

## Original Article

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## Molecular identification of hemoplasmas in free ranging non-human primates in Thailand

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### ABSTRACT

**Objective:** To survey hemoplasmas infection in free ranging non-human primates from 8 provinces in Thailand.

**Methods:** DNA from ethylenediaminetetraacetic acid blood of 262 free ranging non-human primates were identified as hemoplasmas using PCR and phylogenetic analysis based on *16S rRNA* and *rnpB* genes.

**Results:** A total of 148 non-human primates (56.49%) were determined positive for *Candidatus Mycoplasma haemomacae*, including 125 *Macaca fascicularis* and 23 *Macaca mulatta*. Hemoplasmas can cause anemia in monkey but all positive samples were healthy. The positive rates in male and female non-human primates were not significantly different.

**Conclusions:** *Candidatus Mycoplasma* infection is prevalent in free ranging *Macaca fascicularis* and *Macaca mulatta* in Thailand.

## 1. Introduction

Hemoplasmas or hemotropic mycoplasmas are small pleomorphic and uncultivable bacteria that infect erythrocytes and endothelial cells of several mammalian species[1-4], which can cause anemia[5]. Data about the transmission of hemoplasmas is still poor[5]. Only one study has experimentally demonstrated transmission of mycoplasmas by *Rhipicephalus (R.) sanguineus*[6], but the role of this tick in the natural transmission of haemoplasmas is unknown. A recent study investigated a possible trans-stadial route of canine haemoplasma transmission by *R. sanguineus* in field conditions, but no canine haemoplasma DNA was detected in unfed nymphs or adult stages of ticks[7]. Hemoplasma was reclassified in genus *Mycoplasma* from *Haemobartonella* and *Eperythrozoon* based on nucleotide sequences of 16S ribosomal (r) RNA (*16S rRNA*) gene[1].

Recently, RNase P RNA (*rnpB*) gene was recommended to identify and classify hemoplasmas[8]. The hemoplasmas in non-human primates consist of *Candidatus Mycoplasma haemomacae* (*Ca. M. haemomacae*)[9,10], *Candidatus Mycoplasma kahanei* (*Ca. M. kahanei*)[11], and *Candidatus Mycoplasma aoti* (*Ca. M. aoti*)[12].

Wildlife has been a major reservoir of infectious diseases and a source of the trouble that threatening humans and domestic animals health[13]. Non-human primates (NHP), as one of the wildlife species, is suggested to be an important reservoir host of several pathogens[13,14]. The infections of hemoplasmas were reported in free ranging NHP[10,15,16]. In Thailand, the large population

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of free ranging NHP species are long-tailed macaque [*Macaca (M.) fascicularis*] and rhesus macaque (*M. mulatta*)<sup>[17]</sup>. They are closely contact and conflict with humans because of the adaptation of macaque habitats, the human activities and the expansion of human communities<sup>[17,18]</sup>. Thus, the inter-species transmission of hemoplasmas may be occurred. Hemoplasmas in NHP have been reported in Brazil, French, Japan, the United State of America and the United Kingdom<sup>[9,10,12,15,16,19-21]</sup>. Unfortunately, there is no information on the hemoplasmas infection in *M. fascicularis* and *M. mulatta* in Thailand. Therefore, the present study focused on the genetic screening and preformed the phylogenetic analysis of hemoplasmas species in NHP that were identified from free ranging *M. fascicularis* and *M. mulatta* in Thailand.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

A total of 262 samples of ethylenediaminetetraacetic acid bloods were collected from free ranging NHP including 227 *M. fascicularis* and 35 *M. mulatta* from April, 2018 to August, 2018 in 8 provinces of 4 regions in Thailand, including central (Lop Buri and Samut Sakhon), northern (Chiang Rai), southern (Phatthalung and Songkhla), western (Prachuap Khiri Khan), and northeastern (Amnat Charoen and Mukdahan). The NHP were determined healthy by physical examination. All specimens were extracted using DNeasy blood & tissue kit (QIAGEN, Germany) according to the manufacturer's instructions. Each sample was eluted with 50 µL nuclease free water and stored at -20 °C.

