Malaria and Hepatocystis Species in Wild Macaques, Southern Thailand

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Abstract. Southeast Asian macaques are natural hosts for a number of nonhuman primate malaria parasites; some of these can cause diseases in humans. We conducted a cross-sectional survey by collecting 99 blood samples from Macaca fascicularis in southern Thailand. Giemsa-stained blood films showed five (5.1%) positive samples and six (6.1%) isolates had positive test results by polymerase chain reaction. A phylogenetic tree inferred from the A-type sequences of the small subunit ribosomal RNA gene confirmed Plasmodium inui in five macaques; one of these macaques was co-infected with P. coatneyi. Hepatocystis, a hemoprotozoan parasite transmitted by Culicoides, was identified in an isolate that was confirmed by analysis of mitochondrial cytochrome b sequences. All malaria-infected monkeys lived in mangrove forests, but no infected monkeys were found in an urban area. These findings indicate regional differences in malaria distribution among these macaques, as well as differences in potential risk of disease transmission to humans.

INTRODUCTION

The natural habitats of macaques are ecologically diverse, ranging from Asia to northern Africa, and they are the most widely distributed genus of nonhuman primates. Certain macaques are susceptible to a variety of infectious agents, ranging from viruses to parasites, and thus could be the reservoirs responsible for emerging or re-emerging zoonotic diseases in humans.1 The crab-eating or long-tailed macaque, Macaca fascicularis, is found in a wide variety of habitats, including primary lowland rainforests, secondary rainforests, and coastal forests of nipah palm and mangrove. The native range of M. fascicularis includes most of mainland Southeast Asia, including the Malay Archipelago, the islands of Sumatra, Java and Borneo, the islands of the Philippines, and the Nicobar Islands in the Bay of Bengal.2

In Thailand, macaque populations are abundant and have a wide geographic range.3 However, some natural habitats of these macaques have been disturbed by forest destruction, leading to migration of certain macaque troops to nearby human settlements including many Buddhist temples. In some communities of southern Thailand, people keep macaques as pets and also use them for coconut picking. More recently, mangrove reforestation along coastal regions has resulted in a remarkable increase in the number of long-tailed macaques. Because M. fascicularis is highly adaptable to exotic environments with efficient reproductive success, it has not been considered to be an endangered species.

At least 26 malaria species have been known to infect nonhuman primates in their natural habitats.4 Importantly, five of these species, i.e., Plasmodium knowlesi, P. cynomolgi, P. brasilianum, P. simium, and P. inui, can cause apparent diseases in humans.5–9 Of these, P. knowlesi, whose known main natural host is M. fascicularis, has been documented in disease transmission to humans.10,11 Sporadic human infections with P. knowlesi have been reported in the Malaysian Peninsular and southern Thailand, and a large focus of outbreaks has been reported on Sarawak Island.10,11 In addition, P. inui and P. cynomolgi have been implicated in human infections under experimental or accidental conditions and their main natural hosts are Southeast Asian macaques.12–17 Despite the importance and potential hazard to human health, no epidemiologic studies have been performed to evaluate the status of malaria among macaques in Thailand. Therefore, we conducted a cross-sectional survey to determine the prevalence of malaria infections in long-tailed macaques in an urban area of Prachuab Khirikhan Province and mangrove forests in Ranong Province in southern Thailand. Results have shown that malaria was prevalent in macaques living in mangrove forests, but none were found in those living in an urban area.

