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Ecology of malaria parasites infecting Southeast Asian macaques: evidence from cytochrome b sequences

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Abstract

Although malaria parasites infecting non-human primates are important models for human malaria, little is known of the ecology of infection by these parasites in the wild. We extensively sequenced cytochrome b (*cytb*) of malaria parasites (Apicomplexa: Haemosporida) from free-living Southeast Asian monkeys *Macaca nemestrina* and *M. fascicularis*. The two most commonly observed taxa were *P. inui* and *Hepatocystis* sp., but certain other sequences did not cluster closely with any previously sequenced species. Most of the major clades of parasites were found in both *Macaca* species; and the two most commonly occurring parasite infected the two *Macaca* species at approximately equal levels. However, *P. inui* showed evidence of genetic differentiation between the populations infecting the two *Macaca* species, suggesting limited movement of this parasite among hosts. Moreover, coinfection with *Plasmodium* and *Hepatocystis* species occurred significantly less frequently than expected on the basis of the rates of infection with either taxon alone, suggesting the possibility of competitive exclusion. The results revealed unexpectedly complex communities of *Plasmodium* and *Hepatocystis* taxa infecting wild Southeast Asian monkeys. Parasite taxa differed with respect to both the frequency of between-host movement and their frequency of coinfection.

Introduction

Haemosporidians (Apicomplexa: Haemosporida), including the genus *Plasmodium* and related genera such as *Hepatocystis*, are protist parasites of vertebrates; in humans, *Plasmodium* infections cause devastating disease, resulting in approximately one million deaths per year (Greenwood et al. 2008). A number of recent studies have applied polymerase chain reaction (PCR) techniques to the mitochondrial cytochrome b (*cytb*) gene or other genes in order to survey the Haemosporidian parasites infecting natural populations of a number of vertebrate taxa, including lizards (Perkins 2001), bats (Olival et al. 2007), birds (Chasar et al. 2009; Ishtiaq et al. 2007; Loiseau et al. 2010), and African great apes (Ollomo et al. 2009; Prugnolle et al. 2010; Rich et al. 2009). These studies have revealed a remarkable degree of complexity in haemosporidian parasite communities, including

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evidence that coinfection of individual hosts by more than one parasite species may be widespread (Hellgren 2005; Križanauskiene 2010; Pérez-Tris and Bensch 2005).

Theoretical studies of coinfection by parasites have focused on competitive exclusion, particularly on the prediction that a more virulent parasite will out-compete a less virulent parasite when they infect the same host (Mideo 2009; Read and Taylor 2001; Richie 1988). Support for the latter prediction in experimental infections in the laboratory has been equivocal, perhaps because of factors such as genetic diversity in the susceptibility of the hosts (De Roode et al. 2004, 2005; Read and Taylor 2001). Less theoretical attention has been paid to the possibility that two parasites might be mutually beneficial, with infection by one species affecting the environment within the host in such a way that infection by a second parasite is facilitated (Richie 1988; Schall and Bromwich 1994).

Early evidence suggested that in human malaria, coinfection occurs less frequently than expected by chance (Cohen 1973). On the other hand, molecular studies have shown that coinfection with multiple *Plasmodium* species is more frequent in humans than previously supposed (Mayxay et al. 2004; McKenzie and Bossert 1999; Mueller et al. 2007, 2009; Snounou and White 2004). In recent field studies of human malaria infections in Papua New Guinea, Mehlotra and colleagues (2000, 2002) reported evidence that infections with each of the four species *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* occur at random with respect to infection by the other species, implying no competitive or facilitative interactions between them. Another recent study, also in Papua New Guinea, found positive associations in infection between all pairs of the same four species except *P. falciparum* and *P. vivax*, supporting the hypothesis of mutual facilitation in infection by certain species pairs (Mueller et al. 2009).