### 2.2. Hemoplasmas detection

All DNA were initially screened for hemoplasmas using HBT-F (5'-ATACGGCCCATATTC CTACG-3') and HBT-R (5'-TGCTCCACCACTTGTTC-3') primers of PCR based on *16S rRNA* gene<sup>[22]</sup>. The total volume of PCR mixture was 25 µL that consisted of 1 PCR buffer, 3.5 mM MgCl<sub>2</sub>, 0.5 µM of each primer, 0.25 mM of each deoxynucleotide triphosphate and 1.25 U of i-Taq™ DNA polymerase (iNtRON Biotechnology, Korea), 2 µL of DNA template and made up to 25 µL with nuclease-free water. The PCR conditions included a 3 min initial denaturation at 94 °C, followed by 30 cycles at 94 °C for 30 s, 60 °C for 45 s and 72 °C for 45 s with a final extension of 72 °C for 7 min. The PCR products (595 bp) were electrophoresed in 2% agarose gel and visualized with GelRed™ (Biotium, United States) under UV illumination. The positive *16S rRNA* gene was additionally screened by *rnpB* primers (80F1: 5'-GAGGAAAGTCCRYGCTWGCAC-3' and 290R1 5'-TCCCYTACCRAAATTTTGGTTTCT-3')<sup>[8]</sup>. The final concentration of reaction components and condition were similar to the *16S rRNA* screening, except the annealing step was optimized at 44 °C. The *rnpB* primers amplified an approximately 220 bp fragment.

### 2.3. Nucleotide sequence-based analysis

The fragments of *16S rRNA* and *rnpB* genes were purified using QIAquick® Gel Extraction Kit (QIAGEN, Germany) and sent to a private service laboratory (Macrogen Inc., Korea) for nucleotide sequencing. The partial nucleotide sequences of *16S rRNA* and *rnpB* genes were aligned using Clustal W and constructed the phylogenetic relationship of various hemoplasmas using the Maximum Likelihood method in MEGA 7 software<sup>[23]</sup>. The distances were evaluated by the General Time Reversible model and Tamura 3-parameter model for *16S rRNA* and *rnpB* genes, respectively. The data sets were generated using 1 000 bootstrap resamplings. The reference sequences of hemoplasmas species for comparison were obtained from GenBank NCBI (<http://www.ncbi.nlm.nih.gov/genbank>).

### 2.4. Ethnical approval

The present protocol was approved by the Faculty of the Veterinary Science, Mahidol University-Institute Animal Care and Use Committee (Protocol No. MUVS-2018-09-49).

