15.37	0.75
(n = 1)		*Salting out*	1.25	1.58
Museum Zoologicum Bogoriense LIPI	Hair (14)	Chelex	4.07	3.16
		KAPA	12.61	0.53
		Salting out	7.71	2.76
(n = 1)	Skin (1)	Chelex	66.60	1.80
		KAPA	48.75	1.39
		Salting out	41.75	0.93

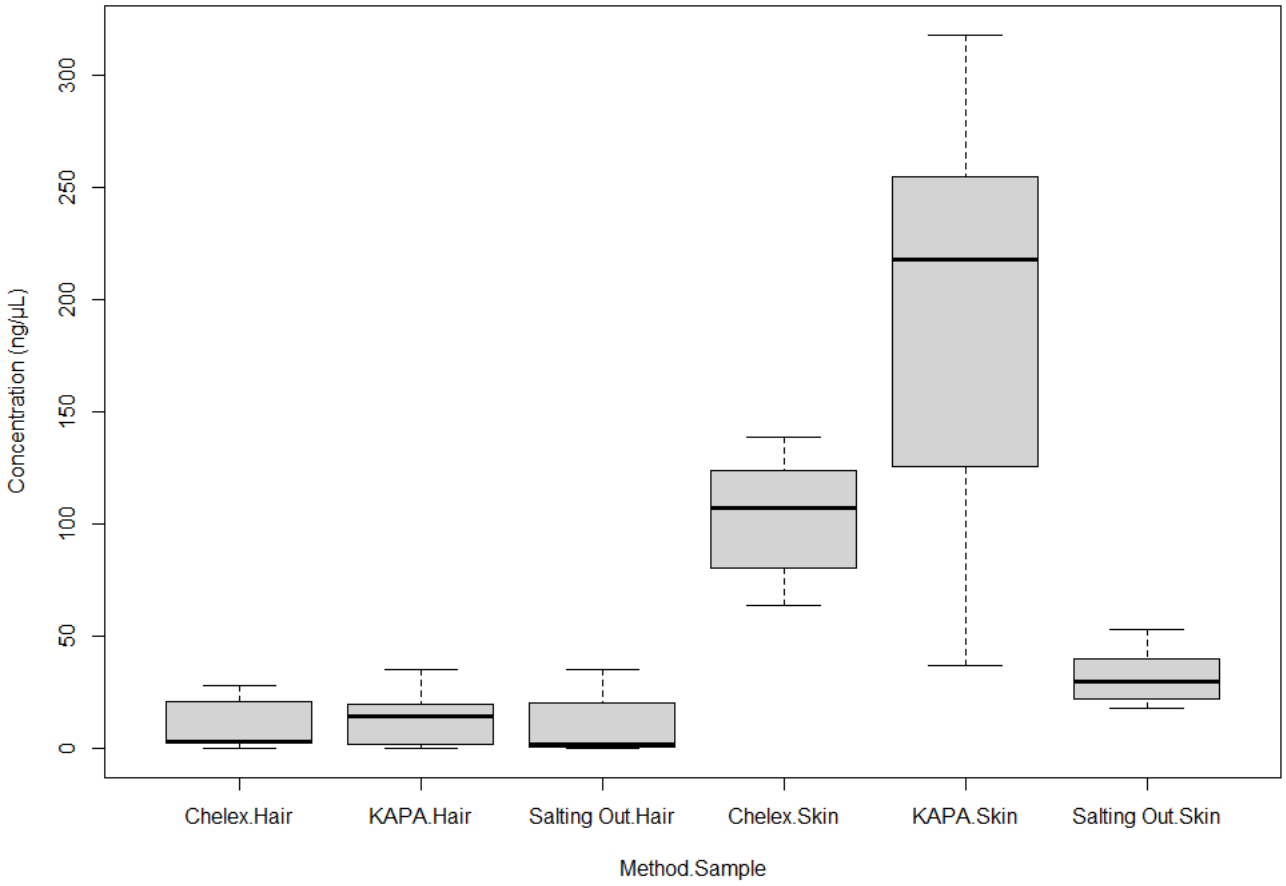


Figure 1. Distribution of DNA concentration from preserved hair and skin samples of Sumatran tiger extracted using three different methods

Kruskal-Wallis test showed that the difference among hair samples' DNA concentration was not significant ($p = 0.146$; $\alpha = 0.05$), but significant for skin samples ($p = 3.69e\text{-}05$; $\alpha = 0.05$). KAPA Express Extract Kit yielded the most optimal DNA concentration from both hair and skin samples, indicated by highest second-quartile values for hair and skin samples and widest interquartile range for skin samples, which correspond to higher DNA concentration distribution (Figure 1).

