GENETIC DIVERSITY OF WHITE-HANDED GIBBON *HYLOBATES LAR* (LINNAEUS, 1771) AT YWAR KAING KAUNG VILLAGE IN DAWNA MOUNTAIN RANGE CORRIDOR*

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Abstract

The white-handed gibbon or *Hylobates lar* is currently classified as Endangered on the IUCN Red List of Threatened Species. Although the total population size is not small, forest fragmentation by human activity has affected the stability of local populations. To uncover the effects of forest fragmentation on the conservation of local white-handed gibbon populations, this study assessed the genetic status of an isolated white-handed gibbon population living in Ywar Kaing Kaung (YKK) village, Kayin State, Myanmar, where 26 individuals were living in nine groups. 631-bp nucleotide sequence consisting of the hypervariable region I (HVR-I) of mitochondrial DNA was analysed. Sequences were determined in nine of 18 samples from adult individuals. Among those, two haplotypes were found. Phylogenetic trees and a haplotype network uncovered that both haplotypes observed in YKK clustered with those of white-handed gibbons living in central Thailand, a subspecies *H. lar entelloides*. Haplotype diversity of the YKK population was low (0.556) compared with those of a white-handed gibbon population in Khao Yai National Park (KYNP), Thailand (0.823) and those of a siamang population in Sumatra, Indonesia (0.886), which suggested the strong bottleneck effect on the YKK population. On the other hand, nucleotide diversity was comparable (0.00357) with that of the KYNP population (0.00238). The low genetic diversity of the YKK population suggested the importance of genetic management at the local population level in white-handed gibbons.

Keywords conservation genetics, *Hylobates lar*, mtDNA, phylogeny

Introduction

Gibbons, or the small apes, belong to the family Hylobatidae within the superfamily Hominoidea. They are found in the various types of forests of Southeast and South Asia. They are arboreal, preferring the upper level of the forest canopy (Hollihn, 1984). In Myanmar, white-handed gibbons (*Hylobates lar*), western hoolock gibbons (*Hoolock hoolock*), eastern hoolock gibbons (*Hoolock leuconedys*) and Gaoligong hoolock gibbons (*Hoolock tianxing* or *Hoolock leuconedys tianxing)* are distributed (Geissmann *et al.*, 2013; Fan *et al.*, 2017). Among those, white-handed gibbons occur only east of the Thanlwin (or Salween) river in the southern part of Myanmar. This includes part of Shan State, Kayah State, Kayin State, Mon State, and Tanintharyi Region (Geissmann *et al.*, 2013). Outside of Myanmar, the species is distributed in Laos (the western side of the Mekong River), a large part of Thailand (except the northeastern region), Peninsular Malaysia, and the northern part of Sumatra, Indonesia (Brandon-Jones *et al.*, 2004; Roos *et al.*, 2014). A small part of Yunnan, China also used to be a distributed zone, but the population has been considered extinct in the last few decades (Grueter *et al.*, 2009). There is no estimate on the total population size of this species; 15,000-20,000 individuals in Thailand (which was based on personal communication, Geissmann, 2007) is the only available estimate so far. Although the exact population size is unknown, their habitat has been decreasing, and thus the species is categorized as Endangered on the IUCN Red List of Threatened Species (IUCN 2022). For many gibbon species, several threats have been recognized, such as habitat loss and fragmentation, habitat degradation, hunting, and illegal trade (Geissmann, 2003; 2007).

