

Genetic characterization based on the D-loop MtDNA and the TSPY genes of the Dwarf Siamang (*Hylobates klossii*) of Mentawai Island, Indonesia

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Manuscript received: 14 December 2019. Revision accepted: 31 May 2020.

Abstract. Hasanah R, Saepuloh U, Perwitasari-Farajallah D, Sinaga W, Hastuti Y, Sajuthi D. 2020. Genetic characterization based on the D-loop MtDNA and the TSPY genes of the Dwarf Siamang (*Hylobates klossii*) of Mentawai Island, Indonesia. *Biodiversitas* 21: 2854-2861. *Hylobates klossii* is an endemic primate on the Mentawai Island, Indonesia that categorized as endangered species due to fragmentation, illegal hunting, and habitat loss. This study aimed to analyze the genetic characterization of *H. klossii* based on maternal and paternal lineages. The samples were collected from TSI (Bogor, Indonesia) cage consist of two males and two females (blood and fecal of four individuals) and Siberut Island, Mentawai, West Sumatra, Indonesia from one unknown gender (one individual fecal sample). PCR amplification was carried out on DNA and cDNA samples using specific primers for D-loop MtDNA and TSPY genes. The amplicon was analyzed with MEGA-7. The dimensional structure model of the TSPY protein was determined using I-TASSER and visualized with PyMol. The maternal analysis showed five samples consisted of four haplotypes revealed that Nam-nam and April were identical with 0% genetic distance. It's revealed that Nam-nam and April were mother and son. The paternal analysis of two *H. klossii* males revealed that April was the closest relative to *H. muelleri* (99.56% identity) and Gou-gou to *H. klossii* (100% identity). Characterization of partial TSPY gene (990 bp) of April and Gou-Gou indicated four amino acids mutation, causing the shift in-amino acid structure position. The results assumed that April was a cross-breeding between *H. klossii* female and *H. muelleri* male. This genetic characterization is expected to support the conservation efforts through recommendations for good management in *H. klossii* preservation.

Keywords: D-loop, hybrid, *Hylobates klossii*, I-TASSER, TSPY

Abbreviations: Ala (A): Alanine, Asn (N): Asparagine, Asp (D): Aspartate acid, bp: basepair, D-loop: displacement loop, Glu (E): Glutamic Acid, Gly (G): Glycine, MtDNA: mitochondrial DNA, Pro (P): Proline, Ser (S): Serine, Thre (T): Threonine, TSI: Taman Safari Indonesia, TSPY: Testis specific protein in the Y chromosome

INTRODUCTION

Dwarf siamang (*Hylobates klossii*) is an endemic primate species on the Mentawai island. It is locally named Bilou. *Hylobates klossii* has a small body with bodyweight ranging from 5.5 to 7.5 kg, body length \pm 45 cm, black hair covering the entire body, brachiation, arboreal and communicates through vocalization with other tribe members (Whittaker 2005). The distribution of *H. klossii* is limited to large islands in Mentawai, such as Siberut, Sipora, North Pagai, and South Pagai. *H. klossii* population is estimated to around 20,000-25,000 individuals, out of which 6,206-17,713 individuals are in the Siberut National Park (Whittaker 2005; Quinten et al. 2015). *H. klossii* is threatened by deforestation and hunting so the habitat has decreased, and its population has declined by 49-59% since

the last survey in 1980 (Whittaker 2005). Dwarf siamang is considered as an endangered species (Whittaker and Geissmann 2008). *Hylobates klossii* is registered in the CITES Appendix I/2001 and protected under the Regulation of the Minister of Environment and Forestry of the Republic of Indonesia Number P.106Menlhk/Setjen/Kum.1/12/2018.

Conservation is an effort to protect the habitat, preserve the diversity and sustainable utilize species population from extinction. Referring to habitat degradation and changing in vegetation composition, primates require habitat, population, and individual management based on scientific understanding of the biology of the species (Singh et al. 2012). The conservation of primates can be done within their habitat (in-situ) or in captivity (ex-situ). Ex-situ conservation aims to support in situ conservation. It

can be done to reduce the threat of extinction in the wild. Species breeding programs one of the programs that can maintain and produce pure offspring aimed at protecting genetic resources and increasing populations. *Hylobates klossii* has a small population so that ex-situ conservation can be done to increase the population in nature (Masy'ud and Ginoga 2016).

