MOLECULAR MARKERS FOR HYLOBATIDAE FOUND IN THAILAND FOR THE IDENTIFICATION IN WILDLIFE CRIME

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Abstract: In Thailand, there are reports of gibbon species on illegal trade. Gibbons are small apes that are threatened to extinction. They are listed in appendix I of CITES. In this study, molecular markers were developed to use as identification tool for 3 gibbons *Hylobates lar, Hylobates pileatus,* and *Nomascus concolor*. Species-specific PCR primers were designed based on polymorphisms of the mitochondrial cytochrome *b* gene. Experiments demonstrated that optimal magnesium concentration for this primer set was 2.0mM. Increase of annealing temperature in the thermocycling condition clearly increased the specificity of all 3 amplification reactions. Each primer pair can specifically amplify its target species when amplification was carried out under high stringent condition. No PCR product were detected when specificity of these primers were tested against human, dog and pig DNA.

Introduction: Illegal wildlife trade is an ongoing problem in Southeast Asia. Gibbons, which are small monogamous territorial apes, are often found to be illegally traded in Thailand. Gibbons belongs to family Hylobatidae. They are divided into four genera, i.e., Hylobates, Symphalangus, Nomascus and Bunopithecus[1]. Their habitat is in subtropical rain forest in Southeast Asia. Gibbon species are classified by morphological and behavioral characters. These include body size, pelage color, and pelage pattern as well. However, variation of coat color makes identification complicated and difficult to accurately identify based only on fur color, even for specialists[4]. There are about 12-14 gibbon species[2]. All of them are on the brink of extinction[3]. Therefore, gibbons are listed in CITES appendix I, thus trades of these species are illegal, strictly prohibited but permitted only under exceptional circumstances. Thailand is home to four gibbon species, *Hylobates agilis, Hylobates lar, Hylobates pileatus, Nomascus concolor* and *Symphalangus syndactylus*. Live gibbons in trade are for the purpose of pets, zoos, wildlife collections and research. Gibbons are also traded for their meat, or ingredient in traditional medicine as well[5]. If the animal is not alive, identification of gibbon species by morphology or vocalization is then not possible. In this case, molecular method is an alternative for identification of gibbon species. The aim of this study is to develop molecular markers to identify gibbon species and its trace by using PCR-based technique.

Methodology: Hair samples of *Hylobates lar*, *Hylobates pileatus* and *Nomascus concolor* were donated from Dusit zoo. DNA was extracted using 10 random hairs of each species by cutting the hair to small pieces (approximately 0.5 cm), including hair roots and digested in extraction buffer containing 100 mM Tris-HCl pH 8, 100 mM NaCl, 3 mM $CaCl_2$, 2%(w/v) SDS, 40 mM DTT, and 250 µg/ml proteinase K, at 56 °C for 2-5 hours prior purification using the Wizard SV Genomic DNA Purification System kit (Promega, USA). DNA was quantified by spectrophotometry, using NanoDrop™ 100 spectrophotometer (Thermo Scientific).

PCR primers were designed from complete cytochrome b DNA sequences of gibbon species which were obtained from GenBank (accession no. shown in table 1). Sequences were initially aligned using ClustalW2 program (EMBL). A forward PCR primer was designed based on the conserved region among the species. Reverse primers were designed based on DNA polymorphisms to be species specific. The designed forward primer sequence is 5'-GGCCGAGGCCTATACTACGG-3'. Reverse primer sequences are as the following; *N.concolor* 5'-GGTTAGTAGGTTTGCTGCCCA-3', *H.pileatus* 5'-GGTTAGTAGGTTTGCTGCCCA-3', and *H.lar* 5'-CTCGTGTAGGAATAGAAGGTGC-3'. Sizes of PCR products were designed to have different lengths of approximately 312, 585, and 708 bp respectively (see figure 1). PCR was carried out in a total volume of 25 µl. Reaction mixture contained 5 pmol of each primer, 10 mM, 25 mM $MgCl_2$, 5X PCR buffer, 5U of Taq DNA polymerase, DNA template, and sterile water. Thermocycling condition was as follow; initial denaturation at 95 ºC for 4 min, followed by 30 cycles of denaturation at 95 ºC for 30 sec, annealing at 59 ºC (otherwise stated) for 30 sec, extension at 72 ºC for 1 min, and final extension was carried out at 72 ºC for 7 min. PCR products were separated in 2%(w/v) ethidium-bromide stained agarose gel.

