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## *Research*

# Mitochondrial Genome of Endangered Hylobatidae, *Hylobates lar*, and *Hylobates agilis*, of Peninsular Malaysia

**Roberta Chaya Tawie Tingga[1,2], Millawati Gani[1,3], Badrul Munir Md-Zain[1]***

1. Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia
2. Centre for Pre-University Studies, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia
3. National Wildlife Forensic Laboratory (NWFL), Ex-Situ Conservation Division, Department of Wildlife and National Parks (PERHILITAN), KM 10 Jalan Cheras, 56100, Kuala Lumpur, Malaysia
*Corresponding author: abgbadd@ukm.edu.my

**ABSTRACT**

*Hylobates agilis* and *Hylobates lar*, members of the family Hylobatidae, are classified as endangered species in Malaysia due to threats such as population decline and habitat loss. The aim of this study was to sequence and characterize the mitochondrial genome of *H. lar* and *H. agilis*. This study reported the total length of mitogenome of *H. agilis* and *H. lar* to be 16,473 and 15,949 base pair (bp), respectively. Each sequence contained 13 protein-coding sequences, 2 rRNAs, 22 tRNAs, and a control region D-loop (*H. agilis*); meanwhile for *H. lar*, there are 13 protein-coding sequences, 2 rRNAs, 19 trNAs, and a control region D-loop. Phylogenetic analysis showed both species formed a strong monophyletic clade within Hylobates grouping. Mitogenomic data of this study is essential for future references in evolutionary biology and conservation management of endangered Malaysian gibbons in captivity.

**Key words:** *Hylobates agilis*, *Hylobates lar*, mitogenome, next-generation sequencing

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## INTRODUCTION

Comprehending an organism's complete mitochondrial genome, or mitogenome facilitates comprehensive genomic studies in systematics and evolution (Chen *et al*., 2021). Whole mitochondrial genomes can give essential information that is useful in elucidating and providing better resolution on phylogenetic relationships and evolutionary history (İbiş, 2020). Complete or partial mitochondrial genome assemblies for each species in the sample are created via the generation of the high-copy mitochondrial fraction that accounts for approximately 1% of all reads (Crampton-Platt *et al*., 2015). The richness of the mitogenome enables better amplification or direct sequencing even from degraded samples (Besnard *et al*., 2016). Previous mitogenomic analysis on primates explored by Finstermeier *et al*. (2013) comprising all 16 recognized families from order Primate throughout South and Central America, Africa, and southern and Southeast Asia, has effectively clarified their evolutionary relationships at different taxonomic levels. This approach proves that a long fragment of mitochondrial sequence can resolve phylogenetic relationships compared to a short fragment of mitochondria or nuclear sequence (Finstermeier *et al*., 2013) based on amplification from high-quality DNA samples (Chan *et al*., 2010).

Hylobatidae consists of four genera, namely, *Nomascus*, *Hoolock*, *Hylobates*, and *Symphalangus* (Roos, 2016). In Malaysia, five species of Hylobatidae have been identified, including, *H. lar*, *H. agilis*, *Symphalangus syndactylus* (Peninsular Malaysia) and *H. funereus* and *H. abbotti* (Sabah

& Sarawak) (Md-Zain *et al*., 2021; 2022). Based on the International Union for Conservation of Nature (IUCN) on the Red List of Threatened Species, all Hylobatidae members in Malaysia are classified as endangered species (Md-Zain *et al*., 2022). One of the critical reasons to emphasize the study of gibbons is that these species are consistently under threat as their populations decrease due to habitat loss for land conversion or hunting for food and also for pet trades (Campbell *et al*., 2015). According to Yimkao and Srikosamatara (2006), the survival of *H. lar* is affected by the hunting of their meat and clearing of the forest for development. As the reproduction rate of Hylobatidae is slow with an estimation of five offspring throughout the female lifetime (Palombit, 1995), thus, it is a challenge for the species to regain back their population size in a short period. In this study, we focused on two species of gibbons which are *H. agilis* and *H. lar*. Therefore, this study was conducted to sequence the mitogenome of *H. agilis* and *H. lar* of Peninsular Malaysia using next-generation sequencing to retrieve its genetic information. Subsequently, phylogenetic relationships are portrayed by constructing a tree of *H. agilis* and *H. lar* based on the mitogenome data.