MATERIALS AND METHODS

Sampling sites and populations. A cross-sectional survey of malaria in wild macaques was conducted in May 2006 on Khon Tee Island (9°57′11.8″ N, 98°35′41.4″ E) and in the Mangrove Forest Research Center (9°52′38″ N, 98°36′9.6″ E) in Prachuab Khirikhan Province, and in Wat Khao Takieb (12°30′51.4″ N, 99°59′9.4″ E) in Prachuab Khirikhan Province in southern Thailand (Figure 1). Khon Tee is a small island consisting of a long strip of mangrove forest where the natural ecosystem is well conserved along the coastal areas facing to the Andaman Sea. By the time of this study, it was estimated that more than 30 macaques were clustered within one troop living in the mangrove forests of this island. This troop fed mainly on fruits, leaves, seeds, other plant parts, and small animals such as crabs, frogs, and insects. However, in time of drought and famine, they might be crop raiders and sometimes were fed by villagers. Ranong Province also has vast mainland mangrove forests that cover approximately 200 km² east of the Andaman Sea that is considered by the United Nations Educational, Scientific and Cultural Organization to be one of the most flourished mangrove forests in Asia-Pacific regions. The study site was approximately 15 km southwest of Ranong Province where the Mangrove Forest Research Center is located. Although the center is open to public, feeding animals and all other activities that would disturb the forest ecosystem are strictly prohibited. Conversely, Wat Khao Takieb is a small hill in an urban area facing the Gulf of Thailand in Prachuab Khirikhan Province where a Buddhist temple is located. Wild macaques have long migrated to live in the vicin-
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Capture-and-release field work. After capturing the macaques in a trap, they were anesthetized by intramuscular injection with 10 mg/kg body weight of ketamine hydrochloride. While the macaques were immobilized, body weights were measured and blood samples were collected and preserved in EDTA buffer. Blood samples isolated from these monkeys from Khon Tee Island, the Mangrove Forest Research Center, and Wat Khao Takieb were labeled WPN, MFRC, and WKT, respectively, and were given an isolate number. All monkeys were released back to the troop after complete recovery. No eventful outcomes were observed among these monkeys during and after the study process. This study was reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

Morphologic study of malaria in blood samples. Aliquots of fresh blood samples were used for both thin and thick blood film preparations, followed by staining with Giemsa. Malaria parasites were examined in at least 400 fields with an Olympus (Center Valley, PA) BX51 light microscope at a magnification of 100 and the parasite density was expressed in terms of percentage of infected erythrocytes in a thin blood film and number of malaria parasites per number of leukocytes in a thick blood film.

Isolation of malaria parasite DNA. Malarial parasite DNA was extracted from 0.2 mL of EDTA-blood samples by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA purification procedure was essentially as described in the manufacturer’s instruction manual. Purified DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at −20°C until used.

Polymerase chain reaction. The DNA fragment spanning the small subunit ribosomal RNA (SSU rRNA) gene of Plasmodium spp. was amplified by a semi-nested polymerase chain reaction (PCR) using primers whose sequences were derived from the 5' and 3' portions of the SSU rRNA gene of the P. vivax Salvador 1 strain (GenBank accession no. U03079). Sequences of the primers used for primary PCR were P18SF0, 5'-AACCTGGTGATCTTGGCCAG-3' and P18SR0, 5'-GAACCTGCGGAAAGGATCATTA-3'. Sequences of the primers for the secondary PCR were P18SF1, 5'-TGTTGATCTTGGCCAGTA-3' and P18SR0. We used the same thermal cycling profiles for both primary and secondary PCR: 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 3 minutes. DNA amplification was performed by using a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). To minimize error introduced in the sequences during PCR amplification, we used ExTaq DNA polymerase (Takara, Shiga, Japan), which has efficient 5' to 3' exonuclease activity to increase fidelity and shows no strand displacement. The size of PCR product was examined by electrophoresis on a 1% agarose gel and visualized with an ultraviolet transilluminator (Mupid Scope WD; Advanced Company Ltd., Tokyo, Japan).

The PCR amplification of the mitochondrial cytochrome b gene (hereafter referred to as the cytochrome b gene) was performed for each isolate using primers MTCbF0, 5'-GTAATGCTAGCATATTCTC-3' and MTCbR0, 5'-ACTCCCTATCATGTCTTGC-3'. The amplification conditions were as follows: an initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes.

