รายงานวิจัยฉบับสมบูรณ์

โครงการ

มาลาเรียในลิงกังและลิงหางยาว และการติดต่อสู่คนในประเทศไทย

โดย

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มีนาคม 2563

สัญญาเลขที่ RSA5980054

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ผู้วิจัย

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สังกัด

ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

และ จุฬาลงกรณ์มหาวิทยาลัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบพระคุณ ผู้ร่วมวิจัยทุกท่าน เจ้าหน้าที่สถานีวิจัยสัตว์ป่าป่าพรุ - ป่าฮาลาบาลา กลุ่มงานวิจัยสัตว์ป่า สำนักอนุรักษ์สัตว์ป่า กรมอุทยานแห่งชาติสัตว์ป่าและพันธุ์พืช นราธิวาส กระทรวงทรัพยากรธรรมชาติและสิ่งแวดล้อม ที่ให้การสนับสนุนช่วยเหลือ จนทำให้งานวิจัยชิ้นนี้สำเร็จลุล่วงด้วยดี และขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัยแห่งชาติและจุฬาลงกรณ์มหาวิทยาลัยที่ให้ทุนสนับสนุนโครงการวิจัย

ผู้วิจัย

มีนาคม 2563

บทคัดย่อ

รหัสโครงการ : RSA5980054
ชื่อโครงการ : มาลาเรียในลิงกังและลิงหางยาว และการติดต่อสู่คนในประเทศไทย
ชื่อนักวิจัย : รศ.ดร. จตุรงค์ พุทธพรทิพย์
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ระยะเวลาโครงการ : 3 ปี

โรคมาลาเรียที่ติดต่อจากลิงสู่คนนับเป็นโรคติดต่อจากสัตว์ที่สำคัญชนิดหนึ่งและอาจเป็นอุปสรรคต่อนโยบายการควบคุมโรคมาลาเรียระดับชาติ ทั้งนี้การติดเชื้อพลาสโมเดียมโนวลิไซนับว่ามีความสำคัญต่อการเจ็บป่วยและการเสียชีวิตที่สำคัญของประเทศต่าง ๆ ในภูมิภาคเอเชียตะวันออกเฉียงใต้ ในการศึกษานี้ได้ทำการสำรวจการติดเชื้อมาลาเรียและชนิดของเชื้อในคน ในลิงแม็กแคกและในยุงก้นปล่องที่มีศักยภาพในการเป็นพาหะนำโรคมาลาเรีย เพื่อเป็นการตรวจสอบว่าเชื้อมาลาเรียที่พบในลิงสามารถก่อให้เกิดความเสี่ยงในการติดเชื้อสู่คนเพียงไร จากการสำรวจผู้ป่วยที่มีอาการไข้จำนวน 3,054 รายพบผู้ติดเชื้อมาลาเรีย 1,336 รายโดยการตรวจหาเชื้อภายใต้กล้องจุลทรรศน์และการตรวจโดยวิธีพีซีอาร์ ทั้งนี้พบการติดเชื้อพลาสโมเดียมไวแวกซ์มากที่สุดคือร้อยละ 59 รองลงมาคือการติดเชื้อพลาสโมเดียมฟัลซิปารัมพบร้อยละ 38.83 ส่วนการติดเชื้อมาลาเรียทั้งสองชนิดนี้ร่วมกันพบร้อยละ 2.17 เมื่อทำการตรวจหาเชื้อมาลาเรียโดยวิธีพีซีอาร์ที่จำเพาะต่อเชื้อมาลาเรียที่พบในลิงที่พัฒนาขึ้นใหม่ในการศึกษานี้ พบว่าผู้ป่วยจำนวน 4 รายที่ติดเชื้อพลาสโมเดียมไวแวกซ์มีการติดเชื้อพลาสโมเดียมโนวลิไซร่วมด้วยจำนวนสองรายและพบการติดเชื้อพลาสโมเดียมไซโนโมลจิและพลาสโมเดียมอินนุยอย่างละราย ดังนั้นการศึกษานี้จึงเป็นการตรวจพบการติดเชื้อมาลาเรียจากลิงสองชนิดหลังเป็นครั้งแรกในคนในประเทศไทย เนื่องจากลิงหางยาวและลิงกังมีถิ่นอาศัยที่ครอบคลุมพื้นที่กว้างในประเทศและยังเป็นรังโรคของเชื้อมาลาเรียบางชนิดที่สามารถติดต่อสู่คนได้ อย่างไรก็ตามการประเมินสภาวะของการติดเชื้อมาลาเรียในลิงเหล่านี้ยังมีอย่างจำกัด ดังนั้นในการศึกษานี้จึงทำการสำรวจอุบัติการณ์ของการติดเชื้อมาลาเรียชนิดต่าง ๆ ในลิงหางยาวและลิงกังที่อาศัยในเขตจังหวัดนราธิวาสการสำรวจโดยวิธีพีซีอาร์ดังกล่าวพบอุบัติการณ์ของการติดเชื้อพลาสโมเดียมอินนุยที่สูงในลิงหางยาวโดยพบร้อยละ 57.14 และพบร้อยละ 39.95 ในลิงกัง นอกจากนี้ยังพบการติดเชื้อเฮปาโตซีสติส พลาสโมเดียมไซโนโมลจิ พลาสโมเดียมโนวลิไซ พลาสโมเดียมโคทนีไอและพลาสโมเดียมฟิลดีในอุบัติการณ์ตามลำดับ ในการติดตามการติดเชื้อมาลาเรียในลิงแม็กแคกอย่างต่อเนื่องเพื่อประเมินว่าการติดเชื้อดังกล่าวเกิดขึ้นเพียงชั่วขณะหรือเกิดขึ้นอย่างต่อเนื่อง จึงได้ทำการเฝ้าระวังและติดตามการติดเชื้อมาลาเรียในลิงกัง 20 ตัวและลิงหางยาว 3 ตัว แม้ว่าโดยทั่วไปลิงแม็กแคกมีอัตราการติดเชื้อมาลาเรียในอุบัตการณ์ที่ต่ำจากการสำรวจเพียงครั้งเดียวแต่ผลการศึกษาติดตามอย่างต่อเนื่องพบว่ากลุ่มลิงเหล่านี้มีการติดเชื้อมาลาเรียเป็นระยะเวลาที่ยาวนาน ดังนั้นลิงแม็กแคกสามารถเป็นแหล่งที่ทำให้วงจรการติดเชื้อมาลาเรียดำรงอยู่ได้อย่างต่อเนื่องและมีศักยภาพในการเป็นแหล่งแพร่เชื้อสู่คนได้ ข้อสำคัญประการหนึ่งคือประชากรของลิงหางยาวมีการขยายตัวเพิ่มขึ้นในประเทศไทยจากการวิเคราะห์ลำดับนิวคลีโอไทด์ในไมโตคอนเดรียโดยวิธีการกระจายของนิวคลีโอไทด์ที่ต่างกัน อนึ่งในการวิเคราะห์ลำดับนิวคลีโอไทด์ในยีนเอปิคอลเม็มเบรนแอนติเจนชนิดที่ 1 ของเชื้อพลาสโมเดียวโนวลิไซที่ได้จากคนและจากลิงแม็กแคกโดยวิธีสร้างสายใยโครงข่ายของยีนให้ข้อสนับสนุนว่ามีโอกาสเกิดการแพร่กระจายของเชื้อมาลาเรียดังกล่าวได้สองช่องทางคือจากลิงแม็กแคกสู่คนและระหว่างคนสู่คน ในการสำรวจยุงก้นปล่องที่มีศักยภาพเป็นพาหะนำโรคมาลาเรียในจังหวัดนราธิวาสพบว่ายุงอะนอฟิลิสไดรัสและยุงอะนอฟิลิสแม็กคูเลตัส มีศักยภาพเป็นพาหะสำหรับเชื้อพลาสโมเดียวโนวลิไซ เชื้อพลาสโมเดียมไซโนโมลจิและเชื้อพลาสโมเดียมอินนุย โดยสรุปพบว่าการที่มีลิงแม็กแคกที่ติดเชื้อมาลาเรีย การมียุงก้นปล่องที่มีศักยภาพเป็นพาหะนำโรคมาลาเรียเหล่านี้จำนวนมากตลอดจนการปรากฏของเชื้อมาลาเรียจากลิงหลายชนิดในคนแสดงว่าวงจรการแพร่กระจายของเชื้อมาลาเรียระหว่างลิงแม็กแคกและระหว่างลิงแม็กแคกสู่คนยังคงดำเนินต่อไปในประเทศไทย

คำหลัก : ไพรเมตมาลาเรีย, มาแคก, การติดต่อ, การตรวจทางชีวโมเลกุล, พาหะ

Abstract

Project Code : RSA5980054
Project Title : Nonhuman primate malarias in pig-tailed and long-tailed macaques and their transmission to humans in Thailand
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Project Period : 3 years

Cross-species transmission of simian malaria from monkeys to humans has been an important emerging zoonotic disease and may complicate nation-wide malaria elimination policy. Of these, *Plasmodium knowlesi* has been known to be incriminated in significant morbidity and mortality among Southeast Asian countries. To investigate whether other simian malaria species can pose an important risk for naturally acquired infections in Thailand, surveys of these malaria parasites in malaria patients, in macaque natural hosts and potential anopheline mosquitoes were performed. Of the 3,054 febrile patients examined, 1,336 harbored malaria parasites in their circulation as detected by microscopy and PCR method. *Plasmodium vivax* was the most prevalent species (59%), followed by *Plasmodium falciparum* (38.83%) and mixed infection by *Plasmodium falciparum* and *Plasmodium vivax* (2.17%). By using newly developed species-specific nested PCR methods in this study, four vivax malaria patients harbored cryptic simian malaria infections. Two of these were infected with *P. knowlesi* and one each with *Plasmodium cynomolgi* or *Plasmodium inui*. Therefore, this is the first study to identify naturally acquired infections in humans with the latter two simian malaria species in Thailand. Meanwhile, long-tailed and pig-tailed macaques inhabit a wide range of geographic locations in this country and are natural hosts for some malaria parasites capable of causing disease in humans. However, assessment of *Plasmodium* infections among these macaques remains largely unknown. In this study, the prevalence of simian malaria in pig-tailed and long-tailed macaques was determined in Narathiwat Province. By using PCR detection, a high prevalence of *Plasmodium inui* was found in long-tailed macaques (57.14%) and pig-tailed macaques (39.95%), followed by *Hepatocystis* spp., *Plasmodium cynomolgi*, *Plasmodium knowlesi, Plasmodium coatneyi* and *Plasmodium fieldi*, respectively. To further determine whether malaria in macaques occurred transiently or persistently which has important implication in disease transmission, longitudinal monitoring of simian malaria was performed in 20 pig-tailed macaques and 3 long-tailed macaques. Despite a low prevalence of simian malaria in these macaques based on a cross-sectional survey, persistent infections have been observed among these infected macaques. Therefore, these macaques can maintain transmission cycles as well as potential cross-transmission between macaques and humans. Importantly, population expansion was observed among long-tailed macaques in Thailand based on mismatch distribution analysis of the mitochondrial sequences. Furthermore, phylogenetic network analysis of human-derived and macaque-derived apical membrane antigen-1 (AMA1) sequences of *P. knowlesi* in Thailand has suggested two potential modes of transmission, i.e. a previously perceived cross-transmission from macaque natural hosts to humans and the other anthroponotic transmission. Surveys of potential anopheline vectors for simian malaria in Narathiwat Province have revealed for the first time that *Anopheles dirus* and *Anopheles maculatus* could serve as potential vectors for *Plasmodium knowlesi*, *Plasmodium cynomolgi* and *Plasmodium inui*. Taken together, the prevalence of simian malaria in macaque natural hosts, the abundance of potential anopheline vectors and the emergence of various simian malaria species in humans suggest that the transmission cycles within macaques and between macaques and humans have been ongoing in Thailand.

Keywords : primate malaria, macaques, transmission, molecular detection, vectors

INTRODUCTION

Plasmodium knowlesi is one of the six *Plasmodium* species naturally infecting humans (Coatney et al. 1971; Sutherland et al. 2010). The main natural hosts of *P. knowlesi* are pig-tailed (*Macaca nemestrina*) and long-tailed macaques (*M. fascicularis*) whose habitats occupy the forests and forest fringes of Southeast Asia (Coatney et al. 1971). The first naturally acquired *P. knowlesi* infection in human was reported in 1965 in which definite diagnosis required experimental inoculation of the patient's blood to other hosts (Chin et al. 1965). Limitation of diagnostic microscopy for *P. knowlesi* in blood smears stems from morphological resemblance of ring stages with those of *P. falciparum* and the band-shape trophozoites with those of *P. malariae*. It was not until 2004 that human infections with this simian malaria were identified in Malaysian Sarawak (Singh et al. 2004) and Thailand (Jongwutiwes et al. 2004) by means of molecular diagnosis. Thereafter, several hundreds of human cases were reported from almost all Southeast Asian countries including Nicobar Islands of India in Andaman Sea (Millar and Cox-Singh 2015; Tyaqi et al. 2013). However, high prevalence of infections caused by this parasite has been observed in Sarawak and peninsular Malaysian, accounting for about half of malaria cases in these areas (Singh et al. 2004; Vythilingam et al. 2008). In Thailand, *P. knowlesi* can be detected in several major malaria endemic areas at relatively low prevalence (Jongwutiwes et al. 2004; Putaporntip et al. 2009; Jongwutiwes et al. 2011). The distribution of *P. knowlesi* seems to overlap these macaque habitats leading to an assumption of zoonotic transmission of this simian malaria (Fooden 1994). However, our recent analysis of the polymorphic merozoite surface protein 1 (MSP-1) locus has shown that the number of haplotypes, haplotype diversity, nucleotide diversity and recombination sites of human-derived isolates remarkably exceeded that of monkey-derived sequences while phylogenetic networks displayed a character pattern that could have arisen from the presence of two independent routes of *P. knowlesi* transmission, i.e. from macaques to human and from human to humans in Thailand (Putaporntip et al. 2013). However, the effects of sampling process could not be entirely excluded because the number of samples included in the analysis was limited and the possibility of differential selective pressure on MSP-1 exerted by different host species could influence the extent of molecular diversity indices, complicating the interpretation of the phylogenetic networks. Therefore, inclusion of more *P. knowlesi* isolates from both humans and macaques along with analysis of other genetic loci including neutrally evolving genetic markers are mandatory to elucidate the transmission pattern of this important simian malaria. If two modes of transmission would de facto occur in Thailand, awareness of potential risk of acquiring knowlesi malaria in this country will need to be reconsidered.

Although *P. knowlesi* infections in macaque natural hosts usually do not cause overt ailments, infections in humans result in febrile symptoms, resembling those caused by other human malaria species. More importantly, severe knowlesi malaria that can be fatal akin to those caused by infection with *P. falciparum* has highlighted the virulence of this simian malaria (Cox-Singh et al. 2008). Meanwhile, almost all severe or fatal knowlesi malaria cases were reported among patients in high prevalent areas of peninsular Malaysia and Sarawak (Cox-Singh et al. 2008; William et al. 2011). However, knowlesi malaria complicated by acute renal failure has been diagnosed in a Thai man and a German traveler to Thailand (Nakaviroj et al. 2015; Orth et al. 2013). Therefore, early definite diagnosis of knowlesi malaria is important for vigilance of potential complications that may ensue regardless of endemicity of this simian parasite. On the other hand, longitudinal epidemiological surveillance of this parasite provides informative data for risk assessment of acquiring the infection in certain endemic areas.

Besides *P. knowlesi* that can be naturally transmitted from macaques to humans, *P. cynomolgi* and *P. inui* reportedly can establish infections in humans under laboratory conditions (Coatney et al. 1971). Importantly, a recent report has revealed that *P. cynomolgi* has been identified as a causative agent of symptomatic malaria in a Malaysian patient (Ta et al. 2014), indicating that this macaque malaria could be cryptically transmitted to human under natural transmission. Failure to detect *P. cynomolgi* in human blood samples in several previous studies could be due to cross-hybridization of *P. vivax*-derived primers with the orthologous sequence of this simian malaria (Ta et al. 2014). To date, it remains to be explored whether these nonhuman primate malarias closely related with *P. vivax* could be naturally infective to humans. Knowledge on definite species of pathogens is important for understanding transmission dynamics, pathogenesis, symptomatology, proper therapeutic intervention and preventive measures.

Both long-tailed and pig-tailed macaques are populated in Southeast Asia (Fooden 1995). In Thailand, particularly southern region, a number of long-tailed and pig-tailed macaques have been kept as pets and used for coconut- and stink bean (*Parkia speciosa*)-picking. Therefore, the presence of animal reservoirs of *P. knowlesi* in close vicinity of human habitats with abundant potential mosquito vectors can undoubtedly maintain transmission cycle of simian malaria. If macaques undergo population expansion, the number of reservoirs of *P. knowlesi* will increase accordingly, resulting in a more threat of infection to humans who live in close vicinity or within the flight range of anopheline vectors. Therefore, information on the interaction between population growth/decline of reservoir hosts and the pathogens carried by them will lead to a better understanding of disease transmission and potential risk to humans.

Natural mosquito vectors of *P. knowlesi* in peninsular Malaysia are *An. hackeri* (Wharton and Eyles 1961), *An. maculatus* (Vythilingam et al. 2014) and *An. cracens* (Jiram et al. 2012) whereas *An. dirus* in Vietnam reportedly harbored messenger RNA transcript of *P. knowlesi* circumsporozoite protein (Marchand et al. 2011), suggesting its role as a vector of this parasite. It is noteworthy that mosquito vectors of *P. knowlesi* belong to members of the *leukosphyrus* group that are prevalent in Southeast Asia; thereby, *P. knowlesi* can be maintained in this region. However, knowledge on species of vectors for *P. knowlesi* in Thailand remains elusive. If the vectors are shared between *P. knowlesi* and other human malaria species, preventive and control measure through reduction in their breeding places could simultaneously control all human malaria species. On the other hand, if the breeding places of *P. knowlesi* vectors differ from others, specific intervention may be required. Meanwhile, when vectors are shared among malaria parasites, non-entomological control measures of one species could benefit other species to occupy the same ecological niche; thereby, reciprocal changes of species distribution in hosts could occur (Mandal et al. 2013). Therefore, knowing malaria vectors undoubtedly forms a basis for preventive and control measures.

Taken together, despite available reports on molecular epidemiology of *P. knowlesi* in macaques and humans in Thailand, fundamental questions remain concerning many aspects of nonhuman malaria: Can other nonhuman malaria parasites closely related with *P. vivax*, especially *P. cynomolgi* and *P. inui*, be infective to humans under natural transmission? Does *P. knowlesi* persistently circulate among major malaria endemic areas of Thailand? Does nonhuman primate malaria undergo population change (expansion/decline) along with their reservoir hosts (pig-tailed and long-tailed macaques)? What species of anopheline mosquitoes serve as the vectors of *P. knowlesi*? Knowledge in these areas is essential for understanding the dynamic of nonhuman primate malaria transmission to humans leading to proper preventive measures.

Significance and historical aspects

Malaria is a scourge of mankind that has been perceived since pre-historical period (Poolsuwan 1995; Sallares 2001). To date, malaria remains and inflicts hundreds of million people annually. It is also a leading cause of mortality worldwide, particularly among children in Sub-Saharan Africa (WHO 2014). Before the turn of this century, four species of the genus *Plasmodium* are known to be the causative agents of human malaria (Bruce-Chwatt 1968). However, application of molecular data has delineated six distinct *Plasmodium* species infecting humans: (i) *P. falciparum*, the most prevalent and most virulent species, (ii) *P. vivax*, the most widespread species with notoriously relapsing behavior, (iii) *P. malariae*, the species of which chronic exposure

incriminating in quartan nephrotic syndrome, (iv, v) *P. ovale* that has been recently verified genetically to contain two cryptic species, i.e. *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland et al. 2010), and (vi) *P. knowlesi*, a zoonotic malaria parasite causing quotidian fever (Chin et al. 1965) and at times culminating in life-threatening infections (Cox-Singh et al. 2008).

The first person who recognized *P. knowlesi* as a distinct malaria species from other nonhuman primate malaria parasites is Franchini in 1927 (Coatney et al 1971). However, detailed morphological description and speciation were reported in 1935 by Mulligan (Mulligan 1935). The species was named in honor of Dr. R. Knowles who, with Das Gupta, demonstrated that *P. knowlesi* could establish symptomatic infection in humans a few years earlier (Coatney et al. 1971). The complete asexual erythrocytic cycle of *P. knowlesi* takes approximately 24 hours. In thin blood films, the ring stages of *P. knowlesi* may possess an accessory chromatin dot reminiscent to double chromatin ring form of *P. falciparum* whereas characteristic band-shaped trophozoite of *P. malariae* can be found in asexual erythrocytic stages of *P. knowlesi* (Chin et al. 1965). Therefore, routine microscopic detection of parasites in blood smears usually cannot provide definite diagnosis of *P. knowlesi*. Before antibiotic era, induction of febrile symptoms by inoculation of blood stage malaria parasites was performed for the treatment of neurosyphilis, formerly referred to as 'general paresis of the insane' (Wagner-Jaurregg, 1922). Besides *P. vivax* and *P. malariae*, *P. knowlesi* was also used as a therapeutic pyrogen in 1935 (van Rooyen and Pile, 1935). The first naturally acquired *P. knowlesi* malaria in human was reported three decades later in a 37-year-old American army officer stationed in a jungle of peninsular Malaysia. Diagnosis was made on the basis of parasite morphology, consistent quotidian periodicity after the patient's blood passages to human volunteers and lethal outcomes after inoculation of the blood samples to rhesus monkeys (Chin et al. 1965). In 1971, a presumptive case of naturally acquired human infection with *P. knowlesi* in Malaysia was reported based on structure of parasites in blood smear and serology (Fong et al. 1971). Later on, despite attempts on searching for knowlesi malaria in humans by the American research teams, none was detected.

Epidemiology

With the advent of polymerase chain reaction (PCR)-based detection of human malaria, a large focus of human infections with *P. knowlesi* in Malaysian Sarawak was reported in 2004 revealing that almost half of malaria parasites circulating in the study community belonged to this species (Singh et al. 2004). Concurrently, the first naturally acquired infection in a Thai patient was published in the same year (Jongwutiwes et al. 2004). Thereafter, knowlesi malaria in humans has been identified from mainland Malaysia, Singapore, Indonesia, Philippines, Myanmar, Vietnam,

Cambodia and Nicobar Islands as either case reports or epidemiological surveys (Millar and Cox-Singh 2015; Tyaqi et al. 2013). Importantly, *P. knowlesi* cases have been reported among travelers from Australia, Finland, France, Germany, Japan, the Netherlands, New Zealand, Scotland, Spain, Sweden and Taiwan after visiting Southeast Asian countries (Millar and Cox-Singh 2015). In Thailand, *P. knolwesi* accounts for approximately 0.5% of human malaria cases (range 0.48% to 0.67%) and circulates in all major malaria endemic areas including Tak, Lampang, Chantaburi, Prachuab Khirikhan, Pang-Nga, Ranong, Yala, and Narathiwat Provinces (Putaporntip et al. 2009; Jongwutiwes et al. 2011; Putaporntip et al, unpublished). A retrospective study has revealed the presence of *P. knowlesi* in blood samples collected in Tak in 1996 (Jongwutiwes et al. 2011). Thereafter, the prevalence of *P. knowlesi* in Thailand has not been extensive explored.

Clinical presentations

Invasion of *P. knowlesi* merozoites into erythrocytes require Duffy antigen receptor for chemokine (DARC) but not reticulocyte receptor; thereby, parasite density in circulation can be highly variable ranging from submicroscopic level to several hundred thousand parasites per microliter of blood (Miller et al. 1975; Chitnis et al. 1996). The periodic fever pattern of knowlesi malaria occurs every 24 hours, known as quotidian fever, coinciding with complete asexual erythrocytic development period. Most of infected cases have uncomplicated malaria symptoms characterized by fever and chills, cephalgia, rigors, malaise, myalgia, cough, nausea, vomiting, abdominal pain and diarrhea akin to other malaria attacks. However, about 9 – 12 % of cases progress to severe complications including acute renal injury, jaundice, hypotension, lactic acidosis, acute and late onset respiratory distress syndrome, severe anemia and hyperparasitemia (more than 100,000 parasites per microliter). Although cerebral malaria has not been documented in knowlesi malaria, the mortality rate of knowlesi malaria is about 1.7 – 1.9 % of *P. knowlesi*-infected individuals or accounting for 14.3 – 20% of complicated cases (Daneshvar et al. 2009; Ahmed et al. 2014). Most of the patients with severe complications and mortality occurred in Malaysian Borneo, the highest endemic area for knowlesi malaria. However, it is interesting to note that acute renal failure with hypotension has recently reported in a Thai patient who acquired the infection around the forest fringe of Chantaburi Province bordering Cambodia (Nakaviroj et al. 2015). Likewise, a German traveler to Thailand who stayed most of the time in Phuket developed acute renal injury (Orth et al. 2013). Therefore, knowlesi malaria in Thailand can be severe, albeit being relatively low prevalence.

Emergence of another nonhuman primate malaria *P. cynomolgi* in human

Although both *P. knowlesi* and *P. cynomolgi* seem to be the most phylogenetically related species with *P. vivax* (Escalante and Ayala 1994), the latter species is biologically more related in terms of asexual blood stage morphology, duration of asexual erythrocytic development, alteration in infected erythrocyte dimension with mature asexual stages, the presence of Schuffner's dots in infected erythrocytes and the presence of hypnozoites in hepatocytes responsible for relapse (Coatney et al. 1971). In 1960s, a number of human infections (more than 50) with *P. cynomolgi* under accidental or experimental inoculations of infective anopheline mosquitoes or blood passages have been recorded. Most of the infected cases developed uncomplicated malaria symptoms and the infections could be transmissible between monkeys to humans, humans to humans and humans to monkeys. However, attempt to search for naturally acquired *P. cynolmogi* infections in Malaysia Peninsula during 1960s failed to detect any positive cases (Coatney et al. 1971). Very recently, a case of naturally acquired infection with this simian malaria was reported in 39-year-old Malay woman who developed non-specific febrile illness with low parasite density. Definite diagnosis was based on the type A 18S rRNA sequence consistent with that of *P. cynolmogi* (Ta et al. 2014). It is noteworthy that the diagnostic primers widely used for *P. vivax* detection developed by Snounou et al (Snounou et al 1993) could cross-hybridize with this isolate, leaving a possibility of underestimation of this simian malaria with this diagnostic method. Meanwhile, *P. inui*, another quartan malaria parasite could be infective to humans under experimental conditions by blood passages and infective mosquito bites (Coatney et al. 1971). Therefore, it is likely that natural transmission of *P. inui* in humans should be possible. Although *P. inui* can establish infections in humans, symptoms are not severe and parasitemia is relatively low. Importantly, long-term infection of rhesus macaques could result in membranoproliferative glomerulonephritis similar to what has been observed in late complication of chronic malariae malaria (Nimri and Lanners 2014). To date, naturally acquired inui malaria in human has not been reported.

Malaria in macaque populations

At least 26 *Plasmodium* species are known to circulate among nonhuman primates (Coatney et al. 1971). Of these, eight *Plasmodium* species found in Southeast Asian macaques containing *P. fragile, P. knowlesi, P. coatneyi, P. inui, P. hylobati, P. simiovale, P. fieldi* and *P. cynomolgi* are phylogenetically closely related with *P. vivax* (Escalante and Ayala 1994), a relapsing human malaria parasite. The main natural hosts of *P. knowlesi* are long-tailed or crab-eating (*M. fascicularis*) and pig-tailed macaques (*M. nemestrina*) while leaf monkeys (*Prebytis melanophos* and some other species) could serve as minor reservoirs. Both pig-tailed and long-

tailed macaques are also hosts for other nonhuman primate malaria transmissible to humans, i.e. *P. cynomolgi* and *P. inui* (Coatney et al. 1971). The prevalence of malaria in monkeys in Thailand exhibits geographic variation with relatively high infection rates in southern region where *P. inui* was found in 36.3% and 38.9% whereas *P. knowlesi* was found in 2.3% and 5.6% of infected pig-tailed and long-tailed macaques, respectively. Mixed species malaria infections were not uncommon among macaques (Seethamchai et al. 2008; Putaporntip et al. 2010). Because malaria in naturally infected macaques usually does not contribute to symptomatic illness; thereby, therapeutic intervention has never been applied. In this regard, it remains unknown how persistence of malaria infections can be in these natural hosts. Meanwhile, the versatile abilities of macaques to thrive in human-altered environments have made them the most prevalent non-human primate species in Asia. Furthermore, cultural beliefs and religions in Southeast Asia have facilitated close interaction between humans and macaques leading to potential cross-transmission of pathogens between these hosts. In Thailand, both macaque species seem to be populated and many of them are reared as pets and sometimes used for coconut- and stink bean (*Parkia speciosa*)-picking, especially in southern areas of the country. It is therefore likely that these natural reservoir hosts of *P. knowlesi* and other nonhuman primate malaria are expanding. Intriguingly, when these reservoirs undergo population expansion, malaria circulating in these hosts would behave in a similar fashion unless being limited by other factors. Understanding the dynamics of population changes among reservoir hosts as well as their infecting malaria parasites has significance implication concerning disease transmission to humans (Levin et al. 1999).

Apical membrane antigen 1

During intraerythrocytic asexual development, malaria parasites express a number of antigens on the surface of infected erythrocytes, the merozoite surface prior to schizont rupture and free merozoites. Of these, some proteins have been implicated in erythrocyte invasion, most of which are merozoite surface proteins (e.g., apical membrane antigen 1 or AMA1, erythrocyte binding antigen 175 or EBA-175 and merozoite surface protein 1 or MSP1); thereby, these proteins are considered to be attractive vaccine targets (Cowman and Crabb 2006). AMA1 is a prime vaccine candidate antigen because antibodies against AMA1 could block merozoite invasion of erythrocytes and conferred protection against challenge infections in experimental immunization in animal models (Remarque et al. 2007). AMA1 is a type I integral membrane protein with 8 disulfide bonds forming three ectodomains (domains I-III) (Healer et al. 2004; Howell et al. 2003). It is synthesized as a 83-kDa precursor protein found most abundantly in micronemes during late schizogony and proteolytically processed to a 66-kDa form prior to relocalisation to the surfaces of

mature merozoites (Bannister et al. 2003; Howell et al. 2001). Recent studies have shown that the interaction between AMA1 and rhoptry neck protein 2 (RON2) is crucial for moving junction formation preceding merozoites invasion into erythrocytes. Inhibition of the binding process of these molecules could disrupt the host cell invasion process (Tonkin et al. 2011). AMA1 exists in all malaria species and exhibits sequence diversity among isolates; thereby, single nucleotide polymorphisms (SNPs) in this locus can be used as a good genetic marker to track parasite populations.

Mosquito vectors

Species of anopheline mosquito vectors of human malaria are diverse and display geographic variation. The main vectors of *P. knowlesi* are diverse mosquito members of the *Anopheles leucosphyrus* group that are prevalent in Southeast Asia. In 1961, Wharton and Eyles discovered that *Anopheles hackeri* is a natural vector of *P. knowlesi* in Malaysia (Wharton and Eyles, 1961). Subsequent studies have shown that *An. vagus, An. sinensis, An. introlatus, An. maculatus, An. kochi*, *An. balabacensis* and *An. quadrimaculatus* are susceptible to infections while oocysts of this simian malaria could develop in the midgut of *An. annularis, An. atroparvus, An. aztecus, An. freeborni, An. labranchiae* and *An. stephensi* in laboratory studies (Coatney et al. 1971). Recent field studies reveal that *An. balabacensis* and *An. latens* serves as the vector for *P. knowlesi* in Sabah (Collins et al. 1967) and Sarawak (Tan et al. 2008), respectively. In peninsular Malaysia, *An. maculatus* (Vythilingam et al. 2014) and *An. cracens* (Jiram et al. 2012) were the predominant vectors. Meanwhile, the primary vector for knowlesi malaria in southern Vietnam was *An. dirus* (Marchand et al. 2011), in which several species of human malaria, i.e. *P. falciparum, P. vivax* and *P. malariae* could co-exist in the same mosquitoes, leading to a possibility of co-infection in humans upon infective bites (Maeno et al. 2015). Nevertheless, mosquito feeding behavior and host preference could further determine the transmission cycle of *P. knowles* (and other nonhuman primate malaria transmissible to humans) as well as host switch from macaques-to-macaques, macaques-to-humans and humans-to-humans. In Thailand, *Anopheles dirus*, *An. maculates* and *An. minimus* are considered to be primary vectors of *P. falciparum* and *P. vivax* (Harrison 1980). There remains a key gap in knowledge of the main vector for *P. knowlesi* in Thailand, an issue that requires active investigation not only for understanding disease transmission cycle but for preventive measures guided by information on entomology.

OBJECTIVES

1. Determine the status of nonhuman primate malaria (*P. knowlesi, P. cynomolgi* and *P. inui*) in humans among major malaria endemic areas of Thailand.
2. Longitudinally monitor the occurrence of malaria in pig-tailed and long-tailed macaques in southern Thailand.
3. Analyze potential transmission cycles of *P. knowlesi* between hosts by phylogenetic analysis of the apical membrane antigen 1 sequences of *P. knowlesi* (and other honhuman primate malaria if detected) derived from humans comparing with those derived from macaque natural hosts.
4. Assess demographic transition (population changes in terms of growth or decline) of malaria and their respective pig-tailed and long-tailed macaque natural hosts.
5. Identify the potential vectors of *P. knowlesi, P. cynomolgi* and *P. inui* in southern Thailand where infected macaques are prevalent.

RESEARCH METHODOLOGY

1. Malaria in humans

***Study Populations*:** Human blood samples, either finger-pricked or venous blood, were obtained from individuals who attend malaria clinics or district hospitals in endemic areas after ethical clearance and obtaining informed consent. The study sites include Tak, Ubon Ratchathani, Chantaburi and Yala/Narathiwat Provinces as shown in Figure 1. This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

***Diagnosis of malaria by microscopy and PCR*:** Thick and thin blood smears are stained with Giemsa solution. Malaria diagnosis by microscopy was performed by experienced microscopists blinded to the results of PCR detection. For each slide, the thick blood film was examined for at least at least 200 leukocytes and the thin blood film for at least 200 microscopic fields, using x100 objective. performance prior to large scale application. For molecular diagnosis, DNA was extracted from the 200 µL of blood by means of a Qiagen DNA Mini Kit. *Plasmodium* species were identified by nested PCR using *Plasmodium* genus–specific outer primers derived from the mitochondria gene as listed in Table 1. Thirty-five and 25 cycles (94°C for 40 s, 55°C for 30 s, and 72°C for 1 min) were performed for the primary and nested PCR, respectively. The PCR products were separated in 1% and 2% agarose gels for primary and nested PCR, respectively; stained with ethidium bromide; and visualized under a UV light

2. Malaria in macaques

Study Population: Monkey blood samples were obtained from Narathiwat province in southern Thailand during October 2017 – October 2019. The areas of capture were located in forest areas of 3 districts (Chanae, Waeng and Sukhirin) as indicated on the map in Figure 2. Venous blood sample about 1–2 mL was collected from each animal either preserved in EDTA for DNA analysis and/or as fresh blood for determination of Duffy's blood group phenotypes. This study was reviewed and approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University. A subset of 10 randomly chosen malaria positive domesticated monkeys diagnosed by PCR were followed every 4 months within two years period and blood samples were collected accordingly; thereby, a total of 6 blood samples from each subject were obtained.

Figure 1. Map of Thailand showing locations of the sample-collection sites in northwestern (Tak Province), northestern (Ubon Ratchathani Province), southern (Yala and Narathiwat Provinces), and eastern (Chanthaburi Province) areas.

Confirmation for the presence of Plasmodium cynomolgi and Plasmodium inui: Blood samples give positive tests for *Plasmodium cynomolgi* or *Plasmodium inui*, additional PCR amplification targeting mitochondria genes were performed. Results from DNA sequencing by a subcloning of the PCR-amplified products were compared with the respective sequences available in the GenBank database.

Table 1 Nested PCR detection of *Plasmodium*.