Blood samples *H. agilis* and *H. lar* were obtained from the Veterinary Officer of the National Wildlife Rescue Centre (NWRC), Department of Wildlife and National Parks (Jabatan PERHILITAN), Peninsular Malaysia. Both samples were preserved in a sterile tube for long-period storage under −20°C (Gaur *et al*., 2017). DNA Extraction of the blood samples was done by using NucleoSpin® Tissue, following the standard manufacturer protocol. The integrity of the extracted DNA was verified using 1% agarose gel electrophoresis, and the DNA concentration was determined using a spectrophotometer (Implen NanoPhotometer® N60/N50). Both genomic DNAs were sent to the Apical Scientific Sdn. Bhd. for library preparation and mitogenome sequencing. DNA sample quality control (QC) was conducted using agarose gel, spectrophotometer (Implen NanoPhotometer® N60), and fluorometric quantification. The samples that pass QC subsequently proceed to amplicon polymerase chain reaction (PCR) using two sets of specific primers of mitochondrial DNA gene that, respectively, produce 8 kb and 10 kb fragments (Finstermeier *et al*., 2013) (Table 1). Mitochondrial DNA amplicon PCR was performed with KOD FX Neo (Toyobo) according to the manufacturer's recommended protocol.

**Table 1.** Sets of mitochondrial DNA gene-specific primer used in this study

| Primer | Sequences |
|---|---|
| Set 1 (generate 8 kb amplicon product) | mt_F1; GGCTTTCTCAACTTTTAAAGGATA<br><br>mt_R1; TGTCCTGATCCAACATCGAG |
| Set 2 (generate 10 kb amplicon product) | mt_F2; CCGTGCAAAGGTAGCATAATC<br><br>mt_R2; TTACTTTTATTTGGAGTTGCACCA |

The purified amplicons generated from the same DNA samples were pooled and proceeded with the native barcoding library preparation method (SQK-LSK109 & EXP-NBD196, Oxford Nanopore Technologies). The quality of the generated amplicon libraries was evaluated using fluorometric quantification. The final DNA libraries that passed QC were subsequently subjected to third-generation sequencing using the Oxford Nanopore Technologies GridION platform. All nanopore raw reads were first trimmed using Porechop 0.2.4 (Wick *et al*., 2017a). All trimmed reads were first subjected to de novo assembly using Unicycler V0.5.0 (Wick *et al*., 2017b) with the bold option for de novo assembly. We aligned selected assemblies to their expected reference by using the map to reference the end-to-end mapping BowTie2 mapper plugin in Geneious Prime 2023.2.1 software (Biomatters, New Zealand) with high-sensitivity setting and reiteration using a published mitochondrial genome of *H. agilis* (LC548012) and *H. lar* (HQ622776) as references. Pairwise alignment of sequences assembled from repeated reference mapping and de novo assembly was carried out in Geneious Prime 2023.2.1, and the consensus sequence from the alignment was selected for sequence annotation. The sequences were annotated using the MITOS web server (Bernt *et al*., 2013) to identify gene regions, protein-coding genes, and ribosomal and transfer RNA genes. Both assembled mitochondrial genome sequences of *H. agilis* (HA) and *H. lar* (HLT973) were annotated for genes/regions individually. The annotation result was then compared with reference mitogenome HQ622776 and LC548012 using Geneious Prime 2023.2.1 to confirm the correct gene annotation order. The constructed mitogenome sequences of *H. agilis* (HA) and *H. lar* (HLT973) were aligned with 21 mitogenome sequences from the genera *Hylobates*, *Symphalangus*, and *Nomascus*, available at the National Center for Biotechnology Information (NCBI) GenBank (Table 2). The Neighbor-joining (NJ) and Maximum Likelihood (ML) phylogenetic trees were constructed in Geneious Prime 2023.2.1. The NJ tree was constructed using the Tamura-Nei distance model, and the ML tree was generated using the PhyML plugin in Geneious Prime 2023.2.1, employing the best phylogenetic model determined by MEGA v11 (Tamura *et al*., 2021). Genetic distances among

the Hylobatidae species included in this study were calculated using the Kimura 2-parameter model implemented in MEGA v11.

