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Original Article

Genetic diversity and relationships among Lyle's flying fox colonies in Thailand

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ABSTRACT

Lyle's flying fox (*Pteropus lylei*) is a large frugivorous bat found in central Thailand that usually roosts in temples in the middle of towns in close proximity to humans. *Pteropus lylei* is considered a reservoir for Nipah encephalitis viral outbreaks reported in Malaysia and Bangladesh. Thailand is bordered to the south by Malaysia. Information on the genetic diversity and genetic relationships of *P. lylei* is limited; therefore, cytochrome *b* (*cytb*) DNA sequences were used to examine the genetic diversity and genetic relationships of *P. lylei*. In total, 52 *P. lylei* individuals from 10 colonies in central Thailand were analyzed. The study identified 25 unique haplotypes and 43 variable sites among the 52 individuals. The results showed that *P. lylei* had high levels of haplotype diversity (0.949, 25 different haplotypes among 52 individuals) but low levels of nucleotide diversity (0.006). The overall pairwise φ_{ST} was 0.006 ($p < 0.05$). The results indicated that high levels of gene flow occurred among *P. lylei* colonies distributed across central Thailand. The sequence data suggested that the overall *P. lylei* population has high levels of haplotype diversity, which may reflect genetic exchange during *P. lylei* movement. These results will help manage populations and assess the risk of outbreaks of the encephalitis (Nipah) virus carried by Lyle's flying fox.

Introduction

Lyle's flying fox (*Pteropus lylei*), a member of the Pteropodidae family, is found in colonies of 100–1000 individuals in Cambodia, Thailand, Vietnam, and in a small disjunct area in southern China (Bumrungsri et al., 2008). In Thailand, 20 colonies have been identified in 12 provinces, with most of the sites reported in the lower central and eastern regions of the country (Duengkae et al., 2015). The largest known colony in Thailand includes approximately 8000 individuals. *Pteropus lylei* is a reservoir of the Nipah virus (NiV), a new viral strain of the Paramyxoviridae family in Thailand (Wacharapluesadee et al., 2005). Infection by this emerging pathogen causes disease in animals and humans. In the first appearance of NiV in Malaysia, the outbreak was preceded by the occurrence of respiratory illness and encephalitis in pigs and was reported among abattoir workers in Singapore who handled pigs originating from the outbreak regions in Malaysia. NiV may have passed directly from bats to pigs and then to humans (Chua et al., 2002). In Bangladesh and India, human-to-human transmission was observed in several NiV outbreaks (Wacharapluesadee et al., 2005). In Thailand, the presence of NiV has been found year round in *P. lylei* colonies, although there has been no evidence of NiV in humans or domestic animals (Wacharapluesadee et al., 2010). Two NiV strains previously identified circulating in

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Malaysia and Bangladesh were found only in *P. lylei* (Wacharapluesadee et al., 2013). Most daytime roosting colonies in Thailand have been found close to temple areas near humans and orchards. Outbreaks of the NiV, of which *P. lylei* is a carrier, may cause harm to humans. In this study, the population genetic relationships of *P. lylei* were examined to help understand viral transmission associated with outbreaks of the NiV carried by *P. lylei*.

Little was known about the connectivity of *P. lylei* roosting sites until Weber et al. (2015) used a high-resolution global positioning system to evaluate the movement and foraging behavior of *P. lylei*. The authors reported that this species travelled foraging distances between day roosts and night visits.

In addition, Weber et al. (2015) reported that *P. lylei* living in two colonies travelled between the colonies, while the movement of *P. lylei* in 18 other colonies was unknown. Hondo et al. (2010) also investigated *P. lylei* movement and showed that the main roosting site had changed from Wat Kaochang to Chainat Province (*P. lylei* can move among sites in a very short time). Therefore, the goal of the current study was to learn more about the genetic relationships among *P. lylei* populations and the genetic exchange associated with their movement. Previous analyses have used the genetic variability of the mitochondrial region of the cytochrome b (*cytb*) gene as a population genetic marker. It is widely used in diversity studies at the species level but has also been used in some studies at the population level for species such as *Eidolon helvum* (Peel et al., 2013), *Epomops buettikoferi*, *Nanonycteris veldkampii*, *Rousettus aegyptiacus*, and *Epomops franqueti* (Hassanin et al., 2016).

To date, there has been no study published on the genetic diversity or genetic relationships of the *P. lylei* populations in Thailand. The current study used 10 *P. lylei* colonies in which NiV had been found throughout the year (Wacharapluesadee et al., 2010). These colonies corresponded to more than half of the *P. lylei* population in Thailand. We determined the genetic diversity and genetic relationships among these *P. lylei* populations and showed that genetic exchange occurred during movement of this species. These findings will aid in bat population management and will facilitate careful risk assessment of outbreaks of the Nipah virus carried by *P. lylei*.