Subcloning. The PCR products were excised from agarose gel, purified by using a QIAquick PCR purification kit (Qiagen), and ligated into pGEM-T-Easy Vector (Promega, Madison WI). After incubation overnight at 4°C, the reaction mixture was precipitated, dissolved in 10 μL of double-distilled water, and transformed into Escherichia coli strain JM109 by electroporation using an E. coli pulser apparatus (Bio-Rad Laboratories, Hercules, CA). Recombinant DNA from positive clones was prepared by using the QIAGEN plasmid mini kit (Qiagen).

DNA sequencing. The DNA sequences were determined from at least 10 plasmid subclones for each isolate. Sequencing analysis was performed from both directions for each template using the BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI3100 Genetic Analyzer (Applied Bio-
systems). Overlapping sequences were obtained by using sequencing primers (available upon request). When required, the sequence was re-determined using PCR products from two independent amplifications from the same DNA samples.

Data analysis. Sequences were aligned by the CLUSTAL X with minor manual adjustment made by visual inspection.\(^{16}\) Phylogenetic construction was performed by the neighboring-joining method using the Kimura-2 parameter, maximum composite likelihood, and Tamura 3-parameter with 1,000 bootstrap iterations as implemented in the MEGA version 4.0 software.\(^{19–24}\) Nucleotide sequences reported in this study have been deposited in the GenBank database under accession nos. EU400384–EU400413. Other SSU rRNA and cytchrome \(b\) sequences were obtained from the GenBank database: \(P.\) \(vivax\), U88336, AF069619; \(P.\) \(inui\), U72541, AF049122, AF049123, AF069617; \(P.\) \(hylobati\), AY579421, AF069618; \(P.\) \(knowlesi\), U72542, L07560, AY722797; \(P.\) \(coatneyi\), AY579420; \(P.\) \(fragile\), M61722, AY722799; \(P.\) \(fieldi\), AY579419, AF069615; \(P.\) \(cynomolgi\), L07559, AF069616; \(P.\) \(simiovale\), AY278221, AF069614; \(P.\) \(gonderi\), AY579416, AF069622, AY800111; \(P.\) \(ovale\), L48986, AF069625, AB182496; \(P.\) \(malariae\), M54897, AF069624; \(P.\) \(brasilianum\), F130735; \(P.\) \(falciparum\), M61722, A276846; \(P.\) \(reichenowi\), Z25819, AF069610; \(P.\) \(elongatum\), AF069611; \(P.\) \(gallinaceum\), AF069612, and \(Hepatocystis\) spp., AF069626.

RESULTS

Macaques. A total of 105 \(M.\) \(fascicularis\) were captured. The age class, sex, and body weight of these monkeys are shown in Table 1. Blood samples were not taken from infant monkeys; six monkeys were excluded from analysis. A total of 99 blood samples were obtained from monkeys on Khon Tee Island (\(n = 15\)), the Mangrove Forest Research Center (\(n = 78\)). Most (64.6%) captured monkeys were male. The average weight of monkeys on Khon Tee Island and at the Mangrove Forest Research Center was 4.7 kg, and the average weight of monkeys in Wat Khao Takeib was 7.0 kg. The distribution of age class did not account for the weight difference between these populations because the proportion of adult and sub-adult to juvenile monkeys of the former populations was not significantly different from that of the latter (0.75:1 and 0.70:1, respectively). Therefore, the average weight of macaques living on foods available in their natural habitats was lower than that of monkeys living mainly dependent on foods from humans.