Primer name	nucleotide sequence (5' -> 3')	parasite targeted	annealing temperature (°C)	amplicon length (bp)
primer (outer primers)				
PMTF0.2	CTTTTAACGCCTGACATGGATGGATAATACTCG	*Plasmodium* spp.	55	1470
PMTR0.2	TCTGGATAATCAGGAATACGTCTAGGCATTAC			
primer (inner primers)				
PCYMTF1	CCAAGCCTCACTTATTGTTAATTTATTTTT	*Plasmodium cynomolgi* Type I	55	314
PCYMTR-V	CTGGAGAACCACATAAAATTGGTAAAAAA			
PCYMTF1.2	CTTATTGTTAATTATATATTGTATTATATATTTTTTG	*Plasmodium cynomolgi* Type II	55	321
PCYMTR-V	CTGGAGAACCACATAAAATTGGTAAAAAA			
PKMTF1	TCATATCCAAGCCTCATTTATGATTTATTT	*Plasmodium knowlesi*	55	308
PK2MTR0	GAACCGCATAAAATTGGTAGAAAATAATTACCG			
PIMTF1	GCCTCACTTATTATTTATTATATTTTCTTTG	*Plasmodium inui*	55	331
PIMTR1	TTCTTGGATATGCAAGTTCTGAG			
PCOMTF0	GCCTCACTTATAATTAATTTATATTTATTTTTTTTTTTG	*Plasmodium coatneyi*	55	324
PCOMTR0	GTTCTGGAGAACCACATAAAATTGGTAGA			
PFI2MTF0	CCAAGCCTCACTTATTATTAATTATATTTTTTTTTG	*Plasmodium fieldi*	55	325
PCYMTR-V	CTGGAGAACCACATAAAATTGGTAAAAAA			
HEPMTF0	CTGTATGGATTCTATCTTACTTATTCATAATC	Hepatocystis	55	340
HEPMTR0	GTTATTAATGAATATCTATTTAAAACAATAAA			

Figure 2. Map of Thailand showing monkey sampling sites in Narathiwat Province.

Microscopy detection: Aliquots of fresh blood samples were used for both thin and thick blood film preparations, followed by staining with 10% Giemsa solution. Malaria parasites were examined in at least 200 fields with an Olympus BX51 light microscope (Center Valley, PA) at a magnification of 1000.

DNA extraction: 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 to further use.

Detection of malaria by nested PCR: Diagnosis of malaria was performed by nested PCR targeting the mitochondria gene of primate malaria species. The outer primers were derived from pan-*Plasmodium*-specific sequences and inner primers from species-specific regions for *Plasmodium knowlesi, Plasmodium cynomolgi, Plasmodium inui, Plasmodium fieldi, Plasmodium coatneyi* and *Hepatocystis* spp. Thermal cycler profiles were essentially as described previously (Putaporntip et al., unpublished data). DNA amplification was performed by using a Gene-Amp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA) and ExTaq DNA polymerase (Takara, Shiga,

Japan). When inner primers yielded negative results in the presence of positive PCR products generated from outer PCR primers, DNA sequence was performed from the purified primary amplified fragments to determine species by using BLAST server.

Sequencing of mitochondrial DNA: The mitochondrial cytochrome *b* of long-tailed and pig-tailed macaques spanning about 1 kb were amplified by PCR and sequenced. Sequences were obtained by a series of forward and reverse primers. Isolates with mixed electropherogram signals were excluded.

3. Malaria in mosquito vectors

Mosquito collection: The study site was a forest fringe near Hala-Bala Wildlife Research Station in Narathiwat Province during March 2018 to November 2019 where *P. knowlesi*-infected macaques have been previously identified and naturally acquired knowlesi malaria in humans occurred in vicinity. The areas surrounding the study site have never been the target of casualty; thereby, these sites were selected because of safety reasons. Mosquitoes were collected by using human landing catch, cattle-bait collection, monkey-bait collection, and CDC light trap method (Table 2). After taking macaques' blood meals, mosquitoes resting at the inner side of the net were caught by using glass tubes and kept in insect collecting bottles. Before preservation in absolute ethanol, preliminary species identification of individual mosquito were performed on the basis of morphology under dissecting microscope. Female anopheline mosquitoes were dissected for salivary glands, midguts and ovaries and these were examined by microscopy for sporozoites, oocysts and parity, respectively. Salivary glands from each mosquito were preserved in absolute ethanol for subsequent DNA extraction. Mosquito collections were performed starting from dusk (18.00) till dawn (6.00) with monthly average collection effort ranging between 5 and 10 person-nights per month over 24 month period. Mosquito collectors are local staff at Hala Bala Wildlife Research Station or native people who usually expose to mosquito bites in daily living environments. All mosquito collectors were trained for mosquito catching. For safety reason, these field workers were regularly screened for malaria and treated appropriately if infected.

Table 2 Distribution of mosquito sampling sites and collection period

No	Province	Location	Collection period			
			Mar - Apr 18	Jul - Aug 18	Jan - Feb 19	Nov - Dec 19
1	Narathiwas	Hala Bala Wildlife Research Station	□	□	□	□
2	Narathiwas	Hala Bala Wildlife Sanctuary	□	□	□	□
4	Narathiwas	Ban Lu Po Pae Lochut, Waeng District	□	□	□	□
5	Narathiwas	Moo 8 Ban Sa wor Lochut, Waeng District	□	□	□	□
6	Narathiwas	Moo 4 Ban Je Dong Lochut, Waeng District	□	□	□	□
7	Narathiwas	Moo 5 Ban Bala Lochut, Waeng District	□	□	□	□
9	Narathiwas	Ban Sa Moo 8 Ban Sa wor Lochut, Waeng District	□	□	□	□
10	Narathiwas	Moo 2 Phukhao Thong, Sukhirin District	□	□	□	□

Molecular detection of malaria and identification of mosquito species: DNA of each female anopheline mosquito were used as templates for PCR-based detection of primate malaria targeting 18S rRNA gene. Amplification of *Plasmodium* mitochondrial and anopheline 18S rRNA from positive samples were done using respective primers in separate reactions and sequenced. Definite species identification of mosquito and malaria parasites were verified by sequence comparison with known species available in the GenBank database.

4. The apical membrane antigen 1 sequences

Subjects and samples: Twenty-four *P. knowlesi*-positive blood samples from humans and macaque natural hosts (12 each) were included for analysis. Seventy *P. inui* samples and 9 *P. cynomolgi* isolates were obtained from naturally infected macaques in Southern Thailand. The 18S nested PCR specific for each of these malaria species were deployed to reaffirm these simian malaria parasites.

DNA preparation: Genomic DNA was extracted from 200 µl of malaria positive blood samples by using a QIAGEN DNA minikit (Hilden, Germany). Procedures of DNA preparation followed essentially the manufacturer's instruction.

PCR amplification: The PCR primers of the *AMA1* genes were designed based on available *AMA1* genes from *P. vivax* (GenBank accession number L27504), *P. knowlesi* (XM_002259303), *P. cynomolgi* (X86099) and *P. inui* (XM_008817929). Forward primers were derived from the 5'uptream sequence before the start codon of each gene and reverse primers were from the 3'

noncoding sequence after the stop codons. Genomic DNA of each malaria sample was used as template for PCR amplification. The PCR products were be analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and imaged under a UV transilluminator. The PCR products encompassed the complete or near complete coding sequences of PkAMA1 (1,689 bp) from humans and macaques as well as *PcyAMA1* (1686 bp) and *P. iui AMA1* (*PiAMA1*) (1686 bp). Purification of PCR-amplified products was done by using QIAquick PCR purification kit following the manufacturer's protocol.

Cloning of samples with mixed alleles: The PCR products that generate superimposed electropherogram signals were reamplified using the same amplification conditions. The positive PCR products were excised from agarose gel, purified by using QIAquick Gel purification kit (Qiagen) and ligated into pGEM-T-Easy Vector (Promega, Madison WI, USA). After incubation at 4°C for overnight, the reaction mixture was precipitated, dissolved in 10 µl of double-distilled water and transformed into *Escherichia coli* strain JM109. Positive recombinant clones were determined by plasmid minipreparation. Recombinant plasmid containing the *AMA1* gene was purified and used as templates for sequencing. At least 20 recombinant clones were prepared for each PCR-amplified sample.

DNA sequencing: All PCR amplified AMA1 genes of *P. knowlesi, P. cynomolgi* and *P. inui* were used as templates for sequencing. DNA sequences were determined directly and bi-directionally from these PCR-purified templates. When sequencing results were unreadable or possessing superimposed signals suggestive of mixed templates, sequences were determined from recombinant plasmid templates. Because the number of mixed alleles could be variable, at least 10 recombinant subclones of each sample were sequenced. Sequencing was performed on an ABI3100 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

5. Statistical analysis

The prevalence of malaria infections in humans, macaques and mosquitoes were determined and expressed in percentage. Detection of natural selection in the AMA1 locus of *P. knowlesi* from humans and macaques were examined by comparing the rate of nonsynonymous substitutions per nonsynonymous site (dN) and the rate of synonymous substitutions per synonymous site (dS). Test for difference was done by Z-test with $p < 0.05$ as a cutoff value for a significant level (Nei 1987). The status of demographic transition of macaques and nonhuman primate malaria were assessed from mitochondrial single nucleotide polymorphisms (SNPs) by

mismatch distribution analysis. Tajima's *D* and its related statistics (Fu and Li's *D** and *F**) were also apply to determine population change as well as to exclude deviation from neutral evolution (Tajima 1983; Fu and Li 1993). Statistical significance for mismatch analysis was done by permutation test and for Tajima's *D* and related statistics by coalescence simulation with 10,000 pseudoreplicates with a cutoff point at 0.05. Phylogenetic tree was constructed using maximum likelihood method with appropriate model that gives the lowest Bayesian Information Criterion (BIC) value. The final tree was selected based on superior log likelihood value. Confidence of tree branch topology was evaluated by bootstrap method using 1000 pseudoreplicates. Phylogenetic network was constructed by the split decomposition method to reduce the visual complexity of the networks using least square optimization of branch lengths (Winkworth et al. 2005). Both sum of differences goodness of fit and least squares goodness of fit were calculated to examine how well the graph represents the distance of the data set (Winkworth et al. 2005).

RESULTS

1. Humans and *Plasmodium* infections

From October 2009 to September 2016, we collected blood samples from 3,054 febrile patients who attended malaria clinics in northwestern (Tak Province, n=437), eastern (Chanthaburi Province, n=157), northeastern (Ubon Ratchathani Province, n=1,120), and southern (Yala/Narathiwas Province, n=1,340) areas of Thailand (Table 3). For malaria diagnosis, Giemsa-stained thick and thin blood films were made, and an additional 200 µL of blood was collected by finger-prick from each patient. Of the 3,054 febrile patients examined in this study, 1,336 harbored malaria parasites in their blood circulation, as detected by both microscopic and PCR examinations. The male-to-female ratio of the patients with malaria was 2.5:1, and the age range was 11–73 years. The majority of the patient population was Thai (84%), and the rest of the patients were cross-border migratory foreigners (13% from Myanmar and 3% from Cambodia). In 1,244 microscopy-positive samples, *Plasmodium vivax* was the most prevalent species (59%), followed by *Plasmodium falciparum* (38.83%) and mixed infection by *Plasmodium falciparum* and *Plasmodium vivax* (2.17%). *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium knowlesi*, *Plasmodium cynomolgi* and *Plasmodium inui* (0.2%) infection were not detected. After we reevaluated the 3,054 blood samples using the nested PCR method (Table 4). Consistent with previous findings, the PCR method offered higher sensitivity in parasite detection. Ninety-two samples were positive by PCR. The most prevalent cryptic infection was with *Plasmodium vivax* (n=20), followed by *Plasmodium falciparum* (n=5) and *Plasmodium knowlesi* (n=2), coinfection with

Plasmodium falciparum and *Plasmodium vivax* (n=53), *Plasmodium falciparum* and *Plasmodium malariae* (n=3), *Plasmodium vivax* and *Plasmodium malariae* (n=3), *Plasmodium vivax* and *Plasmodium cynomolgi* (n=1), *Plasmodium vivax* and *Plasmodium inui* (n=1), and triple infection with *Plasmodium falciparum*, *Plasmodium vivax* and *Plasmodium malariae* (n=2), *Plasmodium falciparum*, *Plasmodium ovale* and *Plasmodium malariae* (n=2)

Table 3 Distribution of *Plasmodium* infections diagnosed by microscopy

Provinces	No. of cases (%)	No. of positive (%)	Species							
			P. f (%)	*P.v* (%)	*P.f + P.v* (%)	*P.m* (%)	*P.o* (%)	*P.k* (%)	*Pcy* (%)	*Pi* (%)
Tak	437 (14.31)	41 (3.29)	29 (6.00)	12 (1.64)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ubon Ratchathani	1,120 (36.67)	706 (56.75)	331 (68.53)	357 (48.64)	18 (66.67)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Chanthaburi	157 (5.14)	15 (1.21)	5 (1.04)	10 (1.36)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Yala/Narathiwas	1,340 (43.88)	482 (38.75)	118 (24.43)	355 (48.36)	9 (33.33)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	3,054 (100)	1,244 (40.73)	483 (38.83)	734 (59.00)	27 (2.17)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 4 Distribution of *Plasmodium* infections diagnosed by nested PCR

Provinces	No. of cases (%)	No. of positive (%)	species											
			P.f (%)	*P.v* (%)	*P.m* (%)	*P.o* (%)	*P.f P.v* (%)	*P.f P.v P.m* (%)	*P.f P.m* (%)	*P.v P.m* (%)	*P.f P.m P.o* (%)	*P.k* (%)	*Pcy P.v* (%)	*Pi P.v* (%)
Tak	437 (14.31)	49 (3.67)	27 (5.53)	12 (1.59)	1 (50)	0 (0)	5 (6.25)	1 (50)	1 (33.34)	1 (33.33)	1 (50)	0 (0)	0 (0)	0 (0)
Ubon Ratchathani	1,120 (36.67)	754 (56.43)	337 (69.06)	368 (48.81)	1 (50)	0 (0)	43 (53.75)	1 (50)	2 (66.66)	1 (33.33)	1 (50)	0 (0)	1 (100)	0 (0)
Chanthaburi	157 (5.14)	16 (1.2)	4 (0.82)	7 (0.93)	0 (0)	0 (0)	3 (3.75)	0 (0)	0 (0)	1 (33.34)	0 (0)	1 (50)	0 (0)	0 (0)
Yala/ Narathiwat	1,340 (43.88)	517 (38.7)	120 (24.59)	367 (48.67)	0 (0)	0 (0)	29 (36.25)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	1 (0)
Total	3,054 (100)	1336 (43.75)	488 (36.53)	754 (56.44)	2 (0.15)	0 (0)	80 (5.99)	2 (0.15)	3 (0.22)	3 (0.22)	2 (0.15)	2 (0.15)	1 (0075)	1 (0.075)

2. Confirmation for the presence of *P. cynomolgi* and *P. inui*

To reaffirm the presence of *Plasmodium cynomolgi* and *Plasmodium inui* in malaria populations of Thailand, we determined the variable sequences of mitochondrial sequence spanning approximately 1,300 bp and compared the results with those reported elsewhere for human or macaque isolates. Phylogenetic analysis placed isolate UBY120 from Ubon Ratchathani within the same clade as most other *Plasmodium cynomolgi* isolates (Figure 3). Isolate YL3178 from Yala province was in the same clade of *Plasmodium inui* (Figure 4). Therefore, *Plasmodium cynomolgi* and *Plasmodium inui* circulating among malaria patients in Thailand were confirmed.

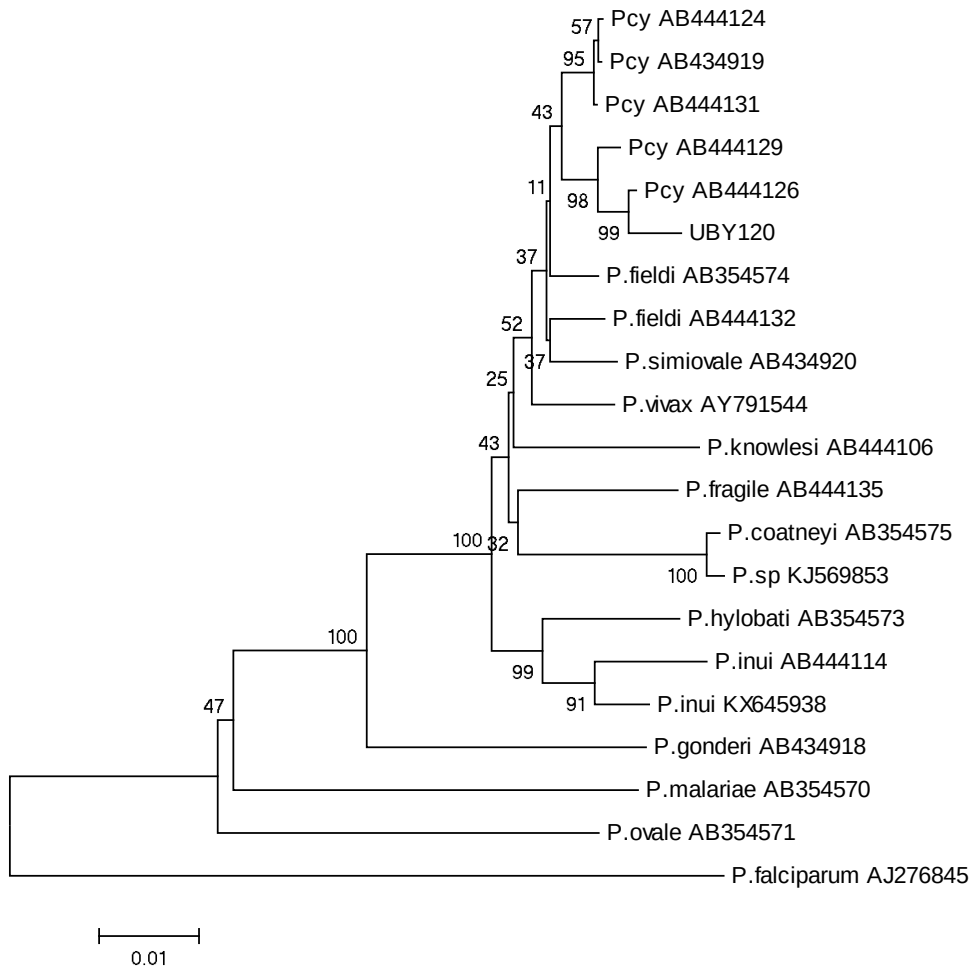


Figure 3. Neighbor-joining tree inferred from the mitochondrial sequence of *Plasmodium cynomolgi* from Thai isolate, compared with those reported elsewhere. Tree was constructed using a maximum composite likelihood model. Support from 1000 bootstrap replicates of >50% is shown along the branches. The scale bar underneath the tree indicates the number of base substitutions per site. Isolate names are listed along with their respective GenBank accession numbers.

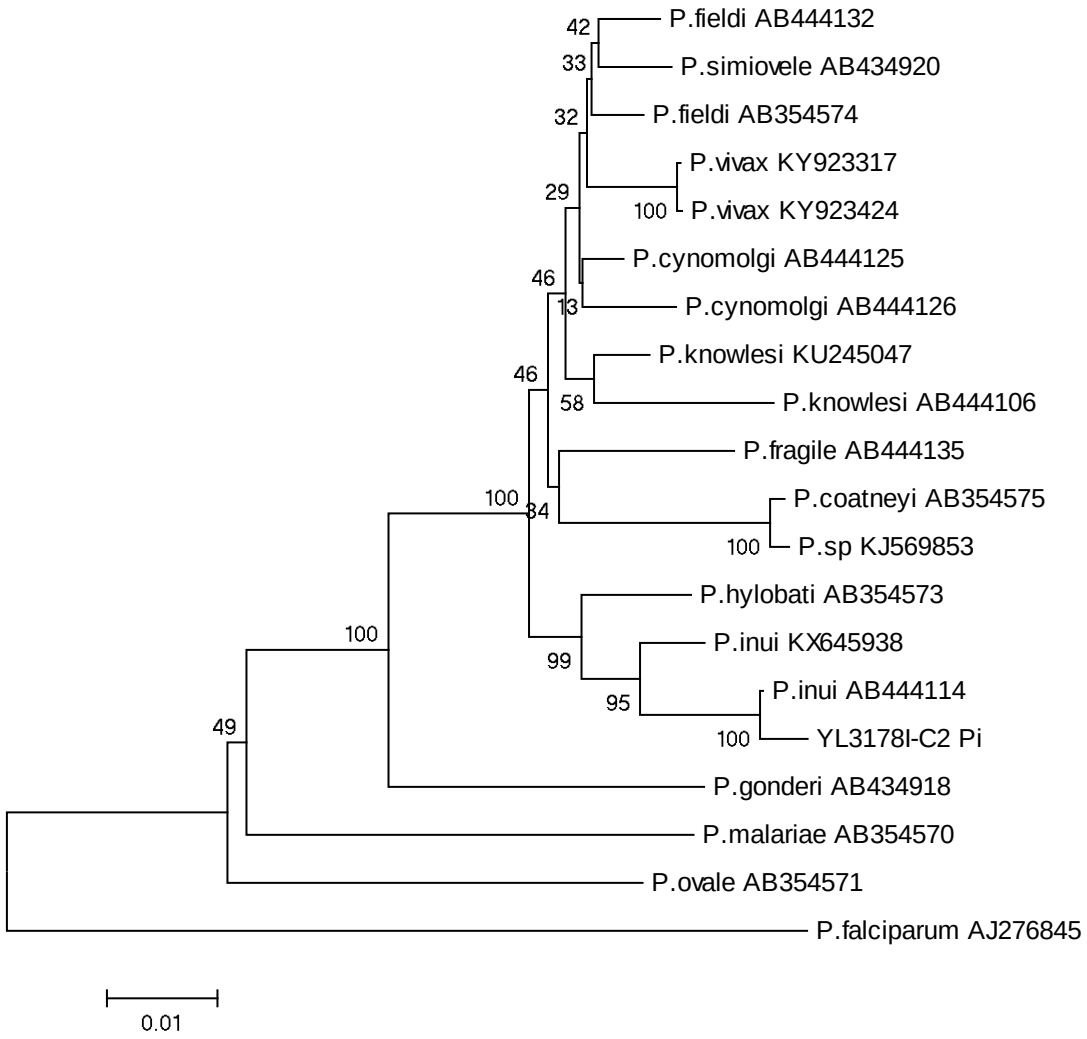


Figure 4. Neighbor-joining tree inferred from the mitochondria sequence of *Plasmodium inui* from Thai isolate, compared with those from macaque isolates using a maximum composite likelihood model. Support from 1000 bootstrap replicates of >50% is shown along the branches. The scale bar underneath the tree indicates the number of base substitutions per site. Isolate names are listed along with their respective GenBank accession numbers.

3. Monkeys and *Plasmodium* infections

In total, 286 monkeys were captured, comprising *Macaca fascicularis* or long-tailed macaques (n=57) and *Macaca nemestrina* or pig-tailed macaques (n=229) (Table 5). Of these, 9 long-tailed and 97 pig-tailed macaques harbored *Plasmodium* and/or *Hepatocystis* spp. in their circulations based on PCR diagnosis. Mixed species infections occurred in 4 long-tailed and 47 pig-tailed macaques, accounting for 44.4% and 48.5% of positive isolates for each monkey species,

respectively. *Plasmodium inui* was the most prevalent simian malaria identified in both macaque species, followed by *Hepatocystis* spp. Although *Plasmodium cynomolgi* was also common among pig-tailed macaques, no long-tailed macaques harbored this nonhuman primate malaria species. Meanwhile, *Plasmodium knowlesi* has been identified in 1 long-tailed and 17 pig-tailed macaques with almost comparable frequency as *Plasmodium coatneyi* (Table 6). *Plasmodium fieldi* was detected in 6 *Macaca nemestrina.* It is noteworthy that microscopy could not efficiently determine malaria infections in macaques because only 3 long-tailed macaques and 47 pig-tailed macaques had *Plasmodium* and/or *Hepatocystis* spp. in their blood smears. The majority of microscopy-positive samples contained ring stages that are not informative for species differentiation. Furthermore, most of the microscopy-positive samples had low parasitemia (<0.01%). The overall prevalence of malaria or *Hepatocystis* spp. infections in long-tailed- and pig-tailed macaques in the study populations was 0.0038, and 0.0279%, respectively.

Table 5 Monkey sampling sites in Narathiwat Province.

Locations	Province	Species	Sex		Total
			Female	Male	
Phukhao Thong, Sukhirin	Narathiwat	*M.fascicularis*	0	2	**2**
Phukhao Thong, Sukhirin	Narathiwat	*M.nemestrina*	6	2	**8**
Mamong, Sukhirin	Narathiwat	*M.fascicularis*	4	3	**7**
Mamong, Sukhirin	Narathiwat	*M.nemestrina*	72	18	**90**
Lochut, Waeng	Narathiwat	*M.fascicularis*	34	9	**43**
Lochut, Waeng	Narathiwat	*M.nemestrina*	72	29	**101**
Chang Phueak, Chanae	Narathiwat	*M.fascicularis*	3	2	**5**
Chang Phueak, Chanae	Narathiwat	*M.nemestrina*	14	8	**22**
Mae Dong, Waeng	Narathiwat	*M.fascicularis*	0	0	**0**
Mae Dong, Waeng	Narathiwat	*M.nemestrina*	6	2	**8**
Total			211	75	**286**

Table 6 Distribution of malaria among macaque populations in Narathiwat Province.

Monkey species	Malaria species*	No. Isolates	% positive**
Macaca fascicularis	*Plasmodium inui*	8	57.14
	Plasmodium cynomolgi	0	0
	Plasmodium knowlesi	1	7.14
	Plasmodium coatneyi	2	14.29
	Plasmodium fieldi	0	0
	Hepatocystis spp.	3	21.43
Macaca nemestrina	*Plasmodium inui*	55	35.95
	Plasmodium cynomolgi	22	14.38
	Plasmodium knowlesi	17	11.11
	Plasmodium coatneyi	10	6.54
	Plasmodium fieldi	6	3.92
	Hepatocystis spp.	43	28.10

* Single and mixed species malaria infections were detected in 5 and 4 long-tailed macaques and in 50 and 47 pig-tailed macaques, respectively.
** Percent of all positive samples in each macaque species.

4. Longitudinal monitoring of parasitemia in pig-tailed and long-tailed macaques

A longitudinal study of simian malaria and *Hepatocystis* infections was performed in 20 pig-tailed macaques and 3 long-tailed macaques in which blood samples were obtained thrice from each monkey at 8-month interval. Microscopic examination of Giemsa-stained thin and thick blood samples detected 7, 5 and 6 *Plasmodium*- or *Hepatocystis*-positives from the first, the second and the third blood sample collections whilst *Plasmodium* species-specific and *Hepatocystis*-specific PCR assays could identify 9, 13 and 11 positive samples, respectively. Eight monkeys were free from *Plasmodium* and *Hepatocystis* infections whereas 5 monkeys were persistently infected with these parasites based on positive results from all 3 blood samples from each monkey. Variation in positive PCR assays across 3 blood samples from each monkey occurred in 10 monkeys. Of these, 5 monkeys had single positive blood samples whereas the remaining 5 monkeys had 2 positives out of 3 blood samples examined (Figure 5).

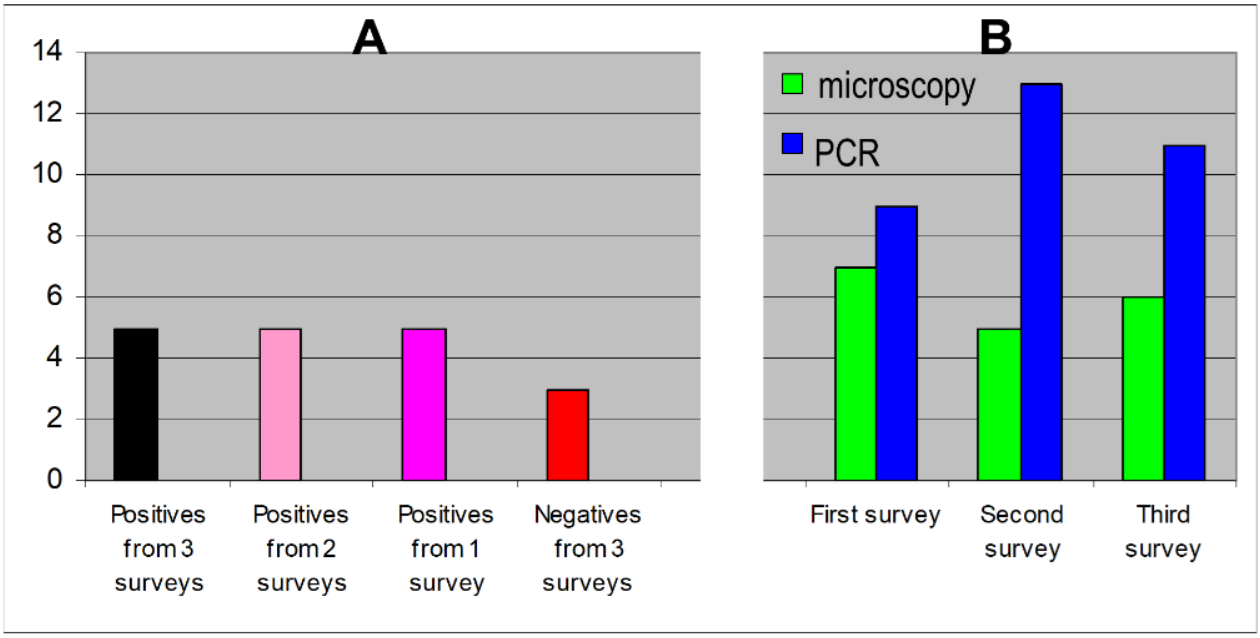


Figure 5. Variation in positive detection rates of simian malaria among 23 macaques across 3 surveys

5. The extent of sequence diversity in malarial AMA1 genes

Genetic diversity in *PkAMA1*, *PcyAMA1*, *PiAMA1* and related malarial AMA1 was determined by using the parameter nucleotide diversity (π) which is the average number of pairwise nucleotide differences between sequences. Although nucleotide substitutions in the *AMA1* locus of simian malaria parasites were observed across the coding region, most of them were concentrated in central region spanning domains I, II and III. Analysis of *PkAMA1* from 24 Thai isolates has revealed 31 nucleotide substitutions resulting in 9 amino acid changes. By inclusion of previously reported sequences from Sabah and Sarawak (total n = 122), 90 nucleotide substitutions and 30 amino acid changes were observed in this locus. The magnitudes of haplotype diversity of *P. knowlesi* isolates from Thailand, Sabah and Sarawak were comparable ranging from 0.92-0.99, indicating that the haplotypes circulating in each endemic area were evenly distributed or almost all haplotypes differed from one another. Likewise, the haplotype diversity of *PcyAMA1*, *PiAMA1*, *PvAMA1* and *PfAMA1* displayed values greater than 0.93, suggesting that malarial AMA1 genes exhibited extensive sequence diversity among isolates and across species. Malarial *AMA1* sequences displayed differential levels of nucleotide diversity across geographic origins. However, the nucleotide diversity of *PcyAMA1* significantly exceeded the AMA1 genes of other *Plasmodium* species ($p<0.05$) (Table 7).

Table 7 Molecular diversity in the *AMA1* genes of *P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. vivax* and *P. falciparum.*

Locus	Geographic origins	n	M	H	*h* ± S.D.	π ± S.E.
PkAMA1	Thailand	24	31	14	0.920 ± 0.038	0.00473 ± 0.00038
	Sarawak	52	58	46	0.995 ± 0.005	0.00501 ± 0.00020
	Sabah	36	36	32	0.994 ± 0.008	0.00417 ± 0.00024
	Total	**122**	**96**	**94**	**0.995 ± 0.002**	**0.00535 ± 0.00018**
PiAMA1	Thailand	70	82	54	0.977 ± 0.011	0.00496 ± 0.00086
	Sarawak	11	143	11	1.000 ± 0.002	0.02109 ± 0.00207
	Total	**81**	**192**	**65**	**0.983 ± 0.009**	**0.00858 ± 0.00119**
PcyAMA1	Thailand	9	111	9	1.000 ± 0.052	0.01760 ± 0.00772
	Sarawak	7	123	6	0.952 ± 0.096	0.03353 ± 0.00686
	Total	**16**	**147**	**15**	**0.992 ± 0.025**	**0.03112 ± 0.00364**
PvAMA1	Thailand	231	62	97	0.936 ± 0.011	0.00744 ± 0.00016
	Brazil	39	39	25	0.951 ± 0.024	0.00656 ± 0.00044
	Indonesia	11	38	11	1.000 ± 0.039	0.00830 ± 0.00078
	Total	**281**	**71**	**133**	**0.956 ± 0.008**	**0.00772 ± 0.00014**
PfAMA1	**Worldwide**	**39**	**73**	**31**	**0.981 ± 0.013**	**0.01127 ± 0.00080**

6. Deviation from Neutrality

Previous study has shown that the rate of nonsynonymous substitutions per nonsynonymous site or dN and the rate of synonymous substitutions per synonymous site or dS has revealed a significant excess of dN in domains I, II and III of *PfAMA1*, suggesting positive selection in these regions (Al-Qatani et al 2016). By contrast, similar analysis has shown a significantly greater dS than dN in domain I and the C-terminal part encompassing transmembrane domain and cytoplasmic tail of *PkAMA1*, implying purifying selection probably exerted by structural or functional constraints of this protein. Likewise, evidence of purifying selection was observed in domains I, II, III and the C-terminal part of *PcyAMA1* whilst no deviation from selective neutrality was detected in *PvAMA1* and *PiAMA1* (Table 8).

Table 8. The rate of synonymous and nonsynonymous substitutions per site in the *AMA1* genes of *P. knowlesi*, *P. inui* and *P. cynomolgi*.

Domain	N#	PkAMA1 (%)		PiAMA1 (%)		PcyAMA1 (%)	
		d_S ± S.E.	d_N ± S.E.	d_S ± S.E.	d_N ± S.E.	d_S ± S.E.	d_N ± S.E.
N-terminal	42	8.41 ± 4.03	0.87 ± 0.34	4.08 ± 2.76	1.70 ± 0.76	4.06 ± 2.31	1.58 ± 0.61
DI	206	2.65 ± 0.74**	0.17 ± 0.10	1.10 ± 0.28	0.50 ± 0.21	6.17 ± 1.52**	1.37 ± 0.29
DII	137	1.35 ± 0.44	0.53 ± 0.23	1.27 ± 0.40	1.67 ± 0.49	14.54 ± 0.26***	0.79 ± 0.30
DIII	102	1.50 ± 0.66	0.63 ± 0.32	1.22 ± 0.87	0.67 ± 0.32	17.76 ± 4.51**	1.68 ± 0.50
C-terminal	76	3.48 ± 1.28*	0.50 ± 0.26	1.67 ± 0.94	0.64 ± 0.35	15.63 ± 4.06**	0.73 ± 0.40
Total	563	1.52 ± 0.35**	0.24 ± 0.08	1.34 ± 0.19	0.91 ± 0.14	10.89 ± 2.00***	1.20 ± 0.21

number of codons. N-terminal: signal peptide and prosequence. C-terminal: transmembrane domain and cytoplasmic tail.
Tests of the hypotheses that dN equals dS: *$p < 0.05$; **$p < 0.005$; *** $p < 0.0001$.

Codon-specific tests for selective neutrality by FEL, MEME and FUBAR methods have shown that most of the amino acid substitutions in PkAMA1, PcyAMA1, PiAMA1 and PvAMA1 evolved under purifying selection. However, 6 amino acid residues (codons 15, 228, 277, 296, 359 and 481) in PkAMA1, 6 amino acids (codons 12, 222, 280, 382, 385 and 493) in PiAMA1 and one amino acid change at codon 6 in PcyAMA1 displayed evidence of positive selection. Meanwhile, 25 amino acid substitutions in PvAMA1 have arisen from positive selection and 10 negatively selected residues were detected. On the other hand, only 2 amino acid residues in PfAMA1 evolved under purifying selection whilst positive selection occurred in 28 codons.