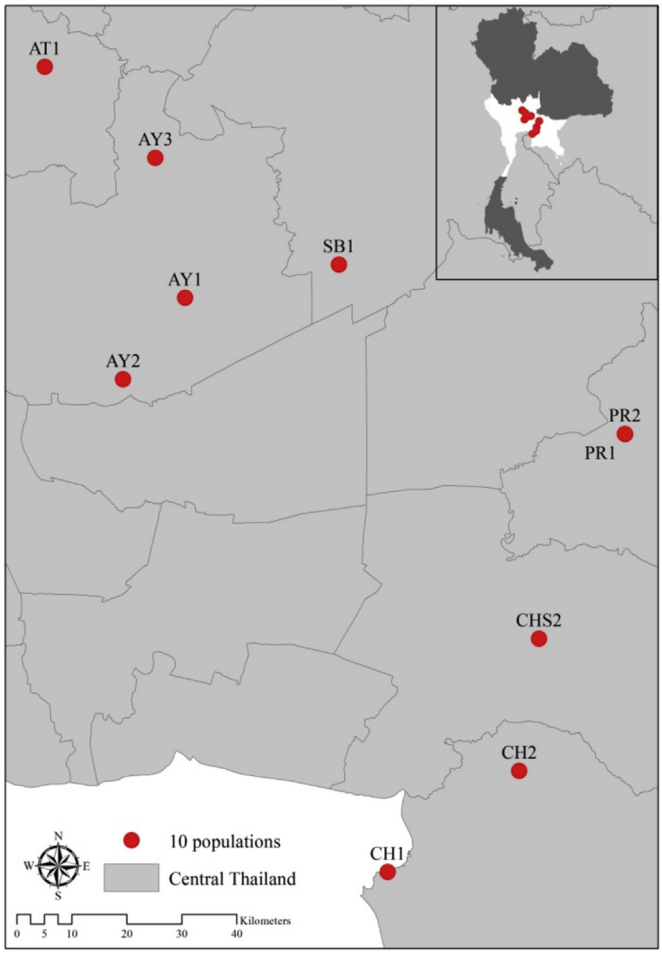

Fig. 1. Map of central Thailand showing collection locations of the 10 colonies of *Pteropus lylei*.

Materials and methods

Sample collection

Sampling was carried out under protocols approved and permitted by the Department of National Parks, Wildlife and Plant Conservation, Thailand (No. 0909.204/2686) and the Animal Use Protocol No.1473001 approved by Chulalongkorn University Animal Care and Use Committee, Bangkok, Thailand. In this study, 52 blood samples were collected from 52 individual from 10 colonies in central Thailand. The roosting sites of *P. lylei* were visited, and a limited number of samples per site were collected (Table 1 and Fig. 1). All of the roosts were sampled in March–June 2014. All of the bats from the 10 colonies were collected using the same protocol. The ages of the bats were similar, ranging from juvenile to adult. The overall health of the bats was considered normal by veterinarians. Immediately following capture, the forearm, hind foot, ear, head, and body of the animals were measured in the field and identified based on a field guide by Francis (2008). A spot of blood from a wing of the bat was placed on an FTA Classic Card WB120205 (Whatman Asia Pacific Pte. Ltd., Singapore) filter paper and allowed to air dry.

Spotting blood samples onto filter paper is useful for sample preparation and allows samples to be kept at room temperature in the field for a few days and stored even longer at lower temperatures. This collection method also allows for storage of DNA for a

Table 1
Population of Lyle's flying fox in roosting sites in Thailand (Duengkae et al., 2015).

No.	Roosting site code	Province of roosting site	Population (at 2015)
1	AY1	Khanon Temple: Phra Nakhon Si Ayutthaya	1319
2	AY2	Thasung Temple: Phra Nakhon Si Ayutthaya	2647
3	AT1	Chantraram Temple: Ang Thong	1293
4	AY3	Tanen Temple: Phra Nakhon Si Ayutthaya	1950
5	SB1	Mongkonteeparam Temple: Saraburi	3164
6	PBR1	Tawabud Temple: Prachin Buri	3852
7	PBR2	Bangkrabao Temple: Prachin Buri	1239
8	CH1	Education Center: Chon Buri	1,000[4]
9	CH2	Luang Phrommawat Temple: Chon Buri	7991
10	CHS2	Pho Temple: Chachoengsao	6128

long period without compromising the DNA quality (Song et al., 2013). The filter paper was placed in a Ziploc bag containing a desiccant and maintained at room temperature prior to DNA extraction. Bats were safely released after measurements and samples had been collected.