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This is also true for gibbons living in Myanmar. Extensive surveys of the hoolock gibbons in Myanmar have revealed that habitat loss and fragmentation were the most serious threats to hoolock gibbons (Lwin *et al.*, 2011; Geissmann *et al.*, 2013). Although there is no detailed report on the status of white-handed gibbons in Myanmar, a similar situation probably exists. White-handed gibbons are legally protected in Myanmar but most of their habitat ranges outside of limited protected areas (Geissmann *et al.*, 2013). Therefore, to conserve endangered white-handed gibbons, it is important to understand the status of this species especially outside of the protected areas. Ywar Kaing Kaung (YKK) village, Kayin State is located at the foot of the western side of Dawna Mountain Range. About 250 ha of fragmented forests exist in the village and the presence of white-handed gibbons has been known. The forests are surrounded and fragmented by crop fields made by the villagers. In Kayin traditions, gibbons are considered respectful animals representing forests, and gibbons are not a target of hunting (Htoo and Grindley, 2010). Therefore, there is no conflict between the gibbons and the villagers. Rather, the major issue on this white-handed gibbon population is the small capacity of the fragmented forests and the isolation, i.e., about 10 km apart from the nearest forest in Dawna Mountain Range. The isolation of animal populations can result in local extinction by stochastic events because of its small local population size. In addition, isolation limits access to mates, which would induce inbreeding. Inbreeding would cause the loss of genetic diversity and expression of maladaptive characteristics related to recessive alleles, i.e., inbreeding depression (Frankham, 2005). Therefore, maintaining the level of genetic diversity is one important issue in conserving a small population of animals. So far, there has been no evaluation of the genetic diversity of white-handed gibbons at a local population level, the assessment of genetic diversity in the YKK village would provide basic information on the genetic diversity of isolated gibbon populations.

In addition, white-handed gibbons are classified into five subspecies, namely *H. lar lar* (Malaysian white-handed gibbons), *H. lar vestitus* (Sumatran white-handed gibbons), *H. lar entelloides* (Central white-handed gibbons), *H. lar carpenteri* (Carpenter's white-handed gibbons), and *H. lar yunnanensis* (Yunnan white-handed gibbons) (Groves, 2001). Some morphological differences such as in pelage colour and hair length between subspecies have been reported (Groves, 1968; 1972; Marshall and Sugardjito, 1986). However, it is difficult to identify the subspecies in captivity only based on the slight morphological differences (Woodruff *et al.*, 2005). A recent study on the cytb gene of mitochondrial DNA (mtDNA) suggested the genetic difference between the five subspecies (Thinh *et al.*, 2010), but because of the limited number of each subspecies analysed in the study, the result was inconclusive. A study focused on the hypervariable region I (HVRI) of mtDNA suggested that *H. lar entelloides* and *H. lar carpenteri* were not distinguishable by mtDNA (Woodruff *et al.*, 2005). YKK village is located at the reported intergrade zone between *H. lar entelloides* and *H. lar carpenteri*, 15°N-17°N (Groves, 1972), and thus it is interesting to know the genetic characteristic of this population by the means of mitochondrial DNA phylogeny, which may contribute the conservation of white-handed gibbons at the subspecies level. Overall, the present research was focused on the following objectives:

- to examine the genetic diversity of the white-handed gibbon population in Ywar Kaing Kaung Village
- to investigate the phylogenetic position of the white-handed gibbons in Ywar Kaing Kaung village among white-handed gibbons.

Material and Method

Study area

YKK village is located at 16°56'14" N and 97°56'12" E and a 2 km^2 area in Pai Kyu sub-township, Hlaing Bwe Township, Hpa An District, Kayin State. Kayin State is bordered by Mae Hong Son, Tak and Kanchanaburi provinces Thailand to the east; Bago Region; Mon State to the west and south; Mandalay Region and Shan State to the north (Fig.1).

Study period

The survey was conducted from January, 2020 to December, 2021.

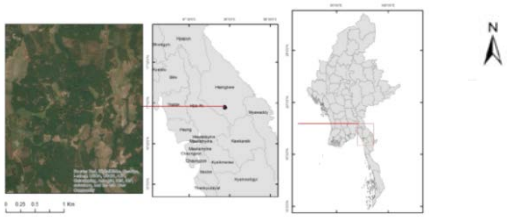

Figure 1 The study area of Ywar Kaing Kaung village

Method and Faecal sample collection

Nine white-handed gibbon groups consisting of 26 individuals, including eight adult males and eight adult females, in the YKK village were the subjects of this study. Faecal samples were immediately collected after observing the defecations from the 18 adult gibbons of the nine groups at YKK village from 2018 to 2019. Each sample was stored in a 50 ml sterile tube containing 30 ml 99.5% ethanol for 24 h, and then transferred into a 50 ml tube filled with 20-30 g silica gel beads (ethanol and silica two-step method, Nsubuga *et al.*, 2004) (Plate 1)