3.2. Molecular Species and Sex Identification of Sumatran Tiger from Hair and Skin Samples

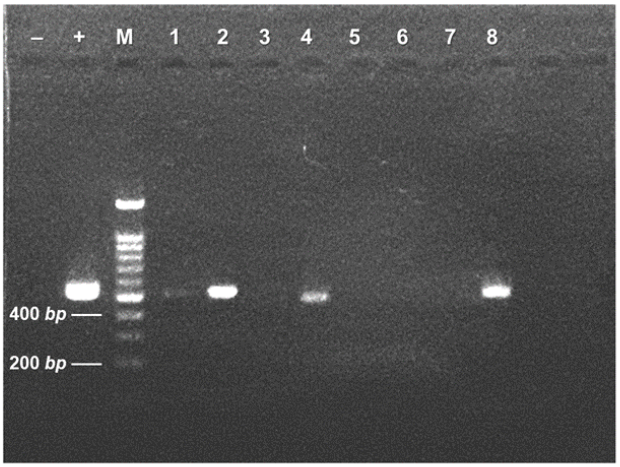
Species identification was successfully performed for the newly aged samples from Aceh and Bengkulu, but unsuccessful for samples obtained from LIPI and all sex identification process. Successful PCR products presented different band intensity on agarose gel due to sample type and amplicon size (Figure 2 and 3). PCR products from hair samples showed a lower success rate at longer amplicon (517 bp; *ND2*) compared to the shorter amplicon (191 bp; *Cyt b*), while skin samples succeeded in giving PCR bands with relatively similar consistency across the sizes.

3.3. Sequence and Phylogenetic Analysis

Mapping results of the sequenced PCR products to the Sumatran tiger mitochondrial DNA (mtDNA) reference sequence (JF357969.1) showed correspondence to the region flanked by the primers' annealing sites. PCR products of *ND2* primers mapped from the mid to the 3' end of the gene (base 5.454 to 5.903), while PCR products of *Cyt b* primers mapped to the region near the 5' end of the gene (base 15.199 to 15.322). These results showed primer and PCR product specificity and verified that the samples originated from tigers.

Multiple sequence alignment showed a single nucleotide variation specific to Sumatran tiger within the sequenced region of each gene. The variations consist of a transversion (T→A) at base 15.223 within

A



B

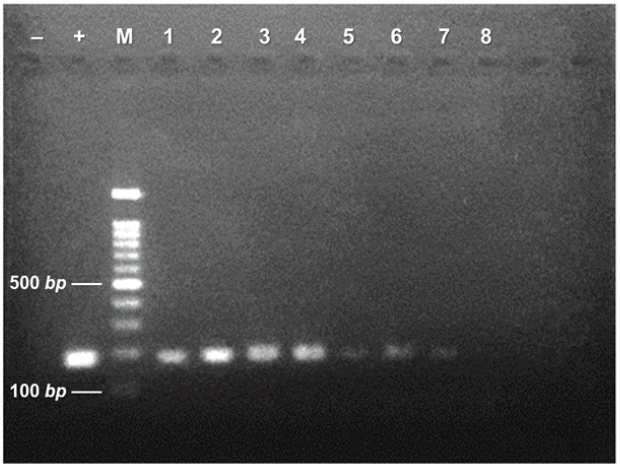


Figure 2. PCR products of partial (A) *Cyt b*, 191 bp and (B) *ND2*, 517 bp target genes from preserved Sumatran tiger hair samples (Aceh and Bengkulu)