Three of the malaria species commonly infecting humans (*P. knowlesi*, *P. malariae*, and *P. vivax*) show evidence of a close relationship with nonhuman primate malaria parasites from Southeast Asia (Escalante et al. 1998, 2005; Hayakawa et al. 2008; Jongwutiwes et al. 2005; Mitsui et al. 2010; Mu et al. 2005). In addition, the discovery that the monkey malaria parasite *Plasmodium knowlesi* causes abundant natural human infections in Southeast Asia suggests a previously unappreciated extent of transmission across host species boundaries in that region (Jongwutiwes et al. 2004; Singh et al. 2004; Putaporntip et al. 2009). Over an evolutionary time frame, phylogenetic analyses suggest that the evolutionary history of malaria parasites of primates in Southeast Asia has been particularly complex, with multiple events of host switching in the past and many instances of extant species infecting more than one primate host species (Garamszegi 2009). These considerations suggest that an understanding of non-human primate malarias in Southeast Asia may yield insights into both the evolutionary origin and the present-day epidemiology of important human parasites; yet to date there have been relatively few studies of haemosporidian parasites in natural populations of Southeast Asian nonhuman primates (Seethamchai et al. 2008).

A previous study of free-living long-tailed or crab-eating macaque *Macaca fascicularis* from Thailand used small subunit ribosomal RNA sequence to survey haemosporidia from the genera *Plasmodium* and one *Hepatocystis* (Seethamchai et al. 2008). Here we report the results of a more extensive survey, using PCR amplification and sequencing of *cytb* sequences of haemosporidia from *M. fascicularis* and the pigtail macaque *M. nemestrina*. We examine the extent to which clades of malaria parasites show specificity to one or other of the two host species. In addition, because we obtained data from a substantial number (N = 171) of individuals of *M. nemestrina*, we test for evidence of competitive exclusion or mutual facilitation between *Plasmodium* and *Hepatocystis* taxa infecting the latter species.

Materials and Methods

Study population

A prospective survey of malaria and *Hepaticystis* among wild monkey populations in southern Thailand was conducted during December 2008 to June 2009. The areas of capture were located in forest areas of 4 provinces (Pattalung, Pattani, Yala and Narathiwat) as indicated on the map in Figure 1. Venous blood (1–2 ml) was collected from each animal and preserved in EDTA. This study was reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University.

Microscopy detection

Aliquots of fresh blood samples were used for both thin and thick blood film preparations, followed by treatment with Giemsa stain. Malaria parasites were examined in at least 200 fields with an Olympus BX51 light microscope (Center Valley, PA) at a magnification of 1000.

Isolation of parasite DNA

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 fragments spanning the entire coding region of both *Plasmodium* and *Hepaticystis cytb* were amplified by a polymerase chain reaction (PCR) using primers, MtCybF0: 5'-GTAATGCCTAGACGTATTCCTG-3' and MtCybR0: 5'-GCAAGACATGATAGGGAGT-3'. The thermal cycling profile contained 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 2 minutes. DNA amplification was performed by using a Gene-Amp 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' → 3' exonuclease activity to increase fidelity and shows no strand displacement. Several studies that require high fidelity of sequence data have used this enzyme for PCR assays (e.g. Atamna-Ismaeel et al. 2008; Seyfang and Jin, 2004; Seyfang et al. 1997). The size of PCR products for *Plasmodium cytb* and *Hepaticystis cytb* were clearly distinguishable as the former had ~1490 bp and the latter ~1660 bp.

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. 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 Biosystems). Overlapping sequences were obtained by using sequencing primers (available upon request). Each unique haplotype was verified by obtaining identical sequences from at least 2 different clones. Isolates containing more than 3 unique haplotypes

were reaffirmed by sequencing of recombinant clones generated from two independent PCR amplifications from the same DNA samples.

Statistical methods

We analyzed a total of 365 *cytb* sequences assigned to the genera *Plasmodium*, *Hepatocystis*, and *Haemoproteus*, 334 from the present study and 31 from Genbank (Supplementary Table S1). Assignments to genus and species of previously published sequences were derived from Genbank annotations. Sequences were aligned using the CLUSTAL X program (Thompson et al. 1997) (Supplementary Figure S1). Phylogenetic trees were reconstructed by the neighbor-joining (NJ) method (Saitou and Nei 1987), using a variety of distances. The NJ method was used, rather than a model-based phylogenetic method, because the number of sites was small; and, when the number of sites is small, a simpler method is likely to be more accurate because it has a lower error of estimation (Nei and Kumar 2000). All distances used in NJ analyses yielded similar results; therefore we report only trees based on the Kimura 2-parameter method, which has a smaller variance than more complex distances (Nei and Kumar 2000). Reliability of clustering patterns was tested by bootstrapping (Felsenstein 1985); 1000 bootstrap samples were used.