A. White-handed gibbon feces

B. Feces preserved in the ethanol

C. Dried up feces with silica gel beads

Plate 1 Collection of faecal samples in the study area (ethanol-silica two-step method)

DNA Extraction

DNA was extracted with the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) with the following modifications. In the first step, a faecal sample was scraped by a sterile surgical blade and measured 100-200 mg particles by a digital scale. The faecal sample was put into a 2 ml tube, 1 ml inhibit EX buffer was added and mixed by vortex for 1 min. The sample was incubated overnight at room temperature. After this step, experimental procedures were conducted following the manufacturer's protocol. At the last step, 200 µl Buffer ATE was applied on the membrane and incubated for 20 min in order to retrieve the larger amount of DNA into the buffer.

PCR amplification of mitochondrial control region

The present study focused on HVRI of the displacement loop (D-loop) region of mitochondrial DNA (mtDNA) that has been used extensively in molecular evolutionary studies

(Vigilant *et al.*, 1989) and thus has a well-studied mutation rate (Tamura and Nei, 1993; Parsons *et al.,* 1997). Approximately 700-base pair (bp) fragment consisting of the HVRI was PCR amplified and sequenced using the following gibbon-specific primers: 5'-CTTCACCCTCAGCACCCAAAGC-3' (Andayani *et al.,* 2001) and 5'-AAGACAGATACTGCGACATAGG-3' (Matsudaira *et al.,* 2013). PCR was conducted following Matsudaira *et al.* (2013). PCR products were detected by agarose gel electrophoresis and were purified using the PCR Clean-up Mini Kit (Favorgen, Taiwan) following the manufacturer's protocol of the kit.

Sequencing

The purified PCR products were used as templates for direct sequence reactions. The cycle sequencing reaction was carried out in a final volume of 10 µl, containing 2 µl of purified PCR product, 0.32 µl of 10 pmol/µl of primer (the forward or the reverse primer), 0.4 µl of BigDye^{TM} Terminator v3.1, 1.8 µl of 5x Reaction buffer and 5.48 µl of distilled water. The reaction mix was purified by ethanol precipitation. Then, sequencing was operated in ABI 3500 Genetic Analyzer (Thermofisher Scientific, USA).

Phylogenetic tree and haplotype network construction

The sequences of HVRI were imported to MEGA-X (Kumar *et al.*, 2018) and were aligned using the Muscle Sequence Alignment Program (Edgar, 2004). Phylogenetic trees were constructed by maximum likelihood (ML; Felsenstein, 1981) and neighbour-joining (NJ; Saitou and Nei, 1987) methods. Tamura-Nei model (TN93; Tamura and Nei, 1993) was used for the evolution model. Bootstrap analysis was carried out for 1000 replications (Felsenstein, 1985). In the phylogenetic analysis, we included the sequences of white-handed gibbons with known wild origins, and thus subspecies, from previous studies (Table 1). They were *H. lar entelloides* from Khao Yai National Park (KYNP), Thailand (Matsudaira *et al.*, 2013; 2022; Markviriya *et al.*, 2022) and Kaeng Krachan National Park (KKNP), Thailand (Matsudaira *et al.,* 2022), and *H. lar lar* from National Wildlife Rescue Centre (NWRC), Malaysia (Gani *et al.*, 2021). The sequences from KYNP also included the sequences obtained from some hybrid gibbons between white-handed gibbons and pileated gibbons (*Hylobates pileatus*). We only included typical white-handed gibbon haplotypes. The individuals of NWRC were all captive individuals, but they were originated from wild and all were from Peninsular Malaysia (Gani *et al.*, 2021). In addition to the white-handed gibbon sequences, outgroup sequences of one agile gibbon (*Hylobates agilis*) and one pileated gibbon (Matsudaira and Ishida, 2010) were included. All the sequences were obtained from GenBank (NCBI; National Center Database for Biotechnology Information). To ensure the results of phylogenetic tree analysis, and to look more detailed relationship among the mtDNA sequences of the white-handed gibbons, haplotype network analysis was conducted. A haplotype network based on the minimum-spanning method (Bandelt *et al.*, 1999) was constructed by using PopART (Leigh and Bryant, 2015). In this analysis, only the sequences of white-handed gibbons were used.