Morphology of malaria parasites. All macaques were examined for malaria parasites and infection rates were determined from Giemsa-stained thin and thick blood films. Five (5.1%) of these macaques harbored malaria parasites and malaria-like parasites in their peripheral blood with varying degrees of parasite density, ranging from 0.001% to 0.112% (Table 2). It is noteworthy that all of these malaria-infected monkeys were from Khon Tee Island and the Mangrove Forest Research Center (prevalence = 23.8%), but parasites were not found in monkeys from Wat Kao Takeib. Isolate WPN4 contained various parasite stages characterized by ring forms with a single prominent and large nucleus, growing trophozoites having amoeboid shape residing in normal erythrocytes with fine intraerythrocytic stipplings similar to Ziemann’s dots, growing schizonts with prominent nuclei and yellow or green-brown malarial pigments, mature schizonts with 12–14 merozoites, and oval-shaped gametocytes occupying almost the entire space of infected erythrocytes with compact cytoplasm and abundant yellow-black pigments (Figure 2). These findings were compatible with \(P.\) \(inui\). Conversely, the structure of malaria parasites in isolate WPN6 was heterogeneous. One parasite population resembled those found in isolate WPN4. However, we could not make an unambiguous diagnosis with the other parasite population. The latter population was characterized by tiny ring forms, ring stages with double unequal nuclei, no definite stipplings in erythrocytes infected with growing trophozoites or schizonts, and multiple infections of parasites in infected erythrocytes (Figure 2).

The low parasite density in isolates WPN5, MFRC15, and MFRC18 precluded definitive diagnosis from blood films because only ring stages were observed without other distinctive characteristics. Isolate MFRC11 contained malaria-like parasites characterized by small ring-like parasites, enlarged trophozoite-like structures with prominent vacuoles, and compact nuclei occupying more than half of the infected erythrocytes, some with elongated nuclei with a substantial amount of fine gold-brown pigment that did not coalesce, some with remarkably enlarged vacuoles in a tenuous or spiky cytoplasmic mass that took up little stain, and some with abundant pigment that were not enclosed by erythrocytes. These characteristics were consistent with gametocytes of \(Hepatocystis\) spp.\(^{25}\)

Analysis of SSU rRNA sequences. Amplification of the SSU rRNA gene by PCR showed positive results in six samples, all from Ranong Province. The amplified SSU rRNA gene of all isolates gave products of nearly identical size (approximately 2.2 kb). All samples positive by microscopy were positive by PCR (Table 2). Because malaria parasites have distinct types of SSU rRNA transcripts that could be simultaneously amplified by primers derived from conserved regions of the gene, we analyzed sequences of each isolate by using recombinant subclones as templates to isolate specific sequence types. We used only the A-type SSU rRNA sequences for orthologous gene comparison. The A-type sequences can be distinguished from the S-types or O types because they have insertions/deletions at specific regions in variable domains as described previously.\(^{26,27}\) The topologies obtained by all 3 parameter models of phylogenetic inference based on variable domain V7, from which information for

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age class*</th>
<th>Mean ± SD body weight, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>Infant</td>
<td>0.40 ± 0.00 (2)</td>
</tr>
<tr>
<td></td>
<td>Juvenile</td>
<td>2.63 ± 1.74 (7)</td>
</tr>
<tr>
<td></td>
<td>Sub-adult</td>
<td>8.42 ± 1.39 (3)</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>7.04 ± 0.86 (4)</td>
</tr>
<tr>
<td>M</td>
<td>Infant</td>
<td>0.20 ± 0.00 (1)</td>
</tr>
<tr>
<td></td>
<td>Juvenile</td>
<td>3.64 ± 1.41 (24)</td>
</tr>
<tr>
<td></td>
<td>Sub-adult</td>
<td>8.87 ± 2.40 (16)</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>12.40 ± 2.50 (21)</td>
</tr>
</tbody>
</table>

* Age classification is based on dental eruption: infant, < 0.5 years of age; juvenile, 1–5 years of age; sub-adult, 3–6 years of age; adult, > 6 years of age.
most malaria species was available, were largely concordant with those reported by other investigators (Figure 3A).28–30 Isolates whose sequences were placed within the same cluster as reference sequences were identified as identical species. Isolates WPN4, WPN5, MFRC15, and MFRC 18 contained *P. inui* and co-infection of *P. inui* and *P. coatneyi* was found in isolate WPN6 (Table 2 and Figure 3A).