7. Intragenic recombination in the malarial AMA1 genes

The presence of sexual development malaria parasites in mosquito vectors has enabled diversification of malarial genes by means of interallelic recombination. Various recombination detection methods have been applied to the sequences of malarial AMA1 genes in this study. Although the RDP package detected evidences of intragenic recombination only in *PiAMA1* and *PfAMA1*, the minimum number of recombination events (Rm) could be detected in all malarial *AMA1* genes, i.e. 4, 8 and 9 sites in *PkAMA1* from Thailand, Sabah and Sarawak, respectively (Table 9). Furthermore, 14 and 15 recombination sites were found in *PiAMA1* and *PcyAMA1*, respectively. For *PvAMA1*, 17, 10 and 7 recombination sites were observed among isolates from Thailand, Brazil and Indonesia, respectively. These results have purported that recombination have conferred an important role in shaping genetic diversity in the AMA loci of diverse malaria species.

Table 9. Recombination in *PkAMA1*

Population	n	Rm	Recombination between sites
Thailand	16	4	(66,357) (357,564) (564,684) (829,986)
Sarawak	52	9	(24,66) (66,72) (72,114) (321,357) (357,492) (630,648) (648,744) (744,886) (1232,1441)
Sabah	35	8	(66,72) (72,321) (357,630) (630,648) (648,684) (684,732) (744,886) (886,1441)
Total#	105	12	(24,66) (66,72) (72,114) (114,174) (321,357) (357,492) (630,648) (648,732) (829,886) (886,1075) (1232,1441) (1441,1530)

Sequences from the H and hackeri strains are included.

Rm = minimum number of recombination events.

8. Analysis of potential transmission cycles of *P. knowlesi* between hosts: Human-derived versus macaque-derived *PkAMA1* in Thailand?

The phylogenetic tree inferred from the malarial AMA1 genes have shown that *P. vivax*, *P. knowlesi*, *P. cynomolgi*, *P. inui*, *P. coatneyi* and *P. fieldi* were clustered together akin to that inferred from the cytochrome *b* and the 18S rRNA genes. Differences in topologies of these trees could stem from post-speciation accumulation of mutations in these loci. Our analysis has shown differential selective pressure on malarial AMA1 genes whilst cytochrome *b* is believed to evolve neutrally. Meanwhile, the 18S rRNA locus could have some functional constraints albeit being a non-protein coding gene (Figure 6). Maximum likelihood phylogenetic analysis of *PkAMA1* revealed that most Thai isolates exhibited a distinct cluster whereas most Sarawak and Sabah samples were distributed together in other clusters of the tree (Figure 7)

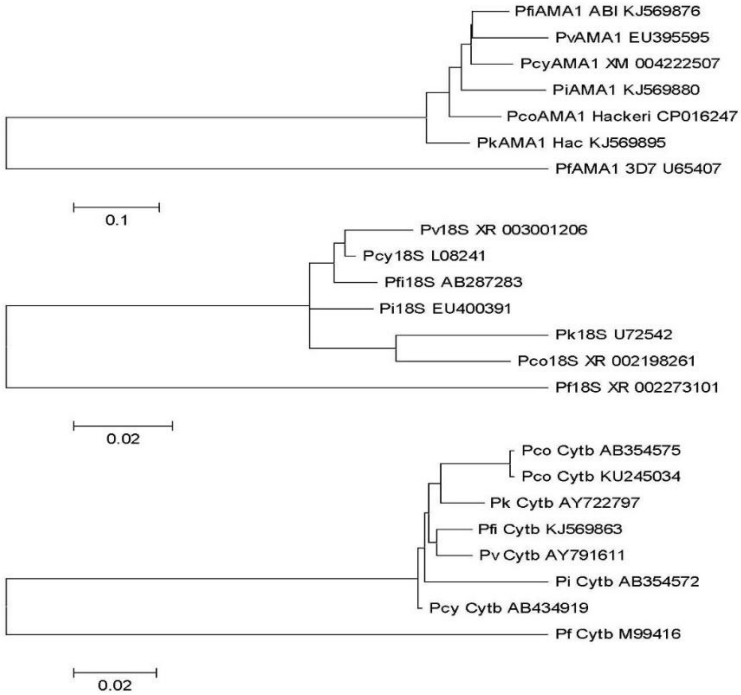


Figure 6. Maximum likelihood trees inferred from *AMA1* (upper), *18S rRNA* (middle) and *cytochrome b* (bottom) of *P. vivax* (Pv), *P. inui* (Pi), *P. cynomolgi* (Pcy), *P. coatneyi* (Pco), *P. knowlesi* (Pk), *P. fieldi* (Pfi) and *P. falciparum* (Pf).

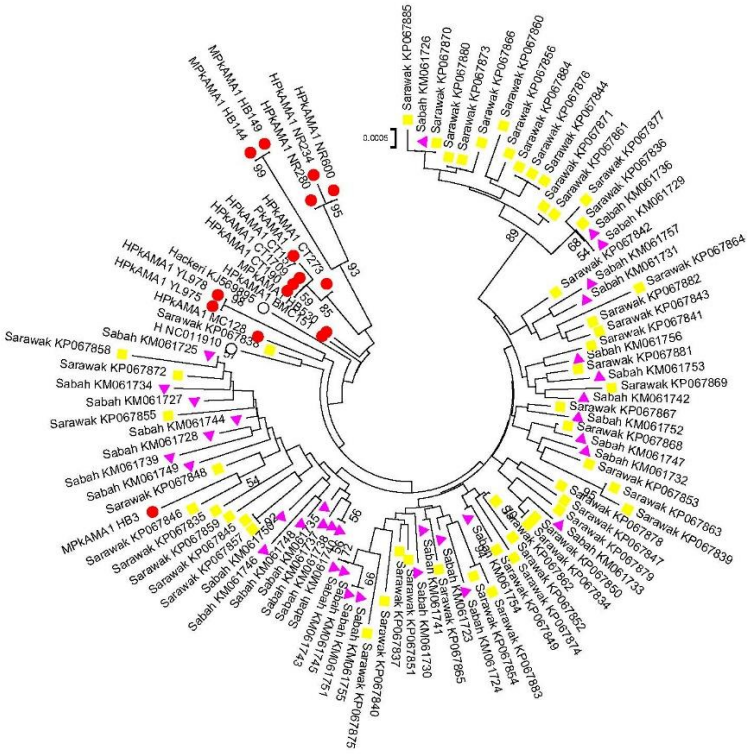


Figure 7. Maximum likelihood tree of PkAMA1 from Thailand, Sarawak and Sabah. Circles and triangles represent human and macaque isolates, respectively.

The number of mutations in PkAMA1 of human-derived and macaque-derived isolates was identical although there were slightly more haplotypes detected among human isolates resulting in higher level of haplotype diversity in human isolates than those derived from macaques. Although the nucleotide diversity of human derived isolates exceeded that of macaque origins, the difference was not statistically meaningful ($p>0.05$)(Table 10). Nevertheless, the minimum number of recombination events in human derived isolates doubled those from macaques. Taken together, these molecular diversity indices seem not to support strict zoonotic transmission of P. knowlesi from macaque to humans in which the greater magnitude of diversity would be expected in macaque derived isolates than those from human infections.

Table 10 Comparison of molecular diversity indices between human-derived and macaque-derived *PkAMA1*

	Human-derived *PkAMA1*	Macaque-derived *PkAMA1*
No. isolates	12	12
No. mutations	20	20
No. haplotypes	8	7
Haplotype diversity (S.D.)	0.924 (0.057)	0.864 (0.078)
Nucleotide diversity (S.E.)	0.00465 (0.00052)	0.00414 (0.0079)
Rm*	4	2

*Minimum number of recombination events

The phylogenetic networks inferred from PkAMA-1 generated similar topology regardless of different nucleotide substitution models. Most terminal branches of the networks received high percentage of bootstrap supports (data not shown); thereby, only a representative networks is shown in Figure 8A. Although the precise location of the root may not be unequivocally located, it could be around interconnecting nodes of most PkAMA-1 sequences from monkeys that are clustered together with short terminal branches. A series of edges were observed along several branches of the networks that could reflect some character conflict or could represent evolutionary history such as recombination or hybridization. Meanwhile, split decomposition has revealed 2 distinct clusters: one containing most monkey-derived sequences displaying short branches and the other containing most human-derived sequences having long branches (Figure 8B).

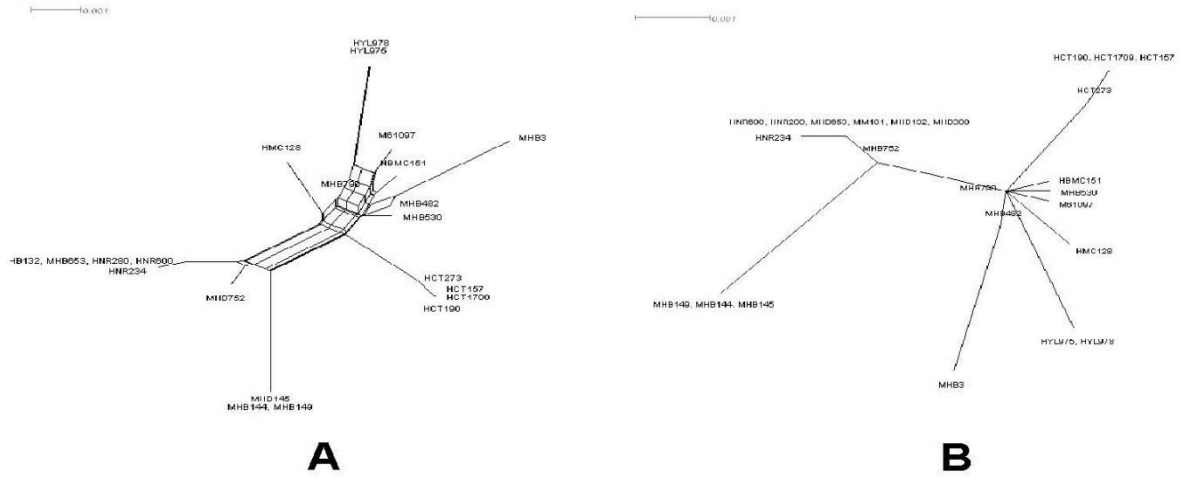


Figure 8 (A) Neighbor-net network and (B) split decomposition network inferred from *PkAMA1* using uncorrected p distance with bootstrap values more than 90%. Human and monkey isolates are shown with initials 'H' and 'M', respectively.

9. Assess population growth/decline of macaque natural hosts.

The population history of reservoir hosts has important impact on the spread and maintenance of zoonotic disease. To assess the population change in macaques in Thailand, the mitochondrial cytochrome *b* sequences of both long-tailed (n = 50) and pig-tailed (n = 26) macaques were determined in this study. Unfortunately, all 26 mitochondrial cytochrome *b* sequences of pig-tailed macaques were nuclear mitochondrial DNA (NUMT), precluding accurate analysis of population history. Therefore, analysis has been carried out for the corresponding gene sequences of long-tailed macaques. By applying, Tajima's *D* and its related statistics, Fu and Li's *D** and *F** tests for 50 sequences from long-tailed macaques. Although a significant deviation from zero for Tajima's *D* and its related statistics can imply deviation from selective neutrality, such significant values may imply population history. However, results have shown no significant deviation from zero for all these tests: Tajima's *D* = -0.48 ($p > 0.1$), Fu and Li's *D** = -1.0091 ($p > 0.1$), Fu and Li's *F** = -0.9069 ($p > 0.1$). On the other hand, mismatch distribution analysis under the assumption of constant population growth model has shown remarkable differences between observed and expected allele frequencies of nucleotide substitutions in the mitochondrial cytochrome b sequences (Figure 9). Although the sum of squared deviation or SSD values did not show significant values under either demographic expansion or spatial expansion models, results from mismatch distribution analysis under demographic expansion model yielded a significant value of the Harpending's raggedness index (0.04, $p = 0.01$). On the other hand, the Harpending's

raggedness index was not significant under spatial expansion model (p = 0.08) (Table 11). Taken together, these analyses have supported that long-tailed macaques in Thailand have been undergone the process of population expansion.

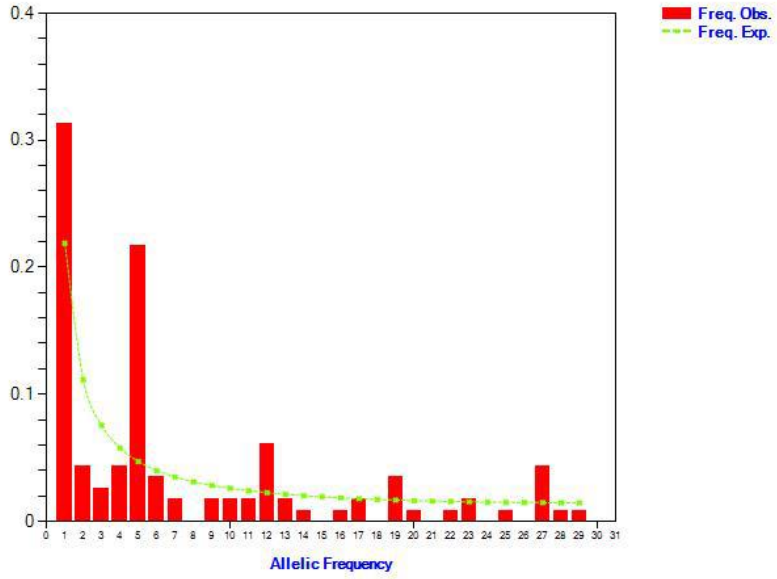


Figure 9 Mismatch distribution of observed and expected values of allele frequencies in the mitochondrial cytochrome b of 50 long-tailed macaques.

Table 11 Estimated demographic expansion and spatial expansion parameters for long-tailed macaques based on the mitochondrial cytochrome *b* sequences

Statistics	**All sequences**	**Mean**
Demographic expansion		
Tau	28.95316	28.95316
Tau qt 2.5%	15.13091	15.13091
Tau qt 5%	17.86727	17.86727
Tau qt 95%	239.9526	239.9526
Tau qt 97.5%	241.9526	241.9526
Theta0	0.00176	0.00176
Theta0 qt 2.5%	0	0
Theta0 qt 5%	0	0
Theta0 qt 95%	0.63632	0.63632

Statistics	All sequences	Mean
Theta0 qt 97.5%	4.14319	4.14319
Theta1	24.89981	24.89981
Theta1 qt 2.5%	15.75674	15.75674
Theta1 qt 5%	16.89077	16.89077
Theta1 qt 95%	64.97786	64.97786
Theta1 qt 97.5%	69.23571	69.23571
SSD	0.02796	0.02796
Model (SSD) p-value	0.11	0.11
Raggedness index	0.03967	0.03967
Raggedness p-value	0.01	0.01
Spatial expansion		
Tau	16.38526	16.38526
Tau qt 2.5%	4.5038	4.5038
Tau qt 5%	10.25929	10.25929
Tau qt 95%	44.8431	44.8431
Tau qt 97.5%	46.2202	46.2202
Theta	10.29392	10.29392
Theta qt 2.5%	0.08793	0.08793
Theta qt 5%	0.33471	0.33471
Theta qt 95%	16.95372	16.95372
Theta qt 97.5%	17.66293	17.66293
M	2.24253	2.24253
M qt 2.5%	0.46656	0.46656
M qt 5%	0.8196	0.8196
M qt 95%	13.10662	13.10662
M qt 97.5%	15.80311	15.80311
SSD	0.02432	0.02432
Model (SSD) p-value	0.34	0.34
Raggedness index	0.03967	0.03967
Raggedness p-value	0.08	0.08

10. Surveys of the potential anopheline vectors for *Plasmodium knowlesi, Plasmodium cynomolgi* and *Plasmodium inui*

Our previous studies have shown that zoonotic malaria in humans were more predominantly encountered among malaria patients in southern part of the country (Putaporntip et al. 2009; Jongwutiwes et al, 2011). Meanwhile, a number of long-tailed and pig-tailed macaques in southern Thailand harbored one or more species of *Plasmodium*, some of which could cross-transmit to humans (Putaporntip et al., 2010). For the feasibility to obtain adequate, longitudinal and informative data, the site for mosquito sample collection was chosen at a suburb of Narathiwat Province.

10.1 Collection using human bait trap

By using human volunteers as baits, adult female anopheline mosquitoes were collected during March-April 2018, July – August 2018, January – February 2019 and November – December 2019. A total of 331 adult anopheline mosquitoes were captured by using human baited trap. Of these, anopheline mosquitoes can be classified into 7 groups, consisting of 15 species based on morphological identification. *Anopheles maculatus* was the most prevalent species, accounting for more than 35% (n=119) of all samples collected (Table 12).

Table 12 Distribution of female anopheline mosquitoes collected during March-April 2018, July – August 2018, January – February 2019 and November – December 2019 by using human bait trap

Anopheles mosquitoes		Human baited trap				Total
Group	species	Mar- Apr 18	Jul - Aug 18	Jan - Feb 19	Nov - Dec 19	
Leucosphyrus	*An. dirus*	0	2	25	1	28
	An. dirus A,B,C	0	0	0	1	1
	An. leucosphyrus A	0	0	0	8	8
	An. introlatus	0	0	3	8	11
	An. nemophilous	0	0	1	2	3
	Dirus unknown	0	0	12	12	24
Maculatus	*An. maculatus*	19	25	48	27	119
	An. willmori	3	1	0	0	4
	An. tessellatus	0	0	1	0	1
	Maculatus unknown	0	0	4	7	11
Barbirostris	*An. barbirostris*	8	0	0	3	11

Anopheles mosquitoes		Human baited trap				Total
Group	species	Mar- Apr 18	Jul - Aug 18	Jan - Feb 19	Nov - Dec 19	
	An. hodgkini	9	32	1	12	54
	An. donaldi	1	4	0	2	7
	Barbirostris unknown	3	3	19	11	36
Umbrosus	*An. umbrosus*	1	1	0	0	2
Hycanus	*An. whartoni*	1	0	0	0	1
	Hyrcanus unknown	1	0	0	1	2
Funestus	*An. aconitus*	0	0	0	2	2
Minimus	*An. minimus*	0	2	0	1	3
	unknown	0	1	0	2	3
	Total	46	71	114	100	331

10.2 Collection using cow bait trap

Using of cow bait trap to capture, anopheline mosquitoes were conducted during March-April 2018, July – August 2018, January – February 2019 and November – December 2019. A total of 2,890 adult anopheline mosquitoes were captured by using this method. According to morphological feature, 10 groups and 25 species of the anopheline mosquitoes were identified. The predominant captured mosquitoes were *Anopheles maculatus* (n=1,450), *Anopheles kochi* (n=180) and *Anopheles kawari (*n=173), respectively (Table 13).

Table 13 Female anopheline mosquitoes collected during March-April 2018, July – August 2018, January – February 2019 and November – December 2019 by using cow bait trap

Anopheline mosquitoes		Collecting adult mosquitoes				Total
Group	species	Mar- Apr 18	Jul-Aug 18	Feb -Jan 19	Nov -Dec 19	
Leucosphyrus	*An. hackeri*	0	0	1	0	1
	An. maculatus	826	300	247	77	1450
	An. sawadwongporni	0	3	0	0	3
Maculatus	*An. willmori*	7	45	0	0	52
	An. karwari	8	2	65	98	173
	An. tessellatus	0	9	151	12	172
	An. barbirostris	8	10	21	1	40
	An. barbumbrosus	0	0	1	0	1
Barbirostris	*An. hodgkini*	0	7	6	4	17
	An. campestris	0	1	1	0	2
	An. donaldi	1	6	0	6	13

Anopheline mosquitoes		Collecting adult mosquitoes				Total
Group	species	Mar- Apr 18	Jul-Aug 18	Feb -Jan 19	Nov -Dec 19	
	Barbirostris unknown	4	24	79	16	123
	An. letifer	2	1	1	0	4
Umbrosus	*An. umbrosus*	8	2	2	0	12
	Umbrosus unknown	1	0	3	0	4
	An. peditaeniatus	37	13	8	1	59
	An. paraliae	1	0	0	3	4
	An. crawfordi	41	52	35	9	137
Hyrcanus	*An. nigerrimus*	1	0	0	0	1
	An. hyrcanus	0	0	4	0	4
	An. nitidus	3	4	9	0	16
	An. sinensis	5	2	3	13	23
	Hyrcanus unknown	1	17	103	13	134
Annularis	*An. nivipes*	0	5	1	5	11
	An. phihippinensis	18	25	29	22	94
Kochi	*An. kochi*	12	50	75	43	180
	An. vagus	49	20	26	0	95
Ludlowae	*An. subpictus*	1	0	1	0	2
	An. indefinitus	1	4	0	1	6
Funestus	*An. aconitus*	2	51	24	35	112
Minimus	*An. minimus*	0	20	0	3	23
	unknown		1	10	1	12
	Total	1037	674	906	363	2980

10.3 Collection using monkey bait trap

Monkey bait trapping was used for collecting anopheline mosquitoes during March-April 2018, July – August 2018, January – February 2019 and November – December 2019. A total of 109 adult anopheline mosquitoes were captured by using this method. By morphological features, the captured anopheline mosquitoes could be classified into 8 groups, comprising 12 species. The predominant mosquitoes were *Anopheles maculatus* (n=38), *Anopheles hodgkini* (n=34) and *Anopheles donaldi* (n=4), respectively (Table 14).

Table 14 Number of female anopheline mosquitoes collected during March-April 2018, July – August 2018, January – February 2019 and November – December 2019 by using monkey bait trap

Anopheles msoquitoes		Collecting adult mosquitoes				Total
Group	species	Mar- Apr 18	Jul-Aug 18	Jan-Feb 19	Nov –Dec 19	
Leucosphyrus	unknown	0	0	0	1	1
Maculatus	*An. maculatus*	24	1	1	12	38

Anopheles msoquitoes		Collecting adult mosquitoes				Total
Group	species	Mar- Apr 18	Jul-Aug 18	Jan-Feb 19	Nov –Dec 19	
Barbirostris	*An. willmori*	0	1	0	0	1
	An. tessellatus	0	0	1	0	1
	An. barbirostris	1	0	0	1	2
	An. hodgkini	7	16	5	6	34
	An. donaldi	0	4	0	0	4
	unknown	0	2	5	2	9
Umbrosus	*An. umbrosus*	1	1	0	0	2
Hyrcanus	*An. peditaeniatus*	1	1	0	0	2
	An. crawfordi	0	0	1	2	3
	unknown	0	1	1	0	2
Kochi	*An. kochi*	0	2	1	0	3
Funestus	*An. aconitus*	0	0	0	3	3
Asiaticus	*An. interuptus*	0	0	1	0	1
	unknown	0	3	0	0	3
	Total	34	32	16	27	109

10.4 Collection using CDC light trap

Besides human and animal bait traps, surveys of anopheline mosquitoes were also done by using the CDC light trapping during March-April 2018, July – August 2018, January – February 2019 and November – December 2019. A total of 40 adult anopheline mosquitoes were captured. Four groups consisting of 8 species of Anopheles were identified baed on morphological features. *Anopheles hodgkini* belonging to Barbirostris group is most prevalent, accounting for more than 52.5 % (n=21) of all mosquitoes collected, followed by *Anopheles barbirostris* (n=8) and *Anopheles barbumbrosus* (n=2), respectively (Table 15).

Table 15 Number of female anopheline mosquitoes were collected during March-April 2018, July – August 2018, January – February 2019 and November – December 2019 by using CDC light trap (CDC)

Anopheline mosquitoes		Collecting adult mosquitoes				Total
Group	species	Mar- Apr 18	Jul-Aug 18	Jan -Feb 19	Nov -Dec 19	
Leucosphyrus	*An. hackeri*	1	0	0	0	1
	An. nemophilous	1	0	0	0	1
Maculatus	*An. maculatus*	1	0	0	0	1
Barbirostris	*An. barbirostris*	8	0	0	0	8
	An. barbumbrosus	1	1	0	0	2
	An. hodgkini	10	10	1	0	21
	An. donaldi	0	1	0	0	1
	unknown	1	1	1	0	3
Umbrosus	*An. umbrosus*	1	1	0	0	2
Total		24	14	2	0	40

During four collection periods (March - April 2018, July - August 2018, January - February 2019 and November - December 2019) using human, animal and CDC light traps, a total of 3,460 adult anopheline mosquitoes belonging to 11 groups and 35 species were identified (Figure 10 & Table 16). The effect of rainfall seems to play an important role for mosquito abundance. The population densities of these potential malaria vectors peaked during March-April 2018 and January – February 2019, and the lowest vector density occurred in November-December 2019. The relationship between rainfall and the number of anopheline mosquitoes is shown in Figure 11. There was an inverse relationship between the average rainfall and the population densities of anopheline mosquitoes.

Primary vector, secondary vector and suspected vector for human malaria species have been previously reported. Based on this information, anopheline mosquitoes collected that belonged to the primary vector species contributed 51.2 % (n=1,773) of all samples, while those belonging to secondary vectors, suspected vector and others were 3.4% (n=117), 9.8% (n=338) and 35.6% (n=1,232), respectively. Primary vectors consist of *Anopheles dirus* (n=79), *Anopheles maculatus (*n=1,668) and *Anopheles minimus* (n=26) while *Anopheles aconitus* (n=117) identified as secondary vector was found. Suspected vectors obtained in these surveys contained 4 species

including *Anopheles barbirostris (*n=61)*, Anopheles campestris (*n=2)*, Anopheles philippinensis (*n=94) and *Anopheles kochi* (n=83) (Table 17 & Figure 12).

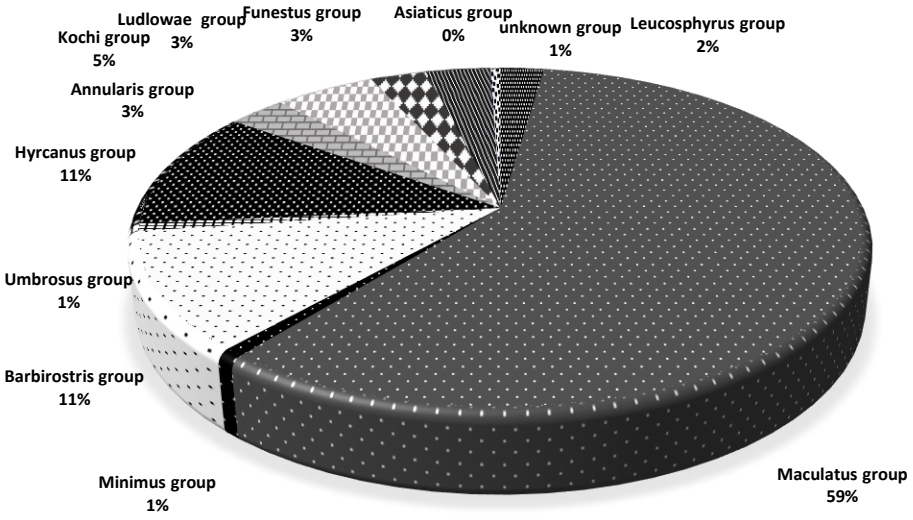


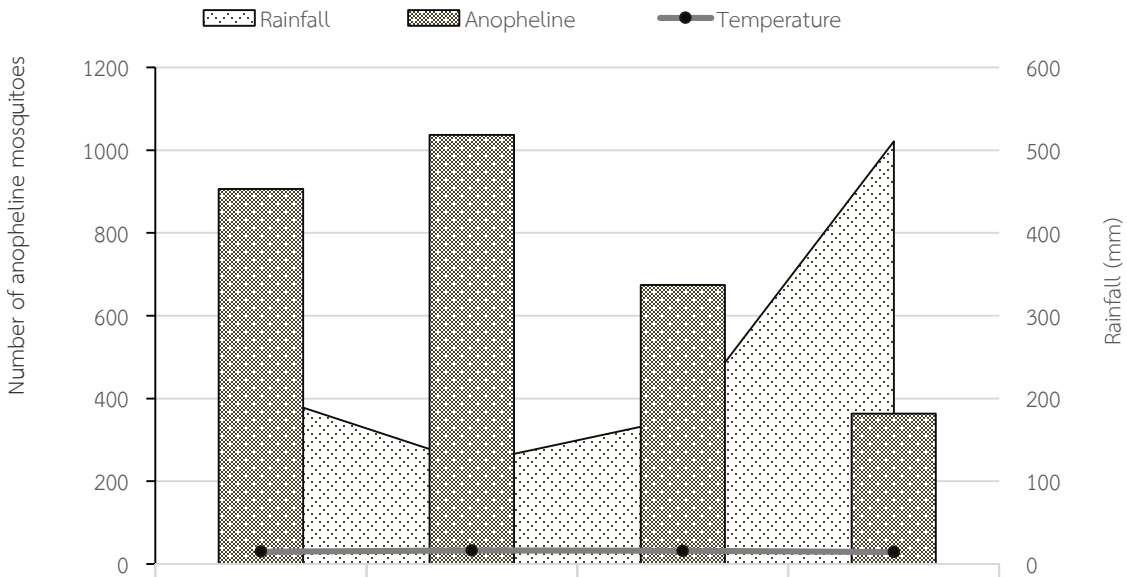
Figure 10. Distribution of *Anopheles* vectors from all 4 survey periods.

Table 16 Number of female anopheline mosquitoes collected during March-April 2018, July – August 2018, January – February 2019 and November – December 2019 by using human bait trap, cow bait trap, monkey bait trap and CDC light trap

Anopheline mosquitoes		Collection period																Total
		Mar- Apr 2018				Jul-Aug 2018				Jan-Feb 2019				Nov -Dec 2019				
Group	species	HBT	CBT	MBT	CDC	HBT	CBT	MBT	CDC	HBT	CBT	MBT	CDC	HBT	CBT	MBT	CDC	
Leucosphyrus	*An. hackeri*	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	2
	An. dirus	0	0	0	0	2	0	0	0	25	0	0	0	1	0	0	0	28
	An. dirus A,B,C	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
	An. leucosphyrus A	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	8
	An. introlatus	0	0	0	0	0	0	0	0	3	0	0	0	8	0	0	0	11
	An. nemophilous	0	0	0	1	0	0	0	0	1	0	0	0	2	0	0	0	4
	unknown	0	0	0	0	0	0	0	0	12	0	0	0	12	0	1	0	25
Maculatus	*An. maculatus*	19	826	24	1	25	300	1	0	48	247	1	0	27	77	12	0	1608
	An. sawadwongporni	3	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	7
	An. willmori	0	7	0	0	0	45	1	0	0	0	0	0	0	0	0	0	53
	An. karwari	0	8	0	0	0	2	0	0	0	65	0	0	0	98	0	0	173
	An. tessellatus	0	0	0	0	0	9	0	0	1	151	1	0	0	12	0	0	174
	unknown	0	0	0	0	0	0	0	0	4	0	0	0	7	0	0	0	11
Barbirostris	*An. barbirostris*	8	8	1	8	0	10	0	0	0	21	0	0	3	1	1	0	61
	An. barbumbrosus	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	3
	An. hodgkini	9	0	7	10	32	7	16	10	1	6	5	1	12	4	6	0	126
	An. campestris	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	2
	An. donaldi	1	1	0	0	4	6	4	1	0	0	0	0	2	6	0	0	25
	unknown	3	4	0	1	3	24	2	1	19	79	5	1	11	16	2	0	171

Table 16 (count.)

Anopheline mosquitoes		Collection period																Total
		Mar- Apr 2018				Jul-Aug 2018				Jan-Feb 2019				Nov -Dec 2019				
Group	species	HBT	CBT	MBT	CDC	HBT	CBT	MBT	CDC	HBT	CBT	MBT	CDC	HBT	CBT	MBT	CDC	
Umbrosus	*An. letifer*	0	2	0	0	0	1	0	0	0	1	0	0	0	0	0	0	4
	An. umbrosus	1	8	1	1	1	2	1	1	0	2	0	0	0	0	0	0	18
	An. baezai	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	unknown	0	1	0	0	0	0	0	0	0	3	0	0	0	0	0	0	4
	An. peditaeniatus	0	37	1	0	0	13	1	0	0	8	0	0	0	1	0	0	61
	An. paraliae	0	1	0	0	0	0	0	0	0	0	0	0	0	3	0	0	4
	An. crawfordi	0	41	0	0	0	52	0	0	0	35	1	0	0	9	2	0	140
	An. nigerrimus	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Hyrcanus	*An. hyrcanus*	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	4
	An. nitidus	0	3	0	0	0	4	0	0	0	9	0	0	0	0	0	0	16
	An. whartoni	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	An. sinensis	0	5	0	0	0	2	0	0	0	3	0	0	0	13	0	0	23
	unknown	1	1	0	0	0	17	1	0	0	103	1	0	1	13	0	0	138
Annularis	*An. nivipes*	0	0	0	0	0	5	0	0	0	1	0	0	0	5	0	0	11
	An. philippinensis	0	18	0	0	0	25	0	0	0	29	0	0	0	22	0	0	94
Kochi	*An. kochi*	0	12	0	0	0	50	0	0	0	75	1	0	0	43	0	0	181
Ludlowae	*An. vagus*	0	49	0	0	0	20	2	0	0	26	0	0	0	0	0	0	97
	An. subpictus	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	2
	An.indefinitus	0	1	0	0	0	4	0	0	0	0	0	0	0	1	0	0	6
Funestus	*An. aconitus*	0	2	0	0	0	51	0	0	0	24	0	0	2	35	3	0	117
Asiaticus	*An. interuptus*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Minimus	*An. minimus*	0	0	0	0	2	20	0	0	0	0	0	0	1	3	0	0	26
unknown	unknown	0	0	0	0	1	1	3	0	0	10	0	0	2	1	0	0	18
	Total	46	1037	34	24	71	674	32	14	114	906	16	2	100	363	27		3460



	Jan - Feb	Mar- Apr	Jul-Aug	Nov -Dec
Rainfall	205.75	122.2	176.49	510.82
Anopheline	906	1037	674	363
Temperature	29.84	32.6	31.89	29.01

Figure 11 Relationship between rainfall and vector densities

Table 17 Distribution of human mosquito vectors during March-April 2018, July – August 2018, January – February 2019 and November – December 2019

Vectors	Mar- Apr 2018		Jul-Aug 2018		Jan-Feb 2019		Nov -Dec 2019		Total	
	number	percent	number	percent	number	percent	number	percent	number	percent
primary vector	882.0	77.3	400.0	50.6	338.0	32.6	153.0	31.2	1773.0	51.2
secondary vector	2.0	0.2	51.0	6.4	24.0	2.3	40.0	8.2	117.0	3.4
suspected vector	55.0	4.8	86.0	10.9	127.0	12.2	70.0	14.3	338.0	9.8
other	202.0	17.7	254.0	32.1	549.0	52.9	227.0	46.3	1232.0	35.6
Total	1141.0	100.0	791.0	100.0	1038.0	100.0	490.0	100.0	3460.0	100.0

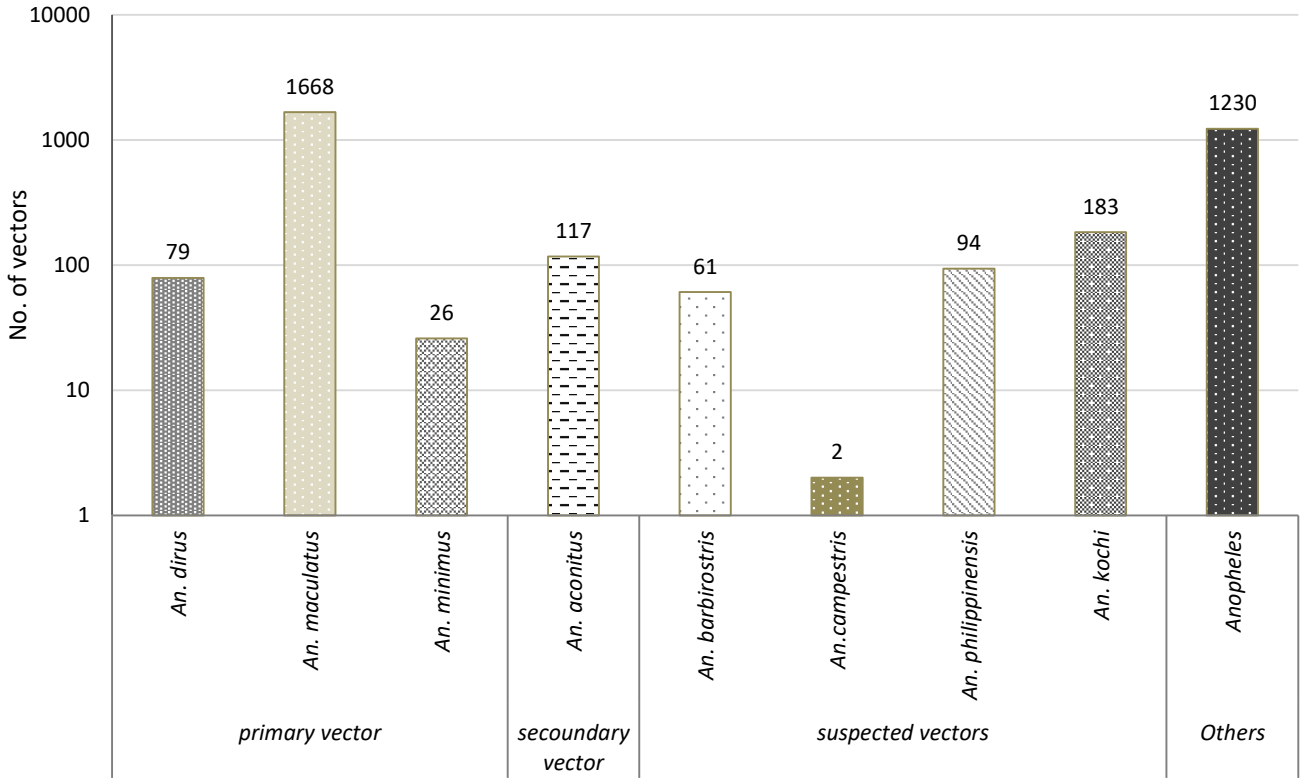


Figure 12 Distribution of primary, secondary and suspected human malaria vectors

Of 3460 anopheline mosquitoes, 510 samples were tested for the present of *Plasmodium* by using PCR-based method. Results revealed that 10 (1.96 %) mosquitoes yielded positive *Plasmodium*-specific PCR products. All but one malaria-infected mosquitoes were *An. dirus (*n=9) and the remaining sample was *An. maculatus* (n=1) (Tables 18).

