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# First molecular investigation of haemosporidian parasites in Thai bat species<sup>☆</sup>

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## ABSTRACT

Malaria parasites in the phylum Apicomplexa (Order: Haemosporida) infect diverse vertebrates and invertebrate hosts. At least seven genera of haemosporidian parasites have been described to exclusively infect bats. Most of these parasites remain enigmatic with a poorly known host range. Here, we investigated 271 bats belonging to 21 species and seven families from six provinces of Thailand. Overall, 124 out of 271 bats (45.8%) were positive for haemosporidian parasites, while none had *Plasmodium*, based on microscopic examination of blood smears and PCR amplification. We obtained 19 distinct cytochrome b (*cytb*) nucleotide haplotypes of *Hepatocystis* from seven bat species (families: Craseonycteridae, Hipposideridae, Pteropodidae, and Rhinolophidae). *Nycteria* was found in four bat species (Craseonycteridae, Emballonuridae, Megadermatidae, and Pteropodidae) and *Polychromophilus* in two species (Emballonuridae, Vespertilionidae). Phylogenetic analysis inferred from *cytb* sequences placed *Hepatocystis* into 2 different clades. Most *Hepatocystis* infections were found in insectivorous bats and clustered together with a sequence from *Hipposideros larvatus* in Cambodia (in subclade 1a). A single sequence of *Hepatocystis* obtained from a frugivorous bat, *Cynopterus brachyotis*, was placed in the same clade with *Hepatocystis* from the same bat species previously reported in Malaysia (clade 2). *Nycteria* in these Thai bats were clearly separated from the African isolates previously reported in bats in the family Rhinolophidae. *Polychromophilus murinus* from *Myotis siligorensis* was placed in a distinct clade (clade 2) from *Polychromophilus melanipherus* isolated from *Taphozous melanopogon* (clade 1). These results confirmed that at least two distinct species of *Polychromophilus* are found in Thailand. Collectively, *Hepatocystis* presented no host specificity. Although *Megaderma spasma* seemed to be infected by only *Nycteria*, its respective parasite does not show specificity to only a single bat host. *Polychromophilus murinus* and *P. melanipherus* seem to infect a narrower host range or are somehow restricted to bats in the families Vespertilionidae and Emballonuridae, respectively.

## 1. Introduction

To date, 1419 species from 21 bat families (Order: Chiroptera) have

been discovered in the world (Simmons and Cirranello, 2020). Bats are the second largest order of mammals after rodents (Burgin et al., 2018). Within Thailand, at least 141 (Simmons and Cirranello, 2020) or 146

<sup>\*</sup> Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under accession numbers **MT136107 – 56** for *Hepatocystis*, **MT136157 – 66** for *Nycteria*, and **MT136167 – 8** for *Polychromophilus*.

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(Karapan et al., 2016) species of bats have been described so far. Among these, the endemic species of bats to Thailand are *Hipposideros pendleburyi* and *Eudiscoderma thongareae* (Soisook, 2017, 2019). In Thailand, bat populations have recently become threatened by human activities, and conservation actions are needed (Bumrungsri et al., 2006; Puechmaile et al., 2009). Despite the fact that bats are known to play an important role in maintaining the dynamics of ecosystems and provide several services for human well-being, they are also known to be carriers of many infectious agents, including vector-borne parasites (Calisher et al., 2006; Lutz et al., 2016).

Malaria parasites belong to order Haemosporida (Phylum: Apicomplexa), which is comprised of many important mammal-infecting genera, including *Plasmodium* in humans. *Plasmodium falciparum* is probably the most well-studied parasite among the other members, due to its lethality in humans. The discovery of chiropteran haemosporidian parasites started over a century ago (e.g. Dionisi, 1899; Manwell, 1946; Garnham, 1953). To date, nine haemosporidian genera have been described in bats: *Biguetiella*, *Bioccala*, *Dionisia*, *Hepatocystis*, *Johnspretia*, *Nycteria*, *Plasmodium*, *Polychromophilus*, and *Sprattiella* (Garnham, 1966; Schaer et al., 2015; Perkins and Schaer, 2016). The original descriptions of these genera were based upon to morphological and biological characteristics without any other studies, such as molecular analysis, in most cases (i.e. Landau et al., 1984). Seven of these nine genera, but not *Hepatocystis* and *Plasmodium*, are thought to exclusively infect bats (Garnham, 1966).

Molecular phylogenetics revealed that *Polychromophilus* and *Nycteria* form basal clades to *Hepatocystis* (Perkins and Schaer, 2016), while *Polychromophilus*, *Nycteria*, and *Hepatocystis* clustered in distinct clades in certain bat species. *Nycteria* has been detected in bats in the Megadermatidae, Rhinolophidae, and Nycteridae families (Schaer et al., 2015). Recent studies, such as on those *Hepatocystis*, have been performed in a few regions of Africa and some in Australia (Schaer et al., 2015; Perkins and Schaer, 2016). However, records of these bat parasites in Thailand remain limited, and are mainly based on microscopic descriptions (Landau et al., 1984; Chumnandee and Pha-obnga, 2018). Therefore, in this study, we aimed to investigate the phylogenetic relationship of haemosporidians from Thai bats, in relation to other haemosporidians. This present study provides an update on haemosporidian species and haplotype infecting bat species based on microscopic examination and molecular (cytochrome b [*cytb*] gene nucleotide sequence) detection and identification.

## 2. Materials and methods

### 2.1. Ethical statements

Permission to trap the bats was granted by Thailand Department of National Parks, Wildlife and Plant Conservation (Permit No. DNP 0907.4/8179 to PS, No. DNP 0909.6/22928 to TN, and No. DNP 0907.4/29038 to MK). This study was reviewed and approved by the Chulalongkorn University Faculty of Veterinary Science Biosafety Committee (IBC, 1931010), and was approved by the Institutional Animal Care and Use Committee in accordance with university regulations and policies governing the care and use of laboratory animals (No. 1931090).

### 2.2. Sampling sites

Sampling sites were chosen according to our cooperation with the local authorities from Department of National Parks, Wildlife and Plant Conservation. Locations in the north and northeast of Thailand were not selected due to logistics reason. Weather and environmental conditions of each sampling sites were compiled from the Thai Meteorological Department (<https://www.tmd.go.th>). In general, Thailand has three distinct seasons namely warm (February–May), rainy (May–October), and cool seasons (October–February). The bats were surveyed, and samples collected during February 2018 to January 2020 in the

Ratchaburi, Phetchaburi, and Kanchanaburi provinces in Western Thailand, and in Saraburi, Songkla, and Trat provinces in Central, Southern, and Eastern Thailand, respectively, as follows.

**Western Thailand:** samples from Ratchaburi were collected from Chompon Cave (13°37' N, 99°35' E) in January 2019, Khun Kunchon Cave (13°23' N, 99°45' E), and Kunchorn Temple (13°29' N, 99°42' E) in January 2020 (Fig. 1). Ratchaburi is located near the Gulf of Thailand with low intensity of rain. Samples from Phetchaburi were collected at Bo Cave (13°20' N, 99°45' E) in January 2020. This province is characterized by highlands with mountains and forests. The average temperature is about 27.9 °C. The surveys in Kanchanaburi were conducted in (i) Daowadung Cave (14°28' N, 98°50' E), (ii) Bat Cave (14°26' N, 98°51' E), (iii) Cave A (14°12' N, 99°09' E), and (iv) Cave B (14°21' N, 98°56' E). Kanchanaburi is mountainous, with annual temperature ranging from 5 °C to 43 °C and abundant rainfall throughout the year. Daowadung Cave and Bat Cave are in Sai Yok National Park, while Cave A and Cave B are in the area administered by the Plant Genetic Conservation Project Under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn. The surveys were conducted in May and December 2018, and in February, July, October, and November of 2019.