We used Nei and Gojobori's (1986) method to estimate d_N and the number of synonymous substitutions per synonymous site (d_S). We computed the mean of all pairwise d_S values, designated the synonymous nucleotide diversity (π_S); and the mean of all pairwise d_N values, designated the nonsynonymous nucleotide diversity (π_N). Pairwise F_{ST} between parasite populations infecting different hosts was estimated using the Arlequin 3.11 software (Excoffier et al. 2005). The hypothesis that two parasite taxa co-occurred in hosts at random was tested by the 2×2 χ^2 test of independence.

Results

Monkeys and *Plasmodium* infections

In total, 655 monkeys were captured comprising *Macaca fascicularis* (n = 195), *M. nemestrina* (n = 449), *Macaca arctoides* (n = 4) and *Semnopithecus obscurus* (n = 7). Of these, 164 monkeys harbored *Plasmodium* and/or *Hepatocystis* sp. in their circulation above microscopic threshold (>50 parasites/ μ l) while 26 monkeys had submicroscopic infections as detected by the PCR method. Subcloning and sequencing of PCR-amplified products reveal that mixed infections between parasites carrying different *cytb* sequences occurred in 72 monkeys (37.9%), yielding 125 *cytb* sequences of *Hepatocystis* (GenBank accession numbers GU92944-GU930068) and 209 sequences of *Plasmodium* (GenBank accession numbers GU930069-GU930277). No *Hepatocystis* or *Plasmodium* infections were found in monkeys in the provinces of Pattalung or Pattani (Figure 1; Supplementary Table S2). Infected monkeys were found only in the two adjacent provinces of Yala (area = 4521 km²) and Narathiwat (area = 4475 km²; Figure 1); and the majority of infected monkeys (88.4%) were from Narathiwat (Supplementary Table S2).

Phylogenetic analysis

A phylogenetic tree was constructed of 365 *cytb* sequences from *Plasmodium* and related genera, including sequences isolated from Southeast Asian monkeys and related sequences from the Genbank database (Figure 2). The tree was rooted following Perkins and Schall (Perkins and Schall 2002). As in certain previous phylogenies (Perkins and Schall 2002; Seethamchai et al. 2008), the genus *Plasmodium*, as currently defined, was shown to be polyphyletic, with taxa assigned to the genera *Haemoproteus* and *Hepatocystis* clustering within *Plasmodium* (Figure 2). A phylogeny based on four genes from nuclear,

mitochondrial and apicoplast genomes found that *Hepatocystis* clustered within *Plasmodium* but *Haemoproteus* did not (Martinsen et al. 2008).

Several clusters in the phylogenetic tree included Genbank sequences assigned to known species or genera, along with sequences from our field collections. The largest of these was a cluster receiving 95% bootstrap support that included a Genbank sequence assigned *Plasmodium inui* (AF069617) along with 129 sequences from our field collections (Figure 2). The next largest cluster, which received 99% bootstrap support, included a Genbank sequence assigned to *Hepatocystis* sp. MFRC11 (EU400408) and 125 field isolates (Figure 2). Other sequences formed clusters with Genbank sequences from the following species: *P. knowlesi* (13 field isolates along with two previously reported sequences, AF069621 and AY722797); *P. coatneyi* (8 field isolates along with one previously reported sequence, EU400407); *P. cynomolgi* (2 field isolates along with two previously reported sequences, AF069616 and AY800108); *P. fieldi* (1 field isolate along with 1 previously reported sequence, AF069615); and *P. simiovale* (1 field isolate along with 1 previously reported sequence, AY800109; Figure 2). Each of the latter five clusters received 86–99% bootstrap support (Figure 2).