The mitogenome of *H. agilis* (HA) and *H. lar* (HLT973) successfully produced a total length of 16,473 and 15,949 base pairs (bp), respectively (Figure 1). The gene arrangement and content of *H. agilis* (Table 3) comprising 40 arrangements include 13 genes, 13 CDS, 2 rRNAs, 22 tRNAs, and a control region D-loop. Meanwhile, *H. lar* (HLT973) only successfully assembled 37 gene arrangements (Table 4) comprising 13 genes, 13 CDS, 2 rRNAs, 19 trNAs, and a control region D-loop. Both *H. agilis* and *H. lar* mitogenomes exhibit overlapping regions situated between the ATP8 and ATP6 genes (39–46 bp in size). Although a complete mitogenome was produced for *H. agilis* (HA), the assembled mitogenome of *H. lar* (HLT973) showed a missing fragment around approximately 550 bp between the ND4 and ND5 genes, including three tRNAs: tRNA-His (69 bp), tRNA-Ser (59 bp), and tRNA-Leu (71 bp). The identification of these missing fragments occurred during the application of end-to-end reference mapping using BowTie2 with the reference mitogenome sequence HQ622776. Consequently, the mitogenome assembly of *H. lar* (HLT973) produced partial ND4 and ND5 gene sequences with lengths of 1340 bp and 1498 bp, respectively.

**Table 2.** List of 18 mitogenome samples obtained from GenBank

| No. | Species | Accession number |
|---|---|---|
| 1 | *Hylobates abbotti* | LC548015 |
| 2 | *Hylobates albibarbis* | LC548013 |
| 3 | *Hylobates agilis* | NC_014042, HQ622758, LC548012 |
| 4 | *Hylobates klossii* | HQ622788 |
| 5 | *Hylobates lar* | HQ622763, HQ622776 |
| 6 | *Hylobates moloch* | HQ622782 |
| 7 | *Hylobates muelleri* | HQ622779, LC548016 |
| 8 | *Hylobates pileatus* | AB504749, NC_014045 |
| 9 | *Nomascus gabriellae* | HQ622807 |
| 10 | *Nomascus leucogenys* | HQ622802 |
| 11 | *Nomascus siki* | HQ622804 |
| 12 | *Symphalangus syndactylus* | AB504750, HQ622791 |

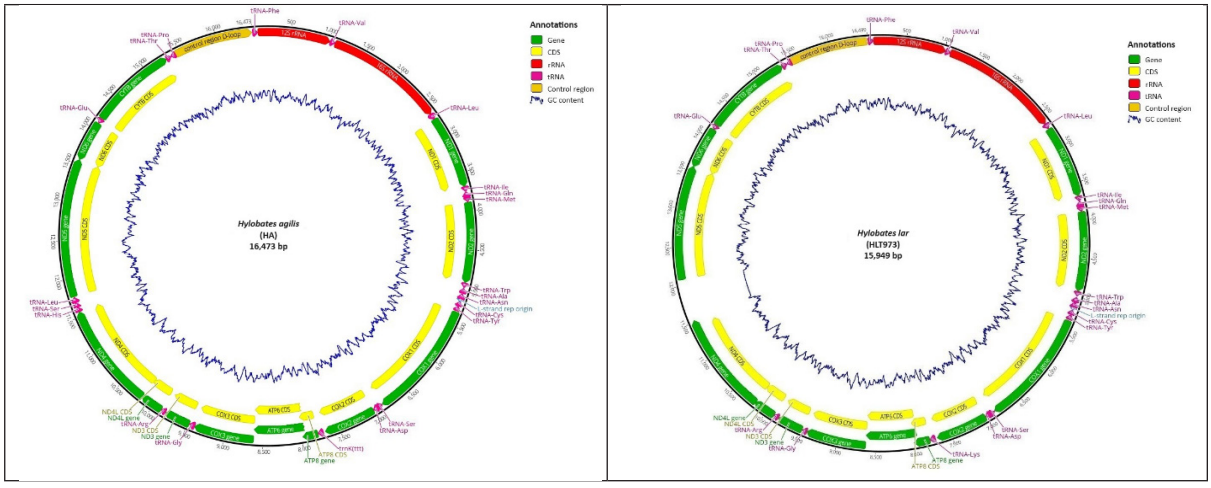


**Fig. 1.** The mitochondrial gene organization of *H. agilis* (left) and *H. lar* (right) with visualization of genes, protein-coding genes, rRNAs, and tRNAs. The blue inner circle showed GC content.

The phylogenetic trees built using NJ and ML methods with 23 mitogenome sequences exhibited consistent topologies validated by strong bootstrap values (Figure 2). The gibbon genus *Hylobates* displayed a well-supported phylogeny, with species forming distinct monophyletic clades and *Nomascus* serving as the basal clade. Notably, in this study, *H. agilis* (HA) is clustered together with the *H. lar* group instead of other *H. agilis* reference species. Moreover, *H. agilis* (HA) was paraphyletic, forming a sister clade with the *H. lar* group. The genetic distance of individual *H. agilis* (HA) in this investigation was observed to be closer to the *H. lar* group compared to the *H. agilis* group acquired from the NCBI GenBank (Table 5).