Molecular analysis can be performed to obtain information regarding the effective population size, heredity, kinship, sex, distribution, population structure, and gene flow (Ferreira et al. 2012). In the analysis of kinship and evolution, the DNA that is often used is mitochondrial DNA (mtDNA). The mitochondrial DNA is maternally inherited as a haploid with the size of is~16.6 kb. Its rapid rate of evolution thus makes it is used in phylogenetic reconstruction, phylogeographic, and population genetic studies (Allendorf et al. 2012; Hu et al. 2015). The D-loop is a non-recombinant and non-coding area, playing a role in the replication and translation process so the mtDNA control region (D-loop region) is the most variable portion of the mammalian mtDNA genome than others (Hu et al. 2015). It has a hypervariable (HV) region consisting of HV-I and HV-II and the most polymorphic region of the human MtDNA genome (Mondal and Ghosh 2013). Those areas are extensively used in the study of molecular evolution at species and sub-species levels (Stoneking 2000).

In contrast to the mtDNA as a larger number of polymorphisms in its region widely used in molecular studies based on maternal (Kundu and Ghosh 2015), the Y chromosome is a non-recombinant gene that can reveal kinship according to paternal lineage (Eriksson et al. 2006). One specific gene on the Y chromosome used to determine paternal inheritance is the TSPY (*Testis-Specific Protein in the Y Chromosome*) gene. This specific Y-chromosome showed relatively high nucleotide diversity indices in varied among the other chromosomal in gibbon (Chan et al. 2012). The complete sequence of the TSPY gene in dwarf siamang has not been documented; hence, it is needed to characterize the gene.

Biparental analysis is needed to analyze both maternal and paternal lineages based on the mtDNA D-loop and the TSPY gene, respectively, and characterize the structure of the TSPY protein-coding gene in dwarf siamang (*Hylobates klossii*) on Mentawai island. This genetic characterization is expected to support the conservation efforts through recommendations for good management in *H. klossii* preservation.

MATERIALS AND METHODS

Time and place

This research was conducted from October 2018 to September 2019. Samples of *H. klossii* were collected from Taman Safari Indonesia (TSI), Cisarua, Bogor, West Java, Indonesia and Siberut, Mentawai Islands, West Sumatra, Indonesia. Laboratory analysis was conducted at the Biotechnology Laboratory of the Primate Research Center (PSSP), LPPM IPB, Bogor, Indonesia.

Procedures

Sample collection

The samples in this study consisted of feces (five individuals) and blood (four individuals) collected in Siberut Island and TSI (Table 1). Fresh fecal samples 180-220 mg were collected in the morning using a cotton bud and put into a tube containing 70% ethanol and Viral Transport Media (VTM) and stored in a cool box. Meanwhile, blood samples were taken at TSI. Two milliliters blood samples were taken and put into a blood tube with EDTA anticoagulant during general examination for *H. klossii*. Blood samples were stored in the freezer at -20°C.

The animal code approved this study of ethics from the Institutional Animal Care and Use Committee (IACUC) Number IPB PRC-19-D007 and the Biorisk Commission of the Primate Research Center IPB Number 010-PL-PSSP-03-2019.

Nucleic acid isolation

The DNA from feces and blood samples was extracted using QiaAmp™ DNA Extraction Stool and Blood mini kit (Qiagen, Hilden, Germany), according to manufacturer's protocol. The RNA extraction from blood samples was carried out using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was reverse transcriptase using the Superscript III cDNA synthesis (Invitrogen) kit. DNA and RNA concentrations were measured with NanoDrop™ One (Thermo Scientific).