**Central Thailand:** samples from Saraburi province were collected in February 2018 at Cave Tiger (14°31' N, 101°02' E). The area is under administration of Chulalongkorn University Saraburi campus. Saraburi is a landlocked province, of which the annual temperature range is 28–30 °C. Rainfall occurs throughout the year except in the south of the province.

**Southern Thailand:** Bats from the southern province of Songkla were trapped in August of 2018 from community forests located in Sadao (6°38' N, 100°23' E) and Saba Yoi (6°33' N, 100°54' E) districts. Songkla is under the influence of two different monsoon winds namely Northeast (October to February) and Southwest (May to October). This province has mild temperature with less seasonal variations.

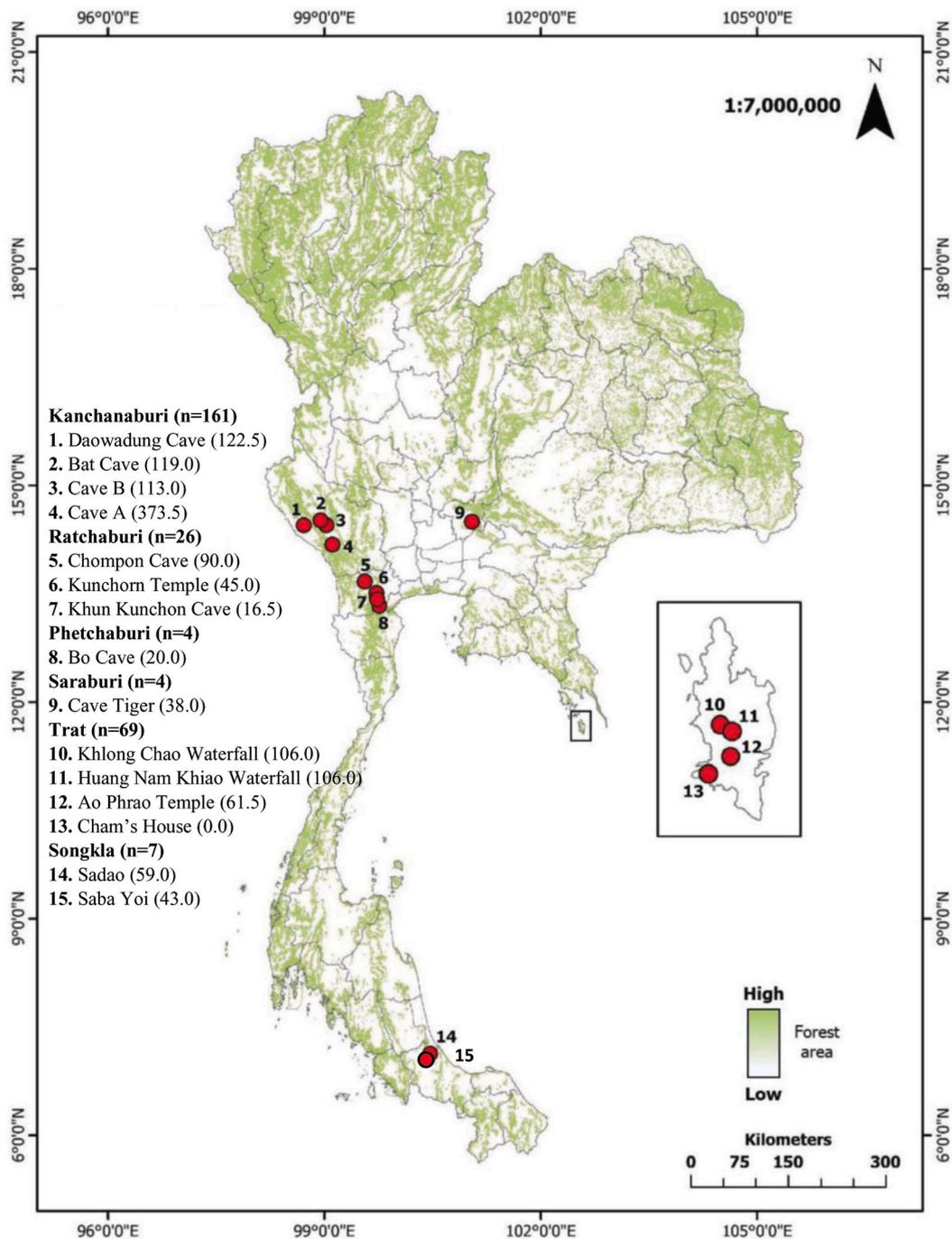
**Eastern Thailand:** The survey in the eastern province of Trat at Ko Kut island was conducted in June 2019 at four sites consisting of Ao Phrao Temple (11°35' N, 102°34' E), Khlong Chao Waterfall (11°38' N, 102°33' E), Huang Nam Khiao Waterfall (11°39' N, 102°33' E), and Cham's House (11°60' N, 102°53' E). Located in the Gulf of Thailand, this mountainous island has dense forest coverage. The average temperature throughout the year is 27.3 °C.

### 2.3. Bat capture and identification

Harp traps or nets were set up in front of the cave's entrance or flying routes before sunset at most sampling sites, but bats in Phetchaburi and Ratchaburi were captured inside the caves using sweep nets. After capturing, the bats were almost immediately transferred to an individual cloth bag and the following data were recorded: sex, body weight (g), forearm length (mm), and reproductive status (juvenile, adult, nulliparous, parous, lactating, and post-lactation). Identification to the family and species level were made based on their external morphology using guide from Francis (2008). If necessary, bat echolocation calls were used to confirm the species.

### 2.4. Blood collections

Bats weighing 2.5 g or more were selected for blood sampling, while those of less than 2.5 g body weight were released without any blood collection. The interfemoral membrane between the hind legs was wiped with 70% ethanol-soaked cotton buds and then a sterile needle (23–27G) was used for pricking the uropatagium vein. For each selected bat, approximately 2–20 µL of blood was collected using a capillary tube pre-coated with acid citrate dextrose solution (BD Franklin Lakes, USA) as an anticoagulant. Samples were transferred into sterile 1.5-mL tubes and transported in dry ice back to the laboratory at the Faculty of Veterinary Science, Chulalongkorn University for the molecular diagnosis.



**Fig. 1.** Map of Thailand depicting the bat sampling sites, forested areas, and altitudes. The map was drawn using ArcGIS version 10.2. Altitudes (in meter scale) are indicated in parentheses after each sampling site and were calculated by DIVA-GIS version 7.5.0.0 using spatial data from SRTM DEM Digital Elevation Database.

Approximately 0.5  $\mu$ L of whole blood was smeared onto a glass slide onsite, air-dried, kept in a slide box and transported to the laboratory for the staining and microscopic examination. After the blood collection, a gentle press with clean tissue paper was performed to stop the bleeding. Finally, bats were released to their original capture sites.

## 2.5. Microscopic examination

Blood smeared slides were fixed in absolute methanol for 2 min and stained with a 10% Giemsa solution (Merck, Germany) in phosphate buffer (pH 7.2) for 30 min. Slides were later washed with clean water and screened at 400 $\times$  magnification. The slides were examined at 1,000 $\times$  magnification under a light microscope (Olympus C $\times$ 31, Tokyo,

Japan) for a minimum of 20–25 min (Valkiunas et al., 2008). Parasitemia level was recorded as percentage of infected erythrocyte in a total number of erythrocytes. The calculation used as follows: total number of infected erythrocytes/average of erythrocyte\*number of observed field (Schaer et al., 2015). In total, 20–100 fields were observed in each blood film. The average of erythrocytes per field was estimated by counting one to three fields with equal density. The negative blood smears were examined at least two times to avoid false negative. The images were recorded with Olympus DP21-SAL digital camera (Tokyo, Japan). Identification of haemosporidian parasites was based on descriptions or images from Garnham (1966), Duval et al. (2012), and Schaer et al. (2013).