Molecular methods

Genomic DNA was extracted from the dried blood spots on the filter paper (FTA Classic Card WB120205) using the FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Seoul, South Korea). The mitochondrial *cyt b* genes were amplified using the primers described in Brown et al. (2011). All of the polymerase chain reaction (PCR) amplifications were performed with the following thermal profile: 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and extension at 72 °C for 45 s with a final extension of 72 °C for 10 min. The primers used were as follows: L14724 5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′and H15915R 5′-GGAATTCATCTCTCCGGTTTACAAGAC-3′. DNA quantity and quality were determined using agarose gel electrophoresis at 100 V for 30 min. PCR products were purified by combining 2 μL exonuclease:phosphatase (25 μL:100 μL) with 20 μL PCR product, and the combined product was incubated for 35 min at 37 °C and for 15 min at 85 °C using the Genetic Analyzer (Macrogen Incorporation; Seoul, S. Korea). Sequences were verified, assembled, and aligned using Autoassembler (version 2.1) (Applied Biosystems; Carlsbad, CA, USA).

Statistics analyses of genetic diversity and relationship

Nucleotide statistics were computed using DnaSP (version 5.0) (Librado and Rozas, 2009) and included the number of haplotypes (*n*h), haplotype diversity (Hd), average number of nucleotide differences (k) and nucleotide diversity (π). Arlequin software (version 3.5) was used to perform an analysis of molecular variance (AMOVA) (Excoffier et al., 1992) on the aligned *cyt b* sequence data to examine patterns of genetic variation within and among individuals. Population variance was measured using the φ_{ST} statistic implemented in Arlequin. Briefly, the φ_{ST} statistic provides a measure of the overall genetic variation within a population. Values ranging from 0 to 0.05 are indicative of low genetic differentiation (frequent gene flow), whereas values greater than 0.25 are considered to represent strong genetic differentiation (very limited or no gene flow). Intermediate values of φ_{ST} (between 0.05 and 0.25) suggest moderate levels of genetic variability within the examined population (Larsen et al., 2014).

Results and discussion

We identified 43 polymorphic sites within the 1113 bp of the amplified *cytb* gene sequence, which included 38 inferred transitions and 5 transversions. No insertions or deletions were observed (Table 2). Among the 52 *P. lylei* individuals collected from 10 colonies in central Thailand, 25 unique haplotypes (haplotypes 1–25, Table 2) were identified. Of the 25 haplotypes, 15 were singletons or represented by one individual (haplotypes 3, 5–9, 12–13, 15, 18, 20–21, and 23–25), and 10 (1–2, 4, 10–11, 14, 16–17, 19, and 22) were shared haplotypes found in two to eight individuals and were also shared between colonies (Table 2 and Fig. 2). Haplotype 1 was the most common haplotype, found in eight (15.38%) individuals of the AY1, AY3, SB1, and PBR2 colonies (Table 2 and Fig. 2). Two other frequent haplotypes (19 and 22) were found in five individuals. Haplotype 19 was found in the AT1, SB1, PBR1, and CHS2 colonies. Haplotype 22 was found in the AY2, SB1, PBR1, and PBR2 colonies. The minimum spanning network of 25 *cytb* haplotypes suggests genetic continuity (Fig. 2). Haplotypes 17, 19, and 22 were also in a

Table 2
Variable nucleotide positions within the 1113 bp sequences of Cyt *b* analyzed in 52 individuals of *Pteropus lylei*, where frequency of the different haplotypes in each colony also is indicated Dots indicate that same nucleotide is present in haplotype 1. The second part of the table indicates the distribution and frequency of these haplotypes in the different colonies.