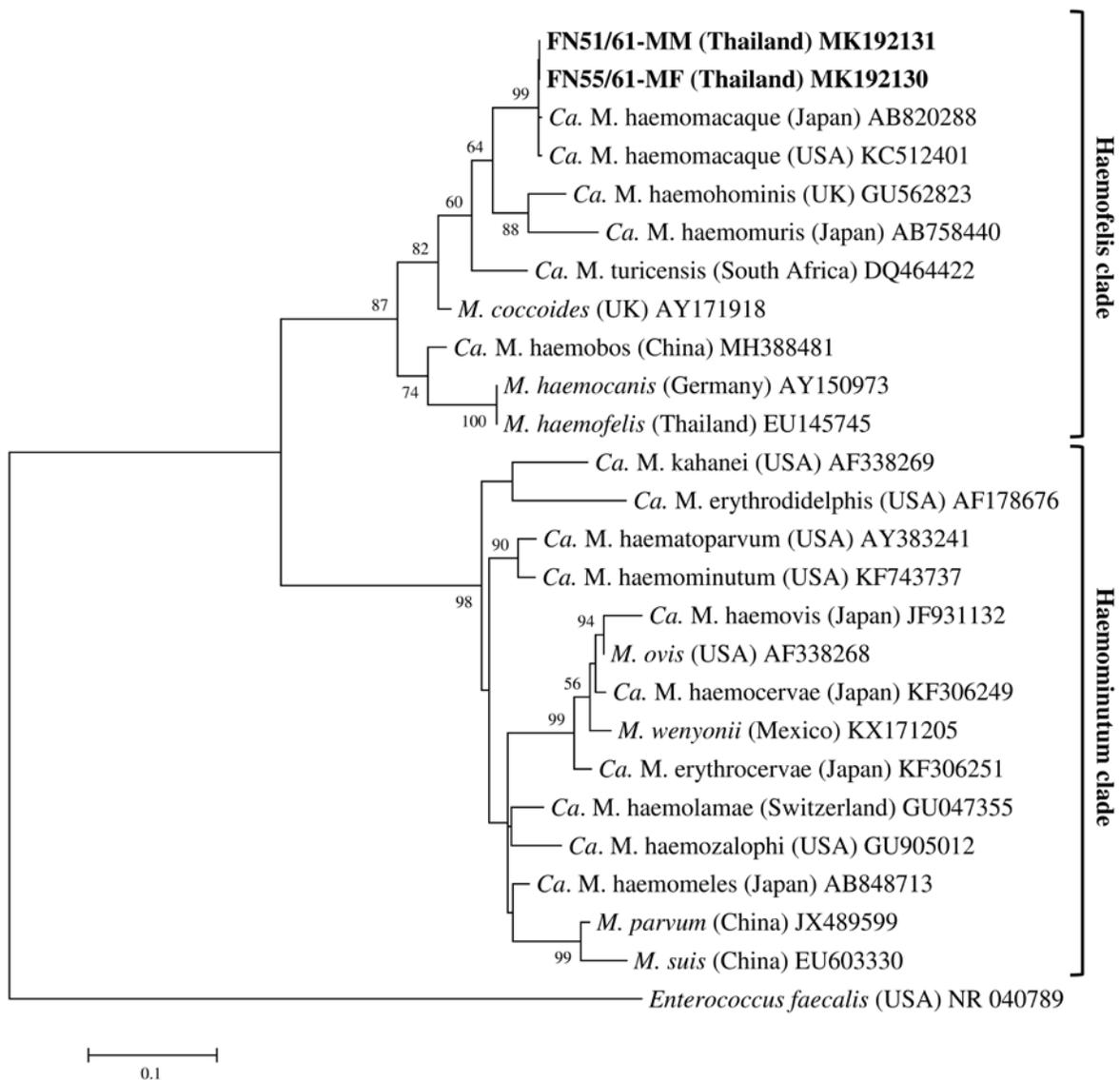
### 2.5. Statistical analysis

The statistical relationships between positive results of hemoplasmas and sex of free ranging NHP was analyzed by Pearson *Chi*-Square test using SPSS 18 for Windows (SPSS Inc., United States). Statistical significance was indicated at *P* value < 0.05.

## 3. Results

One hundred and forty eight (148) of 262 (56.48%) NHP samples were positive for *M. fascicularis* (Table 1), which were collected from Lop Buri (*n*=28), Samut Sakhon (*n*=24), Mukdahan (*n*=14), Phatthalung (*n*=15), Songkhla (*n*=10), Prachuap Khiri Khan (*n*=13), Amnat Charoen (*n*=21) and Chiang Rai (*n*=23). All positive samples were detected in both host species (125 *M. fascicularis* and 23 *M. mulatta*). The positive rate of male NHP was 54.65% (94/172), whereas the positive rate of female NHP was 60.00% (54/90). There is no significant difference in positive rate between male and female NHP (*P*>0.05).

The partial *16S rRNA* and *rnpB* sequences of hemoplasmas derived from both *M. fascicularis* (FN55/61-MF) (Accession No. MK192130 and MK192132, respectively) and *M. mulatta* (FN51/61-MM) (Accession No. MK192131 and MK192133, respectively) were analyzed for sequence homology and phylogeny. The homology between the positive nucleotide sequences and the published nucleotide sequences was analyzed using BLASTn. The obtained sequences of *16S rRNA* gene were highly similar to *Ca. M. haemomacae* (99%) from *M. fuscata* (AB820288) and *M. fascicularis* (KC512401). Conversely, the partial *rnpB* sequences



**Figure 1.** Phylogenetic tree of the partial *16S rRNA* sequences of hemoplasma species in free ranging non-human primates and other hemoplasma species from GenBank.

of positive samples presented low homology (82%) with *Ca. M. haemomuris* (AB973090) and *Ca. M. haemohominis* (GU562825) that isolated from *Apodemus argenteus* and *Homo sapiens*, respectively. The identity rates of *mpB* between the positive and *Ca. M. haemomacaque* sequences were unknown because *mpB* sequence of *Ca. M. haemomacaque* is still unpublished in GenBank.