Specificity of primers was tested with non-target gibbon DNA and DNA of species which are commonly found in forensic investigation, for example, human, dog and pig.

Table 1. Accession number of gibbons cytochrome *b* DNA sequences

Species	**accession number**
Bunopithecus hoolock	Y13304
Hylobates agilis	AJ10583
Hylobates klossii	AJ10581
Hylobates lar	Y13301
Hylobates moloch	AJ010580
Hylobates muelleri	Y13300
Hylobates pileatus	AJ010582
Symphalangus syndactylus	Y13302
Nomascus concolor	GU321249
Nomascus gabriellae	Y13307
Nomascus leucogenys	Y13306

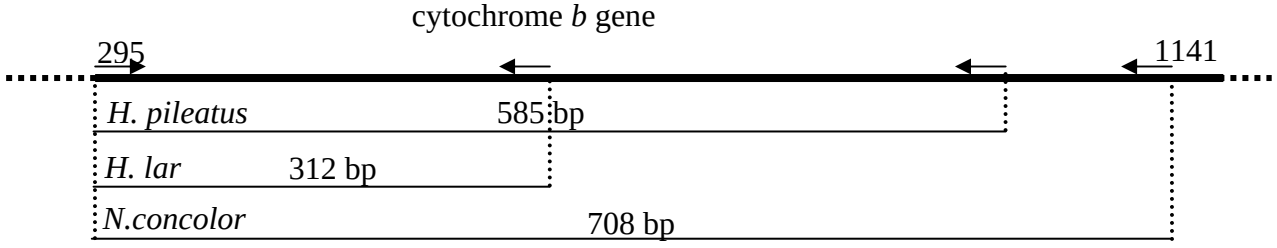
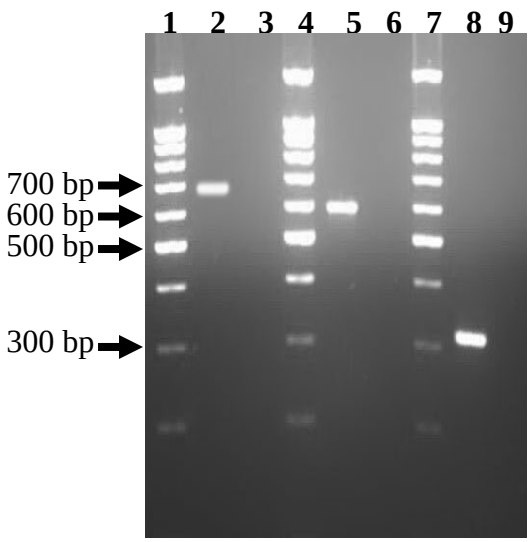


Figure 1. Map depicting position of primers and estimated sizes of PCR products amplified from *H. pileatus*, *H. lar*, and *N. concolor* are 585, 312, and 708 bp.

Results and Discussions:

The designed PCR primers for *N. concolor*, *H. pileatus*, and *H. lar* were initially tested under thermocycling condition at the annealing temperature of 59 ºC to amplify its target DNA. As shown in figure 2, PCR products of the correct sizes were obtained. Sizes of the amplified DNA fragments were approximately 700, 600, and 300 bp for *N. concolor*, *H. pileatus*, and *H. lar*, respectively.