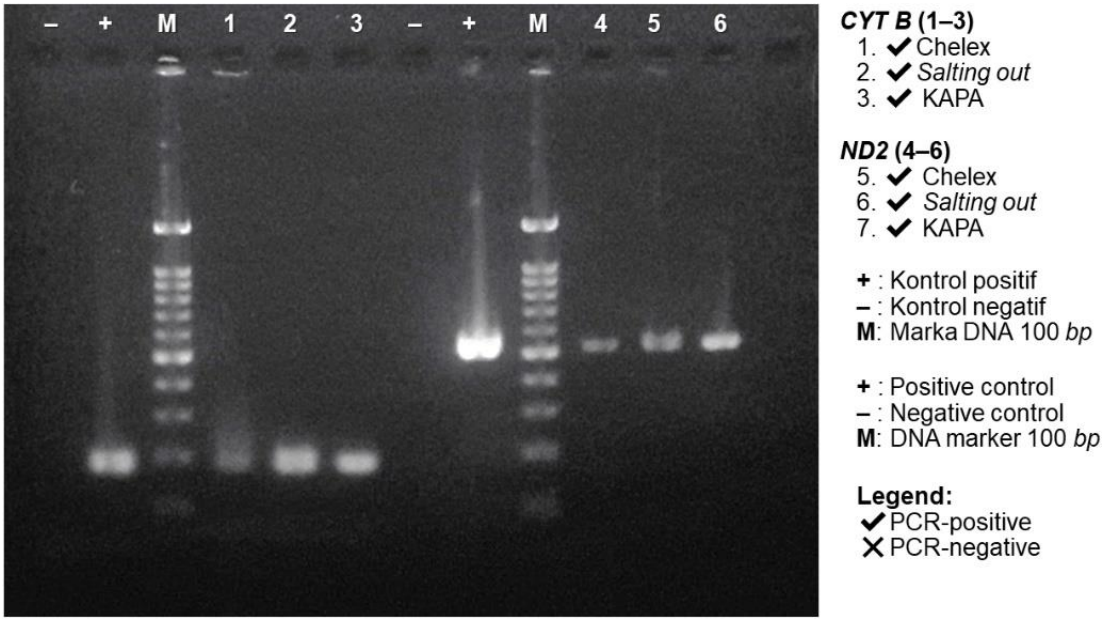


Figure 3. PCR products of *Cyt b* (191 bp) and *ND2* (517 bp) target genes from the preserved Sumatran tiger skin samples (Aceh)

Cyt b (Figure 5A) and a transition (C→T) at base 5.608 within *ND2* (Figure 4B). This finding strengthens the previous statement that the samples originated form tiger and provides potential utilization of both primers in wildlife forensic cases involving Sumatran tiger.

Our identification of Sumatran tiger subspecies is also supported by Bayesian phylogenetic analysis using GTR+I+Γ substitution model. Our PCR products were monophyletic to tiger clade (*Panthera tigris*) and bifurcated into a separated branch comprising the Sumatran tiger reference sequence (*P.t. sumatrae*) and the PCR products of both target genes. A high posterior probability number (1 for *Cyt b* and 0.97 for *ND2*) supported Sumatran tiger's monophyly to the tiger clade (Figure 5).

4. Discussion

The higher concentration distribution yielded by KAPA Express Extract Kit might be resulted by its simple extraction step and protease activity. Protease enzyme catalyzes protein degradation and cell membrane disruption, promoting higher DNA release and yield (Barbosa *et al.* 2016; van Pelt-Verkuil *et al.* 2008). Compared to the protease-based extraction, the ion-exchange method only relies on high incubation temperature. In contrast, as a method with multiple steps, the salting-out may not be the most suitable to recover DNA from relatively small samples, samples with low copy number or degraded DNA due to potential DNA loss during the extraction process (Fahzan *et al.* 2016; Fazzini *et al.* 2018). Moss *et al.* (2003) also

A

```
                                  15210       15220
                                  ....|....|....|....|....|.
Felis catus                       ACACATCAGACACAATAACCGCCTTT
P.t. altaica                      ......................T..C
P.t amoyensis                     ......................T..C
P.t. corbetti                     ......................T..C
P.t. tigris                       ......................T..C
P.t. sumatrae                     ......................A..C
Sampel Aceh_TIG_ASK               ......................A..C
Sampel Bengkulu_TIG BHK           ......................A..C
```