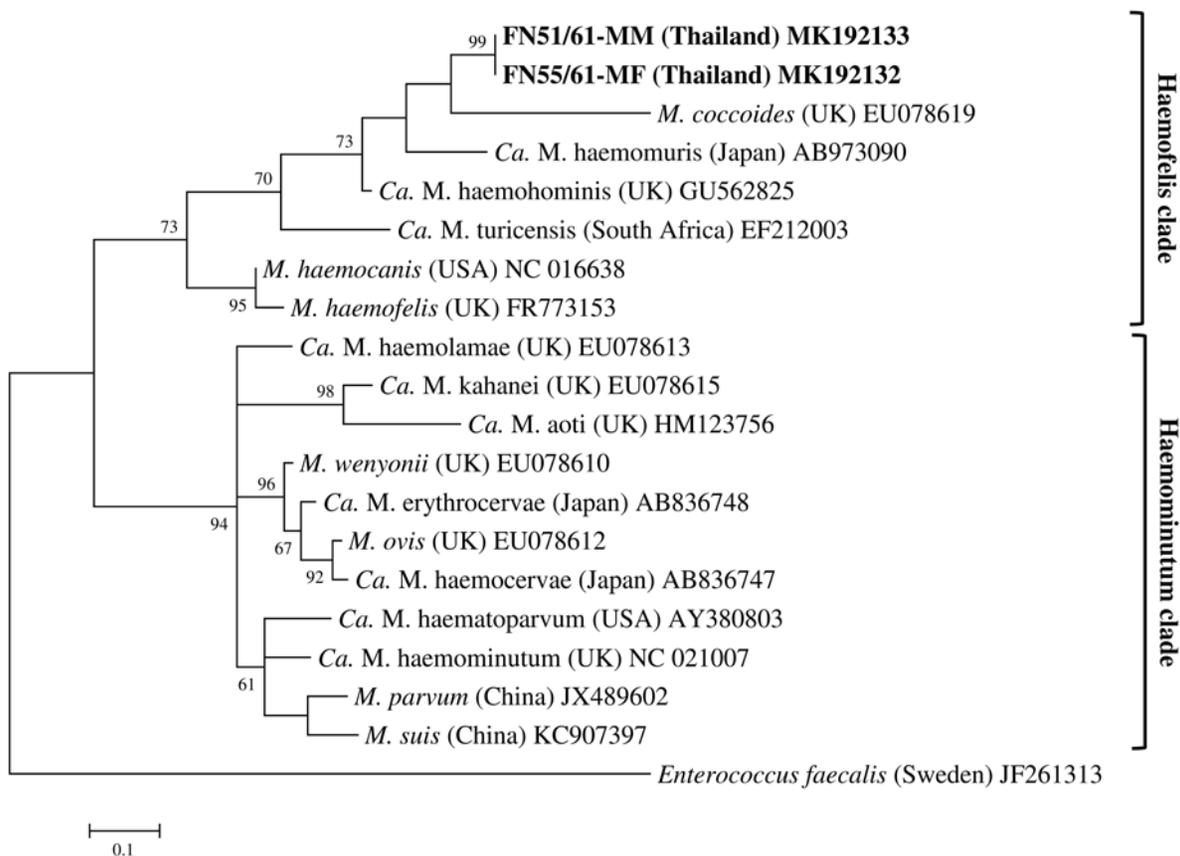
The phylogenetic trees of the partial *16S rRNA* and *mpB* sequences in *M. fascicularis* and *M. mulatta* of the present study were constructed using *Enterococcus faecalis* as an outgroup. Percentages of bootstrap values were obtained on the nodes of the tree, and values over 50% were displayed. The phylogram of both genes were divided into two clades that were haemofelis and haemominutum. In both phylogeny, the positive sequences showed a number of the haemofelis clade, which were positioned in the same cluster as *Ca. M. haemomuris*, *Ca. M. haemohominis*, *Ca. M. turicensis*, and *M. coccoides* (Figure 1 and 2). Moreover, the tree of *16S rRNA* genes

presented the strong relationship between the sequences from the tested NHP and *Ca. M. haemomacaque* sequence from GenBank (Figure 1).