There were two major clusters that included no previously reported sequences and thus could not be assigned to any known taxon; these are designated Clade A and Clade B (Figure 2). Clade A included 6 sequences and received 89% bootstrap support (Figure 1). Clade A clustered with *P. fieldi* (Figure 2), but this pattern received very low (29%) bootstrap support. Clade B included 41 sequences and received 59% bootstrap support (Figure 2). Clade B clustered close to *P. vixax* and *P. simium* (Figure 2), but grouping of this clade with the latter species received very low (8%) bootstrap support. Thus, the phylogeny suggested that Clade A and Clade B did not correspond to any previously sequenced species. In addition to these major groups, there were a total of eight other sequences that did not group with previously sequenced species (Figure 2).

Difference between host species

The single *Semnopithecus obscurus* in our survey was infected by a parasite that clustered with *P. knowlesi*. The parasites infecting the 171 *Macaca nemestrina* and 18 *M. fascicularis* are summarized in Table 1. There was not a significant difference between the two host species with regard to the incidence of infection by either of the two most commonly observed parasite taxa, *P. inui* and *Hepatocystis* (Table 1). On the other hand, *M. fascicularis* was significantly more likely than *M. nemestrina* to be infected by *P. coatneyi* ($P = 0.007$; Table 1). Similarly, when we pooled the data for all *Plasmodium* species except *P. inui*, *M. fascicularis* was over three times as likely to be infected by these taxa as was *M. nemestrina*; and the difference was statistically significant ($P = 0.01$; Table 1).

A phylogenetic tree of the 129 field collected species assigned to *P. inui* revealed two major clusters, separated by an internal branch that received 100% bootstrap support (Figure 3). The representation of these two clusters differed markedly between the two *Macaca* host species (Figure 3). In the smaller cluster, there were 10 sequences derived from *M. fascicularis* (66.6%) and 5 derived from *M. nemestrina* (33.3%; Figure 3). By contrast, the larger cluster included only 3 sequences derived from *M. fascicularis* (2.6%) but 111 (97.4%) derived from *M. nemestrina* (Figure 3). The difference between the two clusters with respect to the proportions of sequences derived from the two host species was highly significant ($P < 0.001$; Fisher's exact test).

Unlike that of *P. inui*, the phylogeny of *Hepatocystis* sp. did not show any major subdivisions (Figure 4). The 9 sequences derived from *M. fascicularis* were identical to one another and to 89 of the 116 sequences derived from *M. nemestrina* (Figure 4). Table 2

shows synonymous nucleotide diversity (π_S) and nonsynonymous nucleotide diversity (π_N) for *P. inui* and *Hepatocystis* sp. derived from the two *Macaca* host species. Within both *P. inui* and *Hepatocystis* sp., π_S was significantly greater than π_N in every case, except for *Hepatocystis* sp. from *M. fascicularis*, which showed no synonymous or nonsynonymous differences (Table 2). This pattern is indicative of purifying selection on the *cytb* gene. Both π_S and π_N were significantly higher in *P. inui* than in *Hepatocystis* sp. both in *M. nemestrina* and in *M. fascicularis* (Table 2). Likewise, both π_S and π_N were significantly higher in *P. inui* than in *Hepatocystis* sp. when sequences derived from two *Macaca* host species were pooled. These results support the hypothesis that the population of *P. inui* infecting these monkeys is more ancient and diverse than that of *Hepatocystis* sp.

The estimate of pairwise F_{ST} between *Hepatocystis* sp. populations infecting the two *Macaca* host species was -0.057 , which was not significantly different from zero. On the other hand, F_{ST} between *P. inui* populations infecting the two host species was 0.586 , which was significantly different from zero ($P < 0.05$). In Sukhirin district, Narathiwat province, 50 isolates of *Hepatocystis* sp. were collected from *M. nemestrina* and 4 from *M. fascicularis*. F_{ST} between the *Hepatocystis* sp. populations infecting the two host species in this location (-0.142) was not significant. These results support the hypothesis of genetic differentiation between the *P. inui* populations infecting the two hosts but not between the *Hepatocystis* sp. populations infecting the same hosts. When we estimated F_{ST} values among collection locations for *Hepatocystis* sp. infecting *M. nemestrina*, there were significant F_{ST} values in several comparisons (Supplementary Table S3). By contrast, *P. inui* infecting *M. nemestrina* showed no evidence of population substructure (Supplementary Table S3).