B

```
                                  5610        5620        563
                                  ....|....|....|....|....|....|
Felis catus                       AATTAATCATAATGCCAACATTACTAGCCA
P.t. altaica                      ..A.........A..C...C.........
P.t. amoyensis                    ..A.........A..C...C.........
P.t. corbetti                     ..A.........A..C...C.........
P.t. tigris                       ..A.........A..C...C.........
P.t. sumatrae                     ..A....T....A..C...C.........
Sampel Aceh ND2_ASK               ..A....T....A..C...C.........
```

Figure 4. Multiple sequence alignment of PCR products from (A) *Cyt b* gene and (B) *ND2* with other tiger subspecies. The sequences were aligned to Felis catus mtDNA sequence (U20753.1) as numbering reference

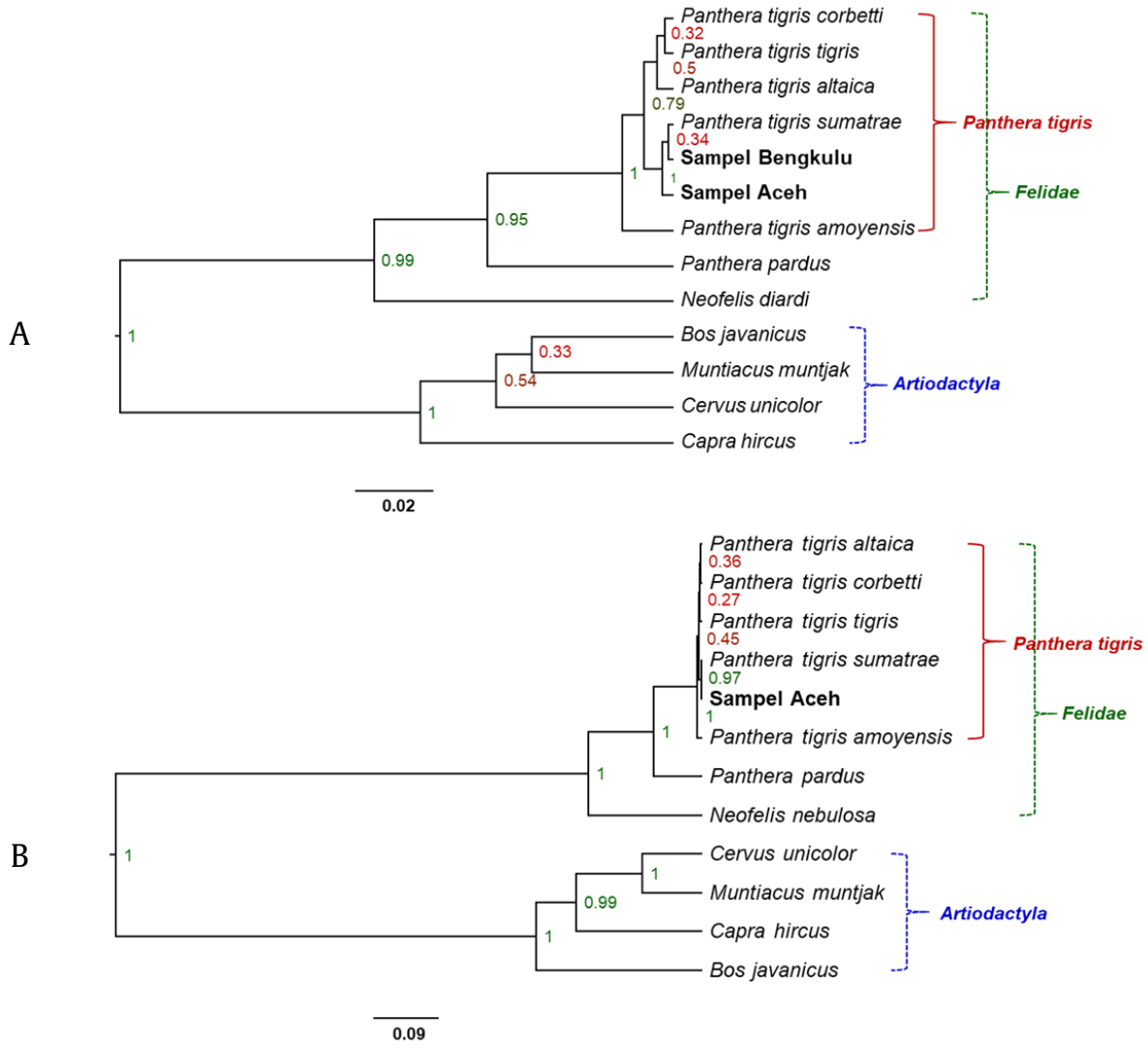


Figure 5. The phylogenetic tree of (A) partial *Cyt b* gene from preserved hair and skin samples and (B) partial *ND2* gene from preserved skin samples of Sumatran tiger. The trees were constructed using Bayesian inference with GTR+I+Γ substitution model