When a comparison was made using nearly complete sequences of the gene, clone a of isolate WPN4 shared identical sequence with clone b of isolate WPN6, but others differed from each another, with the number of nucleotide differences per site based on pairwise comparison (p-distance) ranging from 0.0019 to 0.0226 (mean = 0.0088) (Figure 3B). Clone f of isolate WPN6 was placed within the same cluster as that of *P. coatneyi*. Sequence of isolate MFRC11 was the outgroup from nonhuman primate and human malaria ones, which suggested that they belong to a different genus (Figure 3A). Searches for sequence similarity with those in the GenBank databases failed to identify any identical or nearly identical sequence with the SSU rRNA sequence of isolate MFRC11.

### Analysis of the cytochrome b gene

All isolates that were positive by PCR targeting the SSU rRNA gene also showed positive results when amplified using the primers derived from the cytochrome b gene spanning approximately 1 kb. A phylogenetic tree inferred from the cytochrome b gene has reaffirmed and detected more clonal variation both within and between isolates containing *P. inui*. Although sequences of 13 clones were clustered within the *P. inui* lineage, all displayed sequence microheterogeneity, with p-distance ranging from 0.0019 to 0.0232 (mean = 0.0087). Co-infection between *P. inui* and *P. coatneyi* in isolate WPN6 based on the SSU rRNA locus was also reaffirmed by the cytochrome b gene.

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**Table 2**

Malaria and *Hepatocystis* spp. of *Macaca fascicularis* in southern Thailand diagnosed from blood films and polymerase chain reaction

<table>
<thead>
<tr>
<th>Monkey ID</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Age class*</th>
<th>Microscopy (% parasitemia)</th>
<th>Small subunit ribosomal RNA-based (no. of distinct clones)†</th>
<th>Cytochrome b-based (no. of distinct clones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPN4</td>
<td>M</td>
<td>4.4</td>
<td>Juvenile</td>
<td><em>Plasmodium inui</em> (0.112)</td>
<td><em>P. inui</em> (3)</td>
<td><em>P. inui</em> (4)</td>
</tr>
<tr>
<td>WPN5</td>
<td>M</td>
<td>10.5</td>
<td>Sub-adult</td>
<td><em>Plasmodium spp.</em> (0.001)</td>
<td><em>P. inui</em> (2)</td>
<td><em>P. inui</em> (2)</td>
</tr>
<tr>
<td>WPN6</td>
<td>F</td>
<td>7.8</td>
<td>Adult</td>
<td><em>P. inui</em> and <em>Plasmodium spp.</em> (0.046)</td>
<td><em>P. inui</em> (4), <em>P. coatneyi</em> (1)</td>
<td><em>Plasmodium inui</em> (3), <em>P. coatneyi</em> (1)</td>
</tr>
<tr>
<td>MFRC11</td>
<td>F</td>
<td>4.5</td>
<td>Adult</td>
<td>Malaria-like (0.060) (Hepatocystis?)</td>
<td>Unknown†‡</td>
<td>Hepatocystis spp. (2)</td>
</tr>
<tr>
<td>MFRC15</td>
<td>M</td>
<td>7.1</td>
<td>Adult</td>
<td>–</td>
<td><em>P. inui</em> (1)</td>
<td><em>P. inui</em> (2)</td>
</tr>
<tr>
<td>MFRC18</td>
<td>F</td>
<td>4.3</td>
<td>Adult</td>
<td><em>Plasmodium spp.</em> (0.001)</td>
<td>–</td>
<td><em>P. inui</em> (1)</td>
</tr>
</tbody>
</table>

* Age classification is based on dental eruption: juvenile, 1–3 years of age; sub-adult, 3–6 years of age; adult, > 6 years of age.
† Based on the A-type sequence.
‡ No available sequence of *Hepatocystis* spp. for comparison.