Coinfection

Of the 171 infected *M. nemestrina*, 18 (10.5%) were infected by two different clades, and 3 (1.8%) were infected by three different clades (where clades are defined on the basis of the phylogenetic tree in Figure 2). Whereas 51 of the 171 *M. nemestrina* individuals (29.8%) were infected by *P. inui* alone and 83 (48.5%) by *Hepatocystis* alone, only 11 monkeys (6.4%) were infected by both *P. inui* and *Hepatocystis* sp. The proportion of coinfections with *P. inui* and *Hepatocystis* sp. was significantly less than the random expectation ($\chi^2 = 54.6$; 1 d.f.; $P < 0.001$). Similarly, although 17 of the 171 monkeys (9.9%) were infected by *Plasmodium* from clades other than *P. inui*, only one *M. nemestrina* individual (0.6%) was coinfecting with both *Hepatocystis* sp. and *Plasmodium* from a clade other than *P. inui*. Again, the proportion of coinfection was significantly less than the random expectation ($\chi^2 = 18.4$; 1 d.f.; $P < 0.001$). On the other hand, coinfection with *P. inui* and with *Plasmodium* from a clade other than *P. inui* occurred in four *M. nemestrina* individuals (2.3%), not significantly different from the random expectation ($\chi^2 = 1.3$; 1 d.f.; n.s.).

One factor that might influence the probability of coinfection by two parasite taxa might be the absence of one of the taxa from a given site. We tested whether this factor influenced the results by analyzing data from seven collection sites where both *Hepatocystis* sp. and *P. inui* were found: one site in Yala (Kabang) and six sites in Narathiwat (Chanae, Ra-ngae, Sukhirin, Su-ngai Padi, Tak Bai, and Waeng). Of the 145 *M. nemestrina* from these seven sites, 50 (34.5%) were infected by *P. inui* alone and 74 (51.0%) by *Hepatocystis* alone. By contrast, only 11 monkeys (7.6%) from the seven sites were infected by both *P. inui* and *Hepatocystis* sp. The proportion of coinfections with *P. inui* and *Hepatocystis* sp. was significantly less than the random expectation ($\chi^2 = 71.5$; 1 d.f.; $P < 0.001$).

Discussion

Like previous studies of African apes (Ollomo et al. 2009; Prugnolle et al. 2010; Rich et al. 2009), extensive sequencing of *cytb* sequences of malaria parasites from free-living

Southeast Asian monkeys *Macaca nemestrina* and *M. fascicularis* revealed unexpectedly complex communities of *Plasmodium* and *Hepatocystis* species. Phylogenetic analyses showed that certain sequences corresponded to taxa from which *cytb* sequences have already been reported, the most numerous of which were sequences that appeared to correspond to *P. inui* and to *Hepatocystis* sp. MFRC11. Previous sequencing of SSU rRNA from *M. fascicularis* in Thailand found the latter two taxa to be abundant (Seethamchai et al. 2008). On the other hand, certain *cytb* sequences from *M. nemestrina* and *M. fascicularis* did not cluster close to those from previously sequenced species, raising the possibility that some of these correspond to previously undescribed species.