## 2.6. Blood sample preparation, PCR amplification and sequencing of the *cytb* gene fragment

Blood samples from each site were shipped from the field in dry ice and stored at  $-20^{\circ}\text{C}$  until use. The DNA extractions were performed as previously described in Templeton et al. (2016). Briefly, after thawing for a few minutes, approximately 5–20  $\mu\text{L}$  of sample was treated with 200  $\mu\text{L}$  of 0.15% (w/v) of saponin (Sigma-Aldrich, Germany) in PBS (Takara, Japan) for 1 min to facilitate hemolysis. Samples were then centrifuged at 13,000 g for 2 min and supernatants were discarded. The cell pellets were washed with 200  $\mu\text{L}$  of PBS and centrifuged at 13,000 g for 2 min. The DNA extractions were conducted using NucleoSpin® Blood Kit (Macherey-Nagel, Germany) according to the manufacturer's instruction, except the final elution volume was reduced to 20  $\mu\text{L}$ . Haemosporidian parasites were screened with primers targeting the *cytb* gene as follows; (i) DW2 (5'-TAATGCCTAGACGTATTCCTGATTATCCAG-3') and DW4 (5'-TGTTTGTCTGGGAGCTGTAATCATAATGTG-3') for the primary PCR reaction and (ii) NCYBINF (5'-TAAGAGAATTATGGAGTGGATGGTG-3') and NCYBINR (5'-CTTGTGGTAATTGACATCCAATCC-3') for the nested PCR. Primers and cycling conditions were used as reported in Escalante et al. (1998) and Perkins and Schall (2002). Additional primers targeting the *cytb* gene were used because we observed some mismatch of the oligonucleotide (in NCYBINF and NCYBINR primers) with the bat's haemosporidian sequences. We, therefore, designed specific primers to target the bat haemosporidian *cytb* gene covering the four genera of *Plasmodium*, *Nycteria*, *Polychromophilus*, and *Hepatocystis* (Supplementary Fig. S1). Alignment of 18 *cytb* gene nucleotide sequences of *Plasmodium* spp. (n = 3), *Nycteria* (n = 7), *Polychromophilus melanipherus* (n = 2), *Polychromophilus murinus* (n = 2), and *Hepatocystis* spp. (n = 4) was performed using the MUSCLE software (version 3.8), freely available at [www.ebi.ac.uk](http://www.ebi.ac.uk) (Edgar, 2004a, 2004b). GenBank™ accession numbers of haemosporidian parasites used for the current primer design were KF159710, KF159674, and KF159671 (*Plasmodium*); KP053763–68 and MH177856 (*Nycteria*); MH744503 and MH744505 (*P. melanipherus*); MH744532 and MH744537 (*P. murinus*); and KF159718, KF159712, KF159706, and KF159705 (*Hepatocystis*). The following degenerated primers were selected: Pan\_Haemos\_CytBF (5'-GGGTCAAATGAGTTWYTG GGG-3') and Pan\_Haemos\_CytBR (5'-GAATTAATGTTGTTAAAYRTCTTGTCTGC-3').

The PCR reaction mixture consisted of 6.25  $\mu\text{L}$  of 2X PCR buffer KOD FX Neo, 2.5  $\mu\text{L}$  of dNTPs (0.4 mM each), 0.375  $\mu\text{L}$  of each primer (10 pmol/ $\mu\text{L}$ ), 0.25  $\mu\text{L}$  of KOD FX Neo DNA polymerase (Toyobo, Japan), 1  $\mu\text{L}$  of DNA template and 1.75  $\mu\text{L}$  of sterile distilled water. Thermal cycling was performed in an Axygen® MaxyGene II Thermal Cycler (Life Sciences, USA) at  $94^{\circ}\text{C}$  for 2 min, followed by 40 cycles at  $98^{\circ}\text{C}$  for 10 s and  $62^{\circ}\text{C}$  for 1 min, and then a final  $68^{\circ}\text{C}$  for 3 min before being held at  $12^{\circ}\text{C}$ . Amplicons were resolved in 1.5% (w/v) agarose-0.5 x TAE gel electrophoresis (120 V, 400 mA for 40 min for a  $6 \times 11$  cm gel). The agarose gel was then stained with RedSafe™ (Intron Biotechnology) and PCR products were visualized under a UV transillumination. The expected amplicon size was 524 bp. Genomic DNA of *Hepatocystis* and sterile distilled water were used as positive and negative controls, respectively.

Positive samples were then scaled up to a 25  $\mu\text{L}$  PCR reaction volume for direct sequencing of the PCR amplicon. The PCR products without non-specific bands were treated with ExoSAP-IT™ (GE Healthcare, UK) to remove residual single strand oligonucleotide as per the manufacturer's instruction except we diluted the ExoSAP-IT™ reagent 10-fold. The PCR products from positive samples that contained non-specific bands were cleaned up by excision of the correct sized band from the agarose gel and purified by NucleoSpin® Gel and PCR Cleanup (Macherey-Nagel, Germany). Purified PCR products were sent for commercial sequencing in both directions at Pacific Science (<http://www.pacificscience.co.th/>) using the same primers as for the PCR amplification. However, PCR reactions with faint amplicons were not

included for sequencing. Samples showing mixed infections based on the sequencing results (sample IDs THBat19-111, -112, and -177) were cloned into the pTA2 plasmid supplied in the TArget Clone-Plus kit (Toyobo, Japan). Ligated products were transformed into high competent *E. coli* DH5 $\alpha$  cells (Toyobo, Japan). Plasmids were extracted using NucleoSpin® Plasmid EasyPure (Macherey-Nagel, Germany). Sequencing was performed using the M13F and M13R universal primers.

## 2.7. Phylogenetic and statistical analyses

Base callings were manually assigned where necessary based on the individual chromatograms. The editing and assembling were conducted in the BioEdit software version 7.0.5.3 (Hall, 1999). Primer sequences were removed from the results. Sequences were submitted for a BLASTn search of the GenBank database (<https://blast.ncbi.nlm.nih.gov>). Haplotypes of *Hepatocystis* were determined using the DnaSP software version 6.12.01 (Rozas et al., 2017). The best-fit models for phylogenetic trees were calculated using Find Best DNA/Protein Model in MEGA-X software version 10.0.4 (Kumar et al., 2018). The model with the lowest Bayesian Information Criterion (BIC) score was chosen to construct the phylogenetic trees. The *cytb* nucleotide sequences of *Plasmodium*, *Hepatocystis*, *Nycteria*, and *Polychromophilus* available in the GenBank™ database were included in the analysis. Host origins, localities, and accession numbers of these parasites are summarized in Supplementary Table S1. The trees were inferred using the maximum likelihood (ML) method and Tamura 3-parameter model (Tamura, 1992). The *cytb* sequences of *Leucocytozoon caulleryi* (accession nos. AB302215, MH047560, and LC505517) and *Leucocytozoon sabrazesi* (accession nos. LC506045, and NC\_009336) were used as the out groups. The final dataset included 73 *cytb* haemosporidian sequences of 378 bp. Chi-square analysis was carried out to evaluate the association between bat family, sex, and status with the infection rate. All statistical computations were done using SPSS Ver. 22.0. Kruskal-Wallis test was used to explore the differences of parasitemia level in three bat families (Hipposideridae, Megadermatidae, and Rhinolophidae). Man-Whitney *U* test was conducted to find the influence of sex in parasitemia level.

## 3. Results

### 3.1. Bat samples

A total of 271 blood samples from individual bats (123 females and 141 males or a 1:1.14 ratio) were collected between February 2018 to January 2020. The gender of seven bats was not identified. The proportion of male bat's status between adult: juvenile was 9.54: 1. The proportion in female bats among adult: juvenile: lactating: nulliparous: parous: post lactation: pregnant was 54: 13: 27: 8: 6: 13: 1, respectively. The records regarding the species, sampling site, and date of collection of the bats, and the reproductive status of female bats, are provided in Supplementary Table S2. Collectively, 21 bat species from seven families (Craseonycteridae, Emballonuridae, Hipposideridae, Megadermatidae, Pteropodidae, Rhinolophidae, and Vespertilionidae) were sampled in the present study. Six of the seven bat families in this study are insectivorous, but Pteropodidae is primarily frugivorous. The list of bat species, number, captured sites, prevalence data, and parasitemia are shown in Table 1.