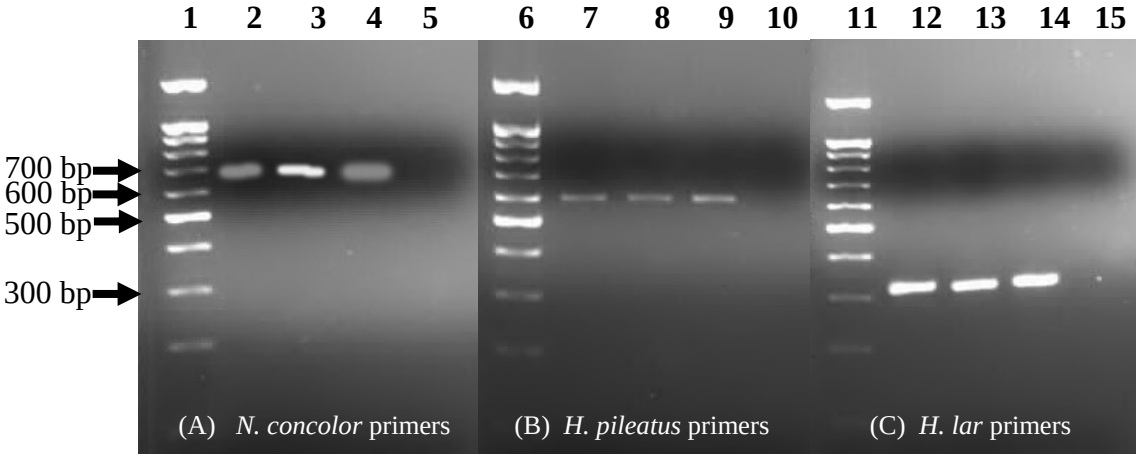
Lane	Description
1	DNA ladder
2	700 bp DNA fragment amplified from *N. concolor* DNA using *N. concolor* primer set
3	Negative PCR control of *N. concolor* primer set
4	DNA ladder
5	600 bp DNA fragment amplified from *H. pileatus* DNA using *H. pileatus* primer set
6	Negative PCR control of *H. pileatus* primer set
7	DNA ladder
8	300 bp DNA fragment amplified from *H. lar* DNA using *H. lar* primer set
9	Negative PCR control of *H. lar* primer set

Figure 2. Primer testing. PCR products of correct sizes were amplified by *N. concolor*, *H. pileatus* and *H. lar* primer pairs when tested with the corresponding target DNA. Sizes were approximately 700 bp (lane 2), 600 bp (lane 5), and 300 bp (lane 8), respectively.

Subsequently, concentration of Mg^{2+} was then varied. Band intensity of PCR products amplified from *N. concolor* primer pair was highest when Mg^{2+} concentration was 2.0 mM (see figure 3). There was no difference in intensity of DNA bands when Mg^{2+} concentrations were varied for *H. pileatus*, and *H. lar* primer pairs. Therefore, amplification reaction was carried out using Mg^{2+} concentration of 2.0 mM.