**Table 1.** Results of hemoplasmas detection in tested non-human primates (%).

Species	Selective provinces	Positive	
		Male	Female
<i>Macaca fascicularis</i>	Lop Buri	62.50 (10/16)	94.74 (18/19)
	Samut Sakhon	89.47 (17/19)	77.78 (7/9)
	Mukdahan	42.31 (11/26)	75.00 (3/4)
	Phatthalung	51.82 (14/27)	33.33 (1/3)
	Songkhla	31.03 (9/29)	10.00 (1/10)
	Prachuap Khiri Khan	40.00 (4/10)	40.91 (9/22)
<i>Macaca mulatta</i>	Amnat Charoen	62.96 (17/27)	66.67 (4/6)
	Chiang Rai	66.67 (12/18)	64.71 (11/17)
Total		54.65 (94/172)	60.00 (54/90)

NHP: non-human primates.



**Figure 2.** Phylogenetic tree of the partial *rnpB* sequences of hemoplasma species in free ranging non-human primates and other hemoplasma species from GenBank.

#### 4. Discussion

In the present study, the positive rate of hemoplasmas infection among the free ranging NHP was rather high (56.48%). The result was higher than the reports in southern (25%) and northeastern Brazil (35.7%) [16,24] but lower than the study in the United State of America (84.2%) [9] and Japan (100%) [10]. In Thailand, hemoplasma infection in NHP has not been previously reported. Result of this research showed the occurrence of hemoplasmas infection in Thai NHP that including *M. fascicularis* and *M. mulatta*. These host species have been found hemoplasmas [9,20,25]. Hemoplasmas infection has presented from no clinical signs to severe anemia in NHP [9,10,19-21,24]. All positive samples in our study did not show clinical signs. Previous reports suggested that anemia may not be associated with hemoplasmas infection in NHP [9,21,24].

The infection rate in male is not significantly different in female, which differ from the previous report [24]. In this study, the greater number of samples was tested, therefore, the results are estimated to be more reliable. Habitats of the tested NHP and most habitats of free ranging NHP in Thailand overlap with communities [17,18], which may cause cross transmission of infection between NHP and humans or domestic animals. Moreover, some species of hemoplasmas were found in humans [26-28], at this point, further studies should be conducted on hemoplasmas infection in humans and other animals in the habitat of NHP, which may effectively

monitor the public health of humans and animals.

The positive sequences showed high similarity in *16S rRNA* of *Ca. M. haemomacaque*. Further, the obtained *16S rRNA* and *rnpB* gene sequences belonged to the haemofelis clade and the cluster of *Ca. M. turicensis* and *M. coccoides*, which is similar to findings in other studies [9]. Unfortunately, the *rnpB* sequence of *Ca. M. haemomacaque* detected in *M. fascicularis* from previous experiment [9] was not available in GenBank, so the identity rate of the *rnpB* sequences of hemoplasmas isolated from the positive NHP samples cannot be determined. Although there is no *rnpB* identity result of *Ca. M. haemomacaque*, we considered that the *rnpB* sequences of the positive samples was *Ca. M. haemomacaque*. This conclusion was supported by the finding that explained above. Previously, *Ca. M. haemomacaque* had been report in *M. fascicularis* from the United State of America [9] and *M. fuscata* from Japan [10]. In Brazil, *Mycoplasma* spp. closely similar to *Ca. M. haemomacaque*, were identified in *Sapajus apella* [16] and *Sapajus flavius* [21].

Overall, the occurrence showed the widespread of *Ca. M. haemomacaque* infection in free ranging *M. fascicularis* and *M. mulatta* in Thailand, and the infection is associated with male sex.

#### Conflict of interest statement

We declare that there is no conflict of interest.

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