### 3.2. Morphology of parasite blood stages, parasitemia, and prevalence

A total of 225 blood smears from individual bats were successfully obtained for the microscopic examinations, and were comprised of 35 slides out of 41 samples collected in 2018, 177 slides out of 217 samples in 2019, and 13 slides out of 13 samples in 2020. Blood smears from four of these samples were later excluded due to their poor quality. Amount of blood samples in 46 bats was not enough to prepare for blood films. Collectively, 24.9% of the slides were found by microscopic

**Table 1**

Bats captured in the present study, distribution, prevalence and parasitemia of haemosporidian parasites..

Family	Species	Sampling site	No. tested	Prevalence (95% CI) *	Parasitemia (%)	
					Mean ± SD	Min - Max
Craseonycteridae	<i>C. thonglongyai</i>	<b>BC, CA, CB</b>	16	50.00 (25.5, 74.5)	-	0.030 - 0.030
Emballonuridae	<i>T. melanopogon</i>	BO, <b>CA, KKC</b>	46	10.87 (1.9, 19.9)	-	0.002 - 0.002
Hipposideridae	<i>H. armiger</i>	<b>BC, CC, DC, KKC</b>	25	44.00 (24.5, 63.5)	0.30 ± 0.33	0.002 - 1.311
	<i>H. bicolor</i>	SBY, <b>CA, CB</b>	14	71.43 (47.8, 95.1)		
	<i>H. larvatus</i>	<b>APT, BC, DC, CC, HNK, KCW, CB, SBY, KT</b>	103	56.31 (46.7, 65.9)		
	<i>H. lekaguli</i>	<b>CC</b>	5	20.00 (-15.1, 55.1)		
	<i>H. pomona</i>	DC, CA	5	0		
	<i>A. stoliczkanus</i>	BC, CA	2	0		
Megadermatidae	<i>M. spasma</i>	CT, <b>DC, CA, CB</b>	14	71.43 (47.8, 95.1)	0.16 ± 0.19	0.006 - 0.576
Pteropodidae	<i>Cy. brachyotis</i>	<b>APT, CH</b>	3	66.70 (13.4, 120.0)	-	-
	<i>E. spelaea</i>	<b>KT</b>	1	100	-	-
Rhinolophidae	<i>R. affinis</i>	SD, SBY	2	0	0.021 ± 0.022	0.004 - 0.066
	<i>R. accumiatus</i>	SD	1	0		
	<i>R. coelophyllus</i>	BC, CB	3	0		
	<i>R. pearsonii</i>	<b>CC, DC</b>	8	62.50 (28.9, 96.1)		
	<i>R. pusillus</i>	APT	1	0		
	<i>R. stheno</i>	SD	1	0		
	<i>R. thomasi</i>	<b>KT</b>	1	100		
	<i>R. malayanus</i>	<b>CC, KKC, CB</b>	4	100		
Vespertilionidae	<i>K. hardwickii</i>	SD	1	0	-	0.006 - 0.006
	<i>My. siligorensis</i>	<b>CC, CB</b>	15	53.33 (28.1, 78.6)	-	
<b>Overall</b>			<b>271</b>	<b>45.75 (39.8, 51.7)</b>	<b>0.22 ± 0.30</b>	<b>0.002 - 1.311</b>

**Note:** Bo Cave (BO), Bat Cave (BC), Chompon Cave (CC), Daowadung Cave (DC), Cave A (CA), Cave B (CB), Cave Tiger (CT), Khun Kunchon Cave (KKC), Kunchorn Temple (KT), Sadao (SD), Saba Yoi (SBY), Ao Phrao Temple (APT), Khlong Chao Waterfall (KCW), Huang Nam Khiao Waterfall (HNK), and Cham's House (CH). Bat species and sampling sites in bold indicate haemosporidian positive. \*Positive results from either microscopic examination or PCRs. Prevalence was expressed as percentage with 95% Confidence Interval (CI). Parasitemia of individual bats are also provided in Supplementary Table 2.

examinations to be positive for haemosporidian parasites, and were derived from the following bat hosts: *Hipposideros larvatus* (26), *Hipposideros armiger* (7), *Hipposideros bicolor* (2), *Rhinolophus pearsonii* (4), *Rhinolophus malayanus* (2), *Craseonycteris thonglongyai* (1), *Megaderma spasma* (10), *Taphozous melanopogon* (1), *Myotis siligorensis* (1), and *Rhinolophus thomasi* (1). In general, the parasitemia level ranged from 0.002% to 1.311% (Table 1 and Supplementary Table 2). Parasitemia level between female (mean  $\pm$  SD = 0.29  $\pm$  0.35) versus male (0.15  $\pm$  0.18) was not significantly different ( $p = 0.07$ ). Compared to the other families, Hipposideridae seems to be the most susceptible to haemosporidian infection ( $p = 0.002$ ). A total of 124 out of 271 blood samples (45.8%) were found to be PCR positive with either one or both sets of primers. The positive samples were detected in blood from host bat species of: *H. larvatus* (58), *H. armiger* (11), *H. bicolor* (10), *H. lekakuli* (1), *R. pearsonii* (5), *R. malayanus* (4), *R. thomasi* (1), *M. spasma* (10), *C. thonglongyai* (7), *C. brachyotis* (2), *E. spelaea* (1), *T. melanopogon* (5), and *My. siligorensis* (8).

We observed three distinct genera of haemosporidian parasites that matched the descriptions of *Hepatocystis*, *Nycteria*, and *Polychromophilus*. No *Plasmodium* was detected from all samples by either microscopic examination or PCR amplification and sequencing. The images and characteristics of *Hepatocystis*, *Nycteria*, and *Polychromophilus* observed in the blood smears together, with their respective hosts, are shown in Fig. 2–4, respectively.

Young gametocytes (Fig. 2j–l) of *Hepatocystis* were found in ring and amoeboid forms with a prominent chromatin dot (black arrowhead). Growing and fully-grown macrogametocytes (female gametocytes) (Fig. 2m–o) were seen in round shape, approximately 5–7  $\mu$ m in diameter, and the cytoplasm stained basophilic with brown pigments distributed all over the gametocyte. The pigment granules appear coarse and approximately 0.1–0.3  $\mu$ m in size. Growing and fully-grown microgametocytes (male gametocytes) (Fig. 2p–r) were seen in a similar size to the macrogametocyte but with an eosinophilic stained cytoplasm with brown pigments distributed all over the gametocyte.

Very early gametocytes of *Nycteria* (Fig. 3e) were seen in a ring form of approximately 2  $\mu$ m in diameter with a prominent chromatin dot (black arrowhead in Fig. 3e). Young and growing macrogametocytes (Fig. 3f–g) were found in an amoeboid form of approximately 3.5–4.5  $\mu$ m; growing and fully-grown macrogametocytes (Fig. 3h–k) were observed in round shape of approximately 5–7  $\mu$ m in diameter. Brown and coarse pigments are visible mostly at the peripheral area of the gametocyte, and the cytoplasm was basophilic, which is a common feature in macrogametocyte. Growing and fully-grown microgametocytes (Fig. 3l–m) were seen in round shape, approximately 5–7  $\mu$ m in

diameter.