presented that protease-based extraction produced better DNA profiling in forensic samples compared to the ion-exchange and phenol-chloroform-based method. This intrigues that the thermostable protease-based extraction has a valuable potency as a simple, fast, and relatively cost-efficient DNA recovery method in wildlife forensic cases.

None of the samples retain intact genomic DNA since it failed to be visualized through electrophoresis, which reflected high degradation rate (Sharma *et al.* 2020). Hair is not generally considered as a reliable sample type for DNA recovery. Furthermore, our hair samples from Bengkulu were no longer retained its root, which is the primary source of DNA in hair (Fulton 2012; Guan *et al.* 2016; Heywood *et al.* 2003; Jun *et al.* 2011).

In contrast, the enriched genomic DNA by multiple displacement amplification (MDA) was successfully visualized by electrophoresis. It indicates that the isolates still contained the required DNA to perform MDA reaction, as REPLI-g Mini Kit recommends using DNA isolates with minimum of 2 kb and several >10 kb double-stranded DNA. Nevertheless, the copy number might be very low since it failed to be visualized by electrophoresis. In addition, the MDA products need to be confirmed by species-specific PCR to verify that the target DNA was co-amplified during the reaction, given the hexamers in MDA reaction annealed randomly instead of the desired region (Schneider *et al.* 2004).

We assume that the amplification results in the Figure 2-4 corresponded to the DNA amount and

reflect the preservation time. Although the hair samples from Bengkulu were preserved in a more recent time, our results demonstrated that hair samples' success rate from Aceh was higher than Bengkulu counterpart. The success rate might be affected by the hair follicle's presence in hair samples from Aceh, increasing DNA yield. Our results also agreed to the common understanding that a shorter amplicon is more considerable to encounter DNA with poor quality (Lopez-Oceja *et al.* 2016; Wandeler *et al.* 2007) and consensus that follicle is the primary source of hair parts for DNA extraction. However, we failed to obtain sex profiles from all samples, including the newly aged samples. We assumed that our methods still need to be optimized further to recover low abundance of sex chromosomes from this kind of samples.

PCR using MDA products showed positive results compared to PCR using pre-enriched DNA, particularly for hair samples from Bengkulu. However, our PCR reaction using MDA products required 7°C increment of annealing temperature due to the lower dilution number of MDA products in TE buffer. We diluted our MDA products in 1:5 ratio since the recommended 1:20 did not yield positive results. Lower dilution number will increase MDA reaction residue (e.g., as hexamers, salts, and polymerase) affecting standard PCR conditions by stabilizing double-stranded DNA bond and contributes to the higher temperature needed to denature the hydrogen bonds (van Pelt-Verkuil 2008). Nevertheless, our results showed the combination of protease-based extraction and multiple displacement amplification has the potency for further application in encountering DNA samples with low copy number.

Using multiple sequence alignment, we also identified Sumatran tiger single subspecies-specific nucleotide variation in the PCR products from both target genes. Single variation in *ND2* gene was C→T transition at base 5.608 of Sumatran tiger mtDNA, corresponded to the nucleotides variations among tiger subspecies described by Luo *et al.* (2004). Single variation in *Cyt b* gene was T→A transversion at nucleotide number 15.223 of the Sumatran tiger mtDNA and corresponded to Liu *et al.* (2018). However, the variation was not described by Wetton *et al.* (2004). Both variations demonstrated its respective potency to be reference in identifying Sumatran tiger subspecies by molecular-based identification. In addition, the M13-tailed Tig117F/Tig231R primers since it produces a shorter amplicon, which is more desired when encountering poor DNA samples.

