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Novel primers of Cyt b sequencing for a non-invasive genetic assessment of the Silvery Gibbon (*Hylobates moloch*)

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Abstract. *Bagasta AR, Sunarto, Susilowati A, Suratman, Mansor MS, Priyono DS, Karyanto P. 2026. Novel primers of Cyt b sequencing for a non-invasive genetic assessment of the Silvery Gibbon (*Hylobates moloch*). Biodiversitas 27 (1): d270101. https://doi.org/10.13057/biodiv/d270101*. Reliable primers for genetic variation assessment of the silvery gibbon (*Hylobates moloch*) are still unavailable. This study aims to design novel primers for the silvery gibbon's complete Cytochrome b (Cyt b) to facilitate a non-invasive genetic variation assessment. Three primer pairs targeting different regions of Cyt b were designed using Primer3Plus and validated through in-silico validation by adjusting a melting temperature (Tm) between 57-63°C and a GC content of 45-65%, and in vivo validation using Sanger sequencing. The primers were optimized for annealing temperature. The optimum annealing temperatures used in this research were 55°C for T1 and T3, and 52°C for T2, and were tested against DNA from ten gibbons' feces collected from the northwestern part of the Dieng Plateau. The gel electrophoresis results showed the amplification success, indicated by the DNA band visualization above 900 bp. Our primers successfully amplified the target, producing 625-1068 bp. De novo assembly on the obtained sequences yielded a range of fragment lengths of 1857-1950 bp and successfully assembled the complete Cyt b gene of 1140 bp. Single-nucleotide polymorphisms (SNPs) were readily detected at sites 67, 222, 234, 271, 286, 364, 372, 378, 501, 563, 918, and 991 (12 sites) along the Cyt b gene of the ten samples of silvery gibbons. We also successfully observed five haplotypes amongst the entire sample. This success indicates that our primers effectively sequence the Cyt b gene of the silvery. Our primers perform a highly resolution ability to assess variation amongst populations, making them a valuable tool for non-invasive population genetic assessment supporting the silvery gibbon's conservation program.

Keywords: Conservation genetics, Cyt b, fecal DNA, haplotype diversity, *Hylobates moloch*, primer design

INTRODUCTION

The silvery gibbon or *Hylobates moloch* (Audebert, 1798) is an endangered small ape endemic to Indonesia (Nijman 2020). This arboreal primate mostly lives in isolated and unprotected forested areas in West and Central Java (Andayani et al. 2001), which are experiencing excessive loss and degradation, encouraging stakeholders to perform conservation efforts for this primate (Widyastuti et al. 2023). Deforestation and habitat degradation have fragmented the remnant habitat of the silvery gibbon, causing them to live in isolated groups with corridor disruption that may prevent group interactions and genetic exchange (Clarke et al. 2024; Gatti 2025). Such habitat interference can lead to breeding depression (Stoffel et al. 2021) when the gibbons interbreed with closely related siblings, it decreases the genetic variation within the population (Kuipers et al. 2021), decline their gene pool heterozygosity (Hill et al. 2022), and increase their susceptibility to genetic drift and bottleneck effect (Wang et al. 2019) causing the silvery gibbon population to face extinction vortex. Unarguably, the inbreeding phenomenon in the silvery gibbon sharpens their population's susceptibility to extinction by reducing genetic variation (Oyarieme et al. 2024). This phenomenon highlights an urgent need for genetic variation studies to provide data for the existing population's genetic condition (Hoffmann et al. 2021). Such a critical study can reveal a profile of the silvery gibbon's gene pool, providing science-based data for their conservation in the linkage between habitat loss and degradation, and the existing population (McDonough and Connallon 2023). The study may include examination of single-nucleotide polymorphisms (SNPs) (Robert and Pelletier 2018) and haplotype diversity (Hd) (Fan et al. 2024) that are important in research regarding conservation genetics. Additionally, since the IUCN status of the gibbon is endangered, such genetic variation assessment should involve a non-invasive sampling technique. This method can enhance data collection opportunities, allowing more frequent sampling without directly impacting the primate.

Since genetic variation studies depend on a highly effective primer with high throughput, developing a specific

and efficient primer is urgently needed for several reasons. Universal or general primer can be inappropriately used in a non-invasive genetic variation assessment because it focuses on the desired conserved region across the sequence of many closely related taxa (Oklander and Soto-Calderón 2024). Indeed, focusing on a shared region across taxa makes this primer unable to specifically recognize and amplify sites or regions that have changed due to SNPs and mutations. The universal primer may also be unable to address the challenge of degraded DNA in the non-invasive sample due to the low quantity and quality of the DNA from the samples (Piel et al. 2022), while this kind of indirect sampling is preferred due to the endangered conservation status of the silvery gibbon. Therefore, there is a need to design a highly effective and specific primer that targets regions capable of facilitating genetic variation assessment, for example, COI (Jackson and Nijman 2020) and Cyt b (Naidu et al. 2012).

One of the genetic markers involved in the variation assessment is Cyt b. The Cyt b gene, located in the mitochondrial DNA, has been widely used as a marker for evaluating genetic variation due to its highly variable sequence. This variability allows the marker to effectively detect genetic variation within the population and facilitate further analysis explaining the linkage between population dynamics and environmental changes, and pressure (Davidović et al. 2022). A previous study on primer design and genetic variation assessment of the silvery gibbon was conducted by Andayani et al. (2001). The primer designed by the author produces only 992 bp, including both Cyt b and 12S genes. Designing a more effective primer producing a longer Cyt b sequence is needed to ascertain a more valid and reliable result for genetic variation measurement. This study aims to develop effective and reliable primers to sequence the Cyt b gene of the silvery gibbon for non-invasive genetic variation analysis. The results of this study will significantly contribute to sequence production and availability in the GenBank, thereby supporting proper analysis for the development of effective conservation strategies and management of the silvery gibbon.

MATERIALS AND METHODS

Fecal sampling location

A non-invasive study to collect DNA samples was conducted from the silvery gibbon (*Hylobates moloch*) in their remaining habitat in the Dieng Plateau. Ten fresh fecal samples were collected from the secondary forest of the northwestern part of Dieng Mountain, Central Java, Indonesia (Figure 1).