Growing and fully-grown macrogametocytes of *Polychromophilus murinus* (Fig. 4c–e) were seen in oval and round shapes, approximately 3–6  $\mu$ m in size and occupying about two-thirds to almost whole of the erythrocyte. Growing and fully-grown macrogametocytes of *Polychromophilus melanipherus* (Fig. 4f–h) were seen in oval and round shapes, approximately 3–6  $\mu$ m in size. Brown and coarse pigments approximately 0.3  $\mu$ m in size were seen in the growing and fully-grown macrogametocytes in both haemosporidian species. Fully-grown microgametocytes of *P. murinus* (Fig. 4i–k) were observed in oval shape, approximately 6  $\times$  8  $\mu$ m in size.

*Hepatocystis* positive slides were seen in the following bat species: *H. larvatus*, *H. armiger*, *R. pearsonii*, *R. malayanus*, *R. thomasi*, and *C. thonglongyai*. *Nycteria* was observed in *M. spasma*, *C. thonglongyai*, *T. melanopogon*, and *E. spelaea*. *Polychromophilus* was detected in blood films of *T. melanopogon* and *My. siligorensis*. Collectively, 10 and 13 out of 21 bat species were found to have positive individuals for haemosporidian parasites by microscopic and molecular analyses, respectively.

### 3.3. Bat haemosporidian phylogenetic analysis based upon *cytb* nucleotide sequence

We obtained a total of 62 partial *cytb* nucleotide sequences, comprised of 50 sequences of *Hepatocystis*, 10 of *Nycteria*, and two of *Polychromophilus*. The residual 378 bp fragment of *cytb* was subjected to the sequence analyses. The sequence located corresponding to nucleotide positions 463 to 840 (amino acid positions 155 to 280) of the *cytb* gene in *Nycteria heischii* (accession no. KX090648). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Among the 50 sequences of *Hepatocystis*, 19 different haplotypes were detected. Haplotype numbers 1 of *Hepatocystis* (originated from *H. larvatus*;  $n = 19$  and *H. armiger*;  $n = 1$ ); and 6 (*H. larvatus*;  $n = 9$ ) were the dominant haplotypes (Supplementary Table S3). Haplotype numbers 3 (*R. pearsonii*;  $n = 2$ ), 4 (*C. thonglongyai* and *R. pearsonii*;  $n = 2$ ), 7 (*H. larvatus*;  $n = 3$ ), and 12 (*H. larvatus*;  $n = 2$ ) were less abundant. The remaining haplotypes were comprised of only one sequence each. Representatives of each haplotype were included in the phylogenetic analysis. Pairwise nucleotide identity among *Hepatocystis* in the present study ranged from 86.7 to 99.7%.

The *cytb* sequences of *Hepatocystis* were divided into 19 haplotypes and clustered into three subclades (1a, 1b, and 2) (Fig. 5). Almost all the *Hepatocystis* in this study were placed in the same subclade (1a) as the *Hepatocystis* previously reported in *H. larvatus* from Cambodia (Daval et al., 2007). This subclade was comprised of *Hepatocystis* isolated from

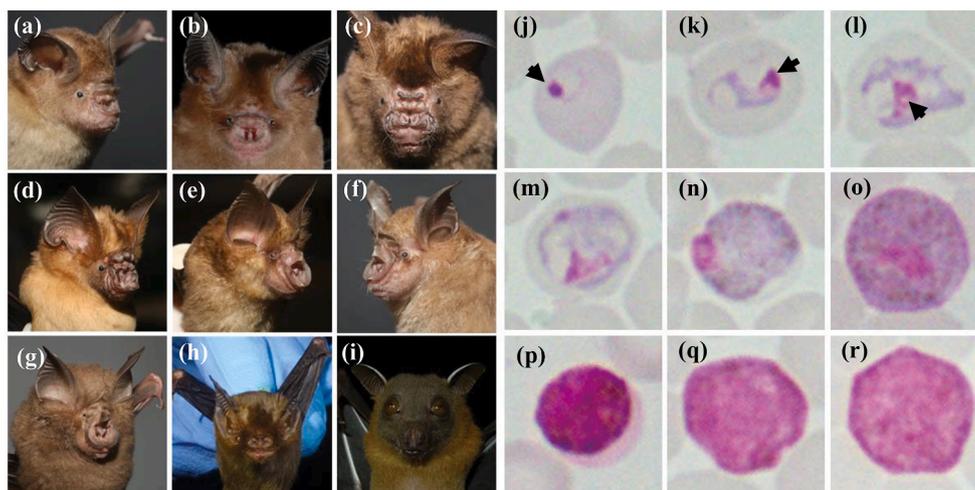
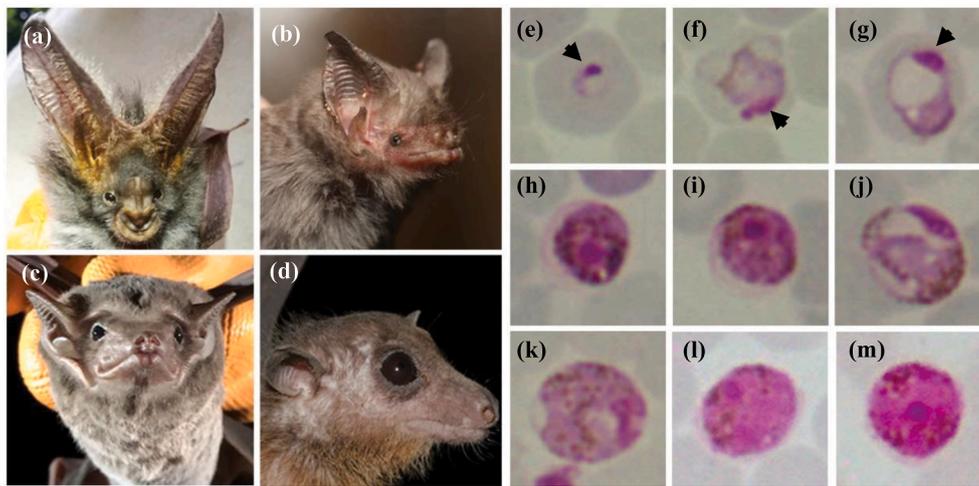
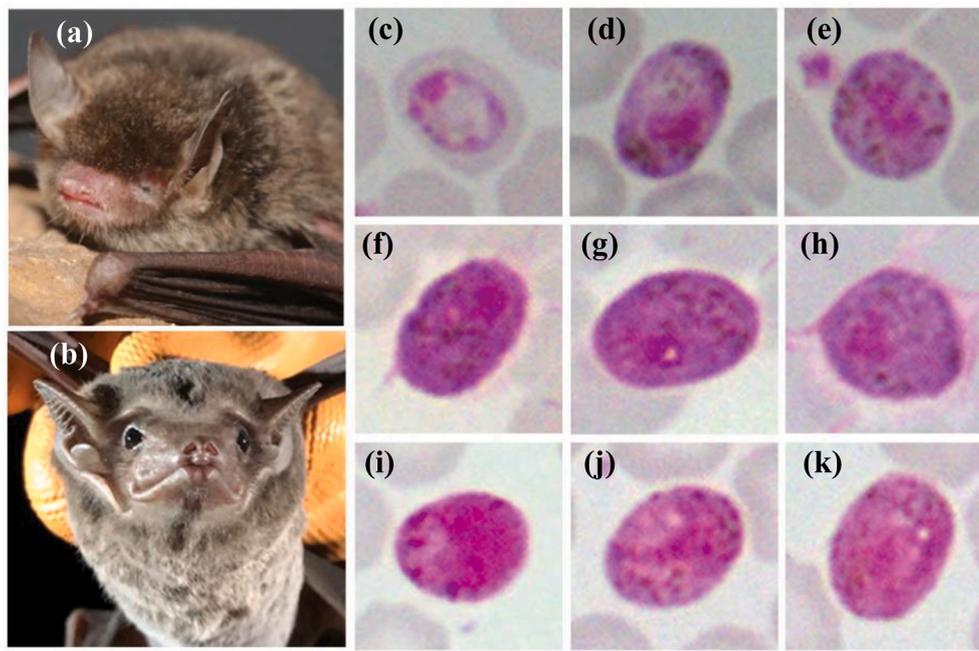