**Table 3.** Gene order of mitochondrial genome of *Hylobates agilis* (HA)

| Name | Type | Start | End | Length (bp) | Direction |
|---|---|---|---|---|---|
| tRNA-Phe | tRNA | 1 | 70 | 70 | forward |
| 12S rRNA | rRNA | 71 | 1020 | 950 | forward |
| tRNA-Val | tRNA | 1021 | 1088 | 68 | forward |
| 16S rRNA | rRNA | 1089 | 2645 | 1557 | forward |
| tRNA-Leu | tRNA | 2646 | 2720 | 75 | forward |
| ND1 gene | gene | 2723 | 3676 | 954 | forward |
| tRNA-Ile | tRNA | 3677 | 3745 | 69 | forward |
| tRNA-Gln | tRNA | 3743 | 3814 | 72 | reverse |
| tRNA-Met | tRNA | 3815 | 3882 | 68 | forward |
| ND2 gene | gene | 3883 | 4925 | 1043 | forward |
| tRNA-Trp | tRNA | 4926 | 4993 | 68 | forward |
| tRNA-Ala | tRNA | 5003 | 5070 | 68 | reverse |
| tRNA-Asn | tRNA | 5072 | 5145 | 74 | reverse |
| L-strand rep origin | rep_origin | 5146 | 5178 | 33 | forward |
| tRNA-Cys | tRNA | 5176 | 5241 | 66 | reverse |
| tRNA-Tyr | tRNA | 5241 | 5306 | 66 | reverse |
| COX1 gene | gene | 5319 | 6860 | 1542 | forward |
| tRNA-Ser | tRNA | 6861 | 6929 | 69 | reverse |
| tRNA-Asp | tRNA | 6933 | 7001 | 69 | forward |
| COX2 gene | gene | 7002 | 7685 | 684 | forward |
| trnK(ttt) | tRNA | 7712 | 7781 | 70 | forward |
| ATP8 gene | gene | 7783 | 7983 | 201 | forward |
| ATP6 gene | gene | 8001 | 8624 | >624 | forward |
| COX3 gene | gene | 8624 | 9407 | 784 | forward |
| tRNA-Gly | tRNA | 9408 | 9475 | 68 | forward |
| ND3 gene | gene | 9476 | 9821 | 346 | forward |
| tRNA-Arg | tRNA | 9822 | 9887 | 66 | forward |
| ND4L gene | gene | 9888 | 10184 | 297 | forward |
| ND4 gene | gene | 10178 | 11556 | 1379 | forward |
| tRNA-His | tRNA | 11557 | 11625 | 69 | forward |
| tRNA-Ser | tRNA | 11626 | 11684 | 59 | forward |
| tRNA-Leu | tRNA | 11685 | 11754 | 70 | forward |
| ND5 gene | gene | 11761 | 13566 | 1806 | forward |
| ND6 gene | gene | 13567 | 14091 | 525 | reverse |
| tRNA-Glu | tRNA | 14092 | 14160 | 69 | reverse |
| CYTB gene | gene | 14165 | 15305 | 1141 | forward |
| tRNA-Thr | tRNA | 15306 | 15371 | 66 | forward |
| tRNA-Pro | tRNA | 15374 | 15441 | 68 | reverse |
| Control region D-loop | D-loop | 15442 | 16472 | 1031 | forward |