Table 18 Detection of simian malaria DNA in anopheline mosquitoes

Anopheles mosquitoes		Total samples collected	Simian malaria molecular detection		
Group	species		Number of observation	Negative	Positive
	An. hackeri	2	1	1	0
	An. dirus	28	28	19	9
	An. dirus A,B,C	1	1	1	0
Leucosphyrus	*An. leucosphyrus A*	8	8	8	0
	An. introlatus	11	11	11	0
	An. nemophilous	4	4	4	0
	unknown	25	25	25	0

Anopheles mosquitoes		Total samples collected	Simian malaria molecular detection		
Group	species		Number of observation	Negative	Positive
Maculatus	*An. maculatus*	1608	158	157	1
	An. sawadwongporni	3	0	0	0
	An. willmori	57	5	5	0
	An. karwari	173	0	0	0
	An. tessellatus	174	2	2	0
	unknown	11	11	11	0
Barbirostris	*An. barbirostris*	61	21	21	0
	An. barbumbrosus	3	2	2	0
	An. hodgkini	126	109	109	0
	An.campestris	2	0	0	0
	An. donaldi	25	12	12	0
	unknown	171	48	48	0
Umbrosus	*An. letifer*	4	0	0	0
	An. umbrosus	18	6	6	0
	An. baezai	0	0	0	0
	unknown	4	0	0	0
Hyrcanus	*An. peditaeniatus*	61	6	6	0
	An. paraliae	4	1	1	0
	An. crawfordi	140	13	13	0
	An. nigerrimus	1	1	1	0
	An. hyrcanus	4	0	0	0
	An. nitidus	16	1	1	0
	An. whartoni	1	0	1	0
	An. sinensis	23	0	0	0
	unknown	138	0	4	0
Annularis	*An. annularis*	0	0	0	0
	An. nivipes	11	0	0	0
	An. philippinensis	94	0	0	0
Kochi	*An. kochi*	183	0	3	0
Ludlowae	*An. vagus*	95	13	13	0
	An. subpictus	2	0	0	0
	An.indefinitus	6	0	0	0
Funestus	*An. aconitus*	117	0	5	0
Asiaticus	*An. interuptus*	1	0	1	0
Minimus	*An. minimus*	26	0	3	0
unknown	unknown	18	0	6	0
Total		**3460**	510	500	10

To determine the species of malaria parasites in positive mosquito samples, nested PCR targeting *Plasmodium* mitochondrial gene was performed. The amplification products of primary PCR were analyzed by 1 % agarose gel electrophoresis. The results of positive PCR products were shown in lanes 1-4 and 6-11 of Figure 13.

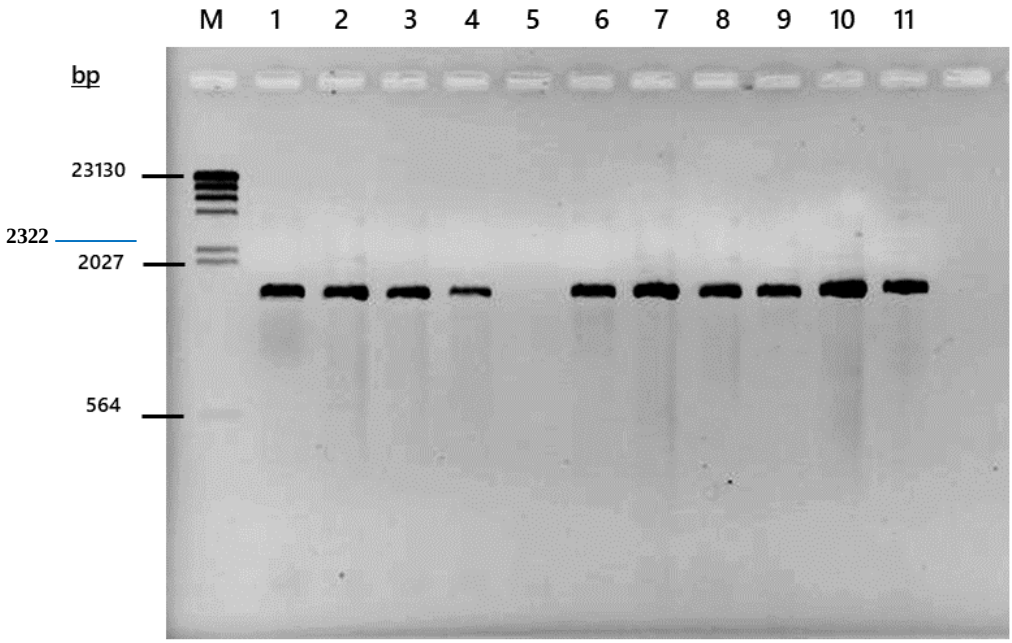


Figure 13 Agarose gel electrophoresis of malaria positive from anopheline samples by nested PCR targeting mitochondrial gene. Molecular marker is shown in lane M.

Sequence and phylogenetic analyses of the mitochondrial gene segments of 10 *Plasmodium* positive mosquitoes have shown that two samples each contained *P. inui* and *P. cynomolgi* and one had *P. knowlesi* (Figure 14 and Table 19). The species of malaria parasites in the remaining 5 samples were not primate *Plasmodium* species It is noteworthy that malaria-positive mosquitoes were found almost all year round with slightly more prevalence during January and February. All these mosquitoes were caught by using human bait trap, suggesting their anthropophilic tendency. Therefore, these mosquitoes could serve as potential mosquito vectors for cross-transmission of these simian malaria to humans.

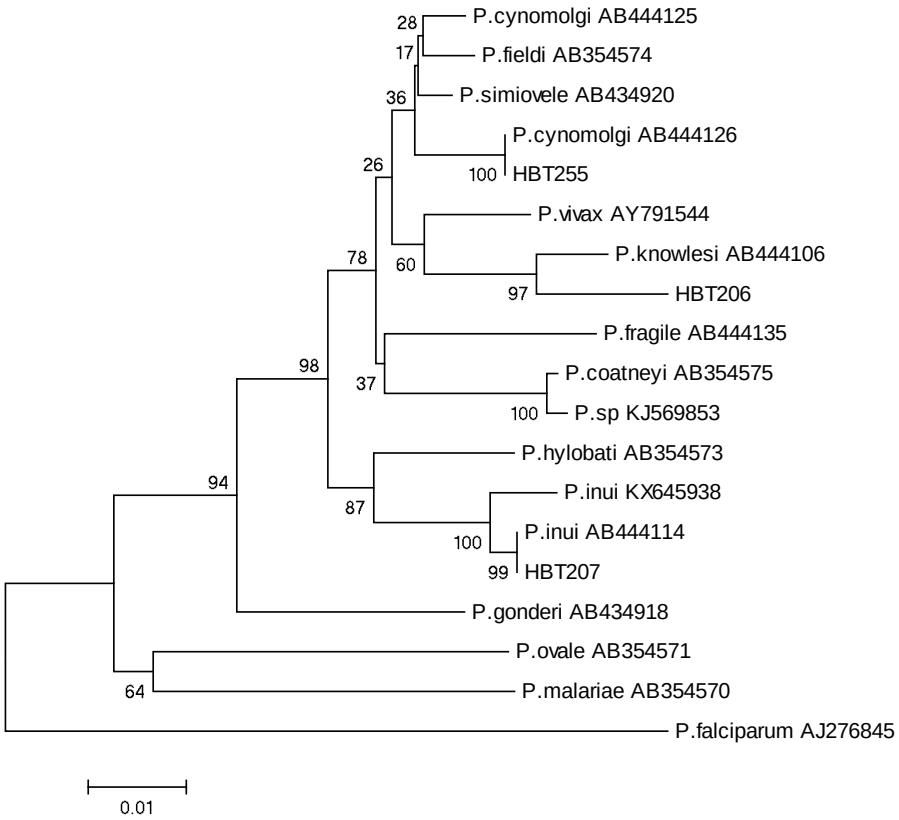


Figure 14 Neighbor-joining tree inferred from the variable sequences of the mitochondria gene of *Plasmodium* sp.from anopheline mosquitoes, compared with those reported elsewhere for human or macaque isolates using a maximum composite likelihood model. Bootstrap values from 1,000 pseudoreplicates of >50% are shown along the branches. The scale bar underneath the tree indicates the number of base substitutions per site. Plasmodium species are listed along with their respective GenBank accession numbers.

Table 19 Distribution of *Plasmodium* infections in anopheline mosquitoes diagnosed by PCR

Date	Location	Trap	Sample	Group	Species	*Plasmodium* spp.
Jan. 2019	Narathiwat	Human bait trap	HBT177	Leucosphyrus	*An. dirus*	*P. sp.*
Jan. 2019	Narathiwat	Human bait trap	HBT179	Leucosphyrus	*An. dirus*	*P. sp.*
Jan. 2019	Narathiwat	Human bait trap	HBT181	Leucosphyrus	*An. dirus*	*P. cynomolgi*
Feb. 2019	Narathiwat	Human bait trap	HBT206	Leucosphyrus	*An. dirus*	*P. knowlesi*
Feb. 2019	Narathiwat	Human bait trap	HBT207	Leucosphyrus	*An. dirus*	*P. inui*
Sep. 2019	Narathiwat	Human bait trap	HBT253	Leucosphyrus	*An. dirus*	*P. sp.*
Oct. 2019	Narathiwat	Human bait trap	HBT255	Leucosphyrus	*An. dirus*	*P. cynomolgi*
Nov. 2019	Narathiwat	Human bait trap	HBT258	Leucosphyrus	*An. dirus*	*P. inui*
Dec. 2019	Narathiwat	Human bait trap	HBT314	Maculatus	*An. maculatus*	*P. sp.*
Dec. 2019	Narathiwat	Human bait trap	HBT329	Leucosphyrus	*An. dirus*	*P. sp.*

DISCUSSION

Primate malaria parasites such as *Plasmodium knowlesi*, *Plasmodium cynomolgi* and *Plasmodium inui* have been circulating among macaque monkeys in Thailand in which long-tailed macaque (*Macaca fascicularis*) and pig-tailed macaque (*Macaca nemestrina*) serve as main natural reservoir hosts (Putaporntip et al. 2010). Importantly, *Plasmodium konowlesi* and *Plasmodium cynomolgi* have been reported to infect humans in several Southeast Asian countries under natural transmission via infected mosquito bites (Singh et al. 2004; Jongwutiwes et al. 2004; Luchavez et al. 2008; Ng et al. 2008; Van den Eede et al. 2009; Nakazawa et al. 2009; Figtree et al. 2010; Jiang et al. 2010; Khim et al. 2011; Ta et al. 2014; Grignard et al. 2019). An experimental and accidental transmissions of *Plasmodium inui* to humans has been incriminated in symptomatic infections (Coatney et al. 1971). Our previous studies of *Plasmodium* infections in both pig-tailed and long-tailed macaques have revealed the presence of *Plasmodium knowlesi*, *Plasmodium cynomolgi*, *Plasmodium inui* and other simian malaria species in Thailand, mainly in southern part of the country (Putaporntip et al. 2010). Most patients infected with *Plasmodium knowlesi* and *Plasmodium cynomolgi* resided in areas where macaques were living in vicinity; therefore, the risk of acquiring this simian malaria could occur upon poaching of macaques' habitats. This study is the first to discover *Plasmodium cynomolgi* and *Plasmodium inui* infections in humans in Thailand where both pig-tailed and long-tailed macaque natural hosts were prevalent. Interestingly, all patients who were infected with *Plasmodium cynomolgi* and *Plasmodium inui* infections harbored *Plasmodium vivax* in their circulations, implying that anopheline vectors for this simian malaria species could be either the same. Human infections with *Plasmodium cynomolgi* seems not to be a newly occurring infection because it has been reported among blood samples collected from a range of time periods since 2007. However, there has not been any report on naturally acquired human infection with *Plasmodium inui*. Morphological similarity between these two simian malaria with other human malaria could compromise conventional microscopic diagnosis (Garnham, 1966). Therefore, nested PCR developed in this study can be a useful technique to differentiate cryptic species of *Plasmodium* in human circulation. The PCR method detected significantly more cases of mixed infections. Mixed-species infections tend to be overlooked by microscopic diagnosis, especially when one parasite species is present at a remarkably lower density than the over-dominant one. For example, in Thailand approximately 33% of cryptic *Plasmodium vivax* infections became clinically patent after treatment for *Plasmodium falciparum* infection, whereas 13% of *Plasmodium vivax* infections coexisted with cryptic *Plasmodium falciparum* infections (Looareesuwan et al. 1987; Siripoon et al. 2002). Our study has shown that the distribution and

prevalence of mixed-species infections varies across geographic regions of Thailand, a variation that is probably correlated with the annual parasite incidence (API) rate.

To date, six species of macaques have been identified in Thailand, i.e. *Macaca fascicularis* (long-tailed macaque), *Macaca nemestrina* (pig-tailed macaque), *Macaca leonina* (northern pig-tailed macaque), *Macaca* mulatta (rhesus macaque), *Macaca arctoides* (stump-tailed macaque), and *Macaca assamensis* (Assamese macaque) (Malaivijitnond et al. 2005). Of these, long-tailed and pig-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., *Plasmodium knowlesi*, *Plasmodium cynomolgi* and *Plasmodium inui* (Bruce-Chwatt, 1968; Fooden, 1994). However, assessment of *Plasmodium* infections among these macaques remains largely unknown. In this study, we determined the prevalence of nonhuman primate malaria in pig-tailed and long-tailed macaques in Narathiwat Province. The study areas were located at forest areas of 3 districts of the province where anopheline mosquitoes were abundant. A high prevalence of *Plasmodium inui* was found in long-tailed macaques (57.14%) and pig-tailed macaques (39.95%), followed by *Hepatocystis* spp., *Plasmodium cynomolgi*, *Plasmodium knowlesi, Plasmodium coatneyi* and *Plasmodium fieldi*, respectively.

Interestingly, our longitudinal monitoring of simian malaria and *Hepatocystis* infections in 20 pig-tailed macaques and 3 long-tailed macaques has shown that the low prevalence of simian malaria in macaques in Thailand based on a cross-sectional survey may not imply the low risk of zoonotic malaria transmission potential whereas long-term infection and/or re-infection of simian malaria in these macaques can maintain transmission cycles within individual infected macaques as well as cross-transmission between macaques and humans.

The AMA1 genes of *P. knowlesi*, *P. cynomolgi* and *P. inui* exhibited extensive sequence diversity among isolates. Most of the nucleotide substitutions occurred in the extracellular domains, i.e. domains I, II and III, akin to what have been observed in the homologous genes in *P. vivax* and *P. falciparum*. Our analysis has revealed differential selective pressure operated on the malarial AMA1 genes in which *P. vivax* and *P. vivax*-related lineage have mainly evolved under purifying selection, probably from structural or functional constraints in these proteins. However, codon-specific selection analysis has revealed that particular codons in *PkAMA1*, *PiAMA1* and *PcyAMA1* were under pervasive or episodic positive selection that could have been masked by recurrent purifying selection in order to restore the function of these proteins. Interestingly, the signal of deviation from selective neutrality was not detected by comparison of dS and dN in each domain of the PvAMA1 gene (Putaporntip et al. 2009). By contrast, a number of positively selected residues were found in this locus by using codon-based analysis of

deviation from selective neutrality, consistent with the fact that PvAMA1 was immunogenic upon natural infection. Importantly, PvAMA1 has been considered to be was a target of invasion inhibitory antibodies akin to PfAMA1; thereby, these proteins are potential candidates for malaria vaccine development (Healer et al. 2004).

Sequence analysis of the *AMA1* locus of *P. vivax* and *P. vivax*-related malaria parasites, e.g. *P. knowlesi, P. cynomolgi, P. inui, P. fieldi and P. coatneyi*, has shown that the evolution of this locus seems to co-diverge with speciation of *Plasmodium* species as viewed from the congruency in clustering of *P. vivax* and *P. vivax*-related malaria parasites in the phylogenetic trees constructed from the *18S rRNA* and *cytochrome b* genes. Subsequent differential selective pressures exerted on each of these loci could have shaped the tree topologies for *P. vivax* and *P. vivax*-related malaria parasites. The positive selective pressure particularly exerted on the extracellular domains of malarial AMA1 implies that these portions of protein are targets of immunological responses from their respective host species, i.e. humans and macaques.

The first naturally acquired *P. knowlesi* infection in human was reported in 1965 in which definite diagnosis required experimental inoculation of the patient's blood into macaque hosts and studies of developmental stages in laboratory reared anopheline mosquitoes (Chin et al. 1965). Limitation of diagnostic microscopy for *P. knowlesi* in blood smears stems from morphological resemblance of ring stages with those of *P. falciparum* and the band-shape trophozoites with those of *P. malariae*. It was not until 2004 that human infections with this simian malaria were identified in Malaysian Sarawak (Singh et al. 2004) and Thailand (Jongwutiwes et al. 2004) by means of molecular diagnosis. Thereafter, several hundreds of human cases were reported from almost all Southeast Asian countries including Nicobar Islands of India in Andaman Sea (Millar and Cox-Singh. 2015; Tyagi et al. 2013). However, high prevalence of infections caused by this parasite has been observed in Sarawak and Malaysian peninsula accounting for about half of malaria cases in these areas (Singh et al. 2004; Vythilingam et al. 2008).

In Thailand, *P. knowlesi* can be detected in several major malaria endemic areas at relatively low prevalence (Jongwutiwes et al. 2004; Putaporntip et al. 2009; Jongwutiwes et al. 2011). The distribution of *P. knowlesi* seems to overlap these macaque habitats leading to an assumption of zoonotic transmission of this simian malaria (Fooden, 1994). However, our recent analysis of the polymorphic merozoite surface protein 1 (MSP-1) locus has shown that the number of haplotypes, haplotype diversity, nucleotide diversity and recombination sites of human-derived isolates remarkably exceeded that of monkey-derived sequences while phylogenetic networks displayed a character pattern that could have arisen from the presence of

two independent routes of *P. knowlesi* transmission, i.e. from macaques to human and from human to humans in Thailand (Putaporntip et al. 2013). However, the effects of sampling process could not be entirely excluded because the number of samples included in the analysis was limited and the possibility of differential selective pressure on MSP-1 exerted by different host species could influence the extent of molecular diversity indices and complicate interpretation of the phylogenetic networks. The present study suggested two modes of transmission of *P. knowlesi* in humans consisting of (i) a previously well-perceived mode from macaques to humans evidenced by neighbor-net analysis showing admixture of *PkAMA1* haplotypes from humans and macaques and (ii) a relatively high molecular indices in human-derived *PkAMA1*, i.e. haplotype diversity, nucleotide diversity and minimum number of recombination events, than those from macaque origins. Taken together, besides being a strong candidate for human malaria vaccines, the *AMA1* sequences of simian malaria parasites provide important information on the evolution and mode of transmission of simian malaria from macaque natural hosts to humans.

Several studies have shown that mosquitoes belonging to Leucosphyrus group were discovered as a vector for *Plasmodium knowlesi* in Southeast Asia countries. According to the study in Malaysian Borneo, *Anopheles latens* mosquitoes were identified as a vector of *Plasmodium knowlesi* (Vythilingam et al. 2006). In Vietnam, *Plasmodium knowlesi* was reported to be transmitted by *Anopleles dirus* in forested areas (Nakazawa et al. 2009). *Anopheles balabacensis* , *Anopheles hackeri* , *Anopheles cracens* and *Anopheles introlatus* have been reported as a primary vector in several areas of Malaysia (Wharton and Eyles 1961; Vythilingam et al. 2014; Wong et al. 2015). However, there has no study on potential vector for simian malaria in Thailand. This study is the first to document that *Anopheles dirus* and *Anopheles maculatus* served as a potential vector for *Plasmodium knowlesi*, *Plasmodium cynomolgi* and *Plasmodium inui* in Narathiwat province southern Thailand.

In conclusion, this study provide a more comprehensive view on the distribution of simian malaria parasites in both humans and macaque natural hosts in Thailand. The presence of *P. knowlesi*, *P. cynomolgi* and *P. inui* co-circulating with other human malaria parasites has suggested that cross-transmission of these simian malaria species between macaques and humans has been ongoing. Further simultaneous monitoring of *Plasmodium* infections in humans and macaques is warranted.

ACKNOWLEDGEMENTS

We thank Napaporn Kuamsab, Surasuk Yanmanee and Urassaya Pattanawong for technical assistance and staff of Hala-Bala Wildlife Research Station, National Park and Wildlife Research Division, Department of National Parks, Wildlife and Plant Conservation for assistance in field work. This research was supported by The Thailand Research Fund and The Asahi Glass Foundation.

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Output จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ผลงานที่เผยแพร่หรืออยู่ในระหว่างการเผยแพร่

1.1 Putaporntip C, Kuamsab N, Pattanawong U, Yanmanee S, Seethamchai S, Jongwutiwes S. Cryptic *Plasmodium cynomolgi* infections among symptomatic malaria patients, Thailand. (manuscript submission)

1.2 Kuamsab N, Putaporntip C, Jongwutiwes S. Polymorphism and natural selection in the merozoite surface protein 3F2 (PVX_97710) locus of *Plasmodium vivax* among field isolates. Infect Genet Evol. 2020;78:104058.

1.3 Buppan P, Seethamchai S, Kuamsab N, Harnyuttanakorn P, Putaporntip C, Jongwutiwes S. Multiple Novel Mutations in *Plasmodium falciparum* Chloroquine Resistance Transporter Gene during Implementation of Artemisinin Combination Therapy in Thailand. Am J Trop Med Hyg. 2018;99:987-994.

1.4 Buppan P, Seethamchai S, Kuamsab N, Jongwutiwes S, Putaporntip C. Episodic positive selection in the Cam734 haplotype and low prevalence of the A144F mutation in *Plasmodium falciparum* chloroquine resistance transporter gene among Thai isolates. Trop Biomed. 2018;35:861-871.

ผลงานที่อยู่ในระหว่างการเตรียมต้นฉบับเพื่อเผยแพร่ต่อไป

1.5 Putaporntip C, et al. First detection of *Plasmodium inui* infection in humans, Thailand.

1.6 Putaporntip C, et al. Molecular epidemiology of simian malaria in long-tailed and pig-tailed macaques in Thailand.

1.7 Putaporntip C, et al. The apical membrane antigen 1 sequences support anthroponotic and zoonotic transmission of *Plasmodium knowlesi*

1.8 Putaporntip C, et al. Identification of anopheline potential vectors for simian malaria transmissible to humans.

2. การนำผลงานวิจัยไปใช้ประโยชน์

เชิงวิชาการ

- พัฒนาการตรวจทางห้องปฏิบัติการเพื่อวินิจฉัยโรคมาลาเรียทั้งชนิดที่พบในคนและชนิดที่ติดจากลิง จากสิ่งส่งตรวจทั้งเลือด น้ำลาย และปัสสาวะ โดยวิธี PCR ซึ่งได้เปิดให้บริการแก่ผู้ป่วยของโรงพยาบาลจุฬาลงกรณ์ และทั่วไป
- พัฒนาการเรียนการสอนการเรียนการสอน รายวิชา Clinical Hematology & Systemic Infection หลักสูตรแพทยศาสตร์บัณฑิต และ Advanced Medical Parasitology หลักสูตรปริญญาโท สาขาปรสิตวิทยาทางการแพทย์
- อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วมระดับปริญญาเอกในหัวข้อวิทยานิพนธ์เรื่อง “การสำรวจยุงก้นปล่องที่มีศักยภาพเป็นพาหะของมาลาเรียในไพรเมตบริเวณภาคใต้ของประเทศไทย”

  ชื่อนิสิต นายสุรศักดิ์ ยานมณี

  หลักสูตรวิทยาศาสตร์ชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยนเรศวร

3. การเสนอผลงานในที่ประชุมวิชาการ

ได้รับเชิญเข้าร่วมประชุมและเป็นวิทยากรบรรยายพิเศษ ในหัวข้อเรื่อง “Malaria in non-human primates” ในที่ประชุม The 79th Annual Meeting of Eastern Branch of Japanese Society of Parasitology ณ Tokai University Yoyogi Campus เมือง Tokyo และในที่ประชุม Annual Meeting of Tokai Medical Association ณ Tokai University Isehara Campus เมือง Kanagawa ประเทศญี่ปุ่น ในระหว่างวันที่ 11-16 ตุลาคม 2562

ภาคผนวก

第 79 回　日本寄生虫学会東日本支部大会

プログラム・講演要旨

PROCEEDINGS OF THE 79TH ANNUAL MEETING OF
EASTERN BRANCH OF JAPANESE SOCIETY OF PARASITOLOGY

期 日：令和元年（2019 年）10 月 12 日（土）

会 場：東海大学 代々木キャンパス

第 79 回日本寄生虫学会東日本支部大会　概要

会　期：令和元年（２０１９年）１０月１２日（土）

会　場：東海大学　代々木キャンパス　２号館１階　２Ｃ１１

東京都渋谷区富ヶ谷２−２８−４

大会長：橘　裕司（東海大学 医学部 基礎医学系 生体防御学領域　教授）

日　程：

9:00	受付開始
9:25	開会　大会長挨拶
9:30〜10:18	一般講演 ４題　線虫・条虫
10:18〜11:06	一般講演 ４題　吸虫
11:15〜12:15	一般講演 ５題　アメーバ・キネトプラスチダ
12:25〜13:20	東日本支部評議員会・総会
13:20〜14:50	招待講演　知っておきたいアジアの原虫感染症

・Somchai Jongwutiwes 博士（チュラロンコン大学医学部）

Of Men and Monkeys: *Plasmodium knowlesi* in humans, Thailand

・Chaturong Putaporntip 博士（チュラロンコン大学医学部）

Of Monkeys and Men: *Plasmodium knowlesi* in macaques, Thailand

・Xunjia Cheng 博士（復旦大学基礎医学院）

Human babesiosis in China

・Windell L. Rivera 博士（フィリピン大学ディリマン校理学部）

Trichomonas vaginalis infections in the Philippines

15:00〜16:00	招待講演　微生物研究の新たな展開

・小林　正規 博士（慶應義塾大学医学部）

腸内寄生原虫感染への腸内細菌の関与

・今西　規 博士（東海大学医学部）

感染症研究を加速するためのゲノムインフォマティクス

16:10〜17:10	一般講演 ５題　マラリア原虫
17:10	閉会
17:20〜	懇親会

大会事務局：〒259-1193 神奈川県伊勢原市下糟屋 143

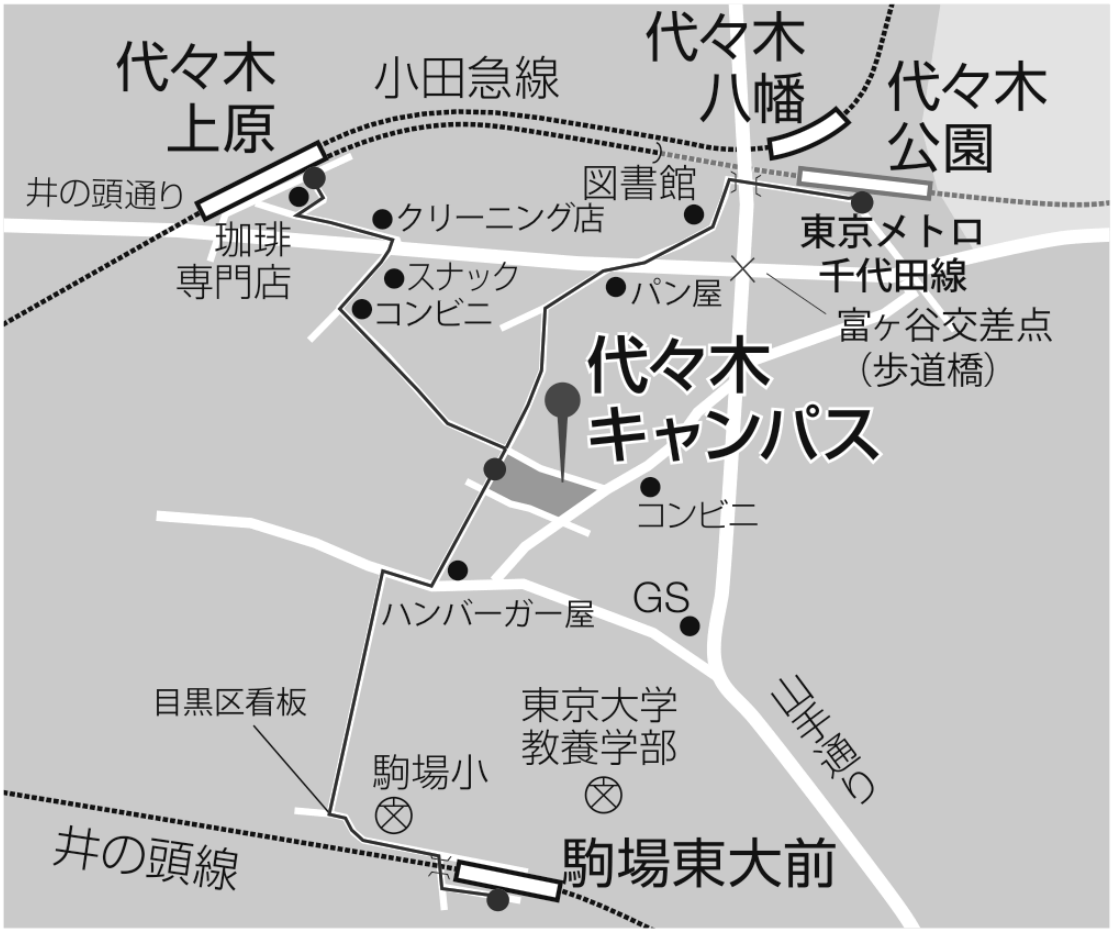
東海大学医学部　基礎医学系生体防御学

TEL：0463-93-1121（内線 2600）　FAX：0463-95-5450

E-mail：jspe79@tsc.u-tokai.ac.jp

会場周辺図・アクセス

東海大学　代々木キャンパス
〒151-8677 東京都渋谷区富ヶ谷２−２８−４



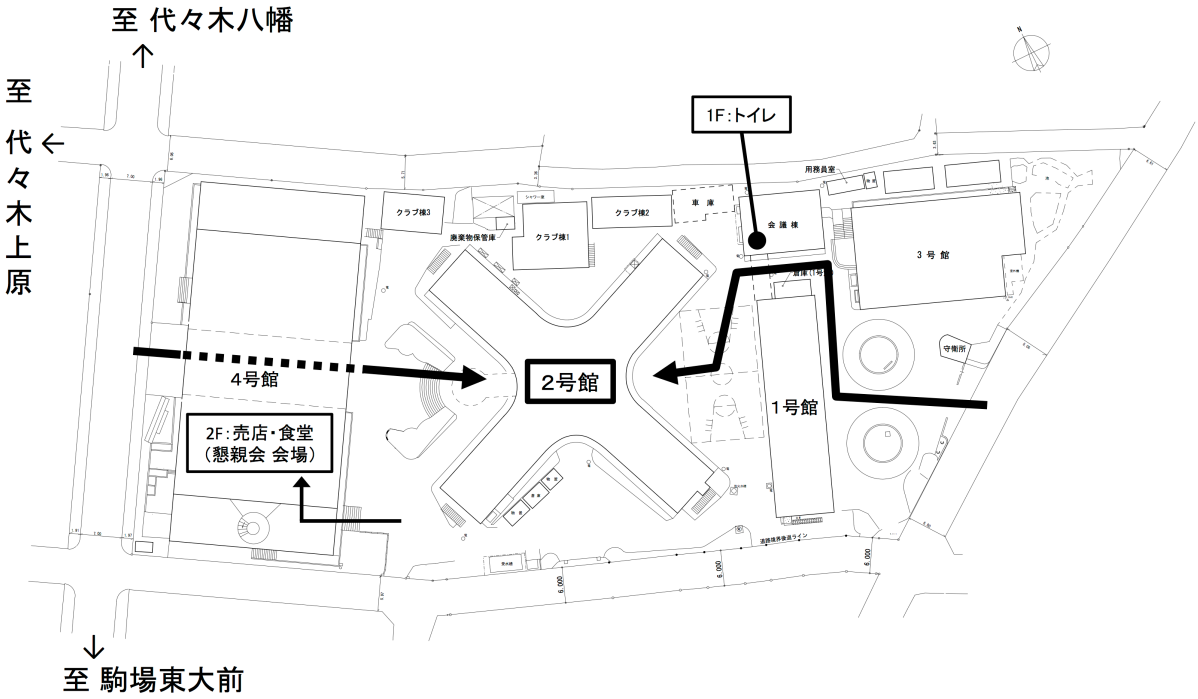
小田急線「代々木八幡」駅（南口出口）または「代々木上原」駅（南口出口）下車
徒歩 10 分

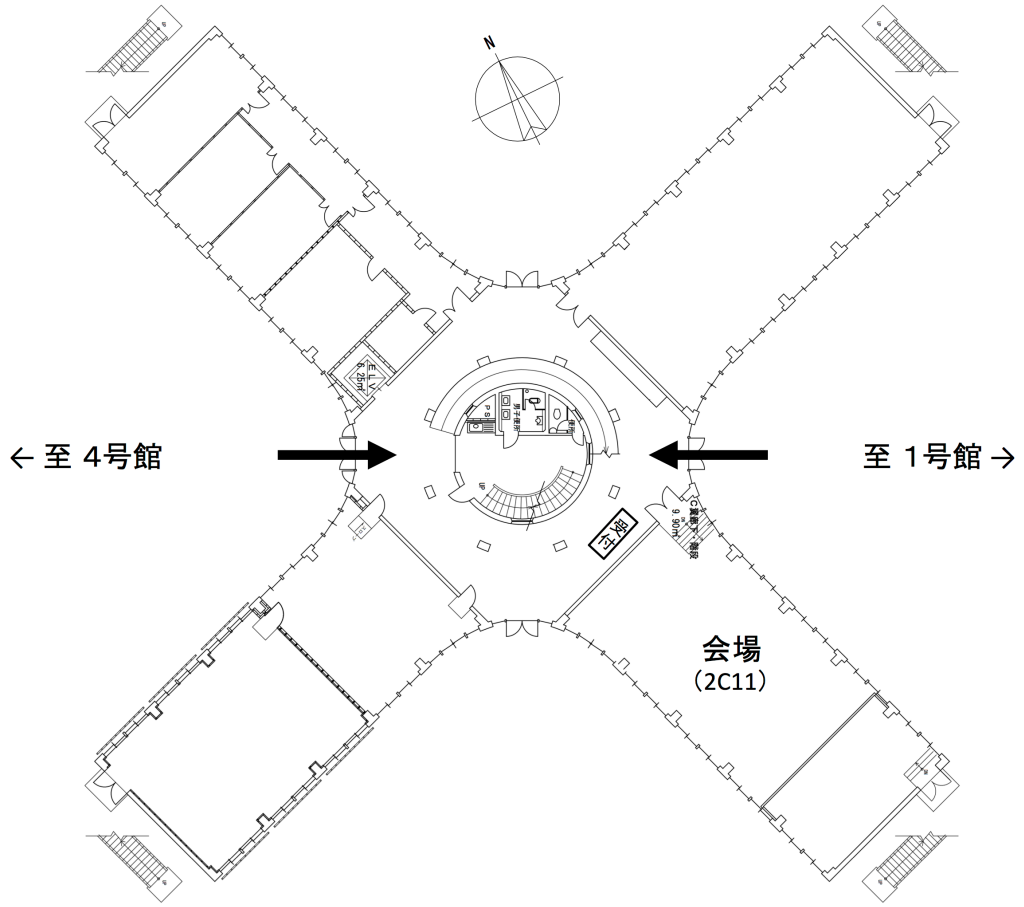
京王井の頭線「駒場東大前」駅（西口出口）下車徒歩 10 分

東京メトロ千代田線「代々木公園」駅（代々木八幡方面出口１）下車徒歩 10 分

会場案内図

２号館１階　２Ｃ１１





参加者へのご案内

大会受付

東海大学代々木キャンパス ２号館 １階 ２Ｃ１１（講演会場）前
（参照：会場案内図）

大会参加費は無料です。受付後、ネームカードとカードホルダーをお受取りください。
また、懇親会費（¥3,000、学生 ¥1,000）、弁当代（¥1,000）は、受付にてお支払いください。お弁当の当日受付は行いません。