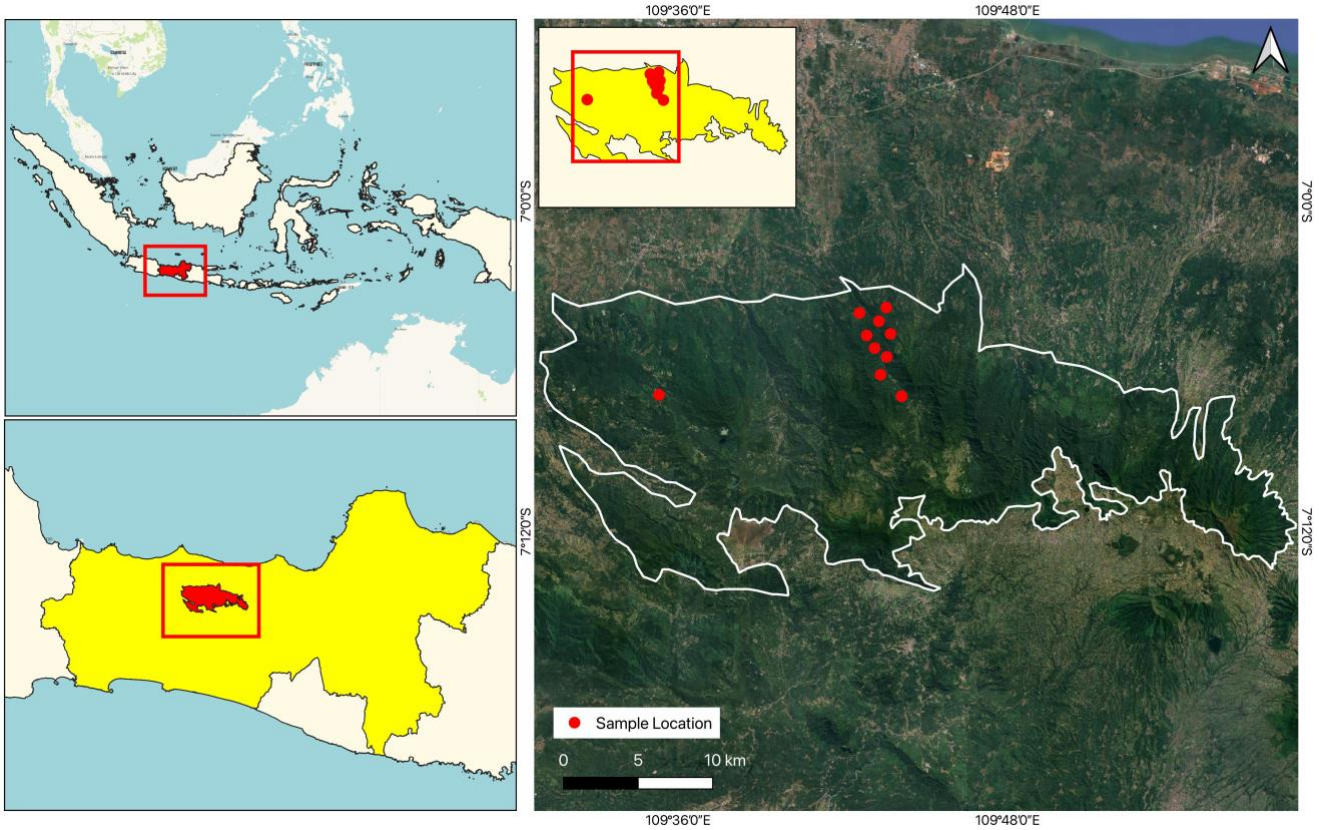


Figure 1. Map of fecal sampling location in the Northwestern part of Dieng Mountain, Central Java, Indonesia

Primer design and specificity check

Our research methodology is focused mainly on designing and validating a reliable primer for non-invasive sequencing of the Cytochrome b (Cyt b) gene in the silvery gibbon (*H. moloch*). The validation process involved in-silico and experimental verification techniques, applying the selected primer to the silvery gibbon's DNA template. The primer design was based on a full mitochondrial genome sequence of the silvery gibbon (*H. moloch*) G81, with the accession number LC548014, provided by the National Centre for Biotechnology Information (NCBI) database, submitted by Matsudaira and Ishida (2021).

Our target gene was the Cyt b, which extends 1,140 base pairs (bp) long. To ensure full coverage, we designed three primer pairs targeting different regions of the gene. These primer pairs were designed to amplify sequences of varying lengths: a minimum of 236 bp for the first region (T1), 767 bp for the middle region (T2), and 137 bp for the remaining region (T3). The spatial arrangement and coverage of these primer pairs in relation to the Cyt b gene are illustrated in Figure 2, providing a visual representation of our experimental design and amplification method. We engaged the Primer3Plus website from https://www.primer3plus.com/ to generate primers ranging from 19 to 25 bp in length, with expected product sizes of 800-1,000 bp for each primer pair. The system settings on the website were adjusted for a melting temperature (Tm) between 57-63°C and a GC content of 45-65%, allowing for an optimum Tm difference, following Zeng et al. (2022). We configured the GC clamp in the Advanced Setting menu to 1 to ensure strong binding between the primer and template, following Chukwuemeka et al. (2020). Some remaining settings in the Primer3Plus system were set as default. We validated ten primer candidates generated by the Primer3Plus based on the oligonucleotide properties of the primers, including secondary structure, hairpins, self-dimers, hetero-dimers, and ΔG values closest to 0 using the Oligoanalyzer tool from https://www.idtdna.com/pages/tools/oligoanalyzer. We performed the in-silico primer validation check using the Primer-BLAST tool on the NCBI website, following Owczarzy et al. (2008). The in-silico primer validation was examined based on the primer's specificity in binding to a particular site using the primer designing tool menu in the https://www.ncbi.nlm.nih.gov/tools/primer-blast/.

Primer annealing optimization and performance test

We conducted multiple PCR reactions with all chosen primer pairs and DNA template extracted from the silvery gibbon Hm3 fecal material while varying the temperature for optimal annealing. The optimized PCR conditions were established based on the optimal annealing temperature, as determined by a PCR gradient optimization assay. The primer performance check was conducted by applying the primer to the DNA template extracted from the fecal sample. We stored the stools in 15 mL tubes containing 96% ethanol following Nsubuga et al. (2004). We extracted the DNA by processing 150 mg of fecal using the ZymoBIOMICS DNA Microprep Kit and following the protocol provided by the manufacturer to produce a DNA template for PCR. The optimum annealing temperatures used in this research were 55°C for T1 and T3, and 52°C for T2. The overall PCR conditions range was conducted with several settings, as shown in Table 1. We employed Geneious Prime Ver. 2025.1.2 to trim and assemble the obtained sequence based on the chromatogram, check the Q-score/Phred to visualize the quantitative value indicating the particular base's confidence level in a base call mode during the sequencing, and assemble the final full Cyt b gene.

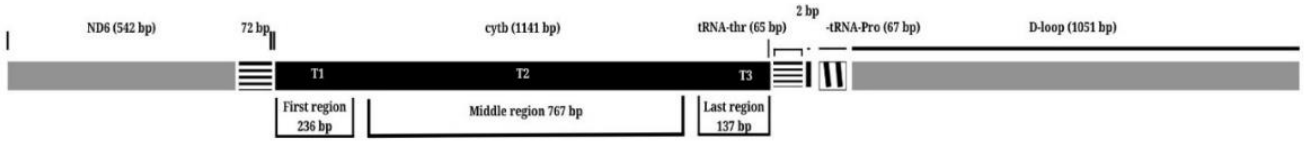


Figure 2. Target regions of the Cyt b gene for primer design

Table 1. The PCR condition range during the primer performance test

Stage	T1	T2	T3
Pre denaturation	95°C, 3 minutes	95°C, 2 minutes	95°C, 3 minutes
Denaturation	95°C, 15 seconds	95°C, 15 seconds	95°C, 15 seconds
Annealing temperature	55°C	52°C	55°C
Elongation	72°C, 45 seconds	72°C, 15 seconds	72°C, 45 seconds
Cycle	35 cycles	35 cycles	35 cycles
Post elongation	72°C, 5 minutes	72°C, 3 minutes	72°C, 5 minutes
Holding	4°	4°	4°

RESULTS AND DISCUSSION

Primer design validation

This comprehensive research method has performed properly, engaging primer validation using both an in-silico approach and experimental verification by applying the primers to the silvery gibbon's DNA template. These two kinds of methods successfully ensured the reliability and specificity of our selected primers. In the in-silico check, after we carefully examined the values of Tm, GC%, hairpin and dimer to prevent the possibility of loop formation and dimer, while considering to scrutinize the optimal product size, we identified the best three primer pairs recommended by Olygoanalyzer targeting the Cyt b gene namely F: TCCCCCTCATAAATCACCCAAC, R: ATTACTGTGGCTCCTCAGAAGG for the initial region of the Cyt b gene (T1), F: TTTCTCCTCAGTAGCCCACATC and R: CTCCGATTCATGTGAGAGTCAG targeting the middle region (T2), and F: CAATCCCTTAGGCATCTCCTCC and R: GCTGGTTGGTTTGTATGTCGTG for the last region of the gene (T3). Oligoanalyzer value results by which the primers were selected are given in Table 2.