Based on this study, KAPA Express Extract Kit outperformed the others due to time efficiency and higher DNA yields. The combination of multiple displacement amplification as post-extraction genome enrichment can be further applied to obtain higher genomic DNA yield when encountering low quality and quantity of DNA samples. The use of Tig117F/Tig231R primer pairs developed by Wetton *et al.* (2004) may be more desired since it yielded a higher success rate of PCR reaction in low DNA samples. However, further studies are needed to obtain sex profiles from newly preserved samples and species profiles from older specimens such as samples from LIPI.

Acknowledgements

We thank to Faculty of Mathematics and Natural Sciences (FMIPA) Universitas Indonesia for financial support under the research grant Hibah Riset FMIPA UI 2021 No. NKB-023/UN2.F3/HKP.0500/2021. We also thank to Museum Zoologicum Bogoriense, Center of Biological Research, Indonesian Institute of Sciences (LIPI), Conservation of Natural Resource Office (BKSDA) of Bengkulu Province, BKSDA of Aceh Province, and Taman Safari Zoo to access the samples. We also thank Dr. Dwi Sendi Priyono and Athena Syarifa from Wildlife Conservation Society-Indonesia Program for supporting this study by providing technical support at the lab and genetic data analysis.

References

Barbosa, C., Nogueira, S., Gadanho, M., Chaves, S., 2016. DNA extraction: finding the most suitable method, in: Cook, N., D'Agostino, M., Thompson, K.C. (Eds.), *Molecular Microbial Diagnostic Methods: Pathways to Implementation for the Food and Water Industries.* Academic Press, USA, pp. 135-154. https://doi.org/10.1016/B978-0-12-416999-9.00007-1

Fazhan, H., Waiho, K., Shahreza, M.S., 2016. A simple and efficient total genomic DNA extraction method for individual zooplankton. *SpringerPlus* 5, 2049. https://doi.org/10.1186/s40064-016-3724-x

Fazzini F., Schöpf B., Blatzer M., Coassin S., Hicks A.A., Kronenberg F., Fendt L., 2018. Plasmid-normalized quantification of relative mitochondrial DNA copy number. *Scientific Reports.* 8, 15347. https://doi.org/10.1038/s41598-018-33684-5

Fulton, T.L., 2012. Setting up an ancient DNA laboratory, in: Saphiro, B., Hofreiter, M. (Eds.), *Ancient DNA Methods and Protocols.* Humana Press, New York, pp. 1-11. https://doi.org/10.1007/978-1-61779-516-9_1

Guan, Z., Zhou, Y., Liu, J., Jiang, X., Li, S., Yang, S., Chen, A., 2013. A simple method to extract DNA from hair shafts using enzymatic laundry powder. *PLoS ONE* 8, e69588. https://doi.org/10.1371/journal.pone.0069588

Heywood, D.M., Skinner, R., Cornwell, P.A., 2003. Analysis of DNA in hair fibers. *Journal of Cosmetic Science.* 54, 21-27.

Jun, J., Han, S.H., Jeong, T.J., Park, H.C., Lee, B., Kwak, M., 2011. Wildlife forensics using mitochondrial DNA sequences: Species identification based on hairs collected in the field and confiscated tanned Felidae leathers. *Genes and Genomics* 33, 721-726. https://doi.org/10.1007/s13258-011-0080-7

Karmacharya, D., Sherchan, A.M., Dulal, S., Manandhar, P., Manandhar, S., Joshi, J., Bhattarai, S., Bhatta, T.R., Awasthi, N., Sharma, A.N., Bista, M., Silwal, N.R., Pokharel, P., Lamichhane, R.R., Sharma, N., Llewellyn, B., Wultsch, C., Kelly, M.J., Gour, D., Waits, L., Hero, J., Hughes, J., 2018. Species, sex, and geo-location identification of seized tiger (*Panthera tigris tigris*) parts in Nepal: a molecular forensic approach. *PLoS ONE.* 13, e0201639. https://doi.org/10.1371/journal.pone.0201639

Liu, Y.C., Sun, X., Driscoll, C., Miquelle, D.G., Xu, X., Martelli, P., Uphyrkina, O., Smith, J.L.D., O'Brien, S.J., Luo, S.J., 2018. Genome-Wide evolutionary analysis of natural history and adaptation in the world's tigers. *Current Biology*. 28, 3840-3849. https://doi.org/10.1016/j.cub.2018.09.019