**Table 4.** The mitochondrial genome organization of *Hylobates lar* (HLT973)

| Name | Type | Start | End | Length (bp) | Direction |
|---|---|---|---|---|---|
| tRNA-Phe | tRNA | 1 | 70 | 70 | forward |
| 12S rRNA | rRNA | 71 | 1022 | 952 | forward |
| tRNA-Val | tRNA | 1023 | 1090 | 68 | forward |
| 16S rRNA | rRNA | 1091 | 2648 | 1558 | forward |
| tRNA-Leu | tRNA | 2649 | 2723 | 75 | forward |
| ND1 gene | gene | 2726 | 3681 | 956 | forward |
| tRNA-Ile | tRNA | 3682 | 3750 | 69 | forward |
| tRNA-Gln | tRNA | 3748 | 3819 | 72 | reverse |
| tRNA-Met | tRNA | 3820 | 3887 | 68 | forward |
| ND2 gene | gene | 3888 | 4930 | 1043 | forward |
| tRNA-Trp | tRNA | 4931 | 4998 | 68 | forward |
| tRNA-Ala | tRNA | 5008 | 5075 | 68 | reverse |
| tRNA-Asn | tRNA | 5077 | 5150 | 74 | reverse |
| L-strand rep origin | rep_origin | 5151 | 5184 | 34 | forward |
| tRNA-Cys | tRNA | 5182 | 5247 | 66 | reverse |
| tRNA-Tyr | tRNA | 5247 | 5312 | 66 | reverse |
| COX1 gene | gene | 5325 | 6866 | 1542 | forward |
| tRNA-Ser | tRNA | 6867 | 6935 | 69 | reverse |
| tRNA-Asp | tRNA | 6939 | 7007 | 69 | forward |
| COX2 gene | gene | 7008 | 7691 | 684 | forward |
| tRNA-Lys | tRNA | 7718 | 7787 | 70 | forward |
| ATP8 gene | gene | 7789 | 7995 | 207 | forward |
| ATP6 gene | gene | 7950 | 8630 | 681 | forward |
| COX3 gene | gene | 8630 | 9413 | 784 | forward |
| tRNA-Gly | tRNA | 9414 | 9481 | 68 | forward |
| ND3 gene | gene | 9482 | 9827 | 346 | forward |
| tRNA-Arg | tRNA | 9828 | 9893 | 66 | forward |
| ND4L gene | gene | 9894 | 10190 | 297 | forward |
| ND4 gene | gene | 10184 | 11523 | 1340 | forward |
| ND5 gene | gene | 12074 | 13571 | 1498 | forward |
| ND6 gene | gene | 13572 | 14116 | 545 | reverse |
| tRNA-Glu | tRNA | 14117 | 14185 | 69 | reverse |
| CYTB gene | gene | 14190 | 15334 | 1145 | forward |
| tRNA-Thr | tRNA | 15335 | 15400 | 66 | forward |
| tRNA-Pro | tRNA | 15403 | 15470 | 68 | reverse |
| Control region D-loop | D-loop | 15471 | 16499 | 1029 | forward |

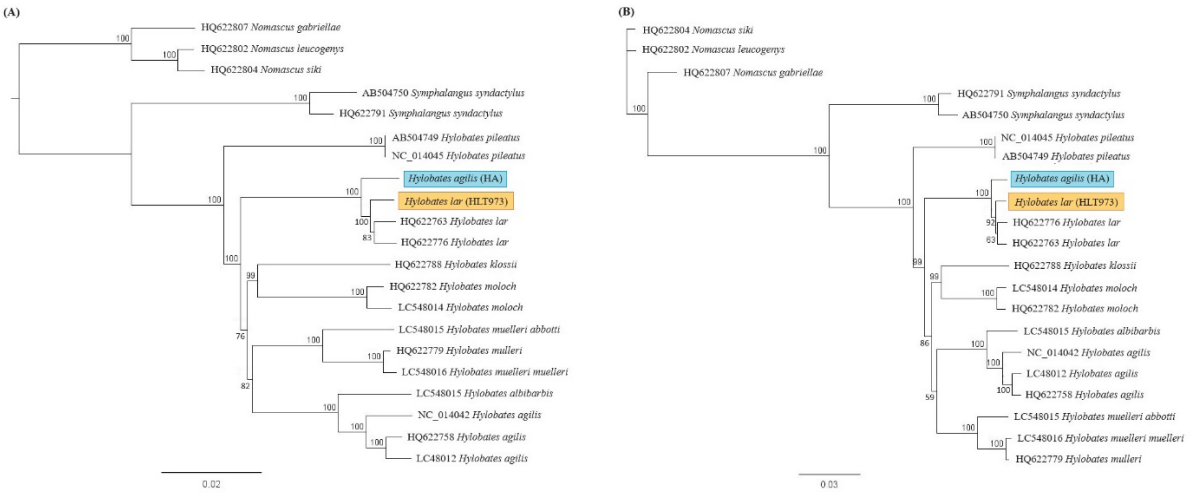


**Fig. 2.** The phylomitogenomic relationships of the Hylobatidae using **(A)** Neighbour-joining (NJ) and **(B)** Maximum Likelihood (ML) methods inferred from the 23 mitogenome sequences with 1000 replicates bootstrap support values.