The optimization of the annealing temperature for amplifying the Cyt b gene in the silvery gibbon resulted in obtaining suitable sequence data across a range of thermal conditions, from 50°C to 59°C. This demonstrates the robustness and effectiveness of the polymerase chain reaction (PCR) protocol employed in our molecular genetic research. This range of optimum temperature performed properly during the PCR protocol. This temperature range enhances flexibility in PCR experimental conditions, where researchers can choose from a wider range of annealing temperatures, allowing easier optimization and settings for different laboratory conditions. This range also indicates that the primers designed to sequence the Cyt b gene of the gibbons are well designed and have high specificity to their target sequence. A 50-50.4% GC content range is generally considered good for most organisms. This range falls within the typical GC content observed in many eukaryotic genomes, including primates. This GC content range is appropriate for efficient PCR amplification and primer design. The hairpin's negative free energy value in the above Table 2 indicates that the process occurs spontaneously under standard conditions. The stability of this hairpin structure is important because it implies that the DNA sequence has a natural tendency to fold into a particular configuration. The results of our research revealed self-dimer values ranging from -1.47 to -4.57. This range indicates varying degrees of potential self-complementarity among the sequences analyzed. Lower negative values imply stronger self-dimer tendencies, with -4.57 representing the most pronounced self-complementarity observed in our data. Conversely, the -1.47 value indicates the weakest potential for self-dimer formation among the examined sequences. These findings provide insights into the stability and specificity of the molecular interactions within this study, which positively influence primer design, PCR efficiency, and other applications sensitive to self-complementarity.

We scrutinized the effectiveness of our designed primers through experimental validation using gibbon fecal samples, employing gel electrophoresis for visual confirmation. The experimental results demonstrated that all tested primers successfully produced amplicons within the anticipated size range of 800 to 1,000 base pairs. This product was clearly shown by the gel electrophoresis visualization in Figure 3, which comprehensively represented the amplicon sizes across all tested primers. The consistent amplification observed across a range of annealing temperatures showed the robustness and specificity of the PCR protocol and primer design in effectively targeting and amplifying the targeted Cyt b sequence. Since all tested annealing temperatures resulted in an appropriate performance in amplifying the Cyt b gene of the silvery gibbon, this study recommends annealing temperatures of 55°C for primers T1 and T3, and 52°C for primer T2 in all experiments for further primer performance under standardized conditions.

The PCR condition of the designed T1, T2, and T3 primers demonstrated their effectiveness in the experiment test on ten fecal samples of *H. moloch* (Table 3). The product sizes estimated using the OligoAnalyzer tool closely match the lengths of sequences yielded from the primer performance test, providing a high level of confidence in the accuracy of our experiment. This match between predicted and actual product sizes indicates that the primers function as intended in sequencing the correct target region. Indeed, the effectiveness of primers is shown by their ability to bind to precise sites within the DNA. Our research shows that the selected primer pairs bind at appropriate sites to amplify the targeted sequences effectively. This precise binding allows for efficient amplification of target sequences. The successful DNA amplification from stool samples is noteworthy, as stool samples often contain degraded DNA, making amplification difficult.

Table 2. In-silico properties of the chosen primer pairs according to OligoaAalyzer results

No		Sequence	Product size	Tm (°C)	GC %	Hairpin (ΔG kcal/mole)	Self-dimer (ΔG kcal/mole)	Hetero dimer (ΔG kcal/mole)
T1	F	TCCCCCTCATAAATCACCCAAC	899	56.7	50	0	-1.47	-5.02
	R	ATTACTGTGGCTCCTCAGAAGG		56.3	50	-1.37	-4.67	
T2	F	TTTCTCCTCAGTAGCCCACATC	833	56.3	50	-0.08	-3.14	-3.17
	R	CTCCGATTCATGTGAGAGTCAG		54.8	50	0.12	-1.47	
T3	F	CAATCCCTTAGGCATCTCCTCC	910	57	54.5	-1.23	-4.67	-3.9
	R	GCTGGTTGGTTTGTATGTCGTG		56.1	50	-0.24	-3.61	

Sequence output and Phred quality

The forward and reverse primer pairs for T1, T2, and T3 produced sequences ranging from 625 to 1,068 base pairs (bp). Sequencing results for *H. moloch* using Cyt b primers on the Sanger sequencing platform produced high-quality output. The obtained sequences showed clear, definitive peaks with minimal background noise, with an appropriate Phred quality score (more than 40 for almost 90% of base calls), indicating effective amplification and sequencing of the target region. The chromatograms showed clear, evenly spaced peaks, allowing accurate base calling along the entire sequence length. These successful results indicate that the selected Cyt b primers were highly effective in amplifying the desired region of the *H. moloch* Cyt b gene. The sequence outputs along with their NCBI accession numbers are presented in Table 4.

Phred quality scores indicated that most sequences were appropriate, as shown by the light blue chromatogram in the Geneious visualization of a representative sample (Hm3) in Figure 4. An appropriate value of Phred score is associated with a lower probability of base calling errors. This higher accuracy results in more reliable sequence information, reducing the likelihood of misinterpretation or incorrect conclusions in downstream analyses. Furthermore, a high-quality score allows researchers to confidently identify and characterize genetic variations, including single-nucleotide polymorphisms (SNPs).