支部評議員会・総会

１２時２５分から講演会場（２Ｃ１１）において、昼食をとりながら支部評議員会・総会を行います。

休　憩・クローク

・講演会場（２Ｃ１１）内後方に飲物・お菓子をご用意しています。
・食堂等も休憩スペースとしてご利用いただけます（参照：会場案内図）。
・クロークの用意はございません。

懇親会

大会終了後、４号館２階食堂にて開催します。事務局の誘導に従って、ご移動ください。

お弁当の引き換え

事前予約された方は、会場内にて引換券をご提示の上、お弁当をお受け取りください。

その他

・キャンパス内には食堂および売店（小規模）がございます。
・キャンパス内には駐車場がございません。公共交通機関をご利用ください。

発表者へのご案内

発表時間

一般講演：発表　9 分 / 質疑応答　3 分

発表ファイル

● 持ち込みPCによる発表

本大会で対応可能な端子は、VGA端子（ミニD-Sub15ピンケーブル）のみです。必要となるコネクターは、各講演者でご用意ください。

●大会事務局が用意するPCによる発表

大会事務局が用意するPC（OS：Windows10、ソフト：Microsoft PowerPoint 2013）の利用を希望される講演者は、PowerPointで作成したファイルをE-mail（jspe79@tsc.u-tokai.ac.jp）またはCDなどの記録媒体で事務局までお送りください。発表ファイルの互換性にご注意ください。

締　切：１０月７日（月）

ファイルの送付先
〒259-1193 神奈川県伊勢原市下糟屋 143
東海大学医学部　基礎医学系生体防御学 内
第79回 日本寄生虫学会東日本支部大会 事務局

動画等を含む大容量ファイル（10MB以上）をオンラインでお送りいただく場合は、ギガファイル便（https://gigafile.nu/）などの無料大容量ファイル転送サービスをご利用ください。Microsoftアカウントをお持ちの方はOneDriveでの転送も可能ですので、大会事務局（jspe79@tsc.u-tokai.ac.jp）までご連絡ください。

当日のデータの受け付けは行いません。また、発表のために送付いただいたデータ・記録媒体は、返却いたしません。講演終了後、事務局で責任をもって消去・廃棄します。

次演者

会場内前方の次演者席にて、PCの準備等を行ってください。

利益相反（COI）の開示

発表時に COI の有無を開示する必要があります。
日本寄生虫学会の HP「利益相反マネージメントに関する申合わせ」
(http://jsp.tm.nagasaki-u.ac.jp/about/conflict-of-interest/) をご覧ください。

プログラム

10 月 12 日（土）

9:00　　受付開始

9:25　　開会 大会長挨拶

【一般講演】

9:30 ～ 10:18　　線虫・条虫

座長：杉山　広（国立感染症研究所）

1. 豚鞭虫卵内服療法における好酸球数の推移について
   保科　斉生（東京慈恵会医科大学 感染制御科）
2. 2019 年に漁獲されたカツオにおけるアニサキス属線虫類の寄生状況
   高野　剛史（目黒寄生虫館）
3. 琉球列島中部に分布するヘリグロヒメトカゲに特異的に寄生する
   Neoentomelas asatoi (Nematoda: Rhabdiasidae)の遺伝的多様性に関する研究
   佐田　直也（目黒寄生虫館）
4. *Spirometra* 属裂頭条虫の遺伝子解析と種同定
   鈴木　淳（東京都健康安全研究センター 微生物部食品微生物研究科）

10:18 ～ 11:06　　吸虫

座長：高本　雅哉（信州大学）

5. 固定法の違いによる吸虫の形態差について
   小川　和夫（目黒寄生虫館）
6. 国内のヘビ類 3 種から見つかった吸虫 *Ochetosoma kansense*
   巖城　隆（目黒寄生虫館）
7. エクアドル産 *Amphimerus* 属肝吸虫のハムスターへの感染試験
   杉山　広（国立感染症研究所 寄生動物部）
8. マンソン住血吸虫症による肉芽腫形成への終末糖化産物受容体(RAGE)の関与
   小泉　頌歌（北里大学大学院 医療系研究科 医科学専攻 国際寄生虫病制御学）

11:06 ～ 11:15　　休憩

11:15 ～ 12:15　　**アメーバ・キネトプラスチダ**

座長：案浦　健（国立感染症研究所）

9. 病原性アメーバ *Balamuthia mandrillaris* のマウス感染モデル樹立と迅速診断法の開発
   花舘 有希（国立感染症研究所 寄生動物部）
10. ライブイメージングによる赤痢アメーバ Peroxiredoxinの動態解析
   今井　達也（東海大学 医学部 基礎医学系 生体防御学領域）
11. 腸内細菌叢との共棲関係に作用する新規赤痢アメーバ感染制御薬の探索
   吉田　菜穂子（慶應義塾大学 医学部 感染症学教室）
12. バイオイメージングによる*T. cruzi* 感染マウスの炎症の検出
   矢澤　祐典（群馬大学大学院 保健学研究科 保健学専攻 地域・国際生体情報検査科学分野）
13. PCR-RFLP法を用いたエクアドルに分布するリーシュマニア原虫種の同定
   加藤　大智（自治医科大学 医学部 感染・免疫学講座 医動物学部門）

12:25 ～ 13:20　　**東日本支部評議員会・総会**

【招待講演】

13:20 ～ 14:50　　**知っておきたいアジアの原虫感染症**

座長：川合　覚（獨協医科大学）

辻　尚利（北里大学）

I-A. Of Men and Monkeys: *Plasmodium knowlesi* in humans, Thailand
   Somchai Jongwutiwes（Chulalongkorn University）

I-B. Of Monkeys and Men: *Plasmodium knowlesi* in macaques, Thailand
   Chaturong Putaporntip（Chulalongkorn University）

II. Human babesiosis in China
   Xunjia Cheng（Fudan University）

III. *Trichomonas vaginalis* infections in the Philippines
   Windell L. Rivera（University of the Philippines Diliman）

14:50 ～ 15:00　　休憩

【招待講演】

15:00 ～ 16:00　　微生物研究の新たな展開

座長：橘　裕司（東海大学）

IV.　腸内寄生原虫感染への腸内細菌の関与
　　小林　正規（慶應義塾大学）

V.　感染症研究を加速するためのゲノムインフォマティクス
　　今西　規（東海大学）

16:00 ～ 16:10　　休憩

【一般講演】

16:10 ～ 17:10　　マラリア原虫

座長：小林　富美恵（麻布大学）

14. ウガンダにおけるマラリア予防政策とその実態
　片山　瑞希（順天堂大学 医学部 熱帯医学・寄生虫病学講座）
15. 熱帯熱マラリア原虫N-アシル化Rab5bとArf1, Rab1bの小胞体近傍における局在解析
　多久　和泉（国立感染症研究所 寄生動物部）
16. マラリア原虫有性ステージ分化におけるRNA結合型ジンクフィンガータンパク質ファミリーの解析
　平山　泰士（東京医科歯科大学大学院 医歯学総合研究科 国際環境寄生虫病学分野）
17. 電子顕微鏡3D構造解析により初めて明らかとなったオーシスト期マラリア原虫の核分裂様式
　荒木　球沙（国立感染症研究所 寄生動物部）
18. マラリア原虫感染赤血球の乳腺組織への蓄積
　新倉　保（杏林大学 医学部 感染症学講座 寄生虫学部門）

17:10　　閉会

17:20 ～　　懇親会

講演要旨

招待講演

Of Men and Monkeys: *Plasmodium knowlesi* in humans, Thailand

Somchai Jongwutiwes

Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Naturally acquired human infections with *Plasmodium knowlesi* are endemic in Southeast Asia. Our previous survey in 2006-2007 has shown a wide-spread and low prevalence of *P. knowlesi* in Thailand, accounting for 0.57% of all malaria cases identified. The follow-up study in the same endemic areas in 2008-2009 and a retrospective analysis of malaria species in patients' blood samples from one of these endemic areas collected in 1996 have shown that *P. knowlesi* contributed 0.67% and 0.48%, respectively, indicating that this simian malaria is not newly emergent human pathogen in this country. Sequence analysis of the complete merozoite surface protein-1 gene of *P. knowlesi* (*Pkmsp-1*) from human and macaque blood samples has revealed considerable genetic diversity among isolates. Importantly, the *Pkmsp-1* sequence from one patient was identical to that from a pig-tailed macaque living in the same locality, suggesting potential cross-transmission of *P. knowlesi* from naturally infected macaques to humans or vice versa. The number of haplotypes, haplotype diversity, nucleotide diversity and recombination sites of human-derived sequences exceeded that of monkey-derived sequences. Phylogenetic networks based on concatenated conserved sequences of *Pkmsp-1* displayed a character pattern that could have arisen from sampling process or the presence of two independent routes of *P. knowlesi* transmission, i.e. from macaque to human and from human to human in Thailand. Importantly, recent life-threatening and fatal infections occurring in Thai patients have highlighted the clinical significance of this simian malaria in this country.

招待講演 - I-B

Of Monkeys and Men: *Plasmodium knowlesi* in macaques, Thailand

Chaturong Putaporntip

Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

The emergence of naturally acquired human infections caused by *Plasmodium knowlesi* in Southeast Asia has emphasized the public health importance of zoonotic malaria in this region. Both long-tailed (*Macaca fascicularis*) and pig-tailed (*M. nemestrina*) macaques that are prevalent in Southeast Asian countries including Thailand serve as main natural hosts for *P. knowlesi*. To explore the potential risk of macaques as reservoir hosts of nonhuman primate malaria potentially transmissible to humans, it is crucial to know the prevalence of this simian malaria parasite and those potentially causing human infections among macaques in Thailand. Because morphological diagnosis of nonhuman primate malaria can be presumptive and some may not be unequivocally determined, we analyzed both the mitochondrial cytochrome *b* gene sequences and the small subunit ribosomal RNA genes of 297 malaria/*Hepatocystis*-positive isolates obtained from 987 monkeys comprising 351 long-tailed and 636 pig-tailed macaques. Species assignment is determined from phylogentic tree construction with reference sequences available in the GenBank database. All simian malaria parasites known to circulate among these macaques were detected in this study, i.e. *P. knowlesi*, *P. cynomolgi*, *P. coatneyi*, *P. inui* and *P. fieldi*. A low prevalence of *P. knowlesi* in both macaque species was observed, mainly clustering in southern Thailand. It is interesting to note that *P. knowlesi* and *P. cynomolgi* are known to be transmissible to humans under natural infection. Hence, some risks emerge for those living in vicinity of or encroaching upon macaques' natural habitats in this region where anopheline vectors are abundant.

Human babesiosis in China

Xunjia Cheng

Department of Medical Microbiology and Parasitology, School of Basic Medical Sciences;
Fudan University; Shanghai, China.

Babesia microti, a tick-borne intraerythrocytic protozoan parasite of the genus *Babesia* in the phylum *Apicomplexa*, is the causative agent of babesiosis in animals and humans worldwide. Although babesiosis predominantly affects animals, increasing cases of babesiosis in humans have raised concerns in the medical community. The clinical spectrums related to babesiosis range from clinical asymptomatic infection or influenza-like illness to fulminant fatal disease. The modest symptoms of human babesiosis include fever, headaches, anemia, chills, myalgia, and fatigue. Severe manifestations can also develop, such as hemolysis, jaundice, thrombocytopenia, hemoglobinuria, and renal-hepatic failure, frequently in an immunocompromised host. The most serious, babesiosis can be life-threatening in certain populations such as neonates/infants or immunocompromised patients.

Babesiosis is endemic in the northeastern and midwestern of United States, the Western Pacific and Europe countries. In China, babesiosis is an emerging disease, more than 150 cases had been reported until 2011. Due to the similar clinical manifestations between babesiosis and malaria, absence of awareness and diagnostic methods could lead to misdiagnosis of babesiosis to malaria which finally result in miss appropriate therapy.

Molecular diagnosis method based on PCR amplification of the 18S ribosomal RNA was used for diagnosis and identify the species to which the parasites belonged. After accurate diagnosis, Malarone, a compound drug which consisted of atovaquone and proguanil hydrochloride, was administered to the patient for 4 weeks at an interval of 1 week. After the patient recovered from high fever and completion of the therapy, nested PCR could be performed to amplify patients' blood gDNA by using genus-specific primer for assess the therapeutic effects.

The latent period or severity of babesiosis varies depending on the condition of host and species of *Babesia*. The parasite can be eradicated by the host immune system or the host can become a carrier without any manifestation, such as an immunocompetent individual. Babesiosis can develop once the host immunity declines, i.e., when the patient becomes weak, undergoes splenectomy, or takes immunosuppressive drugs. Our present cases indicated that development of babesiosis usually contributed to the decreasing of immunity or blood transplant. These findings demonstrated that it would be important to improve the immunity of the patient against babesiosis.

Trichomonas vaginalis infections in the Philippines

Windell L. Rivera

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Trichomonas vaginalis is the causative agent of trichomoniasis, the most prevalent sexually-transmitted infection (STI) in the world. The prevalence of trichomoniasis in the Philippines is low compared to other countries. However, our studies showed an increasing trend in the prevalence of this STI in the Philippines. This can be attributed to the use of nucleic acid amplification techniques (NAATs) which are more sensitive and rapid than the traditional methods of detecting this protozoon. *T. vaginalis* parasitizes the urogenital tract of humans, however, infection in other cell types, like the lung cells, was also observed. Infection with *T. vaginalis* may cause inflammation and this effect was associated with the presence of a virus inside the cell called *Trichomonas vaginalis* virus (TVV). There are four species of TVV and all were detected in *T. vaginalis* isolated from Pampanga, Philippines. Another issue that must be of concern is the occurrence of drug-resistant *T. vaginalis* especially that only two drugs, metronidazole and tinidazole, have the approval for use in treatment of the infection. This is even aggravated by the higher risk of acquiring human immunodeficiency virus (HIV) when one has trichomoniasis. Our country is now challenged by a nearly uncontrollable rise of HIV cases and it is indeed high time that we look into other agents of STI that aid in the spread of HIV.

腸内寄生原虫感染への腸内細菌の関与

小林　正規

慶應大・医学部・感染症

腸内細菌叢のメタゲノム解析の進歩により、腸内寄生原虫感染に関与する腸内細菌も、近年、徐々に特定されつつある。本発表では、*in vitro* 培養系において、実際に、複数の腸内寄生原虫種の増殖促進効果や嚢子内栄養型の高率な脱嚢誘導効果を認めた２種の偏性嫌気性菌［*Bacteroides fragilis*；硫酸還元菌（*Desulfovibrio desulfuricans*）]を用いて、これら細菌の原虫増殖促進効果と病原性原虫の感染への効果を、培養実験と動物感染実験結果を基に考察してみたい。

培養対象とした原虫は赤痢アメーバ、スナネズミ由来の *Entamoeba muris*, ブタとサル由来のアメーバ（*Entamoeba polecki* spp., *Endolimax* sp., 等), ヒト胆嚢から分離された鞭毛虫 *Tritrichomonas foetus,* 陸ガメ由来のメタン菌共生繊毛虫（*Nyctotherus* (*Nyctotheroides*) *teleacus*）などであり、原虫の培養には上記偏性嫌気性菌を大腸菌と共培養させた、Robinson 或いは Balamuth 培地を用いた。*D. desulfuricans* は硫化水素産生菌として知られるが、*B. fragilis* もシステイン濃度に依存して高濃度の硫化水素を産生する。また、これら偏性嫌気性菌は嫌気性原虫と同様、嫌気的なエネルギー代謝に重要な役割を担う電子伝達物質の鉄硫黄蛋白（4Fe-4S フェレドキシン）を有す。また、これら 2 種細菌の培養上清には好中球の殺菌物質のひとつである次亜塩素酸産生にかかわるミエロペルオキシダーゼを不可逆的に阻害する物質も見出されており、原虫の感染成立に貢献する可能性もある。ブタアメーバの嚢子からの脱嚢は硫化水素濃度に依存してみられ、300 -400 μ moles/L で高率に観察された。また、*E. muris, E. polecki* spp., *N. teleacus* 等の培養には硫化水素と共に鉄（鉄硫黄化合物）が必須であった。

感染実験系では、先に、*B. fragilis* の培養上清に赤痢アメーバの CBA マウス盲腸への感染率を上昇させる効果を見出したが、更に、赤痢アメーバと同様、腸粘膜接着因子を有し、免疫不全を伴うヒトへも稀に感染する *T. foetus* の感染実験では、本来、好適宿主ではない CBA マウス盲腸に、*B. fragilis* と共に接種すること、及び、赤痢アメーバを予め *B. fragilis* と共に盲腸に感染させた CBA マウスで、経口的にも、血行性にも盲腸への感染が成立することを見出した。

腸管寄生原虫は嫌気的なエネルギー代謝を利用するものが多く、嫌気性菌と代謝経路の共通点も多い。メタゲノム解析と共に、このような腸内嫌気性細菌と原虫代謝のかかわりを解析することも、細菌叢の変化に伴う原虫症の発症や劇症化メカニズム等の理解に役立つものと考える。

招待講演 - V

感染症研究を加速するためのゲノムインフォマティクス

今西　規

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感染症の迅速なゲノム診断を実現することをめざし、われわれはポータブル型 DNA シークエンサーを使った技術開発に取り組んでいる。これまでに作成した細菌感染症のためのシステムでは、DNA 抽出後から 2 時間以内には細菌種の同定が可能であることを示した。現在は本システムの改良を進め、ウイルスや真菌などの感染微生物を検出可能にするとともに、薬剤耐性菌の迅速検出や絶対定量にも挑戦している。また、上記の感染症研究に必要となるゲノム情報解析について、独自の取り組みを紹介したい。特に、全ゲノム配列と Taxonomy 情報を統合化した独自のデータベースである GenomeSync や、配列データに基づいて生物種を判定するためのソフトウエア群である GSTK を紹介する。さらに、ゲノム配列データベースにおけるヒトゲノム配列のコンタミの問題を指摘したい。

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講演要旨

一般講演

豚鞭虫卵内服療法における好酸球数の推移について

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【背景】欧米では衛生仮説をもとに医療用豚鞭虫卵製剤（TSO）が開発され、さまざまな炎症性疾患、アレルギー疾患への効果が検証されてきた。欧米の自己免疫疾患患者を対象とした臨床試験が複数存在するが、その治療効果についての明確なエビデンスはまだ得られていない。一方、これまでの試験で重篤な有害事象の報告はなく、自己免疫疾患の治療法である免疫抑制療法と比較すれば、安全性は高いと考えられる。ただし、既報は全て欧米での研究で、主に欧米人を対象としており、日本人を含むアジア人における効果と安全性は不明である。以前我々は、日本人における TSO 内服の安全性評価を目的とし、12 人の日本人健康者成人男性を対象に、二重盲検化ランダム化比較試験を実施した。今回、豚鞭虫卵の服用が感染者の好酸球数に及ぼす影響について解析し、その意義と有害事象との関係について検討した。

【方法】臨床研究「医療用豚鞭虫卵製剤の日本人における安全性・認容性」で得られた情報を用いて、豚鞭虫感染前後の好酸球数と、その推移を評価した。先行研究では、12 人の日本人健康成人男性を対象とし、TSO の容量別に 1000 個、2500 個、7500 個の 3 群に分け（それぞれ TSO 1000、TSO 2500、TSO 7500 とする）、各群に 1 人のプラセボを割り当てた。各参加者は TSO 製剤またはプラセボを 1 回内服し、内服 7、14、28、56 日後に血液検査を実施した。介入前と比較し、好酸球数が 200/μl 以上上昇した症例を好酸球増多と定義し、測定時の診療情報と合わせて解析した。

【結果】プラセボ群の 3 人の平均年齢は 37.0 歳（±6.68 SD）であり、TSO 群の 9 人は 34.2 歳（±6.23 SD）であった。試験開始時におけるプラセボ群の平均好酸球数は 366.7/μl（±378.6 SD）で、TSO 群は 166.7/μl（±86.6 SD）であった。TSO 製剤内服 56 日後の平均好酸球変化数は、プラセボ群（33.3/μl）と比較して TSO 1000（33.3/μl）では有意差がなかったが、TSO 2500（766.7/μl）と TSO 7500（300/μl）ではそれぞれ有意な好酸球増多が認められた（p<0.01）。好酸球数増多は、TSO 製剤内服 28 日以降に生じ、TSO 2500、TSO 7500 では TSO 製剤内服後 56 日目に好酸球数が最大となった。経過観察中に自覚症状を認めた 2 例は好酸球数の上昇を伴ったが、自覚症状を伴わない好酸球増多症例を 3 例認めた。

【結論】TSO 内服による好酸球増多は容量依存性であった。好酸球増多は TSO 内服 28 日以降に出現するため、臨床的には十分な観察期間が必要である。また、無症候性の好酸球増多症例も認められ、臨床的意義については更なる評価が必要である。

2019 年に漁獲されたカツオにおけるアニサキス属線虫類の寄生状況

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2018 年、カツオ（特に初ガツオ）の生食が原因と推定されるアニサキス症の報告が急増した。これをうけ、演者らは岩手県沖から南西諸島にかけての 12 地点において、同年 5 月から 11 月に漁獲されたカツオ 59 尾を対象にアニサキス属線虫類の幼虫（以下アニサキス）の寄生状況を調査した（巖城ら、第 88 回日本寄生虫学会大会)。一方、2019 年は初ガツオによるアニサキス症の報告は少なく、厚生労働省の食中毒統計資料によれば、7 月末日までで 7 件にとどまっている。これは昨年同時期までと比べ、1/10 ほどの値である。件数に差が生じた要因として、加工・販売業者が対策を講じていることのほか、カツオのアニサキス寄生状況が昨年と異なることが考えられる。そこで、2019 年も同様の調査を継続し、2018 年の結果と比較することとした。

静岡県、三重県および伊豆諸島において 2019 年 4 月から 6 月に漁獲されたカツオ 41 尾を調べた結果、アニサキス I 型あるいは II 型の形態を示す 182 虫体が見出された。ミトコンドリア DNA の COII 領域の塩基配列比較、もしくは核 DNA の ITS 領域を対象としたリアルタイム PCR 法により、I 型の虫体は *Anisakis berlandi*、*A. pegreffii*、*A. simplex* s. s.あるいは *A. typica*、II 型のものは全て *A. physeteris* と同定され、うち *A. physeteris* が優占した。一方、アニサキス症の主たる原因虫種である *A. simplex* s. s.は少なく、本種の寄生状況を比較すると、2018 年に太平洋沿岸で漁獲されたカツオでは寄生率 84%、平均寄生強度 9.2 虫体/尾であったのに対し、2019 年は寄生率 32%、平均寄生強度 2.6 虫体/尾であった。カツオの筋肉中に寄生する本種虫体も少数であり、これがアニサキス症の件数に影響していることが示唆された。本発表では、今年の戻りガツオにおける寄生状況もあわせて報告する。

琉球列島中部に分布するヘリグロヒメトカゲに特異的に寄生する *Neoentomelas asatoi* (Nematoda: Rhabdiasidae)の遺伝的多様性に関する研究

佐田　直也
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ある動物の遺伝的変異の解明は、進化や生態学研究に資する重要な情報となる。ヘリグロヒメトカゲ（*Ateuchosaurus pellopleurus*）は、琉球列島中部（トカラ列島、奄美諸島、沖縄諸島）に分布し、周辺地域に同属他種がいない遺存固有種である。本トカゲの肺には線虫の *Neoentomelas asatoi* が寄生する。*Neoentomelas* 属は琉球列島中部の固有属であり、*N. asatoi* 1 種のみからなる。本種は、発育に中間宿主を必要としない。宿主のヘリグロヒメトカゲは、先行研究により、形態的に顕著に分化した、沖縄諸島型と奄美諸島以北型が認められている。本研究では、*N. asatoi* について宿主の分化に対応した遺伝的分化が見られるか調べた。

琉球列島中部全域から得られた *N. asatoi* 標本について、mtDNA 塩基配列による分子系統解析を行った。その結果、それぞれの島に固有の個体群が分布し、遺伝的に分化していることが判明した。そして、沖縄諸島の 1 個体群と奄美諸島以北の 1 個体群から成るクレードが 2 つ認められた。両クレードにおいて、沖縄諸島の個体群と奄美諸島以北の個体群間の遺伝距離は小さかった（0.5–1.4%）。近縁な個体群が地理的に離れた島に分布しており、複数回の洋上分散による分布拡大が示唆された。一方のクレードは、他の沖縄諸島個体群が近縁となっており、その樹形から、奄美諸島以北個体群の起源が沖縄諸島にあると推定された。

琉球列島では、黒潮に乗った南から北への分布拡大がいくつかのトカゲ類で示唆されている。本研究は、爬虫類の寄生虫の分布パターン形成においても黒潮が重要な役割を果たしたことを示唆する。

一般講演 - 4

Spirometra 属裂頭条虫の遺伝子解析と種同定

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【目的】　弧虫症は、マンソン裂頭条虫（*Spirometra erinaceieuropaei*）等のプロセルコイドの寄生したケンミジンコが混入した水の摂取や、プレロセルコイドが寄生したヘビ、カエル、ニワトリなどの生食により感染する幼虫移行症である。今回、マンソン弧虫症疑い患者 2 名より摘出されたプレロセルコイド、都内流通の食用ヘビおよびタイ、ミャンマーでのヘビ、カエルから摘出したプレロセルコイドの遺伝子同定を行ったので、その結果を報告する。

【材料および方法】　患者 2 名の皮下組織より摘出されたプレロセルコイド 2 個体、タイのカエル 1 匹から摘出したプレロセルコイド 3 個体、ミャンマーのコブラおよびカエルから摘出したプレロセルコイド 6 個体および 1 個体、都内で食用として市販されていた岡山県産シマヘビ 3 匹から摘出したプレロセルコイド 79 個体、鹿児島県産マムシ 1 匹から摘出したプレロセルコイド 28 個体の計 119 個体を検査対象とした。

市販のキットを用いて各プレロセルコイドから DNA を抽出し、18S rDNA、mtDNA *cox1* および *nad3* 遺伝子のシークエンス解析および系統樹解析により種同定を行った。

【結果および考察】　ヒト、ヘビおよびカエルから摘出したプレロセルコイドの 18S rDNA の 2189bp 中 782bp を解析した結果、供試したすべての個体が *S. erinaceieuropaei* (KY552801) と 100%一致した。一方、mtDNA *cox1* 遺伝子 1566bp 中 709bp および mtDNA *nad3* 遺伝子の全 346bp を解析した結果、両者ともに 6 つのクラスターを形成した。今回、119 個体のヒト、ヘビおよびカエルから摘出したプレロセルコイドの mtDNA *cox1*、mtDNA *nad3* 遺伝子の解析結果から、鹿児島県産のシマヘビから摘出したプレロセルコイドの 92.9%(26/28)が *S. decipiens*（*cox1*: KJ599679、*nad3*: KJ599679）とそれぞれ 100%一致した。また、ヒトから摘出された 1 個体およびタイ、ミャンマーのコブラ、カエルから摘出された 7 個体が *S. ranarum* の mtDNA *cox1*（MH298843）と 100%一致したが、*nad3* 遺伝子に関してはレファレンス配列の登録が確認できなかった。

S. ranarum 感染者は、中国出身者で頻回中国に渡航していること、本調査おけるヘビやカエルの寄生状況から、国内感染ではないことが示唆された。*Spirometra* 属裂頭条虫には、*S. erinaceieuropaei*、*S. decipiens*、*S. ranarum*、*S. houghtoni* および *S. okumurai* などが知られているが、Genbank に登録されている種は *S. erinaceieuropaei*、*S. decipiens*、*S. ranarum* のみである。しかしながら、マンソン弧虫症は、プレロセルコイドの遺伝子解析により、感染地や原因食の特定にもつながる場合もあることが明らかとなった。

固定法の違いによる吸虫の形態差について

小川　和夫
目黒寄生虫館

吸虫の染色標本作製の際には、1）虫体を熱生食に投入後、70%エタノール等で固定（以降、熱固定法）、または、2）虫体をカバーグラスとスライドグラスの間に圧平・伸展し、ＡＦＡ液等で固定する（以降、圧平法）のが一般的である。Cribb & Bray (2010)などは熱固定法を標準法にするべきと主張している。その長所として、設備の整わない場所でも処理できること、虫体が自然で一定の形で固定されることを挙げ、他方、圧平法の短所として、圧平によって虫体の各部位が不自然に「歪んで」(distorted) 固定されること、圧平の程度が実施者によって個人差があり、それによって測定値が異なる（再現性がない）こと、固定の際に実体顕微鏡が必要なことを挙げている。これまで、いずれか一方の方法で作製された標本に基づいて記載がされてきたが、今後は熱固定法に統一すべきなのか？ 圧平法と熱固定法の標本ではどれくらいの形態差が生じるのか？ これらの疑問に答えることを目的とした。

【材料・方法】埼玉県産のツチガエルの直腸に寄生していた Paramphistome 類の *Diplodiscus amphichrus japonicus* Yamaguti, 1936 と広島県産のヒガンフグの内臓血管に寄生していた住血吸虫 *Psettarium japonicum* (Goto & Ozaki, 1929)を材料とした。両種を上記 2 つの方法で固定し、アラムカーミンまたはハイデンハインの鉄ヘマトキシリンで染色、脱水、透徹し、カナダバルサムで封入し、永久標本とした。両種の測定値と形態を熱固定法と圧平法で比較した。

【結果・考察】ツチガエルの吸虫では、熱固定法と圧平法による体長比は 1：1.64、体幅 1：1.72、住血吸虫では体長 1：1.32、体幅 1：1.26 と熱による収縮と圧平による伸長のため、測定値に大きな差が出たが、その影響は吸虫種によって異なった。内部構造については、山口左仲博士が吸虫の形態形質で最も重要とした生殖器の末端構造は、いずれの種についても圧平標本の方がはるかに明瞭に観察された。一方で、住血吸虫では圧平の影響によって雄性生殖器が突出した状態で固定された。熱固定したツチガエルの吸虫では後端の吸盤の形状が一定しなかった。以上のように、いずれの方法によっても形態の一部が不自然に固定されることが判明した。今後は吸虫種を増やしてさらに比較検討すること、単生類など、他の蠕虫類についても検討することが必要と考えられる。

一般講演 - 6

国内のヘビ類 3 種から見つかった吸虫 *Ochetosoma kansense*

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[1]目黒寄生虫館、[2]東京都杉並区

京都産および大阪産シマヘビ *Elaphe quadrivirgata* から採集された吸虫 *Ochetosoma kansense* については第 86 回日本寄生虫学会（2017 年）で報告した。この吸虫が報告された宿主は、今のところ国内ではシマヘビだけだが、他のヘビ類からの未確認の発見情報がある（私信）。今回、シマヘビ、アオダイショウ *Elaphe climacophora* およびヤマカガシ *Rhabdophis tigrinus* から採集された吸虫を観察する機会があったので報告する。

吸虫は 2019 年 6 月、岡山県のシマヘビ、アオダイショウおよびヤマカガシ各 1 匹、福井県のヤマカガシ 1 匹の口腔内から採集され、70%エタノール固定されたものである。

吸虫は全長 2.5‐5.8 mm、最大幅 1.1‐1.9 mm（圧平標本を計測）の楕円形で、形態的に *Ochetosoma* 属に属すると判断され、生殖孔が咽頭のレベルで虫体左側に開口、卵黄腺が体側の中央部にあり前後の 2 群に分かれない、腸盲嚢が体後端に達しないなどの形態的特徴から *O. kansense* と同定された。また、遺伝子解析を行ったところ、28S rDNA（約 1270 塩基対）の配列比較では、今回のヘビ 3 種から採集された *O. kansense* は、北米産ヘビ類 *Drymarchon corais* から採集された *O. kansense*、および京都産シマヘビからの *O. kansense*（2017 年の発表）と完全に一致した。アオダイショウおよびヤマカガシの *O. kansense* 寄生例は初記録と考えられる。

この吸虫は主に北米大陸に分布することが知られているが、この吸虫の生活環と宿主ヘビ類の食性から考察すると、吸虫は北米のウシガエル *Lithobates catesbeianus* とともに日本へ移入され、ウシガエルを捕食したヘビ類に吸虫が寄生したものと考えられた。

なお、*Ochetosoma* が *Renifer* の junior synonym とされている（"Keys to the Trematoda Vol.3" (2008)など）のは誤りで、属名としては *Ochetosoma* が有効と考えられる。

エクアドル産 *Amphimerus* 属肝吸虫のハムスターへの感染試験

杉山　広[1]、森嶋康之[1]、川上　泰[2]、窪田理恵[3,4]、常盤俊大[3]、高木秀和[5]、
板垣　匡[6,7]、平川公昭[8]、M カルボピーニャ[9]
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[5]愛知医大・医、[6]岩手大・農・獣医、[7]岐阜大院・連獣、[8]新日本科学、
[9]アメリカ大・医・エクアドル

後睾吸虫科 Opisthorchiidae に属する肝吸虫は、アジアだけでなく、アメリカ大陸にも分布する。中でも *Amphimerus* 属に分類される肝吸虫は、南アメリカ南部を除く各地から多数の種類が報告されている。演者らはエクアドル北西部・エスメラルダス県で住民に *Amphimerus* 属肝吸虫の感染を発見し、本症の臨床（診断・治療）あるいは疫学（本虫の人獣共通性など）に関する知見を報告した。今回はハムスターへの感染試験を実施したので、成績を報告する。

流行地で採集した淡水魚から本虫のメタセルカリアを分離し、8 頭のゴールデンハムスターに経口投与（130 個/頭）、投与後 293 日まで経時的に安楽殺・剖検した。虫体は胆嚢・胆管より回収し、染色封入標本を作製して、形態観察と計測を行った。肝臓の一部は病理組織標本とし、虫体の寄生状況と病変の発現状況を調べた。

ハムスターの *Amphimerus* 属肝吸虫に対する感受性は高く、今回の検討では、1 頭から平均 42 隻（投与メタセルカリアの 35.5%）の虫体が回収された。感染後 17 日と最も早くに剖検した個体からは、子宮内に虫卵を認めない未成熟虫も検出されたが、虫体後半部にも卵黄腺は分布し、本属の標徴は感染初期より発現することが分かった。病理組織標本の観察では、肝実質に虫道性病変を認めず、幼虫は脱嚢後に胆道系を逆行して胆管・胆嚢に定着すると考えられた。さらに胆管上皮細胞の過形成と胆管の拡張、胆管周囲結合織の増殖、好酸球を主とした細胞浸潤等の所見が、剖検時期を問わずに認められた。しかし長期感染例でも、肝実質への線維化の波及は限定的であり、典型的な肝硬変像の乏しさが本虫感染の特徴と考えられた。

一般講演 - 8

マンソン住血吸虫症による肉芽腫形成への終末糖化産物受容体(RAGE)の関与

小泉　頌歌[1]、坪川　大悟[1,2]、八田　岳士[1,2]、三上　房子[2]、中村　健[2]、
山本　靖彦[3]、辻　尚利[1,2]
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[3]金沢大・医薬保健研究域医学系・血管分子生物