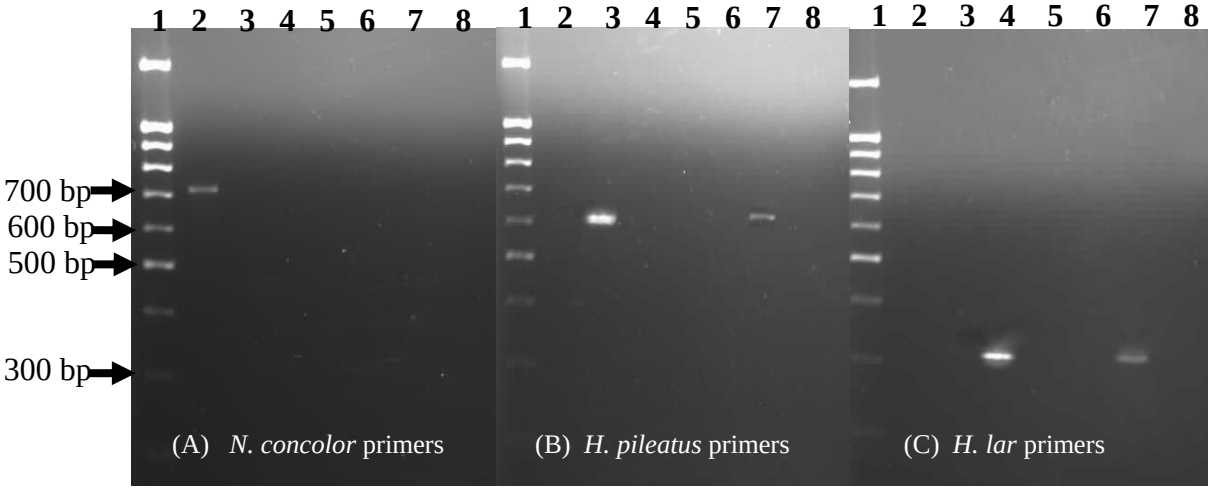
Specificity of *N. concolor*, *H. pileatus*, and *H. lar* primer pairs were then tested with DNA samples of the other 2 non-target gibbon species and 3 other animal species that are commonly found or run possibility of contamination in a forensic investigation, i.e. human, dog and pig. As shown in figure 4, *N. concolor* primer pair did not amplified any DNA sample of the non-target species. For *H. pileatus* and *H. lar* primer pairs, no amplification product was obtained from the non-target gibbon species, human, and dog DNA samples. However, these 2 primer pairs amplified pig DNA (figure 4A and 4B, lane 7). Although, amplification product obtained from pig DNA were approximately the same sizes as PCR products of *H. pileatus* and *H. lar*, intensities of the pig's bands were fainter than the target DNA bands. To increase specificity of PCR, the stringency of reaction was increased by conducting PCR at higher annealing temperatures; 60ºC, 62ºC, 64ºC, 66ºC and 68ºC. As showed in figure 5A, amplification products were obtained from both *H. pileatus* and pig DNA samples when annealing temperatures were 60ºC and 62ºC. No PCR product was detected when using *H. pileatus* primer pair to amplify pig DNA using annealing temperatures of 64ºC, 66ºC and 68ºC. No PCR product was detected when using *H. lar* primer pair to amplify pig DNA at annealing temperatures of 66 and 68ºC. Results suggest

that species-specific detection for *H. pileatus* could be carried out using annealing temperature of 64, 66 and 68ºC, while species-specific detection for *H. lar* detection could be carried out using annealing temperatures of 66ºC and 68ºC.



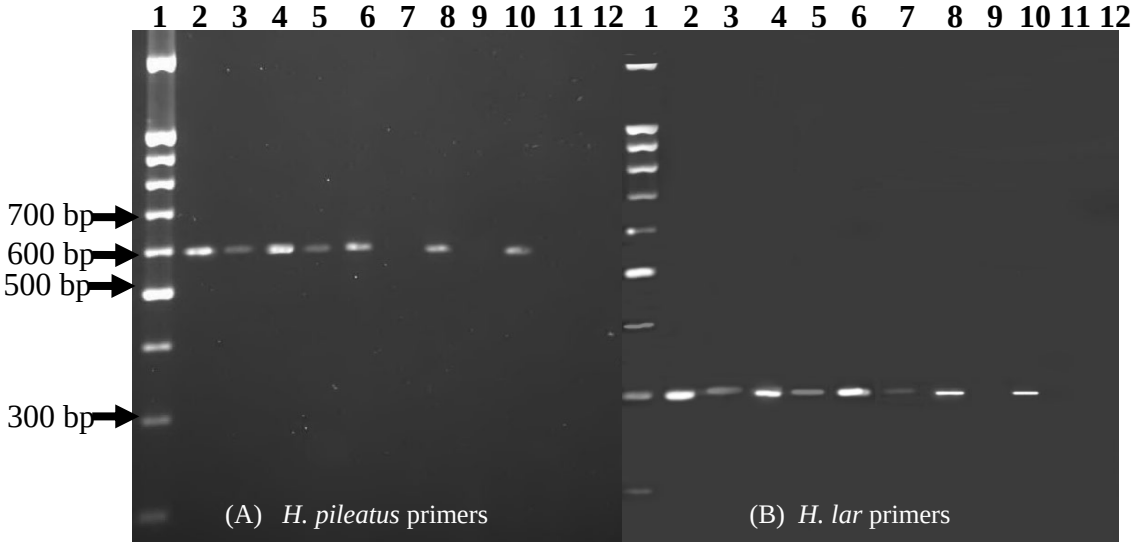
Lane	Description	Lane	Description
1,6,11	DNA ladder		
2,7,12	Mg^{2+} concentration was 1.5 mM	4,9,14	Mg^{2+} concentration was 2.5 mM
3,8,13	Mg^{2+} concentration was 2.0 mM	5,10,15	Negative PCR control