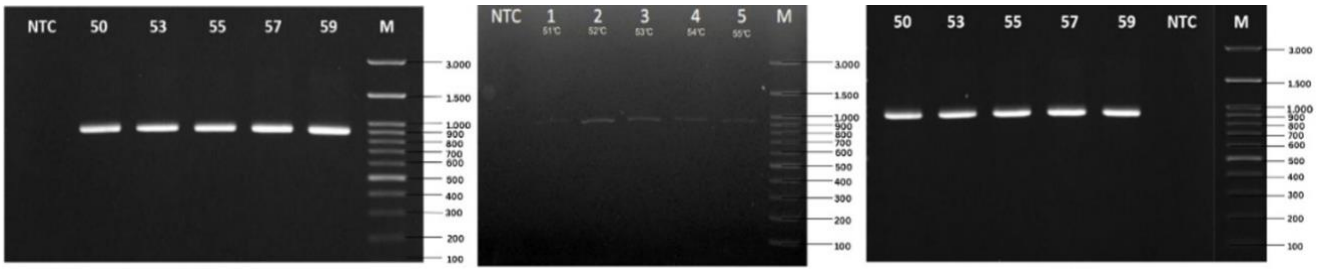
The de novo assembly of sequences generated from the T1, T2, and T3 primer pairs successfully produced a diverse range of sequence sizes, which proved suitable for the comprehensive assembly of the full Cyt b gene. The detailed results of these assemblies, including sequence lengths and coverage metrics, are meticulously summarized in Table 5. This rigorous investigation has unequivocally confirmed the efficacy and specificity of the selected primer pairs in amplifying the target region with high fidelity. This successful amplification and subsequent assembly of the complete Cyt b gene sequence represents a significant achievement, as it provides a robust foundation for conducting further in-depth genetic analyses and phylogenetic studies. The availability of this complete gene sequence will undoubtedly facilitate more comprehensive investigations into evolutionary relationships, population genetics, and molecular systematics within the studied taxa.

Variation profile across Cyt b of ten samples of *Hylobates moloch*

Genetic variation within populations can be effectively evaluated and quantified through the comprehensive analysis of base-pair variations along specific DNA sequences, particularly focusing on Single Nucleotide Polymorphisms (SNPs) within the mitochondrial Cytochrome b (Cyt b) gene, which serves as a valuable molecular marker for assessing genetic diversity and population structure in various organisms (Wang et al. 2019). In the process of elucidating and quantifying genetic diversity within the population under investigation, single-nucleotide polymorphisms (SNPs) proved to be invaluable tools for the researchers, enabling them to meticulously analyze and interpret single-base pair differences along the entire genomic sequence with high precision. Table 6 provides a comprehensive visual representation of the SNPs detected in ten silvery gibbon (*H. moloch*) samples collected from the designated study site, offering a detailed snapshot of the genetic variability present in this primate population. High reliability of base call indicated by Phred quality helped identify base variations at 12 sites along the 1,140 bp Cyt b gene of the silvery gibbon. The high Phred quality scores of the base calls have validated the reliability of these identified variations.

Table 3. The results of the experiment test of the T1, T2, and T3 primers and range of actual size on the performance test on 10 samples

No		Primer	Expected size	Range of actual size from ten samples
T1	F	TCCCCCTCATAAATCACCCAAC	899	868-1068
	R	ATTACTGTGGCTCCTCAGAAGG		625-920
T2	F	TTTCTCCTCAGTAGCCCACATC	833	783-811
	R	CTCCGATTCATGTGAGAGTCAG		801-825
T3	F	CAATCCCTTAGGCATCTCCTCC	910	876-887
	R	GCTGGTTGGTTTGTATGTCGTG		714-934



A **B** **C**

Figure 3. Results of gel electrophoresis showing the in vivo effectiveness of primers in all given annealing temperatures for each primer: A: T1, B: T2, C: T3