DNA Amplification

PCR amplification of the mtDNA D-loop was performed using specific primers, GIBDLF3, and GIBDLR4. The TSPY gene was amplified using TSPY-A and TSPY-B primers, then further analyzed based on the cDNA sample using specific primers (Table 2). The PCR reaction consisted of 1 µL of each 10 pmol µL⁻¹ forward and reverse primer; 12.5 µL of GoTaq Green Master Mix; 2.5-5 µL DNA template and nuclease-free water to a total volume of 25 µL. DNA and cDNA amplification was carried out using a Thermal Cycler machine (GeneAmp® PCR System 9700) with 40 cycles under the following conditions: denaturation at 94°C for 30 seconds, annealing (according to the Ta temperature of each primer) for 30 seconds to 1 minute and extension at 72 °C for 7 minutes. The PCR products were electrophoresed in 1.8% agarose gel and visualized using SYBR Safe (Invitrogen) under UV Gel Doc 2000 (Biorad). The bands were analyzed using the Quantity One program (Biorad). The PCR product was sequenced at 1st BASE Laboratories Sdn Bhd (Malaysia).

Table 1. The list of feces and blood samples collected from *Hylobates klossii*

Name	Sex	Sample	Location
Nam-nam	Adult female	Fecal and blood	TSI, Bogor
April	Juvenile male	Fecal and blood	TSI, Bogor
Lestari	Juvenile female	Fecal and blood	TSI, Bogor
Gou-gou	Juvenile male	Fecal and blood	TSI, Bogor
Bilou	Unknown	Fecal	Bekemen, Siberut

Phylogeny analysis based on mtDNA and TSPY

The nucleotide sequences were manually edited based on the chromatogram using the Bioedit program version 7.2.6 (Hall 1999). The alignment of the nucleotide sequences was carried out using the ClustalW program. The alignment results of the mtDNA, D-loop, and TSPY sequences were analyzed using the NCBI website (<https://blast.ncbi.nlm.nih.gov>) by selecting the BLAST-N to identify the closest relatives. The nucleotide sequences obtained were analyzed using the MEGA-7 program (Kumar et al. 2016). Genetic distance estimation was determined using pairwise distance method with the p-distance model. The phylogenetic tree was constructed based on the NJ (Neighbor-joining) method with 1000 repetitions.

Coding region characterization of the TSPY gene

Nucleotide sequences of the TSPY gene were translated into amino acids through the ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder>). The difference in the nucleotide and amino acid sequences were compared and analyzed using Clustal W. The 3D structure modeling of amino acid was constructed through the I-TASSER program (<https://zhanglab.ccmb.med.umich.edu/I-TASSER>) (Yang and Zhang 2015). The PyMol program was used to visualize the protein structure.

RESULTS AND DISCUSSION

Phylogeny analysis based on maternal lineage

The 512 bp amplicon was obtained from the amplification of *H. klossii*'s d-loop MtDNA. The aligned D-loop MtDNA nucleotide was 449 bp, consisting of 415 conserved regions, 34 variable regions with 12 parsimony regions, and 22 singletons. The gene target was in the HV-I region of 427 base pairs (1-427 of 449 base pairs) (ref. EF363503.1), which was one part of the D-loop with high evolution rate. The HV-I region of the D-loop has a faster mutation rate than other regions. So, it is useful to understand intra-species relationships, studies of population genetic diversity, and the reconstruction of the past demographic history (Whittaker et al. 2007; Hu et al. 2015).

The sequencing of nucleotide bases supported by genetic distance analysis results showed that intra-species variations ranged from 0.00 to 0.047 (Table 3). Nam-nam and April had the smallest genetic distance, which was 0.00. It showed that Nam-nam and April had a very close (identical) relationship based on maternal lineage because they had the same MtDNA nucleotide sequence (conserved). Nam-nam and April are mother and son. They have a smaller genetic distance with Bilou (0.024) compared to Lestari and Gou-gou (0.045). The smaller the genetic distance, the lesser the difference in nucleotide bases. The greater the genetic distance, the greater the differences, and vice versa.

The results of the mtDNA D-loop analysis from five samples showed four haplotypes, as indicated by the distribution of *H. klossii* based on the phylogeny trees (Figure 1). The phylogeny analysis using the neighbor-joining method revealed that Nam-Nam and April were in a cluster with dwarf siamang from the North and South Pagai, Bilou from Sipora, Gou-Gou from Pagai and Lestari from Siberut.