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**Figure 2.** Giemsa-stained thin blood films of parasite isolates WPN4 (A–D) and WPN6 (E–H) showing A, a ring form; B, growing trophozoite with Ziemann’s-like stipplings; C, a mature schizont; D, a female gametocyte; E, a tiny ring form with a prominent nucleus; F, a ring form with double unequal nuclei; G, a growing trophozoite; and H, double growing trophozoites. Scale bar = 5 μm.
sequences. It is apparent that isolate MFRC11 was phylogenetically placed within the *Hepatocystis* lineage, an apicomplexan hemoprotozoan parasite transmitted by biting midges in the genus *Culicoides* (Figure 4).25

**DISCUSSION**

To date, five species of macaques have been identified in Thailand: *M. fascicularis* (long-tailed macaque), *M. nemestrina* (pig-tailed macaque), *M. mulatta* (rhesus macaque), *M. arctoides* (stump-tailed macaque), and *M. assamensis* (Assamese macaque).3 Of these macaques, long-tailed macaques inhabit a wide range of geographic locations in Thailand and are natural hosts for some malaria parasites capable of causing disease in humans, i.e., *P. knowlesi*, *P. cynomolgi*, and *P. inui*.31,32 However, assessment of malaria infection among these macaques remains largely unknown.

In this study, we determined the prevalence of nonhuman primate malaria in long-tailed macaques in Ranong Province with that in Prachuab Khirikhan Province. The habitats of macaques in Ranong Province were mangrove forests where anopheline mosquitoes were abundant and macaques in Prachuab Khirikhan Province were closely related to a Buddhist temple in an urban area. A high prevalence of malaria was found in the former population (23.8%), but no evidence of malaria carriage was observed among the latter, which suggested regional differences in malaria distribution among macaques. It is noteworthy that some anopheline mosquitoes that transmit human malaria in Thailand are also potential vectors for *P. inui* and *P. coatneyi*. Although a survey of mosquito vectors was not performed in this study, the high prevalence of malaria among people living in the vicinity of the habitats of macaques in Ranong Province and none of the persons living at or around Wat Kao Takieb had malaria, infection would indirectly reflect the prevalence of malaria vectors in these regions.33 Experimental or accidental inoculation of the asexual blood stages of *P. inui* has resulted in symptomatic infections in volunteers and laboratory workers.9,14 However, the risk of naturally acquired cross transmission between macaques and humans is unknown.

Identification of nonhuman primate malaria species based on parasite morphology *per se* remains a challenge and sometimes results in an ambiguous conclusion. Although it was possible to differentiate *P. inui* based on developmental stages in blood films in two isolates in this study, three other isolates could not be diagnosed by conventional microscopy. The presence of only ring stages in two isolates has precluded species identification of the parasites in blood films. Furthermore, the structure of nonhuman primate malaria is highly dependent on host erythrocytes. For instance, *P. knowlesi* could resemble *P. vivax* in *M. fascicularis*, *P. falciparum* in rhesus monkeys, and *P. malariae* in humans. Conversely, laboratory-induced *P. vivax* infections in splenectomized chimpanzee exhibit bizarre structures.34 Conversely, results of PCR amplification in this study using either primers that targeted the SSU rRNA or the cytochrome *b* genes have
identified an additional positive sample that included a mixed species infection. A similar study by Fandeur and others who surveyed malaria in monkeys of the families Cebidae and Callitrichidae in the rainforest in French Guiana reported *P. brasilianum*, a closely related or sympatric species with *P. malariae*, in 5.6% of samples based on examination of blood films and a prevalence of 11.3% by a nested PCR method. Likewise, the species-specific PCR-based method for detection of the four human malaria parasites has consistently outperformed those based on conventional microscopy, especially with samples with low-level parasitemias or mixed species infections. Mixed species infections with nonhuman primate malaria in macaques and *Hepatocystis* that resembles malaria parasites further complicate structure-based species determination. Therefore, molecular methods are sensitive and efficient methods for species identification of nonhuman primate malaria in a large-scale survey.