Table 4. The output results

Accession number	Sequence output
>Hm1 (Accession Number: PX252238)	ATGACCCCCCTGCGCAAAACCAATCCACTAATAAAACTAATCAACCACTCACTTATCGACCTTCCAACCCCATCCAACAT TTCTATATGATGAAACTTTGGCTCACTCCTAGGCGCCTGCCTAACCCTCCAAATCATTACAGGGTTATTTTTAGCCATAC ACTACACACCAGATGCCTCCACAGCTTTCTCCTCAGTAGCCCACATCACCCGAGACGTAAACTACGGCTGAATTATCCGC TACCTTCACGCCAACGGTGCCTCAATATTTTTTATCTGCCTATTCCTACACATTGGCCGAGGCCTGTACTACGGTTCGTT CCTCTACCTAGAAACCTGAAATATTGGCGTTATCCTCCTACTCGCAACCATAGCAACAGCCTTCATGGGCTATGTCCTCC CGTGAGGCCAAATATCCTTCTGAGGGGCCACAGTAATCACAAACTTACTGTCCGCTGTCCCATACATCGGAACAGACCTA GTCCAATGAGTCTGAGGCGGTTATTCAGTGGATAACGCCACACTCACACGCTTTTTCACCTTTCACTTCATCCTGCCCTT CACTATCACAGCCCTAGCAACCCTACACCTTCTATTCCTACACGAAACAGGATCAAACAATCCCTTAGGCATCTCCTCCC AGCCAGACAAAATCACCTTCCACCCCTACTACACAACCAAAGACATCCTAGGACTATTTCTCCTCCTCCTCATACTAATG AGTCTAGTACTATTCTCACCCGACCTCCTAGGCGACCCAGACAACTACACCCAAGCTAACCCCTTAAACACCCCTCCCCA CATCAAACCCGAATGATATTTTTTATTCGCATACGCAATCCTACGATCCGTCCCTAATAAATTAGGAGGCGTACTAGCCC TCCTACTATCAATCCTCATCCTAGTAATAATTCCTGCACTTCACACAGCTAAACAACAAAGCATGATATTTCGTCCACTA AGCCAACTCACGTACTGACTCCTAGTAATAGACTTACTGACTCTCACATGAATCGGAGGACAACCAGTGAGCTACCCATT TATCACCATCGGGCAAGTGGCGTCCGTACTATACTTCACCACAATCCTAGTACTAATACCAACCGCCTCCCTAATTGAAA ACAAGATACTCAAATGAACCT
>Hm2 (Accession Number: PX252239)	ATGACCCCCCTGCGCAAAACCAATCCACTAATAAAACTAATCAACCACTCACTTATCGACCTTCCAGCCCCATCCAACAT TTCTATATGATGAAACTTTGGCTCACTCCTAGGCGCCTGCCTAACCCTCCAAATCATTACAGGGTTATTTTTAGCCATAC ACTACACACCAGATGCCTCCACAGCTTTCTCCTCAGTAGCCCACATCACCCGAGACGTAAACTACGGCTGAATTATCCGC TACCTTCACGCCAACGGTGCCTCAATATTTTTTATCTGCCTATTCCTACACATTGGCCGAGGCCTGTACTACGGTTCGTT CCTCTACCTAGAAACCTGAAATATTGGCGTTATCCTCCTACTCGCAACCATGGCAACGGCCTTCATGGGCTATGTCCTCC CGTGAGGCCAAATATCCTTCTGAGGGGCCACAGTAATCACAAACTTACTGTCCGCTGTCCCATACATCGGAACAGACCTA GTCCAATGAGTCTGAGGCGGCTATTCAGTGGATAACGCCACACTCACACGCTTTTTCACCTTTCACTTCATCCTGCCCTT CATTATCACAGCCCTAGCAACCCTACACCTTCTATTCCTACACGAAACAGGATCAAACAATCCCTTAGGCATCTCCTCCC AGCCAGACAAAATCACCTTCCACCCCTACTACACAACCAAAGACATCCTAGGACTATTTCTCCTCCTCCTCATACTAATG AGTCTAGTACTATTCTCACCCGACCTCCTAGGCGACCCAGACAACTACACCCAAGCTAACCCCTTAAACACCCCTCCCCA CATCAAACCCGAATGATATTTTTTATTCGCATACGCAATCCTACGATCCGTCCCTAATAAATTAGGAGGCGTACTAGCCC TCCTACTATCAATCCTCATCCTAGTAATAATTCCTGCGCTTCACACAGCTAAACAACAAAGCATGATATTTCGTCCACTA AGCCAACTCACGTACTGACTCCTAGTAATAAACTTACTGACTCTCACATGAATCGGAGGACAACCAGTGAGCTACCCATT TATCACCATCGGGCAAGTGGCGTCCGTACTATACTTCACCACAATCCTAGTACTAATACCAACCGCCTCCCTAATTGAAA ACAAGATACTCAAATGAACCT
>Hm3 (Accession Number: PX252240)	ATGACCCCCCTGCGCAAAACCAATCCACTAATAAAACTAATCAACCACTCACTTATCGACCTTCCAACCCCATCCAACAT TTCTATATGATGAAACTTTGGCTCACTCCTAGGCGCCTGCCTAACCCTCCAAATCATTACAGGGTTATTTTTAGCCATAC ACTACACACCAGATGCCTCCACAGCTTTCTCCTCAGTAGCCCACATCACCCGAGACGTAAACTACGGCTGAATTATCCGC TACCTTCACGCCAACGGTGCCTCAATATTTCTTATCTGCCTATTCATACACATTGGCCGAGGCCTGTACTACGGTTCGTT CCTCTACCTAGAAACCTGAAATATTGGCGTTATCCTCCTACTCGCAACCATAGCAACAGCCTTCATGGGCTATGTCCTCC CGTGAGGCCAAATATCCTTCTGAGGGGCCACAGTAATCACAAACTTACTGTCCGCTGTCCCATACATCGGAACAGACCTA GTCCAATGAGTCTGAGGCGGTTATTCAGTGGATAACGCCACACTCACACGCTTTTTCACCTTTCACTTCATCCTGCCCTT CACTATCACAGCCCTAGCAACCCTACACCTTCTATTCCTACACGAAACAGGATCAAACAATCCCTTAGGCATCTCCTCCC AGCCAGACAAAATCACCTTCCACCCCTACTACACAACCAAAGACATCCTAGGACTATTTCTCCTCCTCCTCATACTAATG AGTCTAGTACTATTCTCACCCGACCTCCTAGGCGACCCAGACAACTACACCCAAGCTAACCCCTTAAACACCCCTCCCCA CATCAAACCCGAATGATATTTTTTATTCGCATACGCAATCCTACGATCCGTCCCTAATAAATTAGGAGGCGTACTAGCCC TCCTACTATCAATCCTCATCCTAGTAATAATTCCTGCACTTCACACAGCTAAACAACAAAGCATGATATTTCGTCCACTA AGCCAACTCACGTACTGACTCCTAGTAATAGACTTACTGACTCTCACATGAATCGGAGGACAACCAGTGAGCTACCCATT TATCACCATCGGGCAAGTGGCGTCCGTACTATACTTCACCACAATCCTAGTACTAATACCAACCGCCTCCCTAATTGAAA ACAAGATACTCAAATGAACCT
>Hm4 (Accession Number: PX252241)	ATGACCCCCCTGCGCAAAACCAATCCACTAATAAAACTAATCAACCACTCACTTATCGACCTTCCAACCCCATCCAACAT TTCTATATGATGAAACTTTGGCTCACTCCTAGGCGCCTGCCTAACCCTCCAAATCATTACAGGGTTATTTTTAGCCATAC ACTACACACCAGATGCCTCCACAGCTTTCTCCTCAGTAGCCCACATCACCCGAGACGTAAAGTACGGCTGAATCATCCGC TACCTTCACGCCAACGGTGCCTCAATATTTTTTATCTGCCTATTCCTACACATTGGCCGAGGCCTGTACTACGGTTCGTT CCTCTACCTAGAAACCTGAAATATTGGCGTTATCCTCCTACTCGCAACCATAGCAACAGCCTTCATGGGCTATGTCCTCC CGTGAGGCCAAATATCCTTCTGAGGGGCCACAGTAATCACAAACTTACTGTCCGCTGTCCCATACATCGGAACAGACCTA GTCCAATGAGTCTGAGGCGGTTATTCAGTGGATAACGCCACACTCACACGCTTTTTCACCTTTCACTTCATCCTGCCCTT CACTATCACAGCCCTAGCAACCCTACACCTTCTATTCCTACACGAAACAGGATCAAACAATCCCTTAGGCATCTCCTCCC AGCCAGACAAAATCACCTTCCACCCCTACTACACAACCAAAGACATCCTAGGACTATTTCTCCTCCTCCTCATACTAATG AGTCTAGTACTATTCTCACCCGACCTCCTAGGCGACCCAGACAACTACACCCAAGCTAACCCCTTAAACACCCCTCCCCA CATCAAACCCGAATGATATTTTTTATTCGCATACGCAATCCTACGATCCGTCCCTAATAAATTAGGAGGCGTACTAGCCC TCCTACTATCAATCCTCATCCTAGTAATAATTCCTGCACTTCACACAGCTAAACAACAAAGCATGATATTTCGTCCACTA AGCCAACTCACGTACTGACTCCTAGTAATAGACTTACTGACTCTCACATGAATCGGAGGACAACCAGTGAGCTACCCATT TATCACCATCGGGCAAGTGGCGTCCGTACTATACTTCACCACAATCCTAGTACTAATACCAACCGCCTCCCTAATTGAAA ACAAGATACTCAAATGAACCT