Phylogeny of *H. klossii* based on paternal lineage

The TSPY fragment amplification results revealed 780 bp identified in two *H. klossii* male individuals, namely Gou-Gou and April. The nucleotide sequencing of the TSPY gene resulted in the overlapping nucleotide sequences of 746 bp, consisting of 737 conserved regions and 9 variable regions. Referring to the gene reference, the accession number JN871374.1 showed that the TSPY gene consisted of exon 1 (1-72 nt), intron 2 (73-675nt), and exon 2 (676-719nt). This result indicated that the TSPY region consisted of non-coding regions (introns).

Table 3. The genetic distance (*p-distance* %) of the *Hylobates klossii* based on the mitochondrial D-loop sequence

No.	Names of individuals	1	2	3	4	5
1	Nam-Nam (TSI Bogor)	-				
2	April (TSI Bogor)	0	-			
3	Lestari (TSI Bogor)	0.045	0.045	-		
4	Gou-Gou (TSI Bogor)	0.045	0.045	0.040	-	
5	Bilou (Bekemen, Siberut)	0.024	0.024	0.042	0.047	-

Table 2. Specific primers used in this study

Primers	Primer sequence	Ta *)	Amplicon (bp)	References
GIBDLF3	5'CTTACCCTCAGCACCCAAAGC 3'		512	(Andayani et al. 2001)
GIBDLR4	5'GGGTGATAGGCCTGTGAT C 3'	56		
TSPY-A	5'AGCCAGGAAGGCCTTTTCTCG 3'		780	(Chan et al. 2012)
TSPY-B	5'CCATGTAGCTCAGCATGTCTTCAT 3'	60		
TSPY1139F	5'GCTGGTGTCTCACTCAACC 3'	54	1000	(Biotechnology Lab design with reference XM_024240960.1)
TSPY1739R	5'TTGTCAGGGTTCTCTCGTT 3'			
TSPY1509F	5'ACCACCGACTACTTCGAGAC 3'	52	800	
TSPY2109R	5'TCCTTCCTCCTCATAGTTTCC 3'			
TSPY2058F	5'ATCGAGCAAGATGAGGACAT 3'	53	800	
TSPY2798R	5'GAACCTGCTCAGAATTTTATTGG 3'			

Note : *Ta: annealing temperature

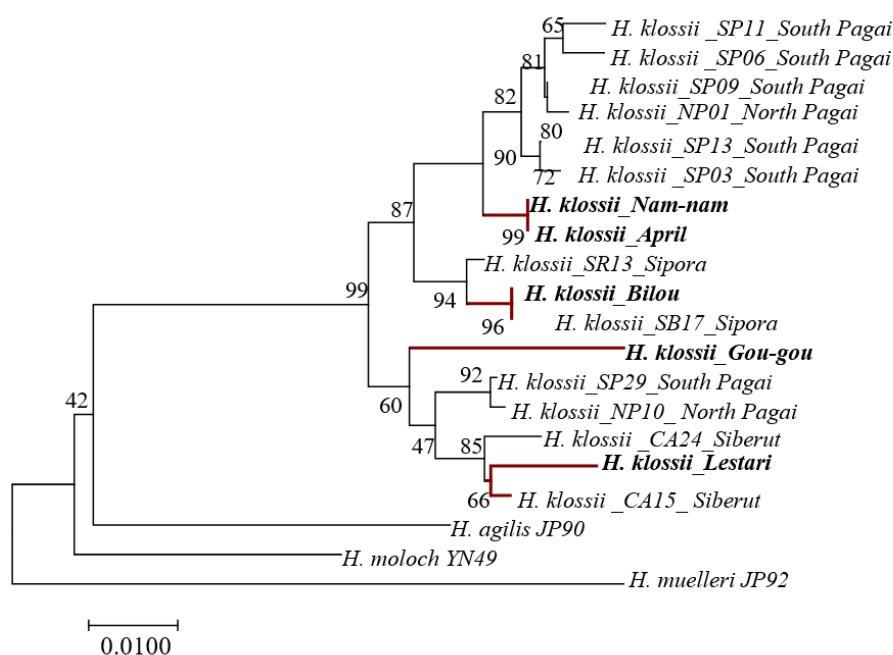


Figure 1. The phylogeny tree of *Hylobates klossii* based on D-loop mtDNA analysis using the Neighbor-joining method