Most of the major clades of parasites were found in both *Macaca* species; and the two most commonly occurring parasite taxa, *P. inui* and *Hepatocystis* sp., infected the two *Macaca* species at approximately equal levels. However, these two parasite taxa showed very different patterns of genetic diversity across the two host species. *Hepatocystis* sp. showed little diversity, and no evidence of genetic differentiation between the isolates derived from *M. nemestrina* and those derived from *M. fascicularis*. By contrast, *P. inui* showed a much greater overall degree of genetic diversity and evidence of genetic differentiation between the isolates infecting *M. nemestrina* and those infecting *M. fascicularis*. On the other hand, *Hepatocystis* infecting *M. nemestrina* showed evidence of geographic differentiation among populations, even within the relatively restricted area of the province of Narathiwat, whereas *P. inui* showed no evidence of population subdivision across Narathiwat and Yala provinces (Supplementary Table S3). The evidence of genetic differentiation between the *P. inui* populations infecting the two macaque species suggests that movement of this particular parasite between these two host species is limited. By contrast, the evidence suggests that *Hepatocystis* sp. moves freely between host species but less freely between geographic localities. These differences may reflect differences in the dispersal and host-specificity of the respective vectors of the two parasite species; namely *Culicoides* midges (Ceratopogonidae) in the case of *Hepatocystis* sp. and anopheline mosquitoes (Culicidae) in the case of *P. inui*.

Because the number of individuals from which parasite sequences were obtained was greatest in the case of *M. nemestrina* (N = 171), we examined the community of malaria parasites inhabiting this species in detail. While coinfection with *P. inui* and *Hepatocystis* sp. occurred in *M. nemestrina*, both of these parasite taxa were found together in the same host significantly less frequently than expected on the basis of the overall occurrence in the host population. This result was found in the overall data set and at collection sites from which both *Hepatocystis* sp. and *P. inui* were collected. Similarly, coinfection with *Hepatocystis* sp. and *Plasmodium* taxa other than *P. inui* occurred significantly less frequently than expected by chance. On the other hand, coinfection with *P. inui* and other *Plasmodium* taxa occurred at a frequency no different from that expected. These results suggest that infection with *Hepatocystis* sp. reduces the likelihood of infection with *Plasmodium* species, and *vice versa*, implying that some sort of competitive exclusion occurs between these taxa.

There has been considerable discussion in the literature regarding potential artifacts of PCR-based methods for surveying haemosporidian diversity (Martinsen et al. 2006; Perkins et al. 1998; Szölli et al. 2008; Valkiúnas et al. 2006, 2009). One problem that has received considerable attention is that PCR may not amplify sequences from rarer parasites in the case of coinfection (Valkiúnas et al. 2006), for which one remedy is the use of haplotype-specific primers. In the present case, we did not use haplotype-specific primers; therefore our conclusion regarding competitive exclusion between *P. inui* and *Hepatocystis* sp. needs to be tested further with primers specific to these two taxa. Although we cannot rule out the possibility that certain haplotypes were missed in some hosts, we feel that our methods were

likely to detect both taxa in most hosts for the reasons: (1) our PCR assay is sensitive enough to amplify a single copy of *cytb* from either *Plasmodium* or *Hepatocystis*; (2) the *cytb* PCR products of *Plasmodium* and *Hepatocystis* differ in size (the former ~1490 bp and the latter ~1660 bp) and are clearly discernible after agarose gel electrophoresis; and (3) all isolates that had 1660-bp PCR product contained clones whose sequences were *cytb* of *Hepatocystis* sp. However, it will be important to subject our methods to further empirical validation; for example, by attempting to PCR from experimentally generated mixed populations of the two haemosporidian taxa.

Another potential problem relates to artifacts due to PCR or cloning errors. We attempted to minimize the effect of such artifacts by verifying each unique haplotype sequences from at least 2 different clones. Evidence that the error rate was low included the observation that π_S was significantly greater than π_N in *cytb* of *P. inui* and *Hepatocystis* sp., as expected under purifying selection (Table 2). If a substantial portion of the observed polymorphism were artifactual, we would not expect to see this pattern, since artifacts would occur at random with respect to the reading frame. One source of sequence errors might be an elevated error rate in AT-rich sequences during recombinant plasmid amplification in bacteria hosts (Frangeul et al. 1999). If such errors contributed substantially to the observed polymorphism, the error rate of base substitutions should be similar for *Plasmodium* and *Hepatocystis*, since their AT content in the amplified region (74% and 72%, respectively) was almost equal. However, our data revealed a remarkable difference in the level of nucleotide diversity at the *cytb* locus between these two genera, which would be difficult to explain as an artifact of AT-richness (Table 2).