Haplotype	Positions of variable nucleotides in base pair (bp)																																											Colony									
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1										
	0	0	1	1	1	1	1	1	1	2	2	2	3	3	3	3	3	4	4	5	5	5	5	5	5	6	7	7	8	8	8	9	9	9	9	9	0	0	0	0	0	0	0										
	4	8	2	2	3	4	6	7	7	4	6	9	6	6	6	7	9	4	7	2	2	3	7	9	9	5	5	9	0	5	6	1	4	5	6	8	1	1	4	6	7	7	9										
	0	9	0	9	8	4	2	2	7	6	4	1	6	7	9	8	9	8	1	2	5	1	3	0	4	1	3	2	4	6	7	8	9	1	1	8	5	8	5	1	0	4	0	AY1	AY2	AT1	AY3	SB1	PBR1	PBR2	CH1	CH2	CHS2
1	G	T	G	C	C	A	C	A	T	C	C	T	T	G	C	A	A	A	G	T	C	A	C	A	T	C	C	T	A	G	T	A	T	G	G	G	A	G	T	T	T	T	G	2			3	2		1			
2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.				1		1				
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6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.						1				
7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.			1							
8	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.		1								
9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.			1							
10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.			1					1		1
11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.						1			1	
12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1									
13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.					1					
14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.								1	1	
15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	1									
16	.	C	A	.	T	.	T	.	C	.	T	.	.	.	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	.									1	1
17	.	.	A	T	.	G	.	T	G	.	T	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.					2		1		1	
18	A	.	A	T	.	G	C	.	.	.	T	A	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.						1				
19	.	.	A	T	.	G	.	.	.	.	T	A	C	.	T	.	G	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.			1		1	2				1
20	.	.	A	T	.	G	.	.	.	.	.	A	C	.	A	.	.	.	.	.	.	.	.	.	.	T	T	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1								
21	.	.	A	T	.	G	.	.	.	.	T	A	C	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.								1		
22	.	.	A	T	.	G	.	.	.	.	T	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	T	C	G	.	A	.	.	.	.	.	.	.	.	.	.	.	.		1			1	1	2			
23	.	.	A	T	.	G	.	.	.	.	T	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	T	C	G	.	.	.	.	.	.	C	.	.	.	.	.	.	.								1		
24	.	.	A	T	.	G	.	.	.	.	T	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	T	C	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.									1	
25	.	.	A	T	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	T	C	G	A	.	.	.	.	.	.	.	.	A	C	A	T	A								1		

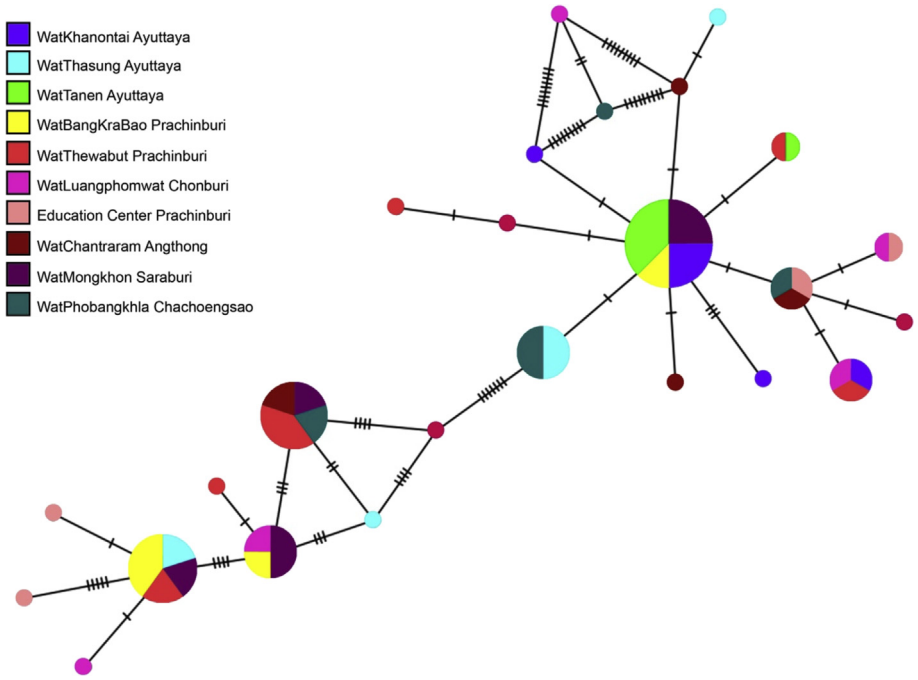

Fig. 2. Minimum spanning tree of *P. lylei* Cyt*b* haplotypes for 52 individuals sampled and 25 haplotypes identified, where colors correspond to the 10 locations and each circle represents a unique DNA sequence (haplotype) with the size of the circles representing the relative number of individuals having the same haplotype.

central position within the network. All unique haplotypes were situated in a distal position within the network.

Molecular diversity statistics for all of the samples and for each colony are summarized in Table 3. The overall Hd in *P. lylei* was uniformly and extremely high (0.974). The Hd values of the colonies were high and ranged from 0.833 to 1.000, with the exception of colony AY3 (Hd = 0.500; Table 3). The average π among all colonies was 0.006 and ranged from 0.000 to 0.009. The pattern of variation across regions was consistent with the level of haplotype diversity, with the greatest diversity in the CH1 and CH2 colonies ($\pi = 0.009$) and the lowest in the AY3 colony ($\pi = 0.000$).