The SSU rRNA transcript of malaria parasites depends on its developmental process in different environments, which uses predominantly A-type during asexual cycle in the human host and S-type in the mosquito vector where sporogonic cycle takes place. An additional distinct SSU rRNA transcript belonging to the ookinet and oocyst stages has been found in *P. vivax*. Nonrandom nucleotide differences between these transcripts could imply differences in ribosomal affinity for subsets of mRNA or suppression of translation termination involved in the developmental progression. Phylogenetic analysis based on the A-type sequences obtained in this study has assisted in speciation of nonhuman primate malaria parasites. Our results reaffirm previous phylogenetic positions of *P. inui* and *P. coatneyi* as being closely related to *P. vivax*, regardless of their differences in the duration of asexual erythrocytic schizogony. Furthermore, these A-type sequences of the SSU rRNA genes of *P. inui* showed sequence variation among subclones both within and between *P. inui* isolates. Analysis of the cytochrome *b* gene has consistently shown sequence difference among clones/isolates of *P. inui*, which indicates that multi-clone infections would occur frequently in natural transmission among these macaques. Although the first human case in Thailand plausibly acquired the infection in another forest area along Thailand-Myanmar border of Prachuab Khirikhan Province, the failure to detect *P. knowlesi* in these macaque populations has suggested its absence or low endemicity. Therefore, this parasite would not be a widespread hazard for human health in the region.

Besides malaria, a malaria-related parasite of the genus *Hepatocystis* has co-circulated among macaques in Thailand. Intraerythrocytic development of *Hepatocystis* is confined to sexual stages, and biting midges in the genus *Culicoides* serve as vectors. Characteristics of merocysts in liver are useful for speciation of *Hepatocystis*, but gametocytes in erythrocytes provide few diagnostic clues. At least four species of *Hepatocystis* (*H. kochi*, *H. simiae*, *H. bouillezi*, and *H. cercopitheci*) are known to infect African monkeys. Infections in Oriental monkeys are caused by *H. semnopitheci* and *H. taiwanensis*. Importantly, *M. fascicularis* is known to be a natural host for *H. semnopitheci*, and *H. taiwanensis* is reportedly restricted to *M. cyclopis* that inhabits Taiwan. Apart from morphologic differences in tissue stages caused by these parasites, the distribution of *H. semnopitheci* seems to extend over southern Asia, west of the latitude of Taiwan. The presence of two distinct cytochrome *b* sequences in isolate MFRC11 that clus-

![Figure 4. Phylogenetic tree based on the mitochondrial cytochrome *b* genes of malaria and *Hepatocystis* spp. inferred using the neighbor-joining method with Kimura 2-parameter model. Percentage of bootstrap supports over 50% are shown along the branches. Scale underneath the tree indicates the number of base substitutions per site. Sequences from known parasite species are listed along with their respective GenBank accession numbers. Sequences of MFRC11 clones A and B were newly obtained in this study.](image-url)
ter within the same lineage as the sequence of Hepatocystis spp. (GenBank accession no. AF069626) could be indicative of multiple clone infections, similar to those found with other malaria parasites. Nevertheless, there is no known hazard for Hepatocystis to cross transmit to humans.

It is noteworthy that the prevalence of human malaria in Thailand displays an annual and almost constant bimodal pattern, peaking in May–July and October–November. The survey period reported herein was restricted to one month when environmental conditions favorable for anopheline vectors could be undoubtedly different from the rest of the year. Whether a remarkable seasonal trend of macaque malaria follows that of human malaria requires further investigation, along with hematologic profiles of infected monkeys, which has not been performed in this study.

In conclusion, our study has identified a remarkable difference in the prevalence of nonhuman primate malaria in populations of M. fascicularis that are prevalent in Thailand. Multiple clone infections of P. inui were commonly encountered among macaques living in the mangrove forest areas. Whether the presence of P. inui in macaques would pose a danger of cross transmission to humans requires further investigation. Determination of these malaria species by structural feature may not be as feasible as determination with molecular tools because these new methods have been shown to be a powerful method for this task.

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REFERENCES

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*... dian inui is not closely related to other quartan Plasmodium species. J Parasitol 84: 278–282.*