Lopez-Oceja, A., Gamarra, D., Borragan, S., Jiménez-Moreno, S., De Pancorbo, M.M., 2016. New *cyt b* gene universal primer set for forensic analysis. *Forensic Science International: Genetics.* 23, 159-165. https://doi.org/10.1016/j.fsigen.2016.05.001

Luo, S., Kim, J., Johnson, W.E., van der Walt, J., Martenson, J., Yuhki, N., Miquelle, D.G., Uphyrkina, O., Goodrich, J.M., Quigley, H.B., Tilson, R., Brady, G., Martelli, P., Subramaniam, V., McDougal, C., Hean, S., Huang, S., Pan, W., Karanth, U.K., Sunquist, M., Smith, J.L.D., O'Brien, S.J., 2004. Phylogeography and genetic ancestry of tigers (*Panthera tigris*). *Plos Biology*. 2, 2275-2293. https://doi.org/10.1371/journal.pbio.0020442

Moss, D., Harbison, S.A., Saul, D.J., 2003. An easily automated, closed-tube forensic DNA extraction procedure using a thermostable proteinase. *International Journal of Legal Medicine.* 117, 340-349. https://doi.org/10.1007/s00414-003-0400-9

Nyhus, P.J., Tilson, R., 2004. Characterizing human-tiger conflict in Sumatra, Indonesia: Implications for conservation. *Oryx.* 38, 68-74. https://doi.org/10.1017/S0030605304000110

Sambrook, J., Russel, D.W., 2001. *Molecular Cloning A Laboratory Manual,* third ed. Cold Spring Harbor Laboratory Press, New York.

Schneider, P.M., Balogh, K., Naveran, N., Bogus, M., Bender, K., Lareu, M., Carracedo, A., 2004. Whole genome amplification—the solution for a common problem in forensic casework? *International Congress Series.* 1261, 24--26

Sharma, V., van der Plaat, D.A., Liu, Y., Wurmbach, E., 2020. Analyzing degraded DNA and challenging samples using the ForenSeq™ DNA Signature Prep kit. *Science and Justice.* 60, 243-252. https://doi.org/10.1016/j.scijus.2019.11.004

Sugimoto, T., Nagata, J., Aramilev, V.V., Belozor, A., Higashi, S., McCullough, D.R., 2006. Species and sex identification from faecal samples of sympatric carnivores, amur leopard and Siberian tiger, in the Russian Far East. *Conservation Genetics.* 7, 799-802. https://doi.org/10.1007/s10592-005-9071-z

Tarditi, C.R., Grahn, R.A., Evans, J.J., Kurushima, J.D., Lyons, L.A., 2011. Mitochondrial DNA sequencing of cat hair: an informative forensic tool. *Journal of Forensic Sciences.* 56, 36-46. https://doi.org/10.1111/j.1556-4029.2010.01592.x

van Pelt-Verkuil, E., van Belkum, A., Hays, J.P., 2008. *Principles and Technical Aspects of PCR Amplification*. Springer Netherlands, Dordrecht. https://doi.org/10.1007/978-1-4020-6241-4

Vipin, V.S., Sharma, C.P., Kumar, V.P., Goyal, S.P., 2016. Pioneer identification of fake tiger claws using morphometric and DNA-based analysis in wildlife forensics in India. *Forensic Science International.* 266, 226-233. https://doi.org/10.1016/j.forsciint.2016.05.024

Wandeler, P., Hoeck, P.E.A., Keller, L.F., 2007. Back to the future: museum specimens in population genetics. *Trends in Ecology and Evolution.* 22, 634-642. https://doi.org/10.1016/j.tree.2007.08.017

Wetton, J.H., Tsang, C.S.F., Roney, C.A., Spriggs, A.C., 2004. An extremely sensitive species-specific ARMS PCR test for the presence of tiger bone DNA. *Forensic Science International.* 140, 139-145. https://doi.org/10.1016/j.forsciint.2003.11.018

Wibisono, H.T., Pusparini, W., 2010. Sumatran tiger (*Panthera tigris sumatrae*): a review of conservation status. *Integrative Zoology*. 5, 313-323. https://doi.org/10.1111/j.1749-4877.2010.00219.x