**Table 5.** Genetic distance (in percentage, %) *H. agilis* and *H. lar* calculated with reference *Hylobates, Symphalangus,* and *Nomascus* mitogenomes using the Kimura 2-paramater model

| | Group/Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| 1. | *H. pileatus* | | | | | | | | |
| 2. | *H. lar* | 6.66 | | | | | | | |
| 3. | *H. muelleri* | 6.66 | 5.86 | | | | | | |
| 4. | *H. klossii* | 6.32 | 6.13 | 5.88 | | | | | |
| 5. | *H. agilis* (HA) | 6.76 | 1.38 | 5.95 | 6.14 | | | | |
| 6. | *H. lar* (HLT973) | 6.63 | 0.96 | 5.90 | 6.18 | 1.41 | | | |
| 7. | *H. agilis* | 7.26 | 6.32 | 5.87 | 5.92 | 6.49 | 6.43 | | |
| 8. | *H. albibarbis* | 7.05 | 6.37 | 5.95 | 6.15 | 6.48 | 6.34 | 2.92 | |
| 9. | *H. moloch* | 6.45 | 6.09 | 5.61 | 5.25 | 6.19 | 6.12 | 6.14 | 6.17 |

Both individuals of gibbons (HA and HLT973) used in this study were surrendered confiscated individuals and were brought to the NWRC at Sungkai, Perak. Thus, the provenance of both individuals *H. lar* and *H. agilis* are unknown. However, the location of the surrendered and confiscated for both individuals is situated in Johor, Malaysia. Based on the phylogenetic trees, the *Hylobates* group established a monophyletic clade of its own, confirmed by a high bootstrap value of 100%. However, notably with our sample, *H. agilis* (HA) is clustered together with the *H. lar* group instead of the reference group of *H. agilis* from GenBank. It is presumed that individual *H. agilis* (HA) is possibly a hybrid species. A similar paraphyletic topology in the mitogenome tree between *H. lar* and *H. agilis* was also reported by Matsudaira and Ishida (2021) based on samples derived from captivity. Further analysis of the study using Cyt *b* discovered that *H. agilis* is closely related to the Sumatran *H. lar vestitus*. The similarity of genetic materials between *H. agilis* and *H. lar vestitus* was reported due to introgression events. Additionally, introgression among other *Hylobates* species (*H. lar* and *H. pileatus*) was also observed in the ongoing hybrid zone (Matsudaira *et al*., 2013). A hybrid is a progeny that arises from sexual reproduction that combines the traits of two organisms of different varieties, species, or genera. The natural process of interbreeding between individuals of genetically divergent taxa may happen at various points during the evolutionary history of the diverging lineage (Cortés-Ortiz *et al*., 2019). Introgression occurs as these hybrids mate with a similar genetic parental type or with one of their parents. Additional primate studies that researched hybrid species include macaques by Kanthaswamy *et al*. (2008, 2009), howler monkeys by Baiz *et al*. (2018), and gibbons by Matsudaira *et al*. (2022).

Reconfirmation of the hybridization status of *H. agilis* in this study is necessary before the translocation of the individual back into its original habitat. The captive gibbons must be confirmed to be pure individuals of the same taxon as those that naturally occur in the area where they will be released. Therefore, taxon identity has to be clarified and confirmed to minimize the impact on the gene flow of the species in the wild (Aifat *et al*., 2021). Apart from that, it is also important to avoid artificial hybridization in rescue centers (Thinh *et al*., 2010). It might be a challenge to accurately identify gibbons taxonomically based on vocal data or pelage colors (Moonick, 2006). In this regard, mtDNA

analysis is a valuable tool to do species identification of these gibbons (Thinh *et al*., 2010). Thus, the mitogenome data of this study gave new insights into the present taxonomic status of captive gibbons in Malaysia apart from relying on their morphological aspects in identifying the species. Further species confirmation on captive gibbons is suggested to be verified with microsatellite analysis with a Delta K method as described by Evanno *et al*. (2005).

## CONCLUSION

Both *H. lar* and *H. agilis* were successfully sequenced and annotated and formed a well-supported distinct monophyletic clade within the *Hylobates* group. Our present mitochondrial sequencing data is crucial as a reference database for taxonomic classification, evolutionary research, as well as conservation and management of gibbons in captivity. Moreover, it is strongly advised for further research on the captive gibbons in Malaysia to conduct a microsatellite study to further clarify the hybridization status of *Hylobates*.

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## ETHICAL STATEMENT

This study was approved by the Department of Wildlife and National Parks (PERHILITAN), Peninsular Malaysia, Malaysia under research permit JPHL&TN(IP):100-34/1.24 Jld 19 (14.4).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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