In summary, analysis of *cytb* sequences revealed a highly complex community of malaria parasites infecting Southeast Asian monkeys, including two numerically dominant species and numerous less common species, several of which were only infrequently recorded. The results were consistent with the hypothesis that competitive exclusion occurs between two most numerically dominant species, but there was no evidence of any mutually facilitative interaction among taxa. If further studies support the hypothesis of competitive exclusion between *Hepatocystis* and *P. inui*, the interaction between these two taxa may provide a useful model for advancing our understanding of the complex question of how different parasite species interact when infecting the same host (Mideo 2009; Read and Taylor 2001; Richie 1988). Moreover, our results imply that the three human malaria parasites of Southeast Asian origin (*P. knowlesi*, *P. malariae*, and *P. vivax*) arose in a complex haemosporidian parasite community. Thus, much of the biology of these parasites is likely to have been shaped by adaptation to coexistence with other haemosporidia. Understanding the immunological and biochemical mechanisms behind interactions between the members of such parasite communities will increase our knowledge of the basic biology of malaria infection and may suggest new avenues for prophylaxis and treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Map of Thailand showing monkey capture sites (*open circles*).

Figure 2. Neighbor-joining tree of *cytb* sequences from *Plasmodium*, *Haemoproteus* and *Hepatocystis* species. Large clades are condensed, with numbers of sequences indicated. Numbers on branches are percentages of 1000 bootstrap samples supporting a given branch; only values ≥ 50% are shown.

Figure 3. Neighbor-joining tree of *Plasmodium inui* cytb field isolate sequences. Isolates from *Macaca nemestrina* are indicated by blue symbols, and those from *P. fascicularis* by red symbols. The bootstrap support (100%) for the branch separating the two major clades is shown.

Figure 4. Neighbor-joining tree of *Hepatocystis* sp. field isolate sequences. Isolates from *Macaca nemestrina* are indicated by *blue* symbols, and those from *P. fascicularis* by *red* symbols.

Table 1Incidence of malaria taxa in infected *Macaca nemestrina* and *M. fascicularis*.

Taxon	M. nemestrina (N = 171)	M. fascicularis (N = 18)	Fisher's exact test of equality of proportions
<i>Plasmodium inui</i>	36.3%	38.9%	n.s.
<i>P. coatneyi</i>	1.2%	16.7%	0.007
<i>P. knowlesi</i>	2.3%	5.6%	n.s.
Clade A	2.9%	0.0%	n.s.
Clade B	11.7%	5.6%	n.s.
<i>Plasmodium</i> other than <i>P. inui</i>	9.9%	33.3%	0.01
Hepatocystis	55.0%	44.4%	n.s.

Table 2

Synonymous (π_S) and nonsynonymous (π_N) nucleotide diversity in *cytb* sequences of *Plasmodium inui* and *Hepatocystis* sp. from *Macaca nemestrina* and *M. fascicularis*.

Parasite and host species	$\pi_S \pm \text{S.E.}$	$\pi_N \pm \text{S.E.}$
<i>P. inui</i>		
<i>M. nemestrina</i> (N = 116)	0.0117 $\pm$ 0.0022***	0.0029 $\pm$ 0.0004***
<i>M. fascicularis</i> (N = 13)	0.0251 $\pm$ 0.0080**	0.0032 $\pm$ 0.0011**
All (N = 129)	0.0181 $\pm$ 0.0045***	0.0033 $\pm$ 0.0006***
<i>Hepatocystis</i> sp.		
<i>M. nemestrina</i> (N = 116)	0.0013 $\pm$ 0.0003	0.0004 $\pm$ 0.0001
<i>M. fascicularis</i> (N = 9)	0.0000 $\pm$ 0.0000	0.0000 $\pm$ 0.0000
All (N = 125)	0.0012 $\pm$ 0.0003	0.0004 $\pm$ 0.0001

Z-tests of the hypothesis that π_S or π_N in *P. inui* equals the corresponding value in *Hepatocystis* sp.:

**
P < 0.01;

P < 0.001.