Fig. 2. Images of bats with *Hepatocystis* positive infection based on either microscopic examination or *cytb* gene PCR amplification and sequencing results: *H. larvatus* (a), *H. bicolor* (b), *H. armiger* (c), *H. lekakuli* (d), *R. malayanus* (e), *R. thomasi* (f), *R. pearsonii* (g), *C. thonglongyai* (h), and *Cy. brachyotis* (i). Giemsa-stained blood smears depicting young gametocytes of *Hepatocystis* in ring and amoeboid forms (j–l), growing and fully-grown macrogametocytes (female) (m–o), growing and fully-grown microgametocytes (male) (p–r) observed in *H. larvatus* (sample ID THBat19-037, *cytb* accession no. MT136132). All parasite images were taken at the same magnification. Scale bar = 5  $\mu$ m. (For viewing the images in color, the reader is referred to the online version of this article.) (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 3.** Images of bats with *Nycteria* positive infection based on either microscopic examination or *cytb* gene PCR amplification and sequencing results: *M. spasma* (a), *C. thonglongyai* (b), *T. melanopogon* (c), and *E. spelaea* (d). Giemsa-stained blood smears depicting very early gametocyte (e) with chromatin dot pointed by arrowhead, young and growing macrogametocytes (female) (f–g), growing and fully-grown macrogametocytes (h–k), and growing and fully-grown microgametocytes (l–m) of *Nycteria* observed in *M. spasma* (sample ID THBat19-177, *cytb* accession no. MT136163). All parasite images were taken at the same magnification. Scale bar = 5  $\mu$ m. (For viewing the images in color, the reader is referred to the online version of this article.) (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** Images of bats with *Polychromophilus* positive infection based on either microscopic examination or *cytb* gene PCR amplification and sequencing results: *My. siligorensis* (a) and *T. melanopogon* (b). Giemsa-stained blood smears depicting growing and fully-grown macrogametocytes of *Polychromophilus murinus* observed in *My. siligorensis* (c–e) (sample ID THBat19-211, *cytb* accession no. MT136168). Growing and fully-grown macrogametocytes of *Polychromophilus melanipherus* observed in *T. melanopogon* (f–h) (sample ID THBat19-170, *cytb* accession no. MT136167). Fully-grown microgametocytes of *P. murinus* (i–k). All parasite images were taken at the same magnification. Scale bar = 5  $\mu$ m. (For viewing the images in color, the reader is referred to the online version of this article.) (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

*H. larvatus*, *H. armiger*, *R. pearsonii*, and *C. thonglongyai*. Another subclade (1b) was unique to the *Hepatocystis* in Australasian small flying fox in the family Pteropodidae and none of our *Hepatocystis* was placed in this subclade. Only one *Hepatocystis* from *Cr. brachyotis* in the current study was placed in the same clade (2) with *Hepatocystis* isolated from the same frugivorous bat species in Malaysia with strong BS support.

We detected *Nycteria* in *M. spasma*, *C. thonglongyai*, *T. melanopogon*, and *E. spelaea* bats that originated from the Phetchaburi, Ratchaburi, and Kanchanaburi provinces. A high nucleotide similarity among the sequences of *Nycteria* was found (97.6–100%). The *Nycteria* observed in our study were placed in the same clade as the *Nycteria* previously reported in *M. spasma* from Cambodia (Duval et al., 2007), but in a distinct clade from the *Nycteria* observed in bats in the family Rhinolophidae from Africa (clade 1) (Fig. 6). *Nycteria* in *M. spasma* was split into three separate subclades (subclades 2a, 2c, and 2d). *Nycteria* in *C. thonglongyai*, *E. spelaea*, and *T. melanopogon* was placed in separate subclades (2b, 2c, and 2d, respectively). *Nycteria* in *T. melanopogon* from Phetchaburi was closely related to *Nycteria* isolated from *M. spasma* in

Kanchanaburi. *Nycteria* in *E. spelaea* from Ratchaburi was closely related to *Nycteria* isolated from *M. spasma* in Kanchanaburi and Cambodia. It is important to note that BS support only in subclades 2c and 2d were greater than 50.

*Polychromophilus* was detected in *T. melanopogon* and *My. siligorensis* bats in Kanchanaburi province. In the present study, the nucleotide similarity between the two sequences of *Polychromophilus* was 97.8%. The BLASTn results of *Polychromophilus* revealed two distinct species comprised of *P. murinus* and *P. melanipherus* (with percent identity 99.47 and 99.74, respectively). The *P. melanipherus* originating from *T. melanopogon* was clustered in clade 1 together with *Polychromophilus* seen in the African insectivorous bats in the family Miniopteridae (Duval et al., 2012) (Fig. 7), while *P. murinus* from *My. siligorensis* was placed in clade 2 with the *P. murinus* found in bats in the family Vespertilionidae in Switzerland (Megali et al., 2011).