Evaluation of genetic diversity

Genetic diversity within each population was estimated by computing haplotype diversity (h; Nei, 1973) and nucleotide diversity (π; Nei, 1987) by using DnaSP (version 6.12.03) (Rozas *et al.*, 2017). The genetic diversity of the gibbon population of YKK was compared with that of other gibbon populations; white-handed gibbons from KYNP (n = 37 adult individuals of 17 groups) (Matsudaira *et al.,* 2018), and siamangs (*Symphalangus syndactylus*) from Bukit Barisan Selatan National Park (BBSNP), Sumatra (n = 15 adult individuals of six groups) (Lappan, 2007). All these sequences were obtained from GenBank, and both haplotype diversity and nucleotide diversity were calculated by referring to the individual haplotypes reported by the

previous studies. In the case of the KYNP population, some individuals showed an mtDNA haplotype introgressed from pileated gibbons (Matsudaira *et al.*, 2013). Because the large sequence difference between white-handed gibbons and pileated gibbons would inflate the nucleotide diversity, these individuals with the pileated gibbon haplotype were excluded from this analysis.

Results

The mtDNA phylogenetic tree and haplotype network

A total of 18 faecal samples were obtained from the 18 adult gibbons (i.e., one sample per individual) in the nine groups. The 631-bp nucleotide sequences of HVRI of the mtDNA were successfully determined in nine of the 18 samples. Two haplotypes (HYKK_1 and HYKK_2) were found among the nine samples. The haplotype HYKK_1 was observed among five individuals (G1F, G2F, G3F, G4F and G4F) and HYKK_2 was observed among four individuals (G5F, G5M, G7M and G8F). Four substitutions were observed between the two haplotypes. The phylogenetic analysis revealed the presence of three distinct clades (Fig. 2). The first clade consisted of all haplotypes from NWRC, Malaysia, which was supported by relatively a high bootstrap value of 81 in the ML tree and 77 in the NJ tree. The second clade consisted of the two haplotypes from YKK, Myanmar, all haplotypes from KYNP, Thailand, and seven haplotypes from KKNP, which was supported by relatively high bootstrap values of 80 in the ML tree and 87 in the NJ tree. The third clade consisted of one haplotype (LC633868: HKK6A) from KKNP. Phylogenetic relationships among the three clades were not well resolved because the position of the third clade was different between ML and NJ trees. While the third clade clustered with the first clade in ML tree, it clustered with the second clade in NJ tree. This ambiguity is consistent with low bootstrap values on the relationships of three clades in both the ML tree (44) and the NJ tree (48). Within the second clade, the two haplotypes from the YKK population did not make their clade. Not only the haplotypes from YKK but also haplotypes from KYNP and KKNP were not distinct from each other in the clade. Similar to the phylogenetic trees, haplotypes from Peninsular Malaysia made one cluster, and haplotypes from YKK, KYNP and KKNP made another cluster (Fig. 2). The haplotype of KKNP which made the third cluster in the phylogenetic trees was located in the middle of the two clusters. The nucleotide difference between this haplotype and the closest haplotype of the Malaysian cluster was nine, and the Myanmar-Thai cluster was 10. This was consistent with the ambiguous position of the third clade in the phylogenetic trees. The haplotype network showed no apparent distinction of the two YKK haplotypes from KYNP and KKNP haplotypes. Only one or three nucleotide differences were observed between YKK haplotypes and the closest KYNP or KKNP haplotype (Fig.3).

Haplotype diversity (h) and nucleotide diversity (π)

In YKK, haplotype diversity was 0.556 and nucleotide diversity was 0.00357. The haplotype diversity of the YKK white-handed gibbon population was lower than that of the KYNP white-handed gibbon population (0.823) and that of the BBSNP siamang population (0.886). On the other hand, the nucleotide diversity was not different from (or slightly larger than) that of KYNP (0.00238), but almost 10 times lower than that of BBSNP (0.03171) (Table 2).