Figure 3. Optimization of Mg^{2+} concentration in PCR. Three concentrations of Mg^{2+} were tested 1.5, 2.0, 2.5 mM. For the *N. concolor* primer pair (figure A), intesity of DNA band was highest when Mg^{2+} concentration was 2.0 mM. Intensities of DNA bands amplified from *H. pileatus* (figure B) and *H. lar* (figure C) primer pairs were similar.



Lane	Description	Lane	Description
1	DNA ladder	5	Human DNA
2	*N. concolor* DNA	6	Dog DNA
3	*H. pileatus* DNA	7	Pig DNA
4	*H. lar* DNA	8	Negative control

Figure 4. Specificity test. Specificity of *N. concolor* (figure A), *H. pileatus* (figure B), and *H. lar* (figure C) primer pairs were tested with non-target gibbon DNA samples, human (lane 5), dog (lane6), and pig (lane7) DNA. Amplification was carried out at the annealing temperature of 59 ºC. PCR products of the correct sizes were obtained from DNA of the target species (lane A2, B3, and C4). All three primer pairs did not amplified non-target gibbon DNA as no PCR product was detected in following lanes; 3A, 4A, 2B, 4B, 2C, and 3C. these three primer pairs did not amplify human and dog DNA. However, faint DNA bands were detected when tested with pig DNA.



Lane	Figure A	Lane	Figure B
1	DNA ladder	1	DNA ladder
2	Ta = 60ºC, *H. pileatus*	2	Ta = 60ºC, *H. lar*
3	Ta = 60ºC, pig DNA	3	Ta = 60ºC, pig DNA
4	Ta = 62ºC, *H. pileatus*	4	Ta = 62ºC, *H. lar*
5	Ta = 62ºC, pig DNA	5	Ta = 62ºC, pig DNA
6	Ta = 64ºC, *H. pileatus*	6	Ta = 64ºC, *H. lar*
7	Ta = 64ºC, pig DNA	7	Ta = 64ºC, pig DNA
8	Ta = 66ºC, *H. pileatus*	8	Ta = 66ºC, *H. lar*
9	Ta = 66ºC, pig DNA	9	Ta = 66ºC, pig DNA
10	Ta = 68ºC, *H. pileatus*	10	Ta = 68ºC, *H. lar*
11	Ta = 68ºC, pig DNA	11	Ta = 68ºC, pig DNA
12	Negative control	12	Negative control

Figure 5. Optimization of PCR specificity for *H. pileatus* and *H.lar* primer pairs. PCR was carried out at annealing temperatures of 60, 62, 64, 66 and 68ºC. PCR products of the correct sizes were obtained from DNA samples of the target species at all condition applied. No PCR product were detected from pig DNA when amplification reactions were carried out at 64, 66 and 68ºC using *H. pileatus* primer pair (figure 5A, lane7,9,11), and 66 and 68ºC using *H. lar* primer pair (figure 5B, lane9,11).

Conclusion: Results demonstrated that the three species-specific primers designed based on the cytochrome B gene sequence can specifically amplify DNA of the 3 target species in family Hylobatdae, i.e. *N. concolor, H. pileatus , H lar* . The optimal concentration of magnesium chloride for these primer sets was 2.0mM. Annealing temperatures that allows specific amplification were 59 °C for *N. concolor* primer pair, at least 64 ºC for *H. pileatus* and at least 66°C for *H. lar*. Specificity tests show that these primer sets did not amplify non-target species, which include human, dog and pig DNA. Species-specific primer pairs developed in this study would benefit forensic identification of *N. concolor, H. pileatus , H lar* in wildlife illegal trade in Thailand.

References:

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