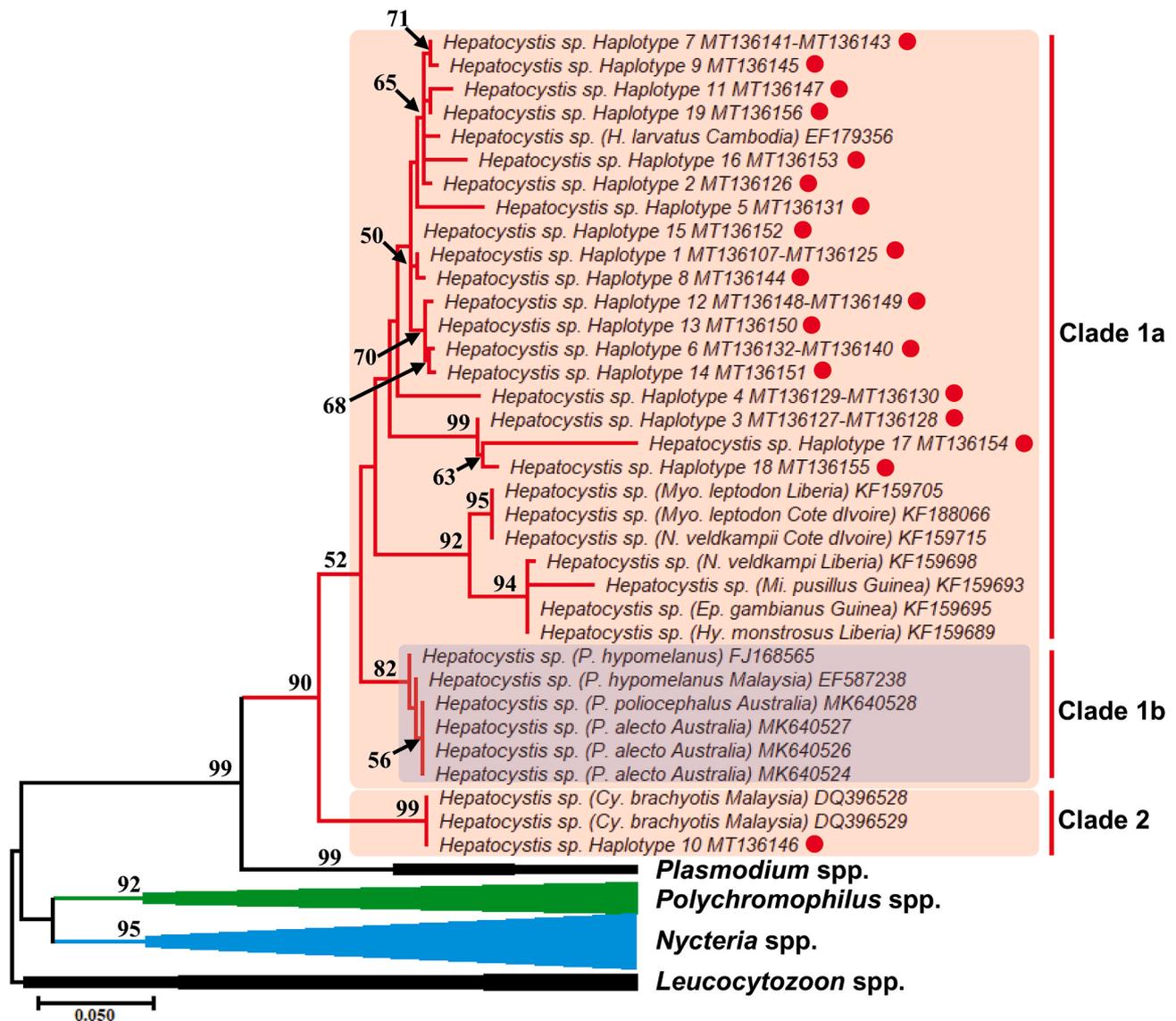


Fig. 5. Phylogenetic (ML) relationship of the *Hepatocystis cytB* gene fragment (378 bp) in the present study (taxa with red circles) and the other global isolates from the GenBank™ database. Clade (number) and subclade (letter) are indicated. Haplotype 10 of *Hepatocystis* in the current study originated from the frugivorous bat *Cy. brachyotis* shares the same clade with Malaysian isolate (in clade 2). The percentage of trees in which the associated taxa clustered together is shown next to the branches. BS values greater than 50% are shown in the figure. The other clades, consisting of *Nycteria*, *Polychromophilus*, *Plasmodium*, and outgroup are collapsed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

#### 4. Discussion

A study of haemosporidians in bats in Thailand was performed almost four decades ago (Landau et al., 1984) and has recently been updated for a few locations only (Chumnandee and Pha-obnga, 2018). Additionally, these observations were made based on microscopic examination. In the present study, we conducted microscopic and PCR-based surveys for haemosporidian parasites in bats from the Western, Eastern, Central, and Southern provinces of Thailand. Most of the samples originated from cave-dwelling bats, except those from Trat and Songkhla provinces, as cave bats are relatively easier to capture compared to forest bats. It is also important to note that surveys in Kanchanaburi were conducted on multiple trips in different seasons of the year. The negative results in Saraburi and Songkla might be explained by the low number of bats that we were able to capture in this study. Almost all of the bats in the present study are insectivorous, with only *Cy. brachyotis* (Pteropodidae) as the exception being frugivorous. The present study added at least eight new bat species into the current

list of bats known to be infected by *Hepatocystis*, where *H. larvatus* was excluded because it had already been documented as a host species (Landau et al., 1984; Duval et al., 2007). However, we did not observe the elongated form of *Hepatocystis* that was previously reported in Australian flying foxes (Family: Pteropodidae) (Schaer et al., 2018).

The phylogenetic analysis of this study was congruent with a previous report (Schaer et al., 2013). The *Hepatocystis* isolates in these Thai bats were genetically diverse with 19 distinct *cytB* haplotypes, which clustered into three different subclades. Most of *Hepatocystis* observed in the present study were phylogenetically placed in a subclade shared with an isolate from *H. larvatus* previously reported from Cambodia (Duval et al., 2007). It is interesting to note that one isolate of *Hepatocystis*, obtained from the frugivorous bat, *Cy. brachyotis*, was positioned in a subclade shared with Malaysian isolate of *Hepatocystis*.

The majority of *Nycteria* (7 out of 10) found in our study were isolated from *M. spasma* (Megadermatidae), with the other *cytB* sequences of *Nycteria* being isolated from *C. thonglongyai*, *T. melanopogon*, and *E. spelaea* (one sequences per bat species). Only one sequence of *Nycteria*

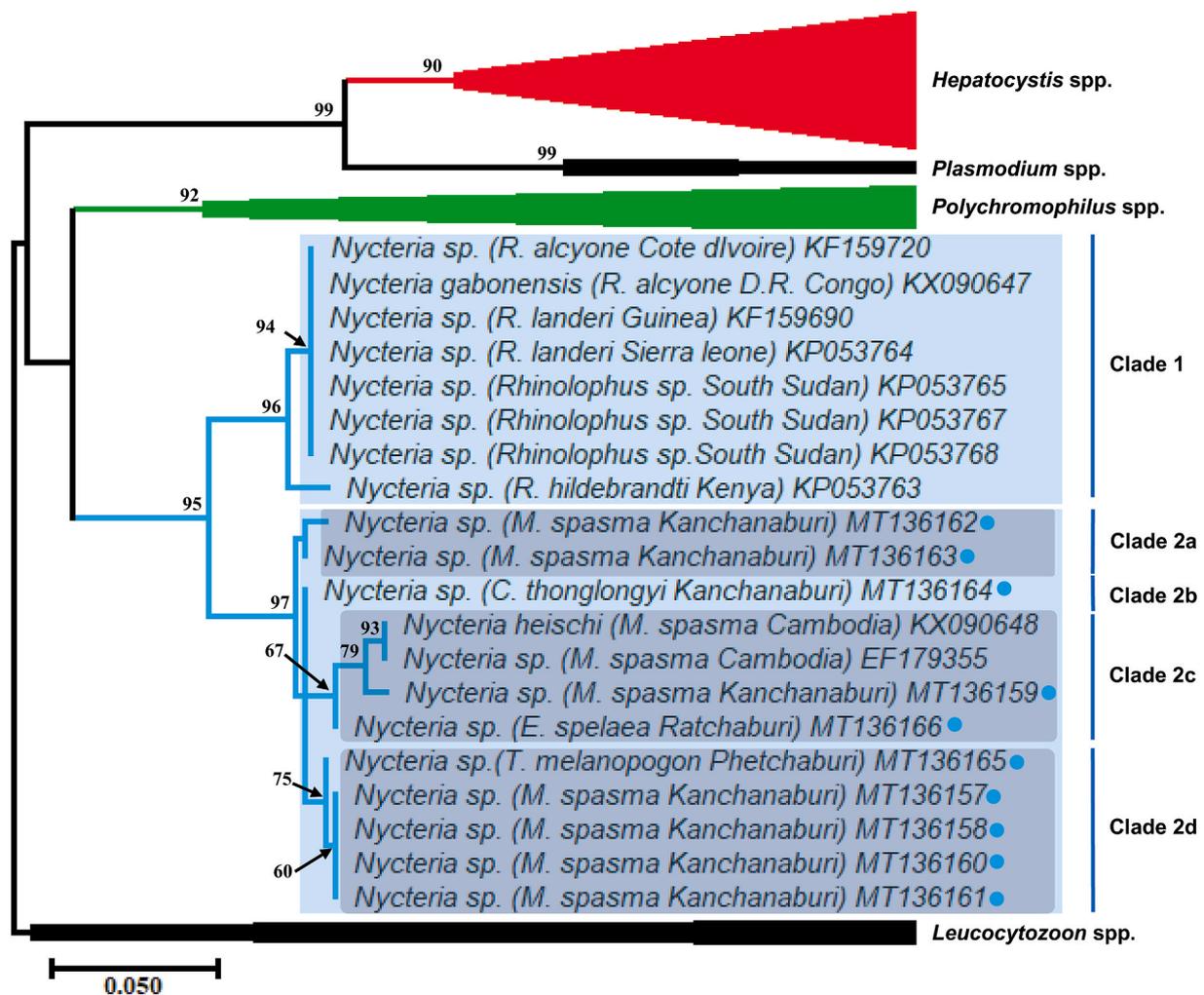


Fig. 6. Phylogenetic (ML) relationship of the *Nycteria cytb* gene fragment (378 bp) obtained in the present study (taxa with blue circles) and the other isolates previously deposited in GenBank™ database. *Nycteria* from Asian bats clustered in clade 2 and a separate clade from the African bat isolates (in clade 1). BS values greater than 50% are shown in the figure. The other clades, consisting of *Hepatocystis*, *Polychromophilus*, *Plasmodium*, and outgroup are collapsed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