Table 1 The sequences of *Hylobates lar* and outgroup species used in the phylogenetic tree analysis

No.	Species	GenBank Accession No.	Haplotype	Location	Reference
1.	*Hylobates lar*	-	HYKK1	Yawr Kaing Kaung, Myanmar	This study
2.		-	HYKK2		
3.		AB720991	HKY1	Khao Yai National Park, Thailand	Matsudaira *et al.*, 2013
4.		AB720992	HKY2		
5.		AB720993	HKY3		
6.		AB720994	HKY4		
7.		AB720995	HKY5		
8.		AB720996	HKY6		
9.		AB720997	HKY7		
10.		AB720998	HKY8		
11.		AB720999	HKY9		
12.		AB721000	HKY10		
13.		LC633853	HKY12A	Khao Yai National Park, Thailand	Matsudaira *et al.*, 2022
14.		LC633854	HKY13A		
15.		LC633855	HKY14A		
16.		LC633856	HKY15A		
17.		LC633857	HKY16A		
18.		LC633863	HKK1A	Kaeng Krachan National Park, Thailand	Matsudaira *et al.*, 2022
19.		LC633864	HKK2A		
20.		LC633865	HKK3A		
21.		LC633866	HKK4A		
22.		LC633867	HKK5A		
23.		LC633868	HKK6A		
24.		LC633869	HKK7A		
25.		LC633870	HKK8A		
26.		MT302850	A05	Khao Yai National Park, Thailand	Markviriya *et al*., 2022
27.		MT302851	A29		
28.		MT302852	A17		
29.		MT302853	J20		
30.		MZ407482	HLL08	National Wildlife Rescue Center, Malaysia (all individuals were originated from Peninsular Malaysia)	Gani *et al.*, 2021
31.		MZ407483	HLL19		
32.		MZ407484	HLL14		
33.		MZ407485	HLL11		
34.		MZ407486	HLL16		
35.		MZ407487	HLL15		
36.		MZ407488	HLL17		
37.		MZ407489	HLL18		
38.		MZ407490	HLL10		
39.		MZ407491	HLL09		
40.		MZ407492	HLL20		
41.		MZ407493	HLL07		
42.	*Hylobates agilis*	AB504748	-	-	Matsudaira and Ishida, 2010
43.	*Hylobates pileatus*	AB504749	-	-	

Table 2 Comparison of Haplotype diversity (*h*) and Nucleotide diversity (π) with other location

Location	Species	Number of Group	Number of adult individuals analysed	Number of Haplotype	Haplotype diversity (*h*)	Nucleotide diversity (π)	Data source
Ywar Kaing Kaung, Myanmar (YKK)	White-handed gibbons	9	9	2	0.556	0.00357	This study
Khao Yai National Park, Thailand (KYNP)	White-handed gibbons	17	37	10	0.823	0.00238	Matsudaira *et al.*, 2018
Bukit Barisan Selatan National Park, Sumatra (BBSNP)	Siamangs	6	15	8	0.886	0.03171	Lappan, 2007