>Hm5 (Accession Number: PX252242)
ATGACCCCCCTGCGCAAAACCAATCCACTAATAAAACTAATCAACCACTCACTTATCGACCTTCCAACCCCATCCAACAT
TTCTATATGATGAAACTTTGGCTCACTCCTAGGCGCCTGCCTAACCCTCCAAATCATTACAGGGTTATTTTTAGCCATAC
ACTACACACCAGATGCCTCCACAGCTTTCTCCTCAGTAGCCCACATCACCCGAGACGTAAACTACGGCTGAATTATCCGC
TACCTTCACGCCAACGGTGCCTCAATATTTTTTATCTGCCTATTCCTACACATTGGCCGAGGCCTGTACTACGGTTCGTT
CCTCTACCTAGAAACCTGAAATATTGGCGTTATCCTCCTACTCGCAACCATAGCAACAGCCTTCATGGGCTATGTCCTCC
CGTGAGGCCAAATATCCTTCTGAGGGGCCACAGTAATCACAAACTTACTGTCCGCTGTCCCATACATCGGAACAGACCTA
GTCCAATGAGTCTGAGGCGGTTATTCAGTGGATAACGCCACACTCACACGCTTTTTCACCTTTCACTTCATCCTGCCCTT
CACTATCACAGCCCTAGCAACCCTACACCTTCTATTCCTACACGAAACAGGATCAAACAATCCCTTAGGCATCTCCTCCC
AGCCAGACAAAATCACCTTCCACCCCTACTACACAACCAAAGACATCCTAGGACTATTTCTCCTCCTCCTCATACTAATG
AGTCTAGTACTATTCTCACCCGACCTCCTAGGCGACCCAGACAACTACACCCAAGCTAACCCCTTAAACACCCCTCCCCA
CATCAAACCCGAATGATATTTTTTATTCGCATACGCAATCCTACGATCCGTCCCTAATAAATTAGGAGGCGTACTAGCCC
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>Hm10 (Accession Number: PX252247)	ATGACCCCCCTGCGCAAAACCAATCCACTAATAAAACTAATCAACCACTCACTTATCGACCTTCCAGCCCCATCCAACAT TTCTATATGATGAAACTTTGGCTCACTCCTAGGCGCCTGCCTAACCCTCCAAATCATTACAGGGTTATTTTTAGCCATAC ACTACACACCAGATGCCTCCACAGCTTTCTCCTCAGTAGCCCACATCACCCGAGACGTAAACTACGGCTGAATTATCCGC TACCTTCACGCCAACGGTGCCTCAATATTTTTTATCTGCCTATTCCTACACATTGGCCGAGGCCTGTACTACGGTTCGTT CCTCTACCTAGAAACCTGAAATATTGGCGTTATCCTCCTACTCACAACCATGGCAACGGCCTTCATGGGCTATGTCCTCC CGTGAGGCCAAATATCCTTCTGAGGGGCCACAGTAATCACAAACTTACTGTCCGCTGTCCCATACATCGGAACAGACCTA GTCCAATGAGTCTGAGGCGGCTATTCAGTGGATAACGCCACACTCACACGCTTTTTCACCTTTCACTTCATCCTGCCCTT CATTATCACAGCCCTAGCAACCCTACACCTTCTATTCCTACACGAAACAGGATCAAACAATCCCTTAGGCATCTCCTCCC AGCCAGACAAAATCACCTTCCACCCCTACTACACAACCAAAGACATCCTAGGACTATTTCTCCTCCTCCTCATACTAATG AGTCTAGTACTATTCTCACCCGACCTCCTAGGCGACCCAGACAACTACACCCAAGCTAACCCCTTAAACACCCCTCCCCA CATCAAACCCGAATGATATTTTTTATTCGCATACGCAATCCTACGATCCGTCCCTAATAAATTAGGAGGCGTACTAGCCC TCCTACTATCAATCCTCATCCTAGTAATAATTCCTGCGCTTCACACAGCTAAACAACAAAGCATGATATTTCGTCCACTA AGCCAACTCACGTACTGACTCCTAGTAATAAACTTACTGACTCTCACATGAATCGGAGGACAACCAGTGAGCTACCCATT TATCACCATCGGGCAAGTGGCGTCCGTACTATACTTCACCACAATCCTAGTACTAATACCAACCGCCTCCCTAATTGAAA ACAAGATACTCAAATGAACCT

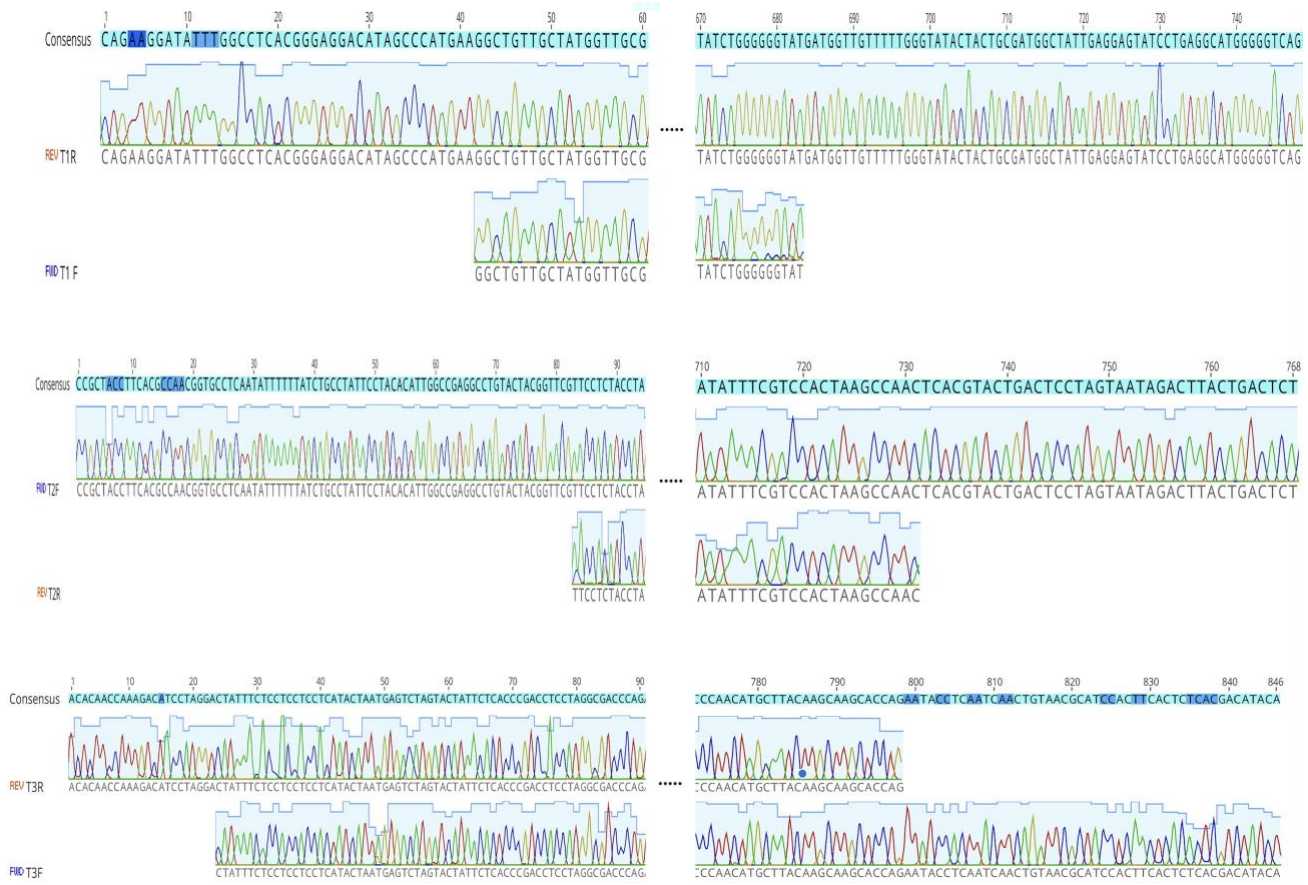

Figure 4. Chromatogram visualization of the Hm3 sample with primers T1 (A), T2 (B) and T3 (C)

Table 5. Quantitative summary of primers' effectiveness (the length of primers and products is presented in bp)

Primer	Expected size	Range of actual size	Success rate indicated by DNA gel electrophoresis	Total range of de novo assembly results	Length of the produced Cyt b	GC content	Sequence variability	Haplotype information
F: TCCCCCTCATAAATCACCCAAC	899	F: 868-1,068	Above 900 bp	1,857-1,950	1,140	0.466	SNIP found at 12 sites along the 1, 140 of the Cyt b (67, 222, 234, 271, 286, 364, 372, 378, 501, 563, 918, and 991)	Three haplotypes were identified
R: ATTACTGTGGCTCCTCAGAAGG		R: 625-920						
F: TTTCTCCTCAGTAGCCCACATC	833	F: 783-811	Above 900 bp					
R: ATTACTGTGGCTCCTCAGAAGG		R: 801-825						
F: CAATCCCTTAGGCATCTCCTCC	910	F: 876-887	Above 900 bp					
R: GCTGGTTGGTTTGTATGTCGTG		R: 714-934						