【背景・目的】住血吸虫症の感染者数は世界で 2 億 4 千万人、死者数はアフリカだけでも年間 28 万人と推定されており、顧みられない熱帯病(NTD)の中でも特に深刻な感染症である。本症の病原体である住血吸虫は、経皮的に宿主体内に侵入し、血行性に体内移行し門脈で成熟・産卵を行う。この過程で生じる本症の典型的な症状は、発熱、下痢、粘血便、肝脾腫大、肝硬変、腹水や虫卵塞栓による脳障害などである。特に慢性期では、肝臓内に塞栓した虫卵周囲の炎症反応と肉芽腫形成が顕著となるが、その病態生理に関する分子論的背景は不明な点が多い。近年我々は、マダニや寄生線虫の分泌物中に、T リンパ球や、単球、マクロファージに発現している終末糖化産物受容体(RAGE)に依存した炎症応答経路を抑制的に制御する物質を発見したことから、寄生虫感染応答に RAGE シグナルが密接に関連していると考えている。そこで本研究では、RAGE 欠損マウスにマンソン住血吸虫 *Schistosoma mansoni* を感染させ、虫卵周囲の炎症および肉芽腫形成と RAGE シグナルの関与について考察を加えた。

【材料・方法】野生型および RAGE 欠損マウスに 150 隻の *S. mansoni* セルカリアを経皮感染させ、約 2 ヶ月後に灌流法により成虫を採取し虫体数を計測した。ELISA 法により *S. mansoni* 成虫可溶性抗原に対する抗体価、感染血清を用いた生化学的機能検査および肝臓の病理組織観察を実施した。

【結果・考察】野生型および RAGE 欠損マウスから得られた成虫数に有意な差は認められなかった。また両系統ではいずれも特異 IgG、IgE 値の上昇が認められたが、有意な差はなかった。興味深いことに、RAGE 欠損マウスの肝肉芽腫のサイズは、野生型よりも有意に小型であったことから、RAGE が虫卵周囲の炎症応答やそれに続く肝肉芽腫の形成を促進していることが強く示唆される。以上の成績から、住血吸虫の虫卵性肉芽腫形成は RAGE シグナルの支配下にあることが考えられるが、RAGE の活性化が虫卵または虫卵分泌物に起因するか否か判別する必要がある。そこで今後は、生虫卵またはホルマリン不活化虫卵を投与したマウスについて、本実験と同様の解析を行う予定である。

病原性アメーバ *Balamuthia mandrillaris* のマウス感染モデル樹立と迅速診断法の開発

花舘 有希、八木田 健司、久枝 一
感染研・寄生動物

近年、自由生活性のアメーバによる髄膜脳炎として、*Naegleria fowleri* による原発性アメーバ性髄膜脳炎や、*Balamuthia mandrillaris* や *Acanthamoeba* 属による肉芽腫性アメーバ性脳炎が世界的に報告されている。これらのアメーバは汚染された水中や土壌に生息しておりそれらを介してヒトに感染するが、感染頻度は高くない。しかし、感染が成立すると極めて重篤な症状を引き起こし、致死率が 90%以上と非常に高いことが問題となっている。その原因として、早期の診断法か確立されていないことや有効な治療法がないことが挙げられる。またアメーバ性脳炎は一般に臨床的特徴が乏しく、他の炎症性中枢疾患との鑑別が難しい。

国内ではこれらのうち *B. mandrillaris* による脳炎発生例が最も多く、当該アメーバによる感染を迅速簡便に行う診断法が不可欠である。また、臓器移植による *B. mandrillaris* の感染も報告されており、移植前の感染予防管理をするための検査手段も求められている。そこで本研究では、*B. mandrillaris* のマウス感染モデルを樹立し、感染における特異的バイオマーカーとして、感染マウスで特異的に変化する抗原の探索を行う。また、重症感染モデルと軽症感染モデルを確立することで、重症感染でのみ増加する重症化因子や軽症感染でのみ増加する防御因子の探索を試みる。以上の成果を応用し、生前検査に利用可能な迅速簡便な診断法の開発を目指す。

一般講演 - 10

ライブイメージングによる赤痢アメーバ Peroxiredoxin の動態解析

今井　達也、牧内　貴志、橘　裕司
東海大・医・基礎医・生体防御

Peroxiredoxin（Prx）は原核生物から高等真核生物まで広く保存されている抗酸化タンパク質である。赤痢アメーバには 2-cys タイプの Prx が存在し、宿主内における虫体の生存や病原性に重要な役割を果たすと考えられている。赤痢アメーバの Prx はゲノム内に 8 遺伝子存在し、アミノ酸配列を比較すると、233 残基で構成される Prx の他に、N 末端側に欠失配列のある Prx、C 末端側に挿入配列のある Prx の 3 タイプに分けることができる。これまでに当研究室では、固定した栄養型虫体を用いて Prx が核や細胞質に局在すると報告したが、他の研究グループからは虫体表面に局在するという報告もあり、3 タイプ間で局在や機能が異なる可能性も考えられる。本研究ではその可能性を検討するため、3 タイプの Prx について、N 末端に緑色蛍光タンパク質（GFP）が融合した Prx（GFP-Prx）を強制発現する株を樹立した。抗 GFP 抗体を用いたウエスタンブロットを行ったところ、GFP-Prx の推定分子量と一致するバンドが 3 株で確認された。生虫体における GFP-Prx のライブイメージングを行った結果、3 株ともに蛍光は細胞質に観察され、特に虫体の運動に伴って、偽足部分に強い蛍光が観察された。また、メトロニダゾールによって酸化ストレスを与えて GFP-Prx の動態解析を行ったが、薬剤添加前後で局在の変化や株間の違いは確認できなかった。従って、3 タイプの Prx 間で局在の違いはないと考えられる。今後、GFP-Prx 発現株に過酸化水素によって酸化ストレスを与えて GFP-Prx の動態解析を行う予定である。また、他種生物の Prx では、酸化ストレスによってペルオキシダーゼ活性のある 10 量体からシャペロン活性のある 20 量体に変化することが報告されているので、3 タイプ間における複合体形成能や酵素活性の違いについても比較する予定である。

腸内細菌叢との共棲関係に作用する新規赤痢アメーバ感染制御薬の探索

吉田　菜穂子[1]、小林　正規[1]、荒井　雅吉[2]
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赤痢アメーバ症は、発展途上国では特に小児下痢症の主要な原因であり、日本においても報告数が急増し重症化や致死的な劇症例が問題となっている疾患である。また、赤痢アメーバ感染は宿主の腸内細菌叢と密接に関連していることが示唆されている。今回我々は、海洋薬用資源（底生海洋生物や海洋微生物の抽出物）を用いて、赤痢アメーバと腸内細菌叢との共棲関係に作用し赤痢アメーバの増殖や組織侵入を阻害し得る、新規活性天然物を探索した。新たな機序による赤痢アメーバ感染制御薬を創出し、またその作用機序を解析することにより、赤痢アメーバ感染、発症および重症化と腸内細菌/細菌叢との関連性を明らかにすることを目的とした。対象とした海洋薬用資源を用いて新たに構築したスクリーニングを行った結果、２種の海洋由来真菌の培養抽出物が、赤痢アメーバ共棲培養系に用いる*Escherichia coli*、アメーバの増殖に強く影響することを見出している*Bacteroides fragilis*、および赤痢アメーバ無菌培養株（YIM-DHA培地;標準株HM1-IMSS）の増殖は阻害せず、共棲培養株（Robinson培地;標準株HM1-IMSS）にのみ増殖阻害活性を示すことを見出した。現在、この2種の抽出物について、各種クロマトグラフィーを用いた活性物質の精製を進めている。本大会では、スクリーニングの詳細と活性成分の精製等、これまでに行った一連の検討結果について報告する。

バイオイメージングによる *T. cruzi* 感染マウスの炎症の検出

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【背景と目的】Chagas 病は細胞内寄生原虫である *Trypanosoma cruzi* によって引き起こされる。原虫がヒトに感染すると、急性期では発熱や発疹などがみられ、成人の大半は慢性期に移行し、十数年という長い経過を経て約 3 割の患者で心筋炎や巨大消化管疾患を発症し、死亡する例もある。しかし、慢性期 Chagas 病の病態形成時期は明らかとなっていない。そこで、感染後の炎症の発生部位と経過をマウスモデルで明らかにすることを目的とし、バイオイメージングにより解析した。

【材料と方法】炎症を可視化できるトランスジェニックマウス（IDOL マウス）を用いた。IDOL マウスは、IL-1β プロモーター制御下に Luciferase をレポーター遺伝子として導入したマウスで、炎症が起きると IL-1β の活性化に伴い、炎症部位から発光が検出される。*T. cruzi* の Colombia 株、Y 株を IDOL マウスにそれぞれ 1×10^5 parasites 感染させ、経時的に発光を測定した。測定時には 3 mg/mouse の D-Luciferin を投与し、10 分後にバイオイメージングシステム（ImagEM X2 EM-CCD カメラおよび HC Image システム Hamamatsu Photonics）を用いて解析を行った。感染から 1 週間毎に測定し、炎症の発生部位とその経過を解析した。また、感染による心機能の変化を心エコーにより解析した。

【結果】両株感染マウスいずれにおいても、感染 5 週までは顕著な発光は検出されなかった。感染 6 週から Colombia 株感染マウスの胸部に発光が検出され、感染 11 週以降、この発光は消失した。一方、Y 株感染マウスでは、胸部には微弱な発光が認められ、腹部にも発光が検出された。また、感染 17 週の心エコーでは拡張型心筋症様の病態が認められた。

【考察と結語】本結果から、Colombia 株感染マウスにおいて、感染後 6 週間で IL-1β が活性化することが明らかとなり、この時期に心筋炎を発症している可能性が考えられた。Y 株感染マウスでは、発光は微弱であったが、拡張型心筋症を呈した。この結果から、病態に先立ち、IL-1β の活性化が起こる可能性が示唆された。今後、マウス心臓の病理切片を作製し、心筋炎が発症しているか否か検証する必要がある。また、原虫の局在と炎症発生との関連性を解析する予定である。

PCR-RFLP 法を用いたエクアドルに分布するリーシュマニア原虫種の同定

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リーシュマニア症は吸血昆虫サシチョウバエに媒介されるリーシュマニア原虫によって引起される疾患である。ヒトに病気を引起すリーシュマニア原虫は約 20 種が報告されているが、本症では感染種が臨床像（皮膚型、内臓型、粘膜皮膚型）を決定することや、感染種によって治療薬への感受性が異なることなども指摘されており、感染原虫種を同定することは感染者の治療や予後判定などに重要である。本研究では、エクアドルの原虫分離株および皮膚型リーシュマニア症の患者検体から原虫の cytochrome *b* (*cyt* b)、heat shock protein 70 (*hsp70*)、mannose phosphate isomerase (*mpi*)および 6-phosphogluconate dehydrogenase (*6pgd*)の各遺伝子の塩基配列の解析を行い、リーシュマニア原虫種の分類への適性について検討した。また、これらの遺伝子を用いた PCR- Restriction Fragment Length Polymorphism (RFLP)法による簡便な種の同定法の確立を試みた。エクアドルで分離された 7 種[*Leishmania (Viannia) guyanensis, L. (V.) panamensis, L. (V.) braziliensis, L. (V.) naiffi, L. (V.) lainsoni, Leishmania (Leishmania) major*-like*, L. (L.) mexicana*]のリーシュマニア原虫株および患者検体を用いて、4 つの遺伝子の原虫種分類への有用性について検証したところ、他の 3 つの遺伝子に比較して *cyt* b 遺伝子の塩基配列の解析では近縁種の分類も可能で、本遺伝子の塩基配列解析が原虫種の同定に有用であると考えられた。次に、より簡便な PCR-RFLP 法による種の鑑別法の確立を目指し、上記 4 つの遺伝子の種間および種内多型を解析し制限酵素切断パターンを比較した。*cyt* b 遺伝子は種内変異が多いこと、*hsp70* 遺伝子は種間で RFLP パターンが類似し短いフラグメントを比較する必要があることから、これらの遺伝子は RFLP 解析には適していないと考えられた。一方、*mpi* 遺伝子および *6pgd* 遺伝子は種内変異が少なく、*mpi* 遺伝子の *Hae*III 切断と *Hap*I 切断、および *6pgd* 遺伝子の *Bsp*1286I 切断と *Hin*fI 切断によってほとんどの種が分類できることが分かった。*mpi* 遺伝子や *6pgd* 遺伝子の PCR-RFLP 解析では区別できなかった近縁種 *L. (V.) guyanensis* と *L. (V.) panamensis* は、*hsp70* 遺伝子の *Bcc*I 切断で分類可能であることが確認できた。今後は、この方法をエクアドルにおける広域疫学調査に応用するとともに、その他の国や地域での調査に応用が可能であるか検討する必要があると考えられた。

ウガンダにおけるマラリア予防政策とその実態

片山　瑞希、山内　祐人、美田　敏宏
順天堂大・医学部・熱帯医学・寄生虫病学講座

ウガンダ共和国は世界で有数のマラリア蔓延国であり、保健省は The Uganda Malaria Reduction Strategic Plan を実施している。そこでは、① 2014 年までに Long Lasting Insecticide Nets（LLINs）を一般的に普及させる、② 2017 年までに 85%以上の国民が正しいマラリアの予防法を身につけることが掲げられている。ウガンダ政府は 2000 年からマラリア予防に関連する世帯調査を Demographic Health Survey（DHS）と Malaria Indicator Survey（MIS）で行っている。そのデータから、これらの目標の達成度やその課題を抽出することが可能だが、調査の地域区分や質問項目が実施年で異なっているため実際には困難となっている。本研究では、媒介蚊対策に関する質問項目の再評価と調査時期毎の地域区分の統一化をした上でデータを再解析し、The Uganda Malaria Reduction Strategic Plan がどこまで達成されているのか、その課題は何か、について検討した。

DHS･MIS のデータセットを The DHS Program（https://dhsprogram.com）から入手し、2006、2009、2011、2014–15、2016 年の計 5 回分について、蚊帳所有率、蚊帳の入手方法、蚊帳を使用しなかった理由を抽出、調査地域区分を年度毎に統一化、さらに地域毎の経年変化を評価した。

その結果、蚊帳所有率が 20%を超えたのは 2006 年には 10 地域中 3 地域のみだったが、2016 年には 1 地域を除く全地域で 70%を超えていた。さらに、2006 年には農村部の方が都心部より保有率が高かった（26% vs 14%）のに対し、2014 年には両者でほぼ同程度となっていた（58.7% vs 60.1%）。蚊帳の入手方法については、2006 年では所有する蚊帳のうち 21.5%が無料配布によるものであったが､2016 年には､その割合が 9 割近くにまで増加した。その一方で、蚊帳を使用しなかった世帯の割合も増加しており、その理由としては、吊るしていないためが最も多かった。

以上の結果から、目標①については達成できているが、目標②については、まだ不十分であると考えられる。今後、蚊帳の重要性の周知に加え、その使用方法についての教育が必要であると考えられる。

熱帯熱マラリア原虫 N-アシル化 Rab5b と Arf1, Rab1b の小胞体近傍における局在解析

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熱帯熱マラリア原虫 *Plasmodium falciparum* は赤血球内に形成した寄生胞内で、数百種のタンパク質を宿主細胞質やその膜表面へ輸送することで、増殖に適した環境や栄養を確保する。熱帯熱マラリア原虫の感染赤血球輸送シグナル PEXEL を含むタンパク質は、他種生物と同様に小胞体からゴルジ体を介して輸送されると考えられている。しかし、原虫内の輸送制御因子についての報告は少ない。

そこで本研究では、陸上植物とアピコンプレキサの一部に保存される N-アシル化 Rab5 (PfRab5b) に着目した。先行研究では、PfRab5b はゴルジ体ではなく小胞体近傍に局在し、一部分は寄生胞膜上にも輸送されることが報告されている。Rab の機能の解明には、局在するオルガネラと結合タンパク質の同定が重要である。そこで PfRab5b の結合タンパク質の同定を試みたところ、他種生物でゴルジ体に局在することが報告されている Arf1 GTPase, Arf1 の活性化因子 Sec7, Rab1b GTPase のホモログを得た。Arf1 と Rab1b が PfRab5b と共局在することを確認するために、恒常的活性型 $PfRab5b^{Q94L}$ 変異と共発現させ、間接蛍光抗体法を行ったところ、Arf1 と Rab1b が PfRab5b と共局在することが観察された。Arf1 と Rab1b は小胞体近傍に局在するのではないかと考え、小胞体マーカーの Bip との局在を超解像顕微鏡 LSM880 with Airyscan で観察した。その結果 Arf1, Rab1b ともに小胞体近傍に位置するとともに、Arf1 の方が Rab1b よりも小胞体に近い位置にいることがわかった。

これらの結果から、PfRab5b は Arf1, Rab1b と共に小胞体近傍で相互作用する可能性が見出された。また、他種生物ではゴルジ体に局在する Arf1, Rab1b が、原虫内では小胞体近傍に局在したことから、他種生物のホモログが熱帯熱マラリア原虫に保存されていても、原虫内での局在性が異なるものがあるとわかり、熱帯熱マラリア原虫の小胞体―ゴルジ体間の輸送制御は、他種生物に比べて多様化していると予想された。

マラリア原虫有性ステージ分化における RNA 結合型ジンクフィンガータンパク質ファミリーの解析

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マラリア原虫は宿主動物及び媒介蚊の間で形態変化を伴いつつ宿主細胞へ侵入、寄生する。赤血球ステージ原虫の内、約１～５％程度の原虫が雌雄の生殖母体へと分化し、宿主動物から媒介蚊へ移行する。生殖母体は媒介蚊への移行後、雌雄生殖体に分化し、受精する。受精したザイゴートは中腸侵入型であるオーキネートへと分化する。成熟生殖母体においては、RNA ヘリケースである DOZI による翻訳抑制機構が働き、オーキネートの発育に必要なタンパク質の mRNA は翻訳されない。媒介蚊への移行後、その翻訳抑制は解除されることが明らかになっている。我々は、しかし、これらは全て同時にタンパク質になるわけではなく、最終的なオーキネート形成までのそれぞれの段階で必要なタンパク質が順番に翻訳されていくべきであると考えた。そこで我々は、マラリア原虫ゲノムの *in silico* 解析から、その転写後制御に関わるタンパク質ファミリーがあると考え、探索を行った。その結果、ステージ特異的に発現しているジンクフィンガータンパク質ファミリーが存在することを見出した。これらは共通して N 末端側に連続した CCCH 型ジンクフィンガードメインを持ち、*Plasmodium* 種間でのドメインの相同性は 95%以上であった。CCCH 型ジンクフィンガータンパク質は他の生物で mRNA と結合するといわれており、mRNA の代謝や翻訳抑制の機構への関与が推察されている。

そこで、本研究では、このジンクフィンガータンパク質ファミリーを Three tandem Zinc Finger protein (TZF)と名付け、生殖母体期からオーキネート期の有性ステージに発現する 5 つの TZF に関して、CRISPR/Cas9 によって遺伝子欠損株及び GFP 融合タンパク質発現株を作出し、TZF の機能及び局在に関する解析を行った。これらの TZF は段階的に発現し、欠損株は生殖母体期からオーキネート期への過程においてステージ特異的に発育が止まることが明らかになった。

以上の結果は、TZF がマラリア原虫の分化・発育に関わるタンパク質発現を制御するタンパク質ファミリーであることを強く示唆している。

電子顕微鏡 3D 構造解析により初めて明らかとなった オーシスト期マラリア原虫の核分裂様式

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真核生物の核の増殖制御は、細胞の機能維持において非常に重要な役割を担う。複雑な生活史を有する原生生物では、より巧妙な制御が必要であると考えられるがその詳細は殆ど明らかとなっていない。マラリア原虫の増殖期は、ヒト体内での肝内期と赤内期の２つの発育ステージとハマダラカ体内でのオーシスト期の発育ステージが存在し、核膜の崩壊を伴わず核だけが繰り返し分裂することが知られている。マラリア原虫の核の制御に関しては赤内期の報告から、核のみが２分裂を繰り返すことで多核体を形成するシゾゴニー様式であることが示されている。しかし、他の肝内期とオーシスト期における核の増殖制御の詳細は不明であり、特にオーシスト期は、有性生殖後の第一世代ハプロイド原虫が産生される興味深い発育ステージであるが、その詳細は明らかとなっていない。

そこで本研究では、2 種のネズミマラリア原虫 *Plasmodium berghei* (Pb)、 *P. yoelii* (Py)と１種のサルマラリア原虫 *P. cynomolgi* (Pcy)をハマダラカに感染させ、得られたオーシストを用いて詳細な解析を行った。走査型電子顕微鏡による観察の結果、Pcy のオーシストは他のネズミマラリア原虫と比較して直径及び表面積が大きく、ハマダラカ中腸からの突出度が極めて顕著であった。更に、原虫の細胞内部構造やオルガネラの分配様式を観察するため、集束イオンビーム/走査型電子顕微鏡を用いて、サイズが最も大きく成長が緩やかな Pcy のオーシストの解析を実施した。得られた連続断層画像群から内部構造の 3D イメージを再構築した結果、驚いたことに、オーシスト期のマラリア原虫の核は１核から複数核へと伸長しており、先端部には中心体が観察された。この増殖様式は、これまで報告された赤内型の増殖機構とは明確に異なり、他の寄生原虫であるサルコシスティスなどの分裂様式である endopolygeny に酷似していることが初めて明らかとなった。本発表では、原虫のライフサイクルに伴う異なる増殖制御機構の意義についても議論したい。

マラリア原虫感染赤血球の乳腺組織への蓄積

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マラリア原虫は, 全世界で約 2 億人に健康被害を与え, 年間 40 万人を超える人々を死に至らしめる人類にとって未だに脅威の病原体である. 特に, 妊娠中のマラリアは母体だけでなく胎児にも重大な影響を与えることから, マラリア流行地において社会問題となっている. 妊娠マラリアによる胎児の流産や死産を未然に防ぐためには, マラリア原虫の早期検出とともに, 流産や死産を予測するバイオマーカーを用いた新たな診断法の確立が必要不可欠である.

近年, 我々は, 妊娠中のマラリアのマウスモデルを用いた研究により, 妊娠マウスの皮下脂肪組織におけるマラリア原虫感染赤血球の蓄積量は, 非妊娠マウスの皮下脂肪組織と比較して有意に増加することを見出した. 一方, 感染赤血球の皮下脂肪組織への集積機序や感染赤血球の集積による脂肪組織への影響については明らかにされていない. そこで本研究では, 妊娠マラリアによる皮下脂肪組織の形態変化と、それに関連する分子発現への影響を明らかにすることを目的とした.

妊娠による脂肪組織の変化を明らかにするために, 非妊娠マウスと妊娠マウスの脂肪組織の比較プロテオーム解析を行ったところ, 妊娠マウスの脂肪組織において, Alpha-S1-casein や Glycam1 などの乳腺に特異的に発現する分子の著しい増加が認められた. 皮下脂肪組織の組織学的解析の結果, 交配後 17 日目の妊娠マウスの皮下脂肪組織では, 非妊娠マウスと比較して, 乳腺の著しい発達が認められた. さらに, マウスマラリア原虫を感染させた妊娠マウスの皮下脂肪組織では, 乳腺周囲の毛細血管に多数のマラリア原虫感染赤血球が集積していることを見出した. これらの結果から, 乳腺組織は妊娠マラリアの早期診断に有用であることが示唆された.

次に, マウスマラリア原虫を感染させた妊娠マウスの脂肪組織と非感染妊娠マウスの脂肪組織の比較プロテオーム解析を行った. その結果, マウスマラリア原虫を感染させた妊娠マウスの脂肪組織において, STAT1 や Iigp1 などの IFN-γによって発現が誘導される分子が著しく増加していることを見出した. これらの分子の増加は, マウスマラリア原虫を感染させた非妊娠マウスの脂肪組織においても認められたが, その発現量は妊娠マウスの脂肪組織と比較して低レベルであった. これらの結果から, マラリア原虫を感染させた妊娠マウスの乳腺組織において, IFNGR1 依存的な炎症反応が誘導されることが示された.

M E M O

日本寄生虫学会東日本支部　大会記録

回	年	大会長	開催地
1	1947	松林久吉	東京都
2	1947	石井信太郎	東京都
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21	1961	坂東丈夫	東京都
22	1962	伊藤二郎	静岡市
23	1963	石井　進	東京都
24	1964	亀谷　了	東京都
25	1965	分島　整	浦和市
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27	1967	飯島利彦	甲府市
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29	1969	大島智夫	松本市
30	1970	石崎　達	東京都
31	1971	常松之典	東京都
32	1972	大塚　裕	東京都
33	1973	小林昭夫	東京都
34	1974	白坂龍曠	東京都
35	1975	岡本謙一	東京都
36	1976	柳沢十四男	相模原市
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39	1979	安羅岡一男	茨城県桜村
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41	1981	佐野基人	浜松市
42	1982	板垣　博	相模原市
43	1983	堀　栄太郎	毛呂山市
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50	1990	小島荘明	東京都
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60	2000	今井　壮一	武蔵野市
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63	2003	南　陸彦	横浜市
64	2004	北　潔	東京都
65	2005	渡邊直熙	東京都
66	2006	遠藤卓郎	東京都
67	2007	小川和夫	東京都
68	2008	石井　明	浜松市
69	2009	狩野繁之	東京都
70	2010	千種雄一	壬生町
71	2011	小林富美恵	三鷹市
72	2012	久枝　一	前橋市
73	2013	倉持利明 小川和夫	東京都
74	2014	松岡裕之	下野市
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御礼

第７９回日本寄生虫学会東日本支部大会開催に際しまして、下記の各位より多大なご支援を賜りました。ここに篤く御礼申し上げます。

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本大会開催に際し、学会関係の皆様をはじめ、多くの方に大変お世話になりました。この場をお借りして御礼申し上げます。

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Cryptic Plasmodium cynomolgi infections among symptomatic malaria patients, Thailand

Journal:	*Emerging Infectious Diseases*
Manuscript ID	EID-19-1660
Manuscript Type:	Dispatch
Date Submitted by the Author:	25-Nov-2019
Complete List of Authors:	Putaporntip, Chaturong; Faculty of Medicine, Chulalongkorn University, Parasitology Kuamsab, Napaporn; Faculty of Medicine, Chulalongkorn University, Parasitology Pattanawong, Urassaya; Faculty of Medicine, Chulalongkorn University, Parasitology Yanmanee, Surasuk; Chulalongkorn University, Parasitology Seethamchai, Sunee; Naresuan University, Biology Jongwutiwes, Somchai; Faculty of Medicine, Chulalongkorn University, Parasitology
Keywords:	Plasmodium cynomolgi, Plasmodium knowlesi, mixed species malaria infection, Thailand, mitochondrial cytochrome oxidase I, cross-transmission, PCR detection
Abstract:	Plasmodium cynomolgi co-existed with P.vivax (n=7), with P.falciparum (n=1) and with P.vivax and P.knowlesi (n=1) among 1,180 symptomatic malaria patients (0.76%) in Thailand. All but one had macaques living in vicinity. Furthermore, human knowlesi malaria was newly identified in northeastern area, suggesting wide distribution of simian malaria in this country.

Cryptic *Plasmodium cynomolgi* infections among symptomatic malaria patients, Thailand

Running Title: cynomolgi malaria in humans, Thailand

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Word counts of the abstract: 50 Word counts of the text: 1,197

Number of references: 15 Figure: 1 Tables: 2

Key words: *Plasmodium cynomolgi*, *Plasmodium knowlesi*, mixed species malaria infection, Thailand, mitochondrial cytochrome oxidase I, cross-transmission, PCR detection

Abstract

Plasmodium cynomolgi co-existed with *P.vivax* (n=7), with *P.falciparum* (n=1) and with *P.vivax* and *P.knowlesi* (n=1) among 1,180 symptomatic malaria patients (0.76%) in Thailand. All but one had macaques living in vicinity. Furthermore, human knowlesi malaria was newly identified in northeastern area, suggesting wide distribution of simian malaria in this country.

An oriental simian malaria parasite *Plasmodium cynomolgi* possesses several biological and genetic characteristics akin to those of the most widespread species of human malaria parasite *Plasmodium vivax*. Although *P. cynomolgi* has been circulating among various monkey species such as long-tailed macaque (*Macaca fascicularis*) and pig-tailed macaque (*M. nemestrina*), experimental and accidental transmissions of this simian malaria parasite to humans have been incriminated in symptomatic infections (*1*). Several mosquito vectors for human malaria can also transmit simian malaria, rendering the risk of cross-species transmission of this simian malaria in areas where monkey natural hosts exist (*1,2*). Both pig-tailed and long-tailed macaques populate in various Southeast Asian countries including Thailand while infections with *P. cynomolgi* are not uncommon (*3,4*). The first natural cynomolgi malaria in human has been reported from eastern Malaysia (*5*). Subsequent surveillances in western Cambodia and northern Sabah, Malaysia have revealed asymptomatic human infections with this simian malaria, *albeit* at low prevalence (*6,7*). Furthermore, symptomatic *P. cynomolgi* infection reportedly occurred in a traveler to Southeast Asia (*8*). Herein, we have identified cryptic *P. cynomolgi* co-infected with other *Plasmodium* species in symptomatic malaria patients in Thailand.

The Study

A total of 1,359 blood samples from febrile individuals who attended malaria clinics or local hospitals in Tak (n=192, during 2007-2013), Ubon Ratchathani (n=239, during 2014-2016), Chanthaburi (n=144, during 2009), Yala (n=592, during 2008-2018) and Narathiwat (n=192, during 2008-2010) Provinces were examined. Of these, 1,152 patients were diagnosed by microscopy to be caused by *P. vivax* (869 patients, 75.43%), *P. falciparum* (272 patients, 23.61%), co-infection of both species (11 patients, 0.96%).

Species-specific nested PCR targeting the mitochondrial cytochrome *b* gene (*mtCytb*) of five human malaria species were deployed for molecular detection as previously described (*9,10*)

including *P. cynomolgi*-specific nested PCR newly developed herein (Appendix). The PCR method diagnosed 1,180 malaria patients with *P. vivax* exceeding *P. falciparum* infections (Table 1). The mean age was 26.3 years (range 7-85 years) and 940 males. All PCR-positive malaria patients developed febrile symptoms for 1 to 7 days (mean ± S.D. = 3.1 ± 1.3 days) prior to blood sample collection. Mixed species infections were detected in 77 patients (0.93%); 55 of these were caused by *P. falciparum* and *P. vivax*. Monoinfections of *P. knowlesi*, *P. malariae* and *P. ovale* occurred in 4, 3 and 1 patients, respectively. In total, *P. knowlesi* was detected in 18 patients; 10 of these were newly identified from Ubon Ratchathani Province bordering Cambodia and Laos. More importantly, *P. cynomolgi* was detected in 9 patients; all of which were co-infected with *P. vivax* (n=7), with *P. falciparum* (n=1) and with *P. vivax* and *P. knowlesi* (n=1). The overall prevalence of *P. cynomolgi* infections was 0.76%. Patients infected with *P. cynomolgi* were found in all provinces in this study. Although 5 of these patients were from Yala Province, the proportion of *P. cynomolgi* infections among malaria cases in each endemic area was almost comparable, ranging from 0.52% to 0.87%.

DNA from 10 *P. knowlesi* isolates from Ubon Ratchathani Province and 9 *P. cynomolgi* isolates were subject to nested PCR amplification spanning 1,318 bp region of *mtCOX1*. Direct sequencing of the purified PCR-amplified template was successfully performed from all *P. knowlesi* and 6 *P. cynomolgi* isolates. All *mtCOX1* sequences of *P. knowlesi* from Ubon Ratchathani Province were different and distinct from the first natural human infection in Thailand (AY598141)(*11*). Likewise, all 6 *P. cynomolgi* isolates contained different sequences and belonged to 2 clades; one was closely related with the Gombak strain (AB444129) and the remaining 5 isolates were clustered with the RO strain (AB444126)(Figure 1).

All but one *P. cynomolgi* infection occurred in male patients with the age range from 15 to 53 years (median = 32 years). The majority of cynomolgi malaria patients resided in areas where domesticated or wild macaques were living in vicinity. Infections with *P. cynomolgi* occurred in different periods of sample collections with more cases detected in rainy season than

in dry season (Table 2). The parasite density of *P. cynomolgi* could not be determined from blood smears because of morphological resemblance to *P. vivax* while an isolate co-infected with *P. falciparum* (YL3634) had very low parasitemia. Six of 8 patients with *P. cynomolgi* co-infection had parasitemia <10,000 parasites/microL or approximately less than 0.2%. Self-reported defervescence among *P. cynomolgi*-infected patients occurred within one to three days after antimalarial treatment with chloroquine plus primaquine or artesunate plus mefloquine per onsite microscopic diagnosis of vivax or falciparum malaria, respectively. However, data on long-term follow up was not available.

Conclusions

This is the first identification of *P. cynomolgi* in humans, Thailand where both pig-tailed and long-tailed macaque natural hosts were prevalent. All patients with *P. cynomolgi* infections harbored either *P. falciparum* or *P. vivax* in their circulations, implying that anopheline vectors for this simian malaria species could be either the same or having both anthropophilic and zoophilic tendencies. The presence of *P. cynomolgi* in diverse malaria endemic areas of Thailand has suggested that cross-species transmission of this simian malaria occurred not infrequently. Human infections with *P. cynomolgi* seems not to be a newly emerging infection because it has been detected among blood samples collected from a range of time periods since 2007. Undoubtedly, morphological similarity between *P. cynomolgi* and *P. vivax* can hamper conventional microscopic diagnosis (*1,5,8*). Cryptic co-existence of simian and human malaria species could further preclude accurate molecular detection when proper diagnostic devices are not included.

Our previous surveys of *Plasmodium* infections in both pig-tailed and long-tailed macaques have revealed the presence of *P. cynomolgi* and other simian malaria species in Thailand, mainly in southern part of the country (*4*). Most patients infected with *P. cynomolgi* resided in areas where macaques were living in vicinity; therefore, the risk of acquiring this

simian malaria could occur upon encroachment to infected macaques' habitats akin to *P. knowlesi*. It is noteworthy that mixed species infection of *P. cynomolgi*, *P. knowlesi* and *P. vivax* occurred in a patient in Yala province whose housing area was surrounded by several domesticated pig-tailed and long-tailed macaques.

Analysis of the *mtCOX1* sequences of *P. cynolmogi* from 6 patients has shown that all isolates possess different sequences, suggesting that several strains or clones of this simian parasite are capable of cross-transmission from macaques to humans. Meanwhile, *P. cynomolgi* seems to contain 2 divergent lineages (*12*), represented by strains RO and Gombak. Importantly, the *mtCOX1* sequences of both lineages were found in human-derived isolates in this study, further supporting that diverse strains of *P. cynomolgi* can infect humans. Likewise, sequence diversity in *mtCOX1* of *P. knowlesi* from Ubon Ratchathani Province has suggested that cross-transmission from macaques to humans may not be restricted to particular parasite strains.

Although human knowlesi malaria may present with asymptomatic to fatal infections, asymptomatic and benign symptoms reportedly occurred in *P. cynomolgi*-infected individuals (*5-8*). Meanwhile, severe and complicated malaria has been observed in rhesus macaque experimentally infected with *P. cynomolgi* (*13*). Whether severe cynomolgi malaria could occur in humans remains to be eluciated. If human infections with *P. cynomolgi* could become public health problems, control measures would be complicated by the presence of hypnozoites akin to vivax malaria (*14,15*). Undoubtedly, further surveillance of this simian malaria in humans is mandatory.

Acknowledgements

We are grateful to all patients for blood samples; local staffs at malaria clinics and local hospitals for assistance in field studies.

This study was funded by The Thailand Research Fund to C.P. (grant no. RSA5980054); The Asahi Glass Foundation to C.P. (grant in fiscal year 2016) and The Thai Government Research Budget for Chulalongkorn University to S.J and C.P. (grant nos. GRB-APS-12593011 and GBA-600093004).