The alignment of nucleotides based on BLAST showed that Gou-Gou had 100% identity similarity with the *H. klossii* (JN871377.1), while April had 99.56% with *H. muelleri* (JN871377.1) (Table 4). The DNA mutations in individuals were determined by the transition (changes in fellow purines or fellow pyrimidines) and transversion (changes in purines to pyrimidines or vice versa) (Brown et al. 1979). The April individual comparison of the *H. klossii* (JN871377.1) and Gou-Gou based on nucleotide sequencing using MEGA revealed seven nucleotide changes in the forms of transition mutations at sites 129 (T/C), 387 (C/T), 510 (G/A), 540 (G/A), 561 (G/A) and 727 (G/A). Whereas, transversion mutations occurred at site 296 from purines to pyrimidines (A/T) (Table 4).

Nucleotide changes were in line with the genetic distance values in the p-distance method in April and Gou-Gou compared with *H. klossii* and data from genbank (Table 5). The *H. klossii* Gou-gou had a genetic distance of 0.00 to the *H. klossii** but had a genetic distance of 0.008 to April. April had a genetic distance value of 0.008 with *H. klossii** and 0.005 to *H. muelleri*⁺. Meanwhile Gou-Gou has a genetic distance value of 0.010 to *H. muelleri*⁺. The resulting genetic distance values showed *H. klossii* April had a smaller genetic distance with *H. muelleri*⁺ than with fellow *H. klossii** (intraspecies).

The phylogeny analysis using the neighbor-joining method (Figure 2) showed that *H. klossii* individuals were divided into two clusters, namely *H. klossii* Gou-gou with similarities to *H. klossii** (JN871377.1) (bootstrap value 98%) and *H. klossii* April being in a group with *H. muelleri*⁺ (JN871374.1) (bootstrap value of 99%).

Characterization of the TSPY protein structure

The analysis of the TSPY gene in the coding region was carried out to characterize the protein structure of the TSPY gene in two male individuals (April and Gou-Gou). The results of the cDNA amplification of the partial TSPY gene produced an amplicon of 990 bp. The alignment based on the nucleotide sequences of cDNA of origin between Gou-gou and April individuals had 99% identity similarity.

The TSPY gene of 990 bp between Gou-Gou and April individuals (Table 6) went through changes in nucleotide bases. The mutations were in the forms of transversions in the 298 bases (C/G) and 841 (A/T) and transitions in the 369, 397, 831 (A/G) bases. Five nucleotide mutations produced changes in the composition of amino acids. The mutations in the nucleotide position 280 caused changes in site 100 amino acids from proline to alanine (Pro100Ala), position 397 changes in amino acids in the site 133 from asparagine to aspartic acid (Asn133Asp), position 831 changes in amino acids site 277 from glycine to glutamine (Gly277Glu) and the position 841 nucleotides undergo the site 281 in amino acids from threonine to serine (Thre281Ser). The position of 369 changes in nucleotides does not cause a change in the site 123 amino acid (Ala123Ala).

The change in amino acids in the TSPY gene between Gou-gou and April caused conformational changes in the predicted three-dimensional structure of the TSPY protein produced. The results of the alignment of the positions between the two structures (superimposed) indicated a change in the location of amino acids (Figure 3).

Discussion

The results of the mtDNA D-loop analysis from five samples showed four haplotypes, as indicated by the distribution of *H. klossii* in four islands. Based on D-loop MtDNA nucleotide, there are no genetic differentiation between *H. klossii* in Siberut, Sipora, North Pagai and South Pagai islands. This assumed that Mentawai Islands

had been not separated in a long time (Whittaker 2005). However, the TSPY gene showed differences which *H. klossii* named April was clustered with *H. muelleri* (JN871374.1). The differences in nucleotide genes in April based on the TSPY gene suggested hybrid mating from *H. klossii* females (Nam-nam) with *H. muelleri* males.