Table 6. Variation in the Cyt b gene across ten selected samples of *Hylobates moloch*

Sample/Base	67	222	234	271	286	364	372	378	501	563	918	991
Hm1	A	C	T	T	C	G	A	A	T	C	A	G
Hm2	G	.	.	.	.	.	G	G	C	T	G	A
Hm3	.	.	.	C	A	.	.	.	.	.	.	.
Hm4	.	G	C	.	.	.	.	.	.	.	.	.
Hm5	.	.	.	.	.	.	.	.	.	.	.	.
Hm6	.	.	.	.	.	.	.	.	.	.	.	.
Hm7	.	.	.	.	.	.	.	.	.	.	.	.
Hm8	.	.	.	.	.	.	.	.	.	.	.	.
Hm9	G	.	.	.	.	.	A	G	C	T	G	A
Hm10	G	.	.	.	.	.	A	G	C	T	G	A

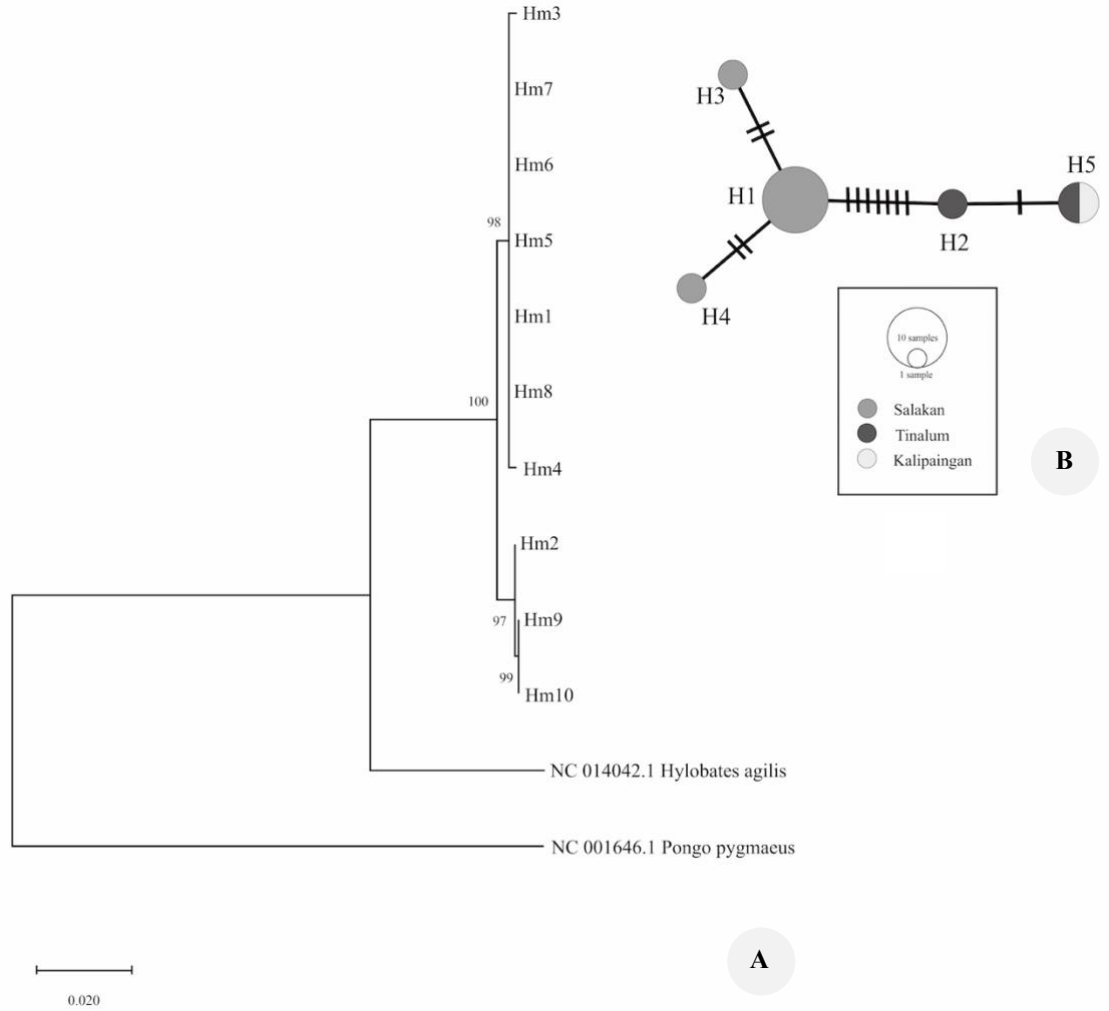

Figure 5. Spanning tree showing the effectiveness of the designed primer in detecting SNIP. Our Cyt b primer identified four haplotypes across the 10 samples of *Hylobates moloch*

The findings derived from our comprehensive research endeavor strongly encourage and empower researchers to confidently and accurately detect genetic variations among the diverse array of silvery gibbon samples, thereby providing invaluable insights into the intricate genetic diversity present within this population. The acquisition of high-quality sequencing data, as evidenced by the robust Phred scores obtained, significantly enhances the accuracy, reliability, and overall integrity of the genetic information collected. This is crucial for understanding the complex genetic structure of this species and unraveling potential conservation implications, which may prove instrumental in formulating effective strategies for the preservation and management of this unique primate population.

Discussion

Primer efficiency to sequence Cyt b of Hylobates moloch

In explaining our amplification success, the appropriate quality and quantity of the Cyt b gene contained in the fecal samples preserved are critical. Effective primer and optimal PCR conditions, as shown in fish metabarcoding research performed by Zhang and his colleagues, are also important (Zhang et al. 2020). The close match between the in-silico and in vivo product sizes indicated that the amplification and sequencing of the Cyt b of the silvery gibbon mitochondrial DNA can be done by the primers effectively due to the complete mitochondrial gene of silvery gibbon in GenBank provided by Matsudaira and Ishida (2021). Indeed, such a complete mitochondrial DNA allows researchers to determine conserved regions and design specific primers targeting the Cyt b gene (Muangkram et al. 2018). Since this region is relatively conserved, it is easier to identify the Cyt b region during the primer design to enhance the specificity of the primer by ascertaining the primer's binding site to the Cyt b region and to reduce the risk of non-specific sequencing to yield accurate sequencing products.

The successful amplification of the silvery gibbon's Cyt b gene within a certain annealing temperature range indicates the robustness of the PCR conditions (Garafutdinov et al. 2020). This wide temperature range indicates that the primers have high specificity in amplifying the Cyt b gene. Consistent amplicon production in the expected size range (800 to 1,000 base pairs) for all primers tested, as confirmed by gel electrophoresis, further validates the effectiveness of the selected primers and the overall PCR strategy. Similar to the study of fish DNA by Min et al. (2023), the consistency of the amplified Cyt b gene across primer and annealing temperature indicates that the amplification performed is target-specific and reliable, allowing further downstream analysis. The arbitrary selection of annealing temperature of 55°C for T1 and T3 and 52°C for T2 in the primer performance test allows us to establish a more appropriate and reliable protocol of Cyt b amplification of the silvery gibbon for the next studies utilizing these primer pairs.