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Table 1. Distribution of *Plasmodium* infections diagnosed by PCR

Species	No. of cases by province					Total	%*
	Tak	Ubon Ratchathani	Chanthaburi	Yala	Narathiwat		
P. vivax	98	57	141	467	59	822	69.66
P. falciparum	72	41	-	87	73	273	23.14
P. knowlesi	-	4	-	-	-	4	0.34
P. malariae	-	2	-	1	-	3	0.25
P. ovale	-	-	-	1	-	1	0.09
P. vivax + *P. falciparum*	21	8	-	11	15	55	4.66
P. vivax + *P. knowlesi*	-	3	2	-	4	9	0.76
P. vivax + *P. cynomolgi*	1	1	1	3	1	7	0.59
P. vivax + *P. knowlesi* + *P. cynomolgi*	-	-	-	1	-	1	0.09
P. falciparum + *P. knowlesi*	-	3	-	1	-	4	0.34
P. falciparum + *P. cynomolgi*	-	-	-	1	-	1	0.09
Negative	-	120	-	19	40	179	-
Total	192	239	144	592	192	1359	-

* Per cent of total malaria cases (n = 1,180).

Table 2. Demographic and parasitologic features of *Plasmodium cynomolgi*-infected patients

Patient	Gender	Age (years)	Ethnicity	Period	Province	Season of infection	Monkey in vicinity	Microscopy	parasites/ microliter	PCR diagnosis
TSY1522	Male	38	Karen	November 2007	Tak	Dry	No	*P. vivax*	12,160	*P. vivax*, *P. cynomolgi*
CT606	Male	30	Cambodian	October 2009	Chanthaburi	Rainy	Yes	*P. vivax*	86,535	*P. vivax*, *P. cynomolgi*
UBY120	Male	32	Thai	August 2015	Ubon Ratchathani	Rainy	Yes	*P. vivax*	570	*P. vivax*, *P. cynomolgi*
NR105	Male	53	Thai	July 2008	Narathiwat	Rainy	Yes	*P. vivax*	4,620	*P. vivax*, *P. cynomolgi*
YL3179	Male	15	Thai	April 2016	Yala	Dry	Yes	*P. vivax*	1,140	*P. vivax*, *P. knowlesi* *P. cynomolgi*
YL3634	Female	40	Thai	December 2016	Yala	Rainy	Yes	*P. falciparum*	60	*P. falciparum*, *P. cynomolgi*
YL3680	Male	49	Thai	December 2016	Yala	Rainy	Yes	*P. vivax*	3,720	*P. vivax*, *P. cynomolgi*
YL3685	Male	18	Thai	December 2016	Yala	Rainy	Yes	*P. vivax*	4,680	*P. vivax*, *P. cynomolgi*
YL4278	Male	21	Thai	October 2017	Yala	Rainy	Yes	*P. vivax*	7,440	*P. vivax*, *P. cynomolgi*

269

270 **Figure legend**

271 **Figure 1.** Maximum likelihood phylogenetic tree inferred from *mtCOX1* of *Plasmodium*
272 *cynomolgi* and *P. knowlesi* spanning 1,318 bp region in relation with other closely related
273 species. Human isolates from Yala, Narathiwat, Chanthaburi and Ubon Ratchathani Provinces
274 are shown as circle, triangle, square and diamond, respectively. GenBank accession numbers of
275 reference sequences are in parentheses. Bootstrap values more than 50% based on 1,000
276 pseudoreplicates are shown on the branches. Scale bar indicates nucleotide substitution per site.

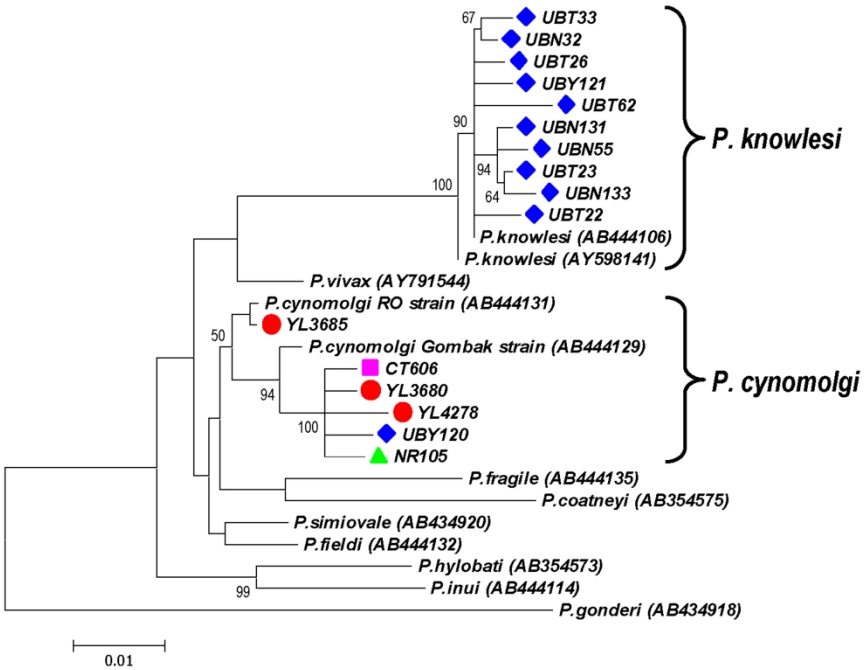

Figure 1. Maximum likelihood phylogenetic tree inferred from mtCOX1 of Plasmodium cynomolgi and P. knowlesi spanning 1,318 bp region in relation with other closely related species. Human isolates from Yala, Narathiwat, Chanthaburi and Ubon Ratchathani Provinces are shown as circle, triangle, square and diamond, respectively. GenBank accession numbers of reference sequences are in parentheses. Bootstrap values more than 50% based on 1,000 pseudoreplicates are shown on the branches. Scale bar indicates nucleotide substitution per site.

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P. ovale	-	-	-	1	-	1	0.09
P. vivax + *P. falciparum*	21	8	-	11	15	55	4.66
P. vivax + *P. knowlesi*	-	3	2	-	4	9	0.76
P. vivax + *P. cynomolgi*	1	1	1	3	1	7	0.59
P. vivax + *P. knowlesi* + *P. cynomolgi*	-	-	-	1	-	1	0.09
P. falciparum + *P. knowlesi*	-	3	-	1	-	4	0.34
P. falciparum + *P. cynomolgi*	-	-	-	1	-	1	0.09
Negative	-	120	-	19	40	179	-
Total	192	239	144	592	192	1359	-

* Per cent of total malaria cases (n = 1,180).

Table 2. Demographic and parasitologic features of *Plasmodium cynomolgi*-infected patients

Patient	Gender	Age (years)	Ethnicity	Period	Province	Season of infection	Monkey in vicinity	Microscopy	Parasites / µL	PCR diagnosis
TSY1522	Male	38	Karen	November 2007	Tak	Dry	No	*P. vivax*	12,160	*P. vivax*, *P. cynomolgi*
CT606	Male	30	Cambodian	October 2009	Chanthaburi	Rainy	Yes	*P. vivax*	86,535	*P. vivax*, *P. cynomolgi*
UBY120	Male	32	Thai	August 2015	Ubon Ratchathani	Rainy	Yes	*P. vivax*	570	*P. vivax*, *P. cynomolgi*
NR105	Male	53	Thai	July 2008	Narathiwat	Rainy	Yes	*P. vivax*	4,620	*P. vivax*, *P. cynomolgi*
YL3179	Male	15	Thai	April 2016	Yala	Dry	Yes	*P. vivax*	1,140	*P. vivax*, *P. knowlesi* *P. cynomolgi*
YL3634	Female	40	Thai	December 2016	Yala	Rainy	Yes	*P. falciparum*	60	*P. falciparum*, *P. cynomolgi*
YL3680	Male	49	Thai	December 2016	Yala	Rainy	Yes	*P. vivax*	3,720	*P. vivax*, *P. cynomolgi*
YL3685	Male	18	Thai	December 2016	Yala	Rainy	Yes	*P. vivax*	4,680	*P. vivax*, *P. cynomolgi*
YL4278	Male	21	Thai	October 2017	Yala	Rainy	Yes	*P. vivax*	7,440	*P. vivax*, *P. cynomolgi*

Appendix Nested PCR detection of *Plasmodium cynomolgi*

Primers	Sequence (5' → 3')	Positions after the RO strain*
Primary PCR		
PCOX1-F0	CTTTTAACGCCTGACATGGATGGATAATACTCG	3,196-3,228
PCOX1-R0	TCTGGATAATCAGGAATACGTCTAGGCATTAC	4,645-4,676
Secondary PCR		
Pcy1COXI-F	CCAAGCCTCACTTATTGTTAATTTATTTTT	3,291-3,320
Pcy2COXI-F	CTTATTGTTAATTATATATTGTATTATATATTTTTTG	**
PcyCOXI-R	CTGGAGAACCACATAAAATTGGTAAAAAA	3,579-3,607

*GenBank accession number AB444131
**Sequence after *P. cynomolgi* from macaques in Thailand (Putaporntip et al. unpublished.)

Primary DNA amplification was done in 30 µL reaction mixture containing template DNA, 2.5 mM $MgCl_2$, 300 mM each deoxynucleoside triphosphate, 3 µL of 10X ExTaq PCR buffer, 0.3 µM of primers PCOX1-F0 and PCOX1-R0 and 1.25 units of ExTaq DNA polymerase (Takara, Seta, Japan). The thermal cycle profile contained preamplification denaturation at 94°C, 1 min followed by 35 cycles of 94°C, 40 s; 50°C, 30 s and 72°C, 1 min, and a final extension at 72°C, 5 min. Secondary PCR was performed in a total volume of 30 µL in 2 separate reactions; one using primers Pcy1COX1-F and PcyCOX1-R, and the other using primers Pcy2COX1-F and PcyCOX1-R. The reaction mixtures were essentially the same as those for primary PCR except primers and 2 microL of primary PCR product as templates. All amplifications were performed in an Applied Biosystem GeneAmp® PCR System 9700 thermocycler (PE Biosystems, Foster City, CA) and were analysed by 2% agarose gel electrophoresis. The expected PCR fragments from primary and secondary PCR were 1,481 bp and 317-320 bp, respectively.

Infection, Genetics and Evolution 78 (2020) 104058

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Polymorphism and natural selection in the merozoite surface protein 3F2 (PVX_97710) locus of *Plasmodium vivax* among field isolates

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ARTICLE INFO

Keywords:
Malaria
Plasmodium vivax
Merozoite surface protein 3F2
Complete coding sequence
Natural selection
Recombination
Thailand

ABSTRACT

Plasmodium vivax, the chronic relapsing human malaria parasite with the most widespread distribution, possesses proteins associated with the merozoite surface that could be targets for host immune responses and potential vaccine candidates. Of these, the merozoite surface protein 3 of *P. vivax* (PvMSP3) is an attractive vaccine target as well as a genetic marker for epidemiological surveillance. PvMSP3 comprises a group of protein members encoded by a multigene family. Although some protein members, i.e. PvMSP3α and PvMSP3β, have been targets for molecular and immunological investigations, the most abundantly expressed protein member during late asexual erythrocytic stages, PvMSP3F2 (PVX_97710), remains unexplored.

To address domain organization and evolution of this locus, the complete coding sequences of 31 *P. vivax* isolates from diverse malaria endemic areas of Thailand were analyzed and compared with 10 previously reported sequences. Results revealed that all *PvMSP3F2* sequences differed but could be divided into 5 repeat-containing domains flanked by 6 non-repeat domains. Repeat domains II and IV at the 5′ portion and domain X at the 3′ portion exhibited extensive sequence and length variation whereas repeat domains VI and VIII located at the central region were relatively conserved. Despite a repertoire of PvMSP3F2 variants, predicted coiled-coil tertiary structure and predicted B-cell epitopes seem to be maintained. Evidence of intragenic recombination has been detected among field isolates in Thailand that could enhance sequence diversity at this locus. Non-repeat domains I and IX located at the 5′ end and at the 3′ portion, respectively, seem to have evolved under purifying selection. Evidence of positive selection was found in non-repeat domains III, V and VII where a number of predicted HLA class I epitopes were identified. Amino acid substitutions in these predicted epitopes could alter predicted peptide binding affinity or abolish peptide epitope property, suggesting that polymorphism in these epitopes conferred host immune evasion. Further studies on PvMSP3F2 are warranted, particularly on interaction with host immune system and the potential role of this PvMSP3 protein member as a vaccine target.

1. Introduction

Plasmodium vivax is a chronic relapsing species of human malaria parasites that incriminates in substantial public health burden outside of Africa. Although a remarkable global reduction in malaria cases by integrated malaria control strategies has been achieved since 2010, a stalling of the progress was observed during the past few years (WHO, 2018). Meanwhile, conventional control of malaria caused by *P. falciparum* could lead to a relative increase in the number of vivax malaria cases as has been observed in Thailand (Delacollette et al., 2009). Therefore, an alternative control measure by development of malaria vaccines is prevailing.

During the complex alternating life cycles between anopheline mosquito vectors and human hosts, erythrocyte invasion by *Plasmodium* merozoites is a fundamental step to initiate and to continue asexual reproductive cycle responsible for pathology and symptoms of malaria. The process involves both non-specific and specific receptors on erythrocyte surface and various proteins on, or associated with the membrane of malarial merozoites (Hadley, 1986; Gaur et al., 2004). Therefore, interruption of this process is of prime importance for malaria vaccine development against blood-stage infection. Several proteins have been identified to be participating in receptor-ligand interaction during merozoite invasion of erythrocytes such as the merozoite surface proteins-1 (MSP-1) adhesion complex (Baldwin et al., 2015) interacting with erythrocyte band 3 and glycophorin A, and the apical membrane antigen 1 (AMA1) binding to rhoptry neck (RON) protein complex (Lamarque et al., 2011). For *P. vivax*, Duffy-binding protein and reticulocyte binding proteins play pivotal roles during reticulocyte

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https://doi.org/10.1016/j.meegid.2019.104058
Received 17 July 2019; Received in revised form 25 September 2019; Accepted 29 September 2019
Available online 06 November 2019

invasion (Gupta et al., 2018). Meanwhile, a number of other merozoite surface proteins of unknown function, such as the merozoite surface protein 3 of *P. falciparum* (PfMSP3), have been identified as potential targets for protective antibodies. Antibodies to PfMSP3 have been shown to act coherently with monocytes to mediate parasite killing by a mechanism known as antibody-dependent cellular inhibition (Bouharoun-Tayoun et al., 1995). Naturally acquired anti-PfMSP3 antibodies among individuals living in endemic areas conferred a significantly lower risk of developing symptomatic and severe malaria. Importantly, MSP3 has been identified in *P. vivax* (PvMSP3) that shares predicted central alpha-helical secondary structure and alanine-rich coiled-coil domains at the central region of these proteins akin to PfMSP3 (Jimenez et al., 2008).

Unlike being encoded by a single gene as in PfMSP3, PvMSP3s are groups of related proteins translated from a multi-gene family, comprising 12 gene members in the Salvador I strain (Jiang et al., 2013; Rice et al., 2014). Variation in the number of gene members in the *PvMSP3* family has been observed among isolates (Rice et al., 2014). Of these, *PvMSP3α* (*PvMSP3H* or PVX_097720) and *PvMSP3β* (*PvMSP3C* or PVX_097680) whose sequences exhibit extensive diversity among isolates have been widely used as genetic markers to differentiate *P. vivax* strains (Bruce et al., 1999; Yang et al., 2006), *albeit* a caveat on their applications has been scrutinized (Rice et al., 2013). Both PvMSP3α and PvMSP3β are immunogenic upon natural malaria exposure. Importantly, Papua New Guinean children who had antibodies to block II of PvMSP3α were at significantly lower risk of acquiring clinical vivax malaria (Stanisic et al., 2013). Although PvMSP3s has been considered a potential vaccine candidate against vivax malaria, it is not well understood which particular protein members could elicit protective immunity. Meanwhile, the *PvMSP3* family exhibits differential timing of gene expression during trophozoite and schizont stages based on stage-specific transcriptional assay (Jiang et al., 2013). At merozoite stage, PvMSP3 were found to be localized on the surface except for a protein member designated PvMSP3F1 (PVX_097700) that has been found to be concentrated at the apical end of the merozoite. Therefore, it has been suggested that individual PvMSP3 could exert unique functions but could also have complementary roles (Jiang et al., 2013).

Among members of the PvMSP3 family, PvMSP3F2 or PVX_097710 is the most abundantly expressed protein (Jiang et al., 2013), contributing to almost half the amount of all PvMSP3 proteins found at the merozoite stage. Despite the lack of comprehensive study on the protein function, the major constituent of PvMSP3F2 on the cell surface could imply its important role, *albeit* currently unknown, at the merozoite stage. To date, little is known about the gene structure and extent of sequence diversity at the *PvMSP3F2* locus. In this study, we analyzed the complete coding sequences of *PvMSP3F2* among Thai isolates collected from symptomatic vivax malaria patients from diverse geographic areas of the country. Results revealed extensive polymorphism at this locus that has been influenced by frequent intragenic recombination. Natural selection observed in some domains of PvMSP3F2 has suggested that further investigations are warranted in terms of biological and immunological aspects of this member.

2. Materials and methods

2.1. Human ethics statement

This study was approved by the Institutional Review Board in Human Research of Faculty of Medicine, Chulalongkorn University, Thailand (IRB No. 546/58 and COA No. 041/2016). Informed consent was obtained from all participants or from their parents or guardians prior to blood sample collection.

2.2. Sources of P. vivax samples

Fifty venous blood samples were obtained from febrile individuals who were diagnosed having vivax malaria by microscopic examination of Giemsa stained blood films. The EDTA-preserved blood samples were collected from Tak province in 2005 and 2013 (n = 13), Ubon Ratchathani province in 2009 and 2016 (n = 10), Chanthaburi province in 2007 and 2011 (n = 12), Narathiwat and Yala province in 2008–2009 (n = 15). Yala and Narathiwat provinces are located next to each other and malaria transmission has been almost similar during the past decades; therefore, these provinces are considered herein to be the same origin. Genomic DNA of blood samples was prepared by using QIAamp DNA mini kit (Qiagen, Hilden, Germany) as recommended in the manufacturer's protocol. DNA was stored at −40 °C until use.

2.3. PCR detection of P. vivax and genotyping

The presence of *P. vivax* DNA in each samples was confirmed by nested PCR method using genus- and species-specific primers derived from the *18S rRNA* gene for primary and nested PCR, respectively, as described (Putaporntip et al., 2009). Allele-specific PCR targeting the polymorphic block 6 of the merozoite surface protein 1 of *P. vivax* was used to determine the number of parasite clones in each isolate as previously reported (Putaporntip et al., 2002; Kosuwin et al., 2014; Cheng et al., 2018).

2.4. Amplification and sequencing of PvMSP3F2

The complete coding region of *PvMSP3F2* from all isolates that contained single PvMSP1 block 6 haplotypes were amplified by PCR using primers PvMSP3.9-F (5′- CGCTAACTGAAAGGTAGTGTATATC -3′) and PvMSP3.9-R (5′- CCACAAACGGGAAACATC -3′) of the Salvador I strain (GenBank accession no. AAKM01000016). These primers have been pre-verified to specifically amplify this locus without cross amplification of the paralogous member *PvMSP3F1* (*PVX_097700*). Amplification was performed in a total volume of 30 µL containing PCR buffer, 200 µM dNTP, 0.2 µM of each primer, nuclease free water, 2 µL of template DNA and 1.25 units of TaKaRa LA Taq™ (Takara, Seta, Japan). The thermal cycling profiles for PCR contained a pre-amplification denaturation at 94 °C, 60 s; followed by 35 cycles of denaturation at 96 °C, 20 s; annealing at 56 °C, 30 s; polymerization at 72 °C, 5 min, and final elongation at 72 °C, 10 min. PCR amplification was carried out in GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). The PCR products were fractionated on 1% agarose gel electrophoresis, stained with ethidium bromide and examined under UV transillumination. Template DNA of isolates used for sequencing was purified by using QIAquick PCR purification kit (Qiagen, Hilden, Germany). DNA sequences were determined directly and from both directions using ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems) and sequencing primers.

2.5. Data analysis

Alignment of nucleotide sequences was performed by using the codon-based option in MUSCLE program with manual adjustment by eye (Edgar, 2004). Besides the complete coding sequence of Salvador I strain, all other available partial sequences of *PvMSP3F2* in the public database were included in this analysis: Vietnam PaloAlto (GenBank accession numbers KC907586), Mauritania I (KC907589), Brazil I (KC907590), Chesson (KC907588), Panama I (KC907591), Vietnam II (KC907592), North Korea I (AFNJ01000104), Sumatra (KC907587) and India VII (AFBK01001357). These partial sequences lack 10 and 9 codons at the 5′ and the 3′ ends of the gene, respectively. Inclusion of these partial sequences did not compromise the integrity and accuracy of analysis because these missing sequences are rather conserved. Furthermore, all sites at which the alignment postulated a gap were eliminated in pairwise comparisons of the analysis. Searching for repetitive DNA sequence motifs was done by scanning each sequence with Tandem Repeats Finder version 4.0 program (Benson, 1999). The

number of haplotype and haplotype diversity including its sampling variance were computed by using the DnaSP version 5.10 program (Librado and Rozas, 2009). The number of nucleotide substitutions per site (d) in the sample sequences was calculated using maximum composite likelihood model (Tamura et al., 2013). The number of synonymous substitutions per synonymous site (d_S) and the number of nonsynonymous substitutions per nonsynonymous site (d_N) were computed by using Nei and Gojobori's model (Nei and Gojobori, 1986) with Jukes-Cantor correction (Jukes and Cantor, 1969). The bootstrap method with 1000 pseudosamplings was carried out to obtain the standard errors of these parameters as implemented in the MEGA 6.0 program (Tamura et al., 2013). A two-tailed Z-test was deployed to determine statistical differences between these parameters and the significance level was set at $p < 0.05$. Exploration of codon-based selection was performed by using the fixed effects likelihood (FEL) (Kosakovsky Pond and Frost, 2005) and fast unconstrained Bayesian approximation (FUBAR) (Murrell, 2013) methods implemented in the Datamonkey Web-Server (Weaver et al., 2018). Significance level settings for these tests were considered per the default values available on the Datamonkey Webserver. Evidences of intragenic recombination were analyzed by using the Recombination Detection Program version 4 (RDP4) (Martin et al., 2015) that included RDP4 (Martin et al., 2005a), GENCONV (Padidam et al., 1999), Bootscan/Recscan (Martin et al., 2005b), the Maximum Chi Square (Maynard Smith, 1992), CHIMAERA (Posada and Crandall, 2001), Sister Scanning (Gibbs et al., 2000) and 3SEQ (Lam et al., 2018) methods. Relationship among sequences was analyzed by phylogenetic tree construction based on the maximum likelihood method using the best substitution model for the sequence data that yielded the minimum Bayesian Information Criterion (BIC) scores (Tamura et al., 2013). Confidence levels of clustering patterns in the phylogenetic tree were assessed by 1000 bootstrap pseudoreplicates. Predicted coiled-coil motif of amino acid sequences was performed using PAIRCOIL program (Berger et al., 1995). BepiPred 2.0 server was deployed to predict B-cell epitopes based on a random forest algorithm trained on epitopes annotated from antibody-antigen protein structures (Jespersen et al., 2017). The PvMSP3F2-derived peptides binding to human MHC molecules were predicted using The Immune Epitope Database (IEDB) webserver (http://www.iedb.org)(Vita et al., 2019) for some common HLA classes I and II among Thai population: HLA-A2, -A24, -A11, -A33, -B46, -B60, -B13, -DRB1*1202, -DRB1*1502, -DRB1*0701, -DRB1*1501, and -DRB5*1602 (Romphruk et al., 1999; Phiancharoen et al., 2004; Kupatawintu et al., 2010). The MHC class I and II predictions were made on 4/17/2013 using the IEDB analysis resource Consensus tool (Kim et al., 2012; Wang et al., 2008; Wang et al., 2010) which combines predictions from Artificial Neural Network (ANN) (Nielsen et al., 2003; Lundegaard et al., 2008), stabilized matrix method (SMM) (Peters and Sette, 2005) and combinatorial peptide library (comblib)(Sidney et al., 2008). The predicted output is given in units of IC_{50} nM, in which peptides with IC_{50} values < 50 nM are considered high affinity, < 500 nM intermediate affinity and < 5000 nM low affinity. Most known epitopes have high or intermediate affinity whilst no known T-cell epitope has an IC_{50} value > 5000 nM (Vita et al., 2019).

3. Results

3.1. Sequence diversity in PvMSP3F2

All 50 *P. vivax* isolates diagnosed by microscopy gave concordant positive results by species-specific nested PCR. Of these, 12 isolates were excluded due to mixed *PvMSP1* genotypes. PCR amplification and sequencing of the *PvMSP3F2* locus was done in the remaining 38 isolates. Seven of these isolates contained mixed and superimposed signals on electropherogram and were omitted from this study. Therefore, 31 *PvMSP3F2* complete nucleotide sequences were available for analysis. The geographic origins of these isolates were as follows: 7 from Tak, 4 from Ubon Ratchathani, 8 from Chanthaburi and 12 from Yala/Narathiwat provinces. All these isolates displayed different sequences and the total number of nucleotides in the complete coding sequences *PvMSP3F2* ranged from 3369 to 4182 bp (mean = 3712 bp; median 3741 bp), resulting in 18 inertion/deletion (indel) haplotypes. All 10 *PvMSP3F2* in the GenBank database displayed different sequences and possessed the total number of nucleotides within the range of these Thai isolates. All previously reported sequences differed from isolates in this study.

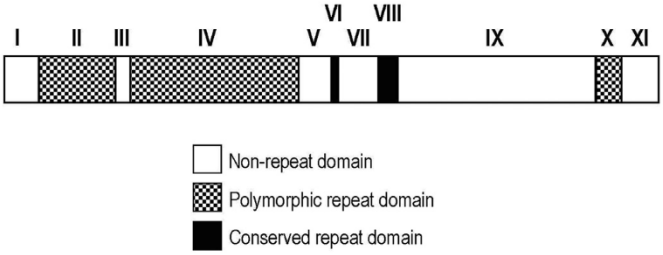

Fig. 1. Domain organization of *PvMSP3F2*.

3.2. Domain organization of PvMSP3F2

Sequence comparison among 31 distinct Thai haplotypes and 10 previously reported sequences has shown that the coding regions of *PvMSP3F2* can be divided into 11 domains, containing 6 non-repeat domains intervened by 5 repeat domains (Fig. 1). The non-repeat domain I at the 5′ end, spanning 249 nucleotides, was the most conserved region (Table 1). No insertion or deletion was observed in non-repeat domains except domain III in which one codon has been deleted from 6 Thai isolates and the Panama I strain. The numbers of nucleotide substitutions per site in non-repeat domains varied from 0.0416 in domain I to 0.1794 in domain III. Among 5 repeat domains, domains II, IV and X were polymorphic (d = 0.1716-0.2708) than other repeat domains due to the presence of repeats with remarkable size variation among isolates. Repeats in domains II and IV of *PvMSP3F2* comprised more complex and degenerate repeat motifs than other repeat-containing domains (Supplementary data 1); therefore, apparent amino acid repeats were barely observed. Repeat domains VI and VIII were less polymorphic without size variation. Although the average numbers of haplotypes for all non-repeat and for all repeat domains were comparable (23 and 22.8 haplotypes per domain, respectively), the numbers of nucleotide substitutions per site in repeat domains significantly outnumbered those in non-repeat domains ($p < 0.01$, Z-test)(Table 1).

3.3. Tests for deviation from selective neutrality

Comparison between the rate of synonymous substitutions per synonymous site or d_S with the rate of nonsynonymous substitutions per nonsynonymous site or d_N was performed for each domain except domains II, IV and X in which perfectly matched sites could not be unambiguously aligned due to indel polymorphism. Among non-repeat domains, d_S was significantly greater than d_N in domains I and IX, suggesting purifying selection in these regions (Table 2). On the other hand, positive selection as evidenced by a significantly greater d_N than d_S was observed in non-repeat domains III, IV and VII. These findings remain consistent when analysis was performed separately among Thai isolates and those previously reported (Table 2). A discordant result occurred only in domain I where departure from selective neutrality was not found among non-Thai haplotypes. No deviation from selective neutrality was observed in other domains analyzed. Meanwhile, codon-based analysis by FEL and FUBAR methods across the coding sequences of *PvMSP3F2* excluding domains II, IV and X has identified 119 positively selected codons and 113 codons under negative selection based the default cut-off values and concordant results from both methods.

Table 1
Haplotypes and nucleotide differences in *PvMSP3F2*.

Domain	No. nt	M	S	H	*h* ± S.D.	*d* ± S.E.
I-Non-repeats	249	35	34	29	0.973 ± 0.013	0.0416 ± 0.0077
II-Repeats	189–453	122	86	29	0.962 ± 0.019	0.2704 ± 0.0546
III-Non-repeats	102–105	81	60	20	0.940 ± 0.019	0.1794 ± 0.0227
IV-Repeats	237–1086	148	112	33	0.954 ± 0.018	0.2708 ± 0.0194
V-Non-repeats	234	121	102	18	0.934 ± 0.019	0.1619 ± 0.0169
VI-Repeats	45	14	10	12	0.902 ± 0.020	0.0836 ± 0.0280
VII-Non-repeats	288	160	129	19	0.933 ± 0.021	0.1705 ± 0.0191
VIII-Repeats	132	62	53	20	0.909 ± 0.030	0.1384 ± 0.0191
IX-Non-repeats	1437	611	537	36	0.989 ± 0.010	0.1133 ± 0.0057
X-Repeats	126–183	52	48	20	0.887 ± 0.036	0.1716 ± 0.0339
XI-Non-repeats	270	118	109	16	0.879 ± 0.031	0.1303 ± 0.0164
All non-repeats	2571–2583	1117	962	39	0.996 ± 0.007	0.1127 ± 0.0060
All repeats	726–1542	381	294	39	0.989 ± 0.010	0.2037 ± 0.0341#
All coding	3342–4182	1515	1271	41	1.000 ± 0.006	0.1265 ± 0.0085

M, the number of mutations; S, the number of segregating sites; H, the number of haplotype; *h,* haplotype diversity; π, nucleotide diversity; S.D., standard deviation; S.E., standard error.
Test of the hypothesis that *d* for all repeat domains equals that for all non-repeat domains: # $p < 0.01$.

The distribution of codons deviated from selective neutrality was not even, i.e. the number of positively selected codons more than two fold exceeding negatively selected codons in domains III, IV and VII whereas the reverse was true for domain I (Fig. 2 and Supplementary datas 2 and 3).

3.4. Recombination

Evidence of intragenic recombination in *PvMSP3F2* was analyzed by using the RDP package for isolates from each endemic area. In total, 94 recombination events were identified. Both *P. vivax* isolates from Chanthaburi and Ubon atchthani provinces had 31 recombination events in this locus, followed by 19 recombination events among parasites from Tak province. Meanwhile, isolates from Yala and Narathiwat provinces possessed 13 recombination events. Recombination breakpoints occurred across all domains of the gene with more prevalence in non-repeat domain I, repeat domains II and X with an average of 6.4, 5.6 and 7.1 sites per 100 bp, respectively (Table 3 and Supplementary data 4). The median distance between recombination breakpoints was similar across endemic areas, varying from 190 to 228 bp.

3.5. Predicted linear B cell epitopes

The central coiled-coil heptad repeat structure in *PvMSP3F2* was predicted to be maintained among different haplotypes regardless of size and sequence variation based on analysis by the PAIRCOIL program (Fig. 3). It has been suggested that the central coiled-coil heptad repeat structure of *PvMSP3* was a target for antibody recognition (Galinski et al., 1999; Mourão et al., 2012; Bitencourt et al., 2013). Therefore, the propensity for B cell epitopes was assessed across the full length of sequences. By using the default cut-off value of 0.5 as suggested in the BepiPred 2.0 web-server for sequence-based epitope prediction (Jespersen et al., 2017), the potential B cell epitopes in the central portion of all PvMSP3F2 variants seem to be maintained (Fig. 3). Further analysis of non-repeat domains III, V and VII with signatures of positive selection has shown some variation in predicted linear B cell epitope scores among haplotypes while the majority of sites in these domains had epitope scores above the predicted threshold (> 0.5) (Supplementary data 5). Therefore, it seems likely that extensive sequence variation might not abolish antigenicity of this protein.

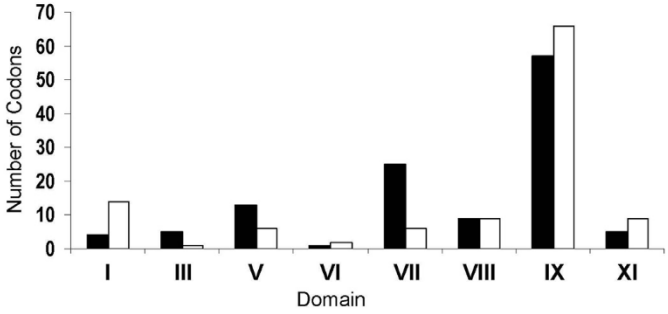

Fig. 2. Numbers of positively selected codons (filled bar) and negatively selected codons (open bar) in each domain of *PvMSP3F2*.

3.6. Predicted helper T-cell epitopes

In silico prediction of CD4+ T cell epitopes based on the allele

Table 2
Numbers of synonymous (d_S) and nonsynonymous (d_N) substitutions per site between *PvMSP3F2* haplotypes.

Domain	Thai isolates (n = 31)		Non-Thai isolates/strains (n = 10)	
	d_S ± S.E.	d_N ± S.E.	d_S ± S.E.	d_N ± S.E.
I-Non-repeats	0.1010 ± 0.0272**	0.0262 ± 0.0076	0.0526 ± 0.0215	0.0306 ± 0.0090
III-Non-repeats	0.0805 ± 0.0284	0.1722 ± 0.0247*	0.0957 ± 0.0360	0.1915 ± 0.0326*
V-Non-repeats	0.0851 ± 0.0190	0.1695 ± 0.0215***	0.0969 ± 0.0233	0.1567 ± 0.0201*
VI-Repeats	0.0433 ± 0.0309	0.0642 ± 0.0277	0.0466 ± 0.0373	0.0588 ± 0.0214
VII-Non-repeats	0.0806 ± 0.0192	0.1829 ± 0.0189****	0.0775 ± 0.0212	0.1901 ± 0.0224****
VIII-Repeats	0.0982 ± 0.0269	0.1257 ± 0.0248	0.1088 ± 0.0407	0.1252 ± 0.0221
IX-Non-repeats	0.1586 ± 0.0119****	0.1030 ± 0.0061	0.1302 ± 0.0139***	0.0879 ± 0.0065
XI-Non-repeats	0.1285 ± 0.0294	0.0808 ± 0.0124	0.0806 ± 0.0248	0.0643 ± 0.0129
All	0.1255 ± 0.0084	0.1084 ± 0.0039	0.1062 ± 0.0086	0.1010 ± 0.0050

Test of the hypothesis that d_N equals the corresponding d_S: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.0005$.

Table 3
Recombination events in *PvMSP3F2* among Thai isolates.

Origin/Province	No. events#	Location of breakpoints		Distance (bp)		
		Non-repeats	Repeats	Range	Mean	Median
Tak (n = 7)	19	34	4	7-3589	776	223
Chanthaburi (n = 8)	31	39	23	22-3796	484	209
Ubon Ratchathani (n = 4)	31	41	21	24-3897	688	228
Yala and Narathiwat (n = 12)	13	19	7	75-2552	445	190
Total	94	133	55	7-3897	605	219

Detection using the RDP package.