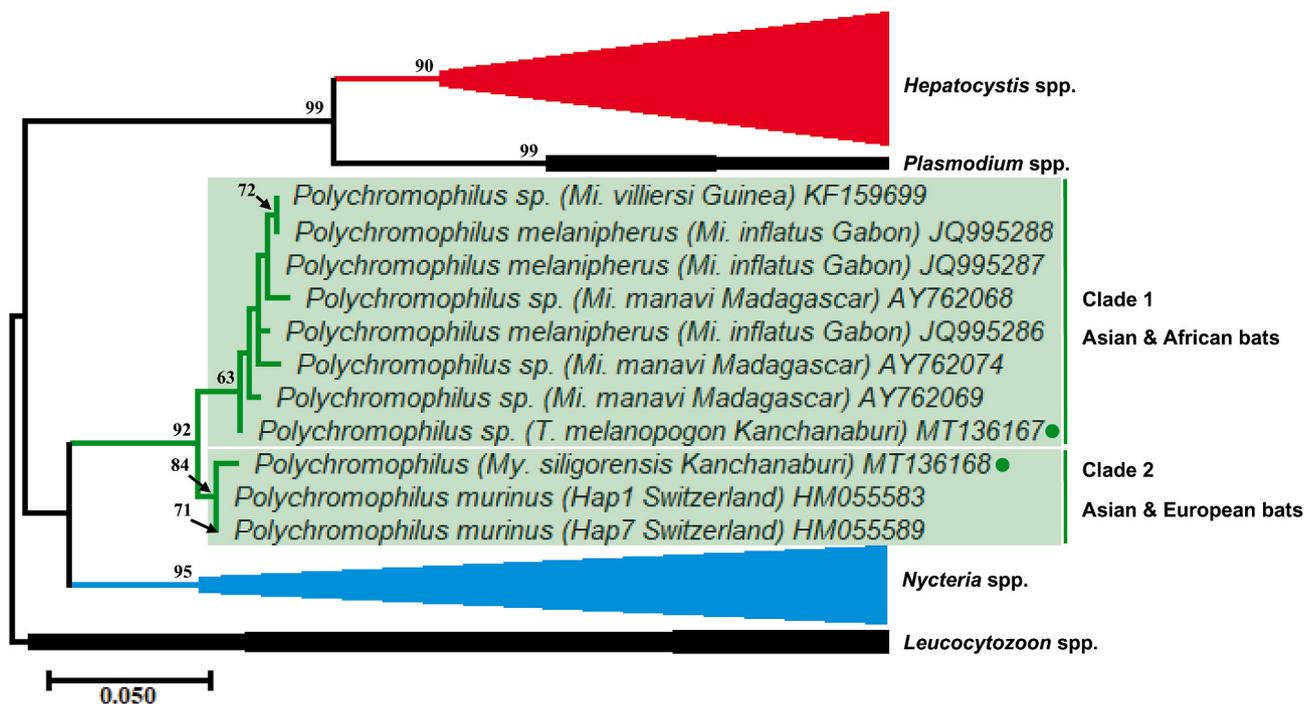
isolated from *M. spasma* in Kanchanaburi was placed in the same clade as *Nycteria heischii* found in *M. spasma* from Cambodia (Duval et al., 2007). The rest of the *Nycteria* isolated from *C. thonglongyai*, *T. melanopogon*, and *E. spelaea* were placed in separate subclades that were distinct from the previous isolates from the insectivorous bats of East and Central Africa (Schaer et al., 2015). Together with previous reports (Duval et al., 2007; Schaer et al., 2015), this study added Craseonycteridae and Pteropodidae into the current list of bat species that act as hosts for *Nycteria* (so far found in Nycteridae, Megadermatidae, Rhinolopidae, and Emballonuridae families). It is interesting to note that *M. spasma* was only found to be infected by *Nycteria* and not by *Hepatocystis* or *Polychromophilus*.

Previous reports of *Polychromophilus* infections in insectivorous bats (Miniopteridae and Vespertilionidae) were from Europe (Megali et al., 2011; Duval et al., 2012), Africa and South-East Asia (Adam and Landau, 1973; Duval et al., 2007), South America (Garnham et al., 1971), and Australia (Mackerras, 1959). This study presented the first molecular evidence (*cytb* nucleotide sequences) together with morphological characterization of *Polychromophilus* in *My. siligorensis* (Vespertilionidae) and added *T. melanopogon* (Emballonuridae) into the current list of its bat host. It is important to note that *P. melanipherus* ex. *T. melanopogon* share the same clade with *Polychromophilus* seen in African insectivorous bats in the family Miniopteridae, suggesting its potential wide distribution range. In addition, *P. murinus* from *My.*

*siligorensis* was placed in the same clade with *P. murinus* found in bats in the family Vespertilionidae in Switzerland (Megali et al., 2011), suggesting a wide distribution range of *P. murinus*, but relatively restricted to bats in the family Vespertilionidae. However, our study mainly targeted cave-dwelling bats, and so we could not rule out haemosporidian infections in bats from the other habitats in Thailand. In addition, North and Northeastern Thailand were not sampled. The results of this study, together with Witsenburg et al. (2012) and Duval et al. (2012), confirmed that *Polychromophilus* could be comprised of at least two distinct species. Further study on the remaining chiropteran haemosporidians, such as *Plasmodium*, *Biguetiella*, *Bioccala*, *Dionisia*, *Johnsprentia*, and *Sprattiella*, which were not seen in the present study, might improve our understanding of their bat hosts and the phylogenetic relationship of these parasites.

## 5. Conclusions

The present investigation confirmed the existence of haemosporidian parasites in bats in Thailand based on both microscopic and molecular (*cytb* nucleotide sequence) analyses. *Hepatocystis* infected diverse bat species, comprised of *H. larvatus*, *H. bicolor*, *H. armiger*, *R. pearsonii*, *H. lekakuli*, *R. malayanus*, *R. thomasi*, *Cy. brachyotis*, and *C. thonglongyai*. *Nycteria* was found in *M. spasma*, *C. thonglongyai*, *T. melanopogon*, and *E. spelaea*, while *P. murinus* was found in blood films of *My. Siligorensis*,



**Fig. 7.** Phylogenetic (ML) relationship of the *Polychromophilus cytb* gene fragment (378 bp) found in this study (taxa with green circles) and the other global isolates. *Polychromophilus* isolated from *T. melanopogon* is placed in the same clade with African bat isolates of *P. melanipherus* in clade 1. *Polychromophilus* isolated from *My. siligorensis* shares the same clade with European bat isolates of *P. murinus* in clade 2. BS values greater than 50% are shown in the figure. The other clades, consisting of *Hepatocystis*, *Nycteria*, *Plasmodium*, and outgroup are collapsed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and *P. melanipherus* was observed in *T. melanopogon*. We observed a high prevalence of haemosporidian parasites in bats in the country with no signature of host specificity especially for *Hepatocystis*.

#### Authors' contributions

**Apinya Arnuphapprasert** and **Elizabeth Riana**: Investigation, methodology, data curation, formal analysis, writing- original draft preparation. **Thongchai Ngamprasertwong**: Resources, validation and funding acquisition. **Monsicha Wangthongchaicharoen**: Investigation and validation. **Pipat Soisook**: Resources and validation. **Suchansa Thane**: Investigation and methodology. **Phanaschakorn Bhodhibundit**: Resources. **Morakot Kaewthamasorn**: Conceptualization, methodology, data curation, formal analysis, validation, writing-reviewing and editing, resources, supervision, project administration, and funding acquisition.

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#### Declaration of competing interest

All authors declare no financial or personal interests that might influence the judgment or decision made with the current study. The final version of article has been read and approved by all authors.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijppaw.2020.07.010>.

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