The close match between the projected product size using the OligoAnalyzer tool and the actual sequence length determined by primer pair efficacy testing indicated the accuracy of the in-silico prediction. The successful binding of the primer pair at the target site demonstrated the primer's specificity toward the desired sequence (Kayama et al. 2021). These results indicate that the primer design can effectively recognize suitable regions for amplification in the Cyt b gene of *H. moloch*. The selection of the best primer candidates with appropriate in-silico properties, as recommended by OligoAnalyzer, may have contributed to the success of our primer design. The in-silico parameters given in Table 1 are appropriate to ensure the complementarity of the primer to the target location, minimizing non-specific binding. The chosen melting temperatures of 56.1-57 optimally support the primer to bind to the DNA template, facilitate the polymerase to amplify the target sequence, prevent primer-dimer formation, and help select the range of efficient annealing temperature during the PCR cycle. The GC content value of 50-54.5% in our primers is appropriate and effectively ensures stable binding and efficient amplification. All of the in-silico properties of our primers are also likely designed to avoid self-complementarity and hairpin formation, improving primer performance.

The successful de novo assembly of high-quality sequences from all three primer pairs (T1, T2, and T3) tells the effectiveness of the chosen primers, allowing the researchers to design primers specific to the full Cyt b gene of *H. moloch*. This would significantly help further studies of this primate's genetic, including phylogeny and population genetics analysis. The Phred quality scores indicate high-quality sequences, as displayed by Geneious' light blue chromatogram. They are also essential in minimizing errors in the final assembled gene sequence and guarantee accurate downstream studies (Crossley et al. 2020). The representative sample HO3 shown in Figure 4 represents such high quality and consistency throughout the dataset. The variation in sequence length (625-1,068 bp) may be due to differences in primer pair binding sites along the Cytb gene or changes in genetic structure between samples. This variation in sequence length can actually be advantageous, as it provides a number of overlapping regions that support more accurate gene assembly and allow identification of specific gene segments relevant for a particular study (Giani et al. 2020). In the context of using potentially degraded or low-quality DNA samples, as in this study, reliable DNA extraction and amplification methods are required, as evidenced by the success of obtaining high-quality sequences from all primer pairs.

Sequence quality and SNPs discovery for genetic variation assessment of Hylobates moloch

In the conservation of the endangered silvery gibbon, genetic variation studies are significant. Genetic variation assessment is helpful since populations with low genetic variation are probably susceptible to extinction (Kardos et al. 2021). The absence of specific and reliable primers for sequencing appropriate genetic markers, in addition, also causes difficulties in genetic assessment research that supports strategies for this endangered primate conservation. Our primer design was aimed to fill the gap by providing primers to sequence the Cyt b of the silvery gibbon using a non-invasive approach. We considered that this marker had been widely employed in the genetic variation assessment, as it could evolve at a faster rate than a nuclear gene, thereby being sensitive to evolutionary change (Weaver et al. 2022). This sensitivity helps the Cyt b marker to be applicable in comparison across fine-scale differentiation within species (Nieves et al. 2021). Besides this high sensitivity, the mitochondria in cells are abundantly available, ensuring the sufficiency of Cyt b for analysis, even from degraded samples (Bhoyar et al. 2024). This makes the marker valuable in investigating the genetic diversity and population structure of the silvery gibbon.

We ensure the sequence quality by performing several procedures from fecal collection to proper functional analysis. Our good sequences are obtained through proper fecal collection. Our fecal collections, performed soon after

defecation, reduce exposure time and avoid the risk of DNA degradation from bacterial activity. Our quick fecal collection has improved PCR DNA yield, as collecting samples soon after defecation generally results in higher yields and better-quality DNA for downstream analyses. The high-quality sequences obtained from our PCR are considered appropriate for the next SNPs analysis.

This research designed three novel, effective, and reliable primer pairs to sequence the Cyt b gene of the silvery gibbon, namely F: TCCCCCTCATAAATCACCCAAC, R: ATTACTGTGGCTCCTCAGAAGG targeting the first region of the Cyt b gene (T1), F: TTTCTCCTCAGTAGCCCACATC and R: CTCCGATTCATGTGAGAGTCAG targeting the middle region (T2), and F: CAATCCCTTAGGCATCTCCTCC and R: GCTGGTTGGTTTGTATGTCGTG for the last region of the gene (T3). All these primer pairs amplified the full Cyt b of the silvery gibbon (1140 bp) successfully across multiple fecal samples. Such a finding indicates that they are effective in sequencing the target gene. They can produce high-quality sequences, ensure accurate base calls, and provide a reliable SNP profile to support a reliable assessment of the silvery gibbon genetic variation through a non-invasive approach.

Conservation implications

Our effective novel primer sequencing of the silvery gibbons' full Cyt b gene contributes significantly to the gibbons' monitoring and conservation program. The primers effectively avoided false positive and negative results and performed a high resolution in identifying the genomic variability of the gibbons in their low population density (Theissinger et al. 2023). This effectiveness enables the primers to be utilized in the non-invasive genetic variation assessment to inform particular adaptive responses of the gibbons' population to an existing environmental threat (Wu et al. 2023). Using our primers, the genetic variation of the silvery gibbon can be accurately assessed to reveal the possibility of mating with closely related siblings due to habitat fragmentation (Bercovitch 2023). The effectiveness of our primer to be used as a non-invasive marker is shown by its ability to detect the SNIP along the Cyt b gene, as visualized in Figure 5. Fragmented habitat hinders physical movement and the genetic flow amongst existing populations and causes inbreeding stress (Solórzano-García et al. 2021).

Indeed, further sequence analyses using our novel primers can help identify gene flow patterns and the possibility of interbreeding and population declines, enable quick detection of health risk, and reveal the species' geographic history regarding current habitat loss and degradation. This information aids in evaluating population persistence, which is valuable for conservation and promoting an appropriate conservation program. Such insights help inform captive breeding programs in ex-situ conservation through recommendations to prioritize for genetic variation (Oklander and Soto-Calderón 2024), assist in selecting individuals for translocation efforts (Smith et al. 2023), and enable assessment of management intervention effectiveness, leading habitat restoration initiatives through corridor design and restoration programs in the in-situ conservation program (Ruiz-López et al. 2022).

In conclusion, this designed primer can serve as an effective tool that is valuable to facilitate a better conservation program for the silvery gibbon (*H. moloch*), offering data-driven insights that inform and enhance conservation strategies and decision-making processes. This approach enables stakeholders to implement more effective and targeted interventions, ultimately contributing to the long-term preservation of this endangered primate species and its habitat. Our meticulously designed primer has the potential to facilitate consistent and reliable data collection over an extended temporal period, enabling researchers to track population trends in the dynamic and evolving ecosystem effectively. This approach is particularly valuable for both short-term and long-term monitoring initiatives focused on the population dynamics of the silvery gibbon. By employing non-invasive monitoring techniques, our highly effective primer plays a critical role in maximizing the benefits and outcomes of conservation management strategies specifically tailored to the preservation and protection of this endangered primate species. The implementation of this primer can significantly contribute to the development of evidence-based conservation policies and interventions, ultimately enhancing the long-term survival prospects of the silvery gibbon population in its natural habitat.

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