Table 4. Variations of the TSPY nucleotide substitution between *Hylobates klossii* (BLAST) individuals

Names	Nucleotide Substitution							Identity (%)
	1	2	3	5	5	5	7	
Gou-gou	T	A	C	G	G	G	G	100
<i>H. klossii</i> (JN871377.1)	T	A	C	G	G	G	G	
April	C	T	T	A	A	A	A	99.56
<i>H. muelleri</i> (JN871374.1)	C	T	T	A	A	G	A	

Table 5. The genetic distance (p-distance %) of *Hylobates klossii* to other non-human primates species based on the TSPY gene

No.	Species names	1	2	3	4	5	6	7	8	9	10
1	<i>H. sapiens</i>	-									
2	<i>P. troglodytes</i>	0.504	-								
3	<i>M. fascicularis</i>	0.159	0.521	-							
4	<i>S. syndactylus</i>	0.077	0.501	0.153	-						
5	<i>H. lar</i>	0.074	0.496	0.144	0.025	-					
6	<i>H. klossii_April</i>	0.076	0.494	0.149	0.026	0.015	-				
7	<i>H. klossii_Gou-gou</i>	0.076	0.499	0.144	0.023	0.010	0.008	-			
8	<i>H. klossii*</i>	0.076	0.499	0.144	0.023	0.010	0.008	0.000	-		
9	<i>H. muelleri</i> ⁺	0.077	0.496	0.151	0.028	0.016	0.005	0.010	0.010	-	
10	<i>H. agilis</i>	0.077	0.499	0.148	0.028	0.013	0.011	0.007	0.007	0.013	-

Note: *,⁺ is a comparative individual that use to compare with April dan Gou-gou (data were taken from Gene Bank for comparison).

Table 6. Differences in the nucleotide base and individual amino acids of Gou-Gou and April of the TSPY gene cDNA

Names	Type of changes	Nucleotide positions				
		298	369	397	831	841
Gou-gou	Nucleotide	CCC	GCA	AAT	GGG	ACG
	Amino Acid	Pro (P)	Ala (A)	Asn (N)	Gly (G)	Thr (T)
April	Nucleotide	GCC	GCG	GAT	GAG	TCG
	Amino Acid	Ala (A)	Ala (A)	Asp (D)	Glu (E)	Ser (S)

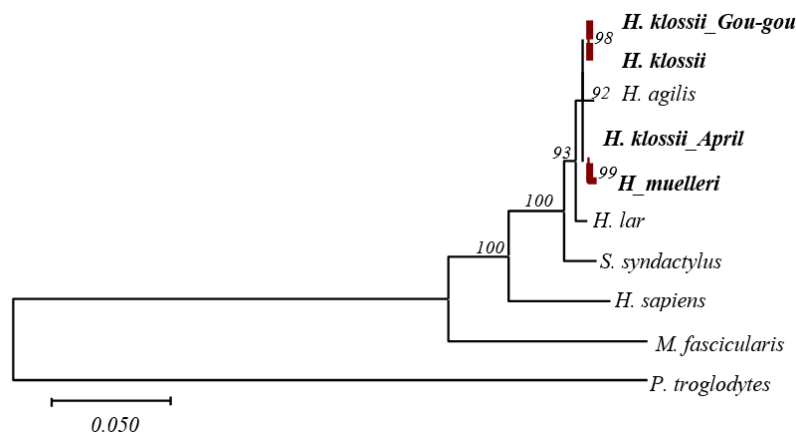


Figure 2. The phylogeny analysis of the TSPY gene based on the neighbor-joining method