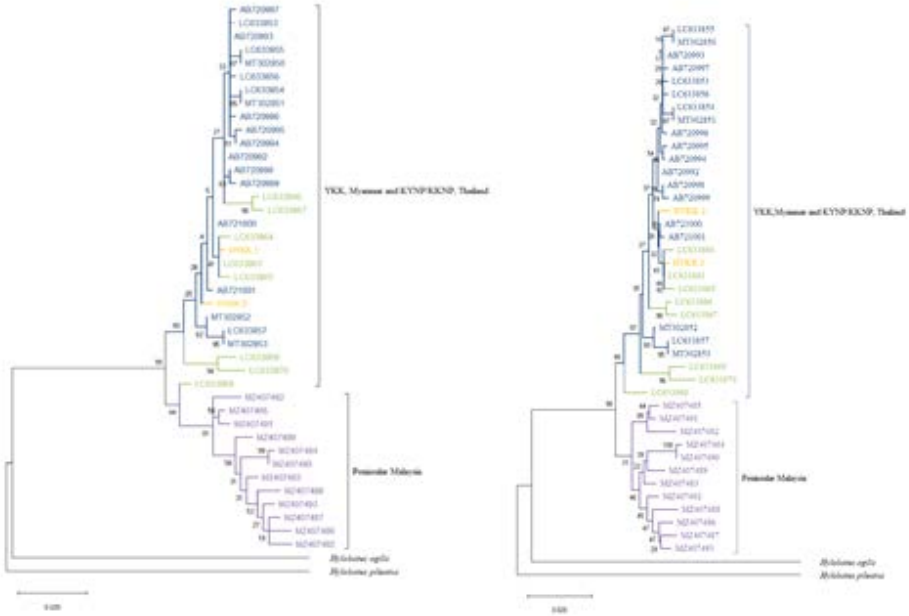

Figure 2 ML and NJ tree constructed from the 631-bp partial mtDNA sequences of *H. lar* in Ywar Kaing Kaung village (orange), Khao Yai National Park(blue), Kaeng Krachan National Park (green), Wildlife Rescue Centre (violet) and two outgroup species. The tree was constructed using Tamura-Nei model (TN93). Bootstrap analysis was carried out 1000 replications.

Figure 3 Minimum joining haplotype network of the 631-bp partial mtDNA sequences of *Hylobates lar* in Ywar Kaing Kaung village (orange), Khao Yai National Park (blue), Kaeng Krachan National Park (green), Wildlife Rescue Centre (violet)

Discussion

Phylogenetic position of the study population within white-handed gibbon

In this study, a total of nine partial mtDNA sequences were successfully obtained. The nine sequences showed two haplotypes in YKK population. Both phylogenetic tree and haplotype network showed that YKK, KYNP and KKNP white-handed gibbons were genetically

not distinguishable at the population level. White-handed gibbons living in KYNP and KKNP have been recognized as subspecies *H. lar entelloides* (Groves, 2001). Therefore, the most plausible subspecies status of YKK population is *H. lar entelloides*. Alternatively, YKK population might be *H. lar carpenteri*, which has been reported to be distributed to northern Thailand and a part of Myanmar. We could not include reference haplotypes from *H. lar carpenteri*, and thus it was not possible to completely deny this alternative hypothesis. However, if YKK population is *H. lar carpenteri* and not *H. lar entelloides*, our results rather oppose this classification, and *H. lar carpenteri* may better to be integrated into subspecies *H. lar entelloides*. The future studies including samples from the northern side such as Shan State, Myanmar, and the northern part of Thailand would solve this question. Regardless of the subspecies status, the genetic similarity between YKK population and KYNP/KKNP populations suggested that this group of white-handed gibbons are widely distributed and originally shared a maternal gene pool.

Genetic diversity among white-handed gibbon in Ywar Kaing Kaung village

The low haplotype diversity of mtDNA in YKK population suggested that this population has experienced a strong bottleneck. This is consistent with the known history that this population was separated from the Dawna Mountain Range population because of the fragmentation of the forests due to anthropogenic activities. Different from the haplotype diversity, the nucleotide diversity was not largely different between YKK and KYNP populations. This is probably because of the small sample size of YKK population and the stochastic process that randomly inherited the mtDNA haplotypes from the gene pool of the source population in Dawna Mountain Range. Nucleotide diversity of mtDNA was largely different between the white-handed gibbon populations and the siamang population. This was probably derived from the species difference. In the case of gibbons, the comparison of nucleotide diversity of mtDNA may be valuable only when it is performed for the same species.
One limitation of this study was that the sequences of only nine of the 18 adult individuals (50%) were determined. Although nine sequences seemed to reflect well the genetic status of the population, the future study should include all adult individuals of the population to precisely evaluate the effect of the forest fragmentation and the isolation on the genetic diversity. This study revealed the quite low genetic diversity in the isolated small population of white-handed gibbons in YKK village. Because of its small population size and the isolation from the neighboring Dawna Mountain Range population, the genetic diversity of the YKK population will decrease rapidly. To maintain the population healthier, the level of genetic diversity should be maintained at a certain level. To accomplish this, the translocation of some animals from the nearby population, or connecting the forest habitat to the source population might be better to planning before this population would locally extinct. The continuous monitoring of the population is important.

Conclusion

The phylogenetic analysis confirmed that the genetic affinity of gibbons in the YKK population does not deviate from other white-handed gibbon populations, and suggested the plausible subspecies status as *H. lar entelloides*. The genetic diversity of the study population was lower than the genetic diversity of the populations in other protected areas. To conserve the YKK white-handed gibbon population, maintaining their genetic diversity is important. For this purpose, translocation of some animals to the population might be required in the future. Keeping monitoring the both ecological and genetic status of the population is inevitable.

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