The overall pairwise φ_{ST} statistic was 0.006 ($p < 0.05$) and ranged from 0.002 to 0.009 among colonies (Table 4). The Tamura three-parameter model of evolution was identified as the best-fit model for the data and was implemented in the Arlequin software. AMOVA showed that 0.05% of the overall genetic variation occurred among the 10 populations. The overall pairwise values indicated low genetic differentiation (frequent gene flow and strong genetic relationships) (Larsen et al., 2014) among the colonies.

This study represents, based on the authors' knowledge, the first study of the genetic diversity and genetic relationships of *P. lylei* in central Thailand. The results revealed high haplotype diversity in *P. lylei* (Hd = 0.949). Compared with studies of other bat *cytb* gene sequences, *E. franqueti* and *R. aegyptiacus* exhibited higher haplotype diversity (Hd = 0.988 and 0.997, respectively; Hassanin et al., 2016), while other species exhibited lower diversity than that of *P. lylei* [0.870 for *E. helvum* (Peel et al., 2013), 0.891 for *N. veldkampii*, and 0.922 for *E. buettikoferi* (Hassanin et al., 2016)]. Furthermore, among the 10 colonies, the strongest genetic relationship among colonies was found between AY1 and AY3 ($\varphi_{ST} = 0.002$), while the weakest was observed between CH1 and CH2 ($\varphi_{ST} = 0.009$) (Table 4). Several haplotypes were shared among and within colonies. This was supported by lower levels of pairwise genetic differentiation (Table 4). According to Weber et al. (2015), this species travels 2–3 km between day roosts and feeding areas and appears to develop satellite colonies, supporting the occurrence of gene flow among the populations of *P. lylei* while traveling or foraging. The number of samples collected in this study resulted in the high number of haplotypes identified. However, the authors maintain that if more samples could have been collected, still higher numbers of haplotypes would have been identified. The 25 haplotypes observed in the 10 colonies indicated that the colonies were genetically closely related, although the geographic distances between the colonies were large. No significant correlations were identified between gene flow and the distance between different *P. lylei* colonies.

Pteropus lylei has been identified as a reservoir of NiV, and the nature of the *P. lylei* population together with increased interaction with the resident human population may pose some level of risk. Hence, the results of this study complement the results of two previous studies (Wacharapluesadee et al., 2010; Weber et al., 2015). The current results show that different colonies are genetically closely related, which may reflect inter-colony movement or

Table 3
Number of individuals sampled (N), haplotypes observed, haplotype diversity (Hd), average number of nucleotide differences (K) and nucleotide diversity (π) in each colony of *Pteropus lylei* (Cyt*b* sequence).

Colony	n	Haplotypes observed	Hd	k	π
AY1	5	4	0.900	2.400	0.002
AY2	5	4	0.900	7.600	0.007
AT1	5	5	1.000	5.600	0.005
AY3	4	2	0.500	0.500	0.000
SB1	7	5	0.905	6.762	0.006
PBR1	7	6	0.952	8.095	0.007
PBR2	4	3	0.833	5.833	0.005
CH1	5	5	1.000	10.000	0.009
CH2	5	5	1.000	10.400	0.009
CHS2	5	4	0.900	8.400	0.008
Overall	52	25	0.949	6.945	0.006

Table 4
Pairwise estimates of φ_{ST} values for 10 colonies of *Pteropus lylei* in central Thailand based on 1113 bp of Cyt*b*.

Colony	AY1	AY2	AT1	AY3	SB1	PBR1	PBR2	CH1	CH2	CHS2
AY1										
AY2	0.005									
AT1	0.004	0.006								
AY3	0.002	0.005	0.003							
SB1	0.006	0.006	0.006	0.005						
PBR1	0.007	0.007	0.007	0.006	0.006					
PBR2	0.008	0.006	0.007	0.007	0.005	0.006				
CH1	0.008	0.007	0.007	0.007	0.007	0.008	0.007			
CH2	0.007	0.008	0.007	0.007	0.007	0.008	0.007	0.009		
CHS2	0.005	0.006	0.006	0.005	0.007	0.008	0.008	0.008	0.008	

even the same population. In forthcoming work, the mitochondrial D-loop region sequence will be analyzed, and more samples will be collected for analysis of the D-loop and *cytb* regions in combination. Population genetic information will not only help with better management of *P. lylei* populations but also with outbreaks of the NiV carried by *P. lylei*.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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