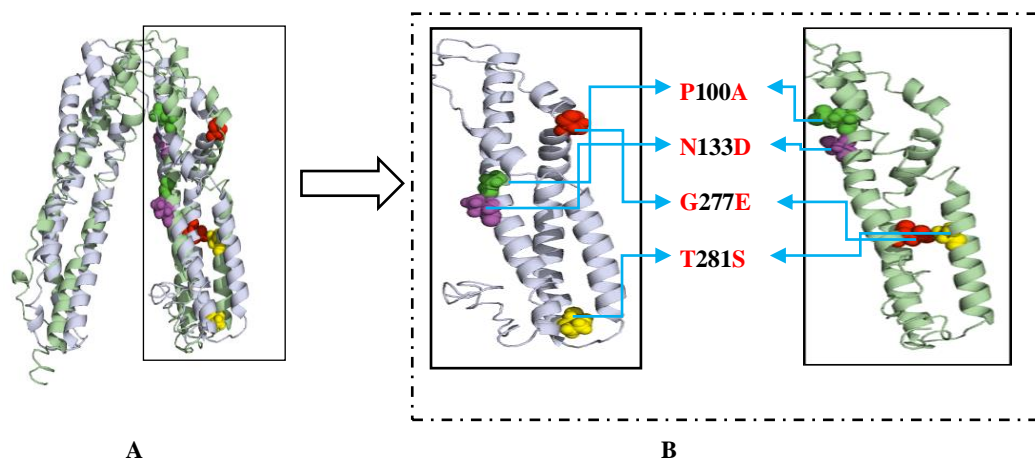


Figure 3. Prediction of three-dimensional structure models of partial cartoon TSPY forms using the I-TASSER program visualized with the PyMol program. A. Position alignment (superimposed) TSPY protein structure of individual Gou-gou (gray) and April (light green), B. Position of amino acids undergoing mutations (spherical shape)

Morphologically, *H. klossii* April has the same characteristics as *H. muelleri* individuals in terms of face and head shapes (Figure 4). The genus *Hylobates* has many similarities in character such as skull shape, intermembrane index, and genital features (Groves 2001) such as same humeral long with bowed humeral shafts (Holliday and Friedl 2013). *H. klossii* has a grey-brown hair color, blackish underside, crown with a dark patch surrounded by a ring of bright fur around its face while *H. klossii* had all black hair and monochromatic (Groves 1971). As a result of hybrid tillers, April was a black monochromatic short-haired with white eyebrows. April has the same characteristics as elder males, namely the presence of white eyebrows, relatively thick back hair like those of *H. muelleri* (male elder), and larger body size than normal dwarf gibbons. Based on observation, April has the bodyweight about 6.6 kg with height about 77 cm while others *H. klossii* has bodyweight about 1.7-5.7 kg with a height 55-76 cm. *H. muelleri* has a bodyweight of about 5.8 kg with a height of about 54 cm. Body mass and height of April were above the average of *H. klossii*, thus it makes more evidence that he is a hybrid mating between *H. klossii* female and *H. muelleri* male (Figure 4).

Hybridization is the marriage of individuals from two different populations or groups which are differentiated based on one or more inherited characters. The success of producing offspring from hybridization is due to individual elders that are still in one genus *Hylobates* and have the same number of chromosomes ($2n = 44$) (Groves 2001). Hybridization between species can occur due to forest fragmentation and habitat destruction, making it difficult for a species to find the same species (Detwiler et al. 2005). Hybrid cases in captivity occur due to the limited number of similar individuals and the location of adjacent species in cages.

Hybrid results occur with and without introgression. Fertile hybrid results from introgression of the parental

gene. Infertile hybrid cause introgression, which removes the pure genes of the elders. Sterile hybrid yields cause a decrease in reproductive capacity resulting in a decrease in population and support species extinction (Allendorf et al. 2012). If the hybrid results are sterile, a decrease in reproductive capacity results in spermatogenesis. The analysis of sperm quality requires further analysis.

TSPY protein structure analysis showed some changes in amino acids that affect changes in protein function and require further analysis. These changes must pay attention to various properties of amino acids, the level of sustainability/conserved, and the percentage of changes (Chavez 2010). Protein is an important component of biological organisms but is prone to errors when DNA replication can cause mutations. Mutations can change phenotypes and have beneficial, damaging, or neutral effects on individual fertility rates (Studer et al. 2013).

TSPY gene expression is limited to male genital cells and neoplastic of the testes (Vinci et al. 2009). The differentiation of genital cells and the maturity of spermatozoa is a complex regulatory process where deregulation results in tumor formation or infertility (Schnieders et al. 1996). Mutations in the TSPY gene can be associated with abnormalities in the development and function of the testes (Vinci et al. 2009). The TSPY region, characterized by protein binding, plays an important role in spermatogenesis through targeting histone for ubiquitination. Ubiquitin is involved in the regulation of transcription, epigenetic modification, and other processes that are important for the formation of normal gametes (Sheng et al. 2014). However, to completely understand changes in the structure of these proteins that cause changes in the function of the TSPY protein which affect fertility (e.g., being sterile/fertile) in hybrid individuals, requiring further research.

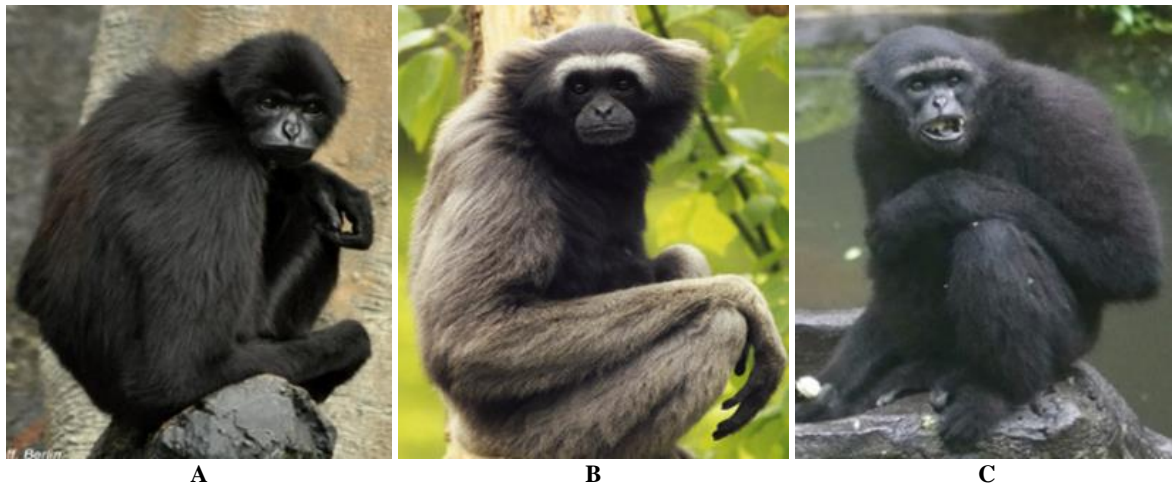


Figure 4. Morphologically appearances of: A. *Hylobates klossii* Nam-Nam (adult female), B. *H. muelleri* (adult male), C. *H. klossii* April. Source: A. Klaus Rudolf, Germany; B. <http://zoo.avantia.net/animals/179>, C. Personal documentation of RH

Our study gives new insights about paternal and maternal lineage markers by using D-loop MtDNA and TSPY gene to obtain phylogenic between intraspecies and interspecies of *Hylobates*, specifically. The finding of three-dimensional TSPY protein structures can be used to determine this gene further and its function on male primates. This hybridization case can suggest good management for *H. klossii* preservation in captivity. Efforts to support conservation in the form of genetic information are expected to help conserve species. Genetic information obtained based on male and female parent lineages can provide suggestions for conservation management in maintaining the integrity of species. This is done through the prevention of inbreeding and separating hybrid animals that are indicated to prevent mating with origin species.

ACKNOWLEDGEMENTS

The authors would like to thank Jansen Manansang. This research is a collaboration between Taman Safari Indonesia (TSI), Bogor, Indonesia and the Primatology Study Program, IPB Graduate School, Bogor, Indonesia. Laboratory analysis was carried out at the Primate Research Center, Lembaga Penelitian dan Pengabdian kepada Masyarakat, Institut Pertanian Bogor (PSSP, LPPM-IPB), Bogor, Indonesia.

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