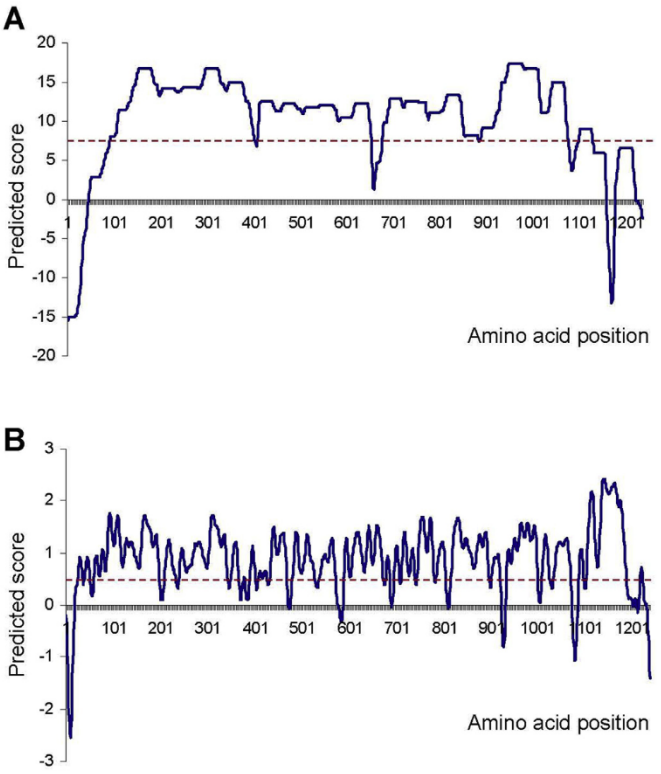

Fig. 3. Plots of predicted scores for coiled-coil motif (A) and B-cell epitopes (B) across the complete amino acid sequence of PvMSP3F2. Broken lines indicate default cutoff values.

independent CD4+ T cell immunogenicity at population level (Paul et al., 2015) has identified 12–14 potential epitopes in PvMSP3F2 (Supplementary data 6). None of these predicted epitopes occurred in repeat domains. However, the propensity of peptides binding to some common HLA-DR haplotypes in Thai population, i.e. DRB1*1202, DRB1*1502, DRB1*0701, DRB1*1501, and DRB5*1602, was not found in PvMSP3F2. Meanwhile, a number of predicted peptides binding to some common MHC class I molecules in Thai population, i.e. HLA-A2, -A24, A11, -A33, -B46, -B60 and -B13, have been identified (Supplementary data 7). Importantly, amino acid substitutions in these HLA class I-binding peptides could affect their predicted binding affinities, varying from slight reduction in binding scores to complete loss of binding affinities. For example, peptides AVQEATYAK and AVNQATSAK from non-repeat domain III were predicted to possess high affinity to HLA-A*11.01 whereas variant peptides AVKQATYAK, AVKQATYAK, AVQQATDAK, AVEQATYAK, AVQQATDEK, VVKKVTDAK, AVKLASDAK, AVKQATDAK and VVEQATSAK were considered to have intermediate binding affinity. Furthermore, other variant peptides AVEQATGAK and AAKQATTAK displayed low binding affinity scores whereas predicted binding affinity was abolished in peptides VEKQATNAK and AVEQATGAQ. Some other examples are listed in Table 4.

3.7. Phylogenetic analysis

A phylogenetic tree of all *PvMSP3F2* sequences was constructed using the maximum likelihood method with the *PvMSP3D1* (PVX_097685) sequence of the Salvador I strain as an outgroup. The tree topology did not reveal discernible clade in *PvMSP3F2*. Furthermore, sequences of Thai isolates did not cluster according to their geographic origins (Fig. 4).

4. Discussion

Among the *PvMSP3* gene family, *PvMSP3α* and *PvMSP3β* have been extensively analyzed for the extent of sequence diversity and gene organization (Rayner et al., 2002; Rayner et al., 2004; Ord et al., 2005; Rice et al., 2013; Putaporntip et al., 2014). Both of these loci exhibit extensive sequence variation among isolates; thereby, these genes have been deployed for genotyping of *P. vivax* among field isolates (Bruce et al., 1999; Yang et al., 2006). Herein, sequence analysis of the complete coding region of distinct *PvMSP3F2* sequences has revealed a high level of sequence diversity (d = 0.1275, n = 41) greater than those observed in both *PvMSP3α* (n = 48) and *PvMSP3β* in which the d values of these genes were, 0.0443 (n = 48) and 0.1017 (n = 65), respectively, based on the same calculation parameter (data not shown). Therefore, *PvMSP3F2* could be an additional genetic marker for strain-typing of *P. vivax*. It is noteworthy that these 3 genes shared similar predicted protein secondary structure and motifs, characterized by signal peptides and conserved NLRNG motif at the N-terminal region, central coiled-coil tertiary peptide structure containing heptad repeat motifs and glutamine-rich motif near the C-terminal part (Jimenez et al., 2008; Jiang et al., 2013). However, the locations of polymorphic repeats domains of *PvMSP3α* and *PvMSP3β* occupied approximately the central portion of the genes whereas *PvMSP3F2* contained 5 repeat-containing domains. The first two repeat domains near the 5′ portion of *PvMSP3F2* seem to be reminiscent to the central repeat regions of *PvMSP3α* and *PvMSP3β* in terms of length and long stretches of insertion or deletion of sequences. Coding repeat arrays are abundant in malarial proteins and are prone to expansion and contraction by slipped-strand mispairing or recombinational mechanisms such as gene conversion or unequal crossing-over (Levinson and Gutman, 1987). Phylogenetic analysis also suggested that *PvMSP3F2* was not closely related to these two gene members in the *PvMSP3* family (Rice et al., 2013). Differences in the magnitude of sequence diversity of individual genes encoding protein family could be due to genetic drift and selection pressure (Ohta, 1983; Hughes, 1994). Variation in tandem repeats can accelerate evolution of coding sequence and regulatory regions that may be associated with phenotypic diversity (Caburet et al., 2005; Gemayel et al., 2010). Therefore, it is likely that repeats in the *PvMSP3* gene family could enhance sequence diversity within gene member and accelerate divergence between paralogues through the process of slipped-strand mispairing and/or recombinational mechanisms. The lower levels of sequence divergence between paralogous gene members in *PvMSP3* (*PvMSP3D1* and *PvMSP3D2*, *PvMSP3E1* and *PvMSP3E2*, and *PvMSP3F1* and P*vMSP3F2*) than those between their orthologues in *P.*

Table 4
Amino acid substitutions in some predicted HLA class I-binding peptides in PvMSP3F2.

Domain	HLA	Peptide	IC_{50} nM#	n	Domain	HLA	Peptide	IC_{50} nM#	n
III	A*11.01	AVQEATYAK	21	1	VII	A*11.01	VTDDSVTKAK	157	3
		AVNQATSAK	38	3			VTEKSISDAK	306	1
		AVKQATYAK	73	7			VETKSVSEAK	312	4
		AVKQATYAK	77	1			VTEDSVTKAK	314	14
		AVQQATDAK	92	2			VTEESLSEAK	414	2
		AVEQATYAK	102	2			VTEESVSEAK	424	3
		AVQQATDEK	122	3			VTEDSVTKGK	479	1
		VVKKVTDAK	132	3			LTEDSVTKAK	501	2
		AVKLASDAK	201	2			LEEGSVSDAK	1765	3
		AVKQATDAK	306	8			VEEDSVSGAK	15,779	2
		VVEQATSAK	316	3			VEEKSVSEAK	16,602	6
		AVEQATGAK	668	1					
		AAKQATTAK	755	1	VII	B*44.02	AENAKEKAK	418	2
		VEKQATNAK	13,214	2			AENAKKKAE	1166	14
		AVEQATGAQ	27,157	2			AENAKKKVE	1650	1
							VEKAKEKAK	5883	6
V	A*11.01	ASDKVTIANK	65	8			AEKAKEKAK	11,159	1
		ASDKVTSESK	147	1			VEKAKVKAK	14,565	1
		ISDKMTIANK	167	9			ADKAKEKAK	23,801	11
		ASDKVTSENK	176	3			AKNAKEKAK	33,357	2
		ISDKMSIANK	200	4			TKNAKEKAK	37,964	3
		ASDKVTSEDK	207	3					
		ASKKVTSEDK	285	3	VII	B*46.01	KFAKEVSSAEY	345	6
		ISDKIKIANK	440	3			ELAKDVSSAEF	1981	1
		ASDKVTSENE	28,941	7			ELAKDVSSAEY	3123	2
							KLAKEVSSAEY	6387	6
V	A*11.01	STKAKEAEK	179	7			ELAKEVSSAEY	6406	8
		STKAKDAEK	291	6			SLAKEVSKAEY	8377	5
		SPKAKEAEK	12,087	1			KLAKKVSSAEY	8389	2
		STKAKGAET	22,800	3			KFAKEVSSAEC	8837	1
		STKAKDAET	26,861	3			KLAKEVSESEY	9015	2
		SQKAKDAET	33,289	8			ALAKKVSKAEY	10,779	4
		SQKAKEAET	33,041	2			SLAKQVSKAEY	11,348	3
		SKKAKDAEI	38,361	1			ELQKKVSSAEY	24,482	1
		SKKAKDAET	40,992	10					
					VII	B*58.01	KAAEVATHI	7	9
V	A*11.01	ASKRADEALYK	90	4			KAAKVATHI	7	8
		ASKRAEEAFEK	571	1			KAADVATHI	11	3
		ASNRAGKAFDK	587	1			KAANVAKHI	80	5
		ASNRAKEALGK	641	8			KAANVVTDI	95	5
		ASKRADEALGK	683	1			KAADVAKHI	96	5
		ASKRAEEAFGK	878	2			KAANVATDI	98	3
		ASKRAEEALEK	956	6			KAANIATDI	270	2
		ASNRAKEALDK	960	2			KAENVAKHI	4031	1
		ASKRADEALDK	1026	3					
		ASKRAEEALDK	2214	6					
		ASKRADEALGR	4929	7					

#Binding prediction score by artificial neural network (ANN) method. IC50 values < 50 nM indicates high affinity peptides, < 500 nM intermediate affinity, < 5000 nM low affinity and > 5000 nM non T cell epitope.
Total n = 41.

cynomolgi MSP3 (*PcyMSP3*) could suggest post-speciation duplication of these gene members and imply their recent origins. However, the magnitude of sequence divergence between *PvMSP3E1* and *PvMSP3E2* was remarkably lower than those between other paralogues, implying differential evolution of these duplicated gene members (Rice et al., 2013).

Like other proteins on the surface of malarial merozoites, *PvMSP3α* and *PvMSP3β* are immunogenic upon natural *P. vivax* infections (Mourão et al., 2012; Stanisic et al., 2013; Bitencourt et al., 2013). Despite no seroepidemiological study on PvMSP3F2, antibodies raised against protein members of PvMSP3 family displayed both cross-reactive and member-specific responses, suggesting the presence of both shared and unique B cell epitopes among protein members (Jiang et al., 2013). B cell epitopes in PvMSP3α have been investigated by using naturally acquired antibodies from *P. vivax*-exposed individuals and located mainly at the central repeat region (Lima-Junior et al., 2011). Meanwhile, prediction of linear B cell epitopes across PvMSP3F2 has indicated a number of regions with high epitope scores, spanning > 70% of the protein and sparing only short regions of both N- and C-termini (Fig. 3). Although extensive sequence and length variation in this gene were observed among field isolates leading to variation in predicted linear B cell epitope scores (e.g. domains III, V and VII), the predicted B cell epitopes as well as the predicted coiled-coil tertiary structure in this protein seems to be maintained. The presence of abundant negatively selected codons distributed across the protein has suggested that PvMSP3F2 could be influenced by some structural constraints and probably be exposed to host immune system. Meanwhile, most malarial surface proteins seem to have evolved under positive selection, probably exerted by host immune pressure (Hughes and Hughes, 1995). Likewise, positive selection has been detected in a number of codons almost comparable to negatively selected codons. However, signature of positive selection seems to be more predominant in domains III, V and VII than other domains, indicating heterogeneity in selection pressure across the *PvMSP3F2* locus. Despite variation in predicted linear B cell epitope scores was envisaged in these positively selected domains, Although PvMSP3F2 was predicted to contain CD4+

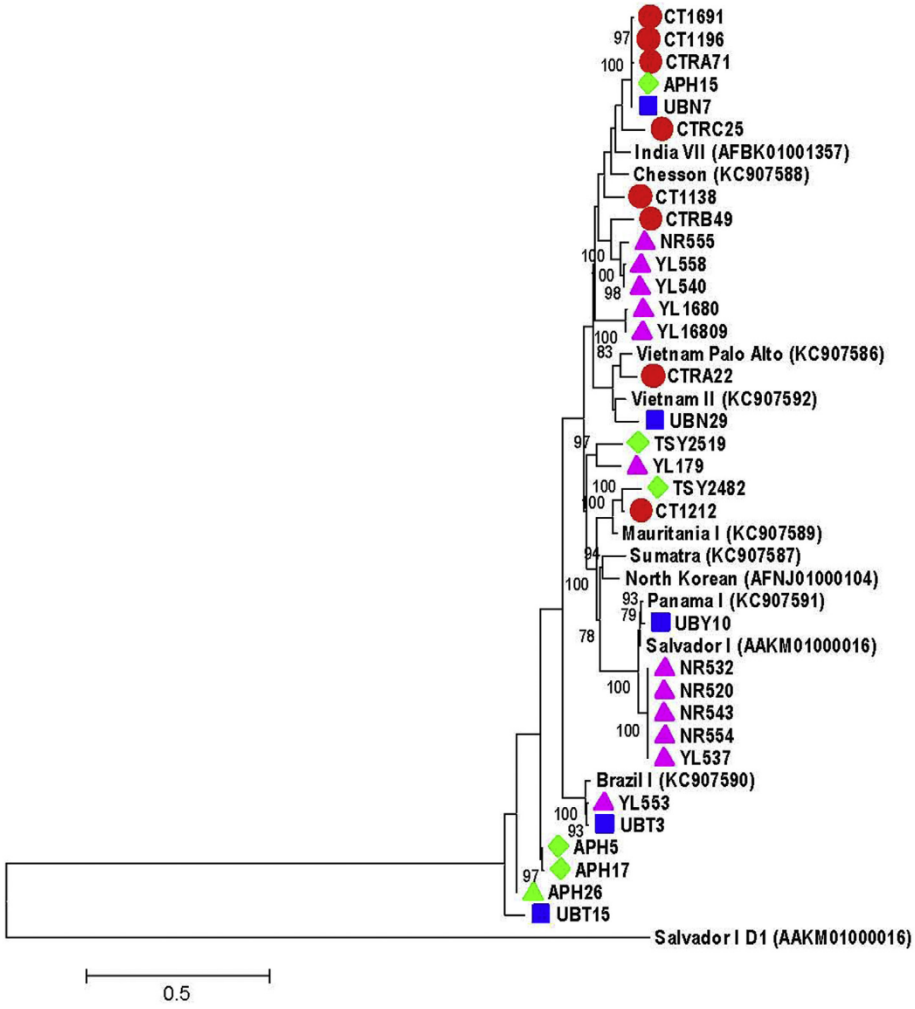

Fig. 4. Maximum likelihood tree of *PvMSP3F2* based on the General Time Reversible with gamma distribution (BIC score = 68,149.878). Numbers on the branch represent the percentage of 500 bootstrap samples supporting the branches; values > 70% are shown.

T cell epitopes (Supplementary data 6), none of them could be potential epitopes for some common HLA-DR alleles in Thai population. Therefore, positive selection in this molecule seems not be conferred by avoidance of being recognized by HLA-class II-mediated immunity. By contrast, several predicted HLA class I-binding peptides were detected in this protein based on some common alleles in Thai population. Importantly, amino acid substitutions in these epitopes could alter their predicted peptide binding affinity, varying from no obvious change, reduction in binding affinity to loss of binding propensity. Alteration in HLA-class I-binding affinity could be a mechanism for malaria parasite to escape from host immune destruction by narrowing the repertoire of cellular immune response which has been reported from analyses of the circumsporozoite protein of *Plasmodium falciparum* (Zevering et al., 1990; Plebanski et al., 1999). Positive selection that maintains a polymorphism (balancing selection) in PvMSP3F2, particularly in domains III, V and VII where remarkable changes in predicted peptide binding affinity to HLA-class I molecule, could imply its role in immune evasion process. On the other hand, despite no drastic change in predicted scores for linear B cell epitopes in these domains, it is intriguing to address whether amino acid sequence diversity could alter three dimensional structures of B cell epitopes in PvMSP3F2 and confer differential effects on protective immunity against *P. vivax* infections. Undoubtedly, further experimental studies on the effects of variants in this protein on antibody responses are required.

Genetic recombination in malaria parasites has been identified in a number of malarial genes and genomes (Ranford-Cartwright and Mwangi, 2012; Miles et al., 2016; Putaporntip et al., 2001; Putaporntip et al., 2002). The magnitude of sequence diversity and the efficacy of selection can be influenced by the effects of recombination (Charlesworth and Campos, 2014). Our previous analysis has shown that intragenic recombination has contributed to sequence variation in *PvMSP3β*, in which 45 recombination breakpoints occurred among 45 *P. vivax* isolates from Tak province while none was found among 28 isolates from Yala and Narathiwat provinces (Putaporntip et al., 2014). By using much fewer number of isolates with the same recombination prediction program, as many as 31 recombination events were detected among 8 isolates from Chanthaburi and the same number of recombination events were found among 4 isolates from Ubon Ratchathani whilst 19 recombination events occurred among 7 isolates from Tak. It is noteworthy that recombination sites were detected at almost comparable frequencies in non-repeat domains (mean = 0.0516/sites) and repeat domains (mean = 0.0556/sites), suggesting that recombination uniformly occurred and played an important role in the generation of sequence diversity at this locus. More importantly, 13 recombination events were observed in *PvMSP3F2* among 12 isolates from Yala and Narathiwat provinces. Because *P. vivax* isolates from these provinces used for analysis of *PvMSP3F2* and *PvMSP3β* were sampled just a few years apart (2006 versus 2007–2008), a stark contrast in recombination events in these loci could rather stem from intrinsic difference in recombination propensity of each locus than simply from temporal variation in parasite population.

In conclusion, *PvMSP3F2* has different domain organization from

those of *PvMSP3α* and *PvMSP3β* albeit sharing predicted coiled-coil tertiary structure, conserved NLRNG motif and variable glutamine-rich region. Extensive sequence diversity in *PvMSP3F2* has been observed among clinical isolates of *P. vivax*. Our analysis of both nucleotide and amino acid sequences of this locus provide evidence of structural or functional constraint on this molecule. Evidence of positive selection and alteration of predicted peptide binding affinity to HLA class I of some common alleles in Thai population has suggested that PvMSP3F2 could be a target for host immune responses. However, further experimental studies are undoubtedly required to address whether this protein member in the PvMSP3 family could be a target for vaccine against *P. vivax*.

Acknowledgements

We are grateful to all patients who donated their blood samples for this study. We thank the staff of the Bureau of Vector Borne Disease, Department of Disease Control, Ministry of Public Health, Thailand, for assistance in field work and Urassaya Pattanawong for excellent technical assistance. This work received support from Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University (Grant No. RA60/012) to CP, SJ and NK; The Thai Government Research Budgets to Chulalongkorn University (GRB-APS-12593011 and GBA-600093004) to SJ and CP; The Thailand Research Fund (RSA5980054) to CP; The Royal Golden Jubilee Ph.D. Programme Scholarship (RGJ) to NK; and The 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship to NK.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.104058.

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Am. J. Trop. Med. Hyg., 99(4), 2018, pp. 987–994
doi:10.4269/ajtmh.18-0401

Multiple Novel Mutations in *Plasmodium falciparum* Chloroquine Resistance Transporter Gene during Implementation of Artemisinin Combination Therapy in Thailand

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Abstract. Mutations in the chloroquine resistance transporter gene of *Plasmodium falciparum* (*Pfcrt*) are associated with drug susceptibility status of chloroquine and other antimalarials that interfere with heme detoxification process including artemisinin. We aim to investigate whether an increase in duration of artemisinin combination therapy (ACT) in Thailand could affect mutations in *Pfcrt*. The complete coding sequences of *Pfcrt* and dihydrofolate reductase (*Pfdhfr*), and size polymorphisms of the merozoite surface proteins-1 and 2 (*Pfmsp-1* and *Pfmsp-2*) of 189 *P. falciparum* isolates collected during 1991 and 2016 were analyzed. In total, 12 novel amino acid substitutions and 13 novel PfCRT haplotypes were identified. The most prevalent haplotype belonged to the Dd2 sequence and no wild type was found. A significant positive correlation between the frequency of *Pfcrt* mutants and the year of sample collection was observed during nationwide ACT implementation ($r = 0.780$; $P = 0.038$). The number of haplotypes and nucleotide diversity of isolates collected during 3-day ACT (2009–2016) significantly outnumbered those collected before this treatment regimen. Positive Darwinian selection occurred in the transmembrane domains only among isolates collected during 3-day ACT but not among those collected before this period. No remarkable change was observed in the molecular indices for other loci analyzed when similar comparisons were performed. An increase in the duration of artesunate in combination therapy in Thailand could exert selective pressure on the *Pfcrt* locus, resulting in emergence of novel variants. The impact of these novel haplotypes on antimalarial susceptibilities requires further study.

INTRODUCTION

A worldwide effort to control malaria has been highly reliant on antimalarial chemotherapy; therefore, drug resistance in malaria parasites can substantially preclude effective therapeutic and preventive interventions. One of the most devastating impacts of this phenomenon has been widely concerned since the emergence and spread of chloroquine resistance in *Plasmodium falciparum*, the most prevalent and malignant malaria species in the tropics. To date, the global dispersal of chloroquine-resistant *P. falciparum* has rendered the drug ineffective in almost all disease-endemic countries.[1,2]

Plasmodium falciparum chloroquine resistance transporter or chloroquine resistance transporter of *P. falciparum* is a 48.6-kDa transmembrane protein on the digestive vacuolar membrane that confers chloroquine resistance.[3] It is encoded by a single copy 13-exon gene, *Pfcrt*, comprising 424 codons. A point mutation at codon 76 (K76T) is a key determinant of chloroquine resistance phenotype.[3–5] However, the hitherto mutation per se may not be the sole predictor of drug resistance, whereas specific substitutions beyond codon 76 could modulate drug susceptibility status or compensate for the fitness costs of chloroquine-resistant parasites.[3,5,6] To date, at least 30 amino acid substitutions and ~51 haplotypes were identified in PfCRT of natural isolates.[7,8] In Thailand, all *P. falciparum* isolates collected during the past two decades exhibited chloroquine-resistant phenotypes, and the key mutations at codons 72–76 of the *Pfcrt* gene revealed CVIET as the most predominant haplotype.[9–11]

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Antimalarial treatment in Thailand has been relied on parasite-based diagnosis by microscopy, whereas antimalarial drug policies have been implemented and changed in response to therapeutic efficacy based on national surveillance system. Chloroquine was introduced to treat malaria caused by all *Plasmodium* species in 1945 and was implemented nationwide in 1965 despite the first report on drug resistance in falciparum malaria patients in 1957.[12,13] Because of the widespread occurrence of chloroquine resistance in *P. falciparum*, national treatment policy was changed to the combination of sulfadoxine and pyrimethamine (SP) in 1974 and was implemented until 1981 when antifolate resistance deteriorated in most endemic areas of the country.[14] During 1982 and 1985, quinine plus tetracycline were used as a standard treatment regimen and was replaced by the combination of mefloquine and SP because of the problem of drug compliance. Because the lack of evidence for delaying mefloquine resistance in combination therapy and the adverse effects from SP, mefloquine monotherapy was initiated in 1990, fully implemented in 1992, and used until 1994 when severe deterioration of mefloquine efficacy occurred.[14–16] During 1995 and 2008, artemisinin combination therapy (ACT) with mefloquine as a partner drug was deployed as a 2-day treatment regimen (Figure 1). Because of emergence and increasing tendency in the treatment failure rate of 2-day ACT, treatment with 3-day ACT regimen was initiated in 2008 and completely implemented nationwide in 2009.[17] However, a recent increase in treatment failure of 3-day ACT has prompted the change in treatment regimen to dihydroartemisinin plus piperaquine in 2017 and fully implemented in 2018. Nevertheless, chloroquine has been used as the first-line drug to treat vivax and other non-falciparum malaria since its first implementation in 1965. Importantly, about 8–10% of vivax malaria patients had

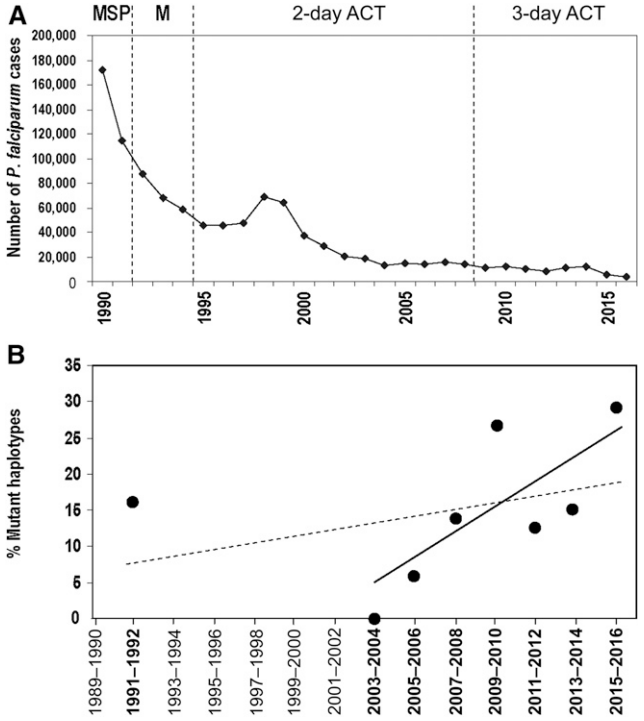

FIGURE 1. (**A**) Annual numbers of *Plasmodium falciparum*–infected cases in Thailand during 1990 and 2016. Vertical broken demarcation lines indicate the period of nationwide implementation of antimalarial treatment regimens: MSP, mefloquine combined with sulfadoxine and pyrimethamine; M, mefloquine monotherapy; 2-day artemisinin combination therapy (ACT), artesunate plus mefloquine for 2 days; 3-day ACT, 2-day ACT plus artesunate on day 3. (**B**) Biannual frequencies of mutant *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) haplotypes (spots) relative to the Dd2 sequence (GenBank accession no. AF030694). Correlation between frequencies of mutant haplotypes and years of sample collection (during 1991 and 201 represented in broken line: Pearson's correlation coefficient, $r = 0.382$; $P = 0.350$, and during 2003 and 2016 shown in solid line: $r = 0.780$; $P = 0.038$).

submicroscopic *P. falciparum* infection and were treated with chloroquine.[18,19] Therefore, it is likely that a small proportion of *P. falciparum* populations in Thailand could have been exposed to chloroquine.

Although mutations in *Pfcrt* are not considered to be the key molecular markers for resistance of *P. falciparum* to other antimalarial drugs, transfection-based approaches have shown that certain mutations at this locus conferred alteration in susceptibilities to artemisinin and some partner drugs in ACT, particularly those that interfere with heme detoxification process in the parasite's digestive vacuole such as mefloquine and piperaquine.[6,20–22] Importantly, changing antimalarial treatment regimens to ACT have remarkable impacts on *P. falciparum* drug resistance markers, including *Pfcrt* of parasite populations in Uganda and Kenya where chloroquine resistance deteriorated around the turn of the century.[23–25] To determine whether nationwide implementation of ACT and its modification could influence mutations in *Pfcrt*, we analyzed the complete coding sequences of *Pfcrt* and the dihydrofolate reductase gene (*Pfdhfr*) along with length polymorphisms in the merozoite surface proteins-1 and 2 (*Pfmsp-1* and *Pfmsp-2*) genes of *P. falciparum* isolates in Thailand. Results revealed multiple novel *Pfcrt* haplotypes with greater molecular diversity indices among isolates collected during implementation of the 3-day ACT regimen than those collected before this period, consistent with ongoing selective pressure on this locus.

METHODS

Parasite populations. Venous blood samples were obtained from 383 *P. falciparum*–infected patients initially diagnosed by microscopy and confirmed by species-specific polymerase chain reaction (PCR) assay.[26] Blood samples were collected during 1991, 2003–2006, 2008–2011, and 2013–2016 from seven provinces of Thailand bordering Myanmar, Cambodia, and Malaysia (Supplemental Figure 1). The parasite density determined from thick blood films ranged from 35 to 217,800 parasites/μL (geometric mean = 2,761 parasites/μL). Each blood sample was aliquoted; one preserved in EDTA anticoagulant and the other in RNA preservative for DNA and RNA extractions, respectively. The annual *P. falciparum* cases and the national treatment regimens for falciparum malaria in Thailand during sample collection period are depicted in Figure 1.

Detection of multiple-clone infections. Clonality of samples was determined by PCR analysis of size polymorphisms in block 2 of the *Pfmsp-1* and the central repeats of the *Pfmsp-2* of *P. falciparum* (Supplemental Material 1).

Sequencing of *Pfcrt*. The complete coding region of *Pfcrt* was obtained from direct sequencing of the PCR products amplified from cDNA and genomic DNA of each isolate (Supplemental Material 2). All singleton substitutions were redetermined using PCR products from independent amplifications of newly synthesized cDNA template from the same isolate.

Sequencing of *Pfdhfr*. The complete *Pfdhfr* sequence was amplified by PCR using genomic DNA of each isolate (Supplemental Material 3). Sequences were determined directly from purified PCR products.

Ethics. Written informed consent was obtained from all participants or from their parents or guardians before blood sample collection. The ethical aspects of this study were reviewed and approved by the Institutional Review Board on Human Research of Faculty of Medicine, Chulalongkorn University (IRB No. 257/57).

Statistical analysis. The sequences were aligned against the corresponding gene of the 3D7 clone. Haplotype diversity (*h*) and its sampling variance were calculated using the DnaSP program.[27] Nucleotide diversity (π), the rates of synonymous substitutions per synonymous site (d_S), and nonsynonymous substitutions per nonsynonymous site (d_N) and phylogenetic tree were analyzed by using the MEGA 6.0 program.[28] Evidence of genetic recombination was determined by various recombination tests implemented in the RDP4 package.[29] Tajima's *D* statistics was used for computing deviation from neutrality.[30] Tajima's *D* test determines the differences between the average number of nucleotide differences and an estimate of θ from the number of segregating sites where $\theta = 4Ne\mu$, in which *Ne* and μ are the effective population size and the mutation rate, respectively.[30] Under an equilibrium model or no influence from population history, significant positive Tajima's *D* values imply balancing or positive selection, whereas significant negative values suggest purifying or negative selection. Analysis of population genetic structure was performed by using molecular variance approach implemented in the Arlequin 3.5 software.[31]

RESULTS

Novel *Pfcrt* coding sequences. Analysis of size polymorphisms in *Pfmsp-1* and *Pfmsp-2* revealed that 242 of 383 isolates harbored single alleles of both loci. The complete coding sequences of *Pfcrt* could be obtained from 189 of these 242 isolates (78.1%). Compared with the 3D7 sequence, 26 nucleotide substitutions were detected among 189 *Pfcrt* sequences, resulting in 21 amino acid changes. Most nucleotide substitutions (84.6%) occurred in the putative transmembrane domain-encoding regions. Newly identified amino acid substitutions included 76I, 78L, 97R, 147D, 166L, 172M, 218F, 220P, 320T, 333I, 337N, and 351V (Table 1). In total, 21 genotypes were identified, yielding 18 different amino acid haplotypes. Haplotype I was most prevalent (87.83%) that shared the same sequence with the Dd2 clone (GenBank accession no. AF030694). Single synonymous substitutions were detected in three isolates at 411T>C, 543A>G, and 567T>C, all belonged to haplotype I. Isolates in haplotype I were heterogeneous based on analysis of the *Pfmsp-1* and *Pfmsp-2* loci. Haplotype II was found in four isolates (2.1%) that were identical with a Thai isolate TM93 collected two decades ago.[32] Haplotype III possessed the same sequence as the K1 strain first isolated from Kanchanaburi in 1979[33] and the Colombian FCB strain.[34] Haplotypes IV and V shared perfect sequence identity with the Cambodian isolate 734 and the Ghanaian isolate GB4, respectively.[7,35] The remaining haplotypes VI-XVIII were novel and occurred as single isolates (Table 1). The wild-type *Pfcrt* was not found. Although the number of isolates collected from each endemic area and from different period in this study was uneven, the overall frequencies of mutant codons relative to the wild-type (3D7) and the most common Dd2 haplotypes were very low, ranging from 0.5% to 2.1% (Table 2).

The complete *Pfdhfr* coding sequences. The complete *Pfdhfr* sequences from the same 189 isolates contained four nonsynonymous nucleotide substitutions, resulting in amino acid changes at N51I, C59R, S108N, and I164L. In total, four amino acid haplotypes were identified, characterized by IRNL (64.02%), IRNI (31.75%), NRNL (2.64%), and NRNI (1.59%).

Population genetics inferred from *Pfmsp-1* and *Pfmsp-2*. We observed 13 *Pfmsp-1*, 9 *Pfmsp-2*, and 73 combined *Pfmsp-1* and *Pfmsp-2* alleles among these 189 isolates. The number of haplotypes and haplotype diversity of these loci were almost comparable for populations bordering Myanmar and Cambodia, whereas lower levels of these parameters occurred for parasites near Malaysia (Supplemental Table 1). Genetic structuring was observed because all pairwise *Fst* values deviated significantly from zero, suggesting limited gene flow across these distant endemic areas and consistent with previous analyses using other loci (Supplemental Table 2).[36]

Variations in *Pfcrt* and other loci relative to change in ACT regimens. There was a significant positive correlation between frequency of *Pfcrt* mutants and the year of sample collection during implementation of ACT (during 2003 and 2016) ($r = 0.780$; $P = 0.038$) but not when all sample collection period was considered (during 1991 and 2016) ($r = 0.382$; $P = 0.350$) (Figure 1). For further analyses, these samples were, therefore, assigned in the "pre-3-day ACT" and "3-day ACT" groups for those collected during 1991 and 2008, and 2009 and 2016, respectively. The number of *Pfcrt* haplotypes was significantly increased in the 3-day ACT group in comparison

TABLE 1

Distribution of *Plasmodium falciparum* chloroquine resistance transporter haplotypes among 189 clinical isolates in Thailand

Haplotype	Amino acid substitution											No. isolates							
				* *				*											
	12	3777779	11111111111	11222	2	222	3333	3333	3										
	04	9245687	24444566677	99012	7	777	2233	3555	7										
			34578203624	48580	1	357	0634	7016	1										
3D7	QD	SCMNKFH	HAFGLTLSIFI	IETIA	Q	HPN	ANTS	ICII	R	Tak	Ubon Ratchathani	Chantaburi	Trat	Ranong	Yala	Narathiwat	*Total (%)*
Known variants	KY	PSIET Q	RFI IAY*RV*	TKA S	E	N*LD*	SSN	R T	I								
		KA L	Y P	S			DA	L	T								
		D*I*	H					*V*									
		N	T														
I (**Dd2**,TSY164)	..	..IET..	.....	....S	E	...	.S..	...T	I	78	21	3	32	13	13	6	*166(87.83)*
II (**TM93**,CT449)	..	..IET.L	.....	....S	E	...	.S..	...T	I	–	–	1	3	–	–	–	*4(2.12)*
III (**K1**,UBT2150)	..	..IET..	.....	....S	E	...	.S..	....	I	–	2	–	–	–	–	–	*2(1.06)*
IV (**Cam734**,UBN82)	..	..IDT..	.F..I.....	T...S	E	...	..S.	....	.	–	1	–	–	–	–	–	*1(0.53)*
V (**GB4**,CT448)	..	..IET..	.....	....S	E	...	....	....	I	–	–	1	–	–	–	–	*1(0.53)*
VI (UBN73)	..	..IDT.	.F..I.....	T...S	E	...	..S.	....	.	–	1	–	–	–	1	–	*2(1.06)*
VII (UBT1650)	..	..IET..	..... .	... S	E	...	.S..	...T	I	–	1	–	–	–	1	–	*2(1.06)*
VIII (TSY2473)	..	..IET..	.....	....S	E	...	.S..	...T	I	1	–	–	–	–	–	–	*1(0.53)*
IX (AP2064)	..	..IET..	.....	....S	E	...	....	...T	I	1	–	–	–	–	–	–	*1(0.53)*
X (AP2425)	..	..IET..	..... .	....S	E	...	.S..	...T	I	1	–	–	–	–	–	–	*1(0.53)*
XI (AP2427)	..	..IET..	.....	....S	E	...	.S..	..T	I	1	–	–	–	–	–	–	*1(0.53)*
XII (AP2466)	..	..IET..	.....	....S	E	...	S..	.. T	I	1	–	–	–	–	–	–	*1(0.53)*
XIII (AP2474)	..	..IET .	.....	....S	E	...	.S..	...T	I	1	–	–	–	–	–	–	*1(0.53)*
XIV (UBT2860)	..	..IET..	... .	....S	E	...	.S..	...T	I	–	1	–	–	–	–	–	*1(0.53)*
XV (CT467)	..	..IET..	.....	....S	E	...	.S .	...T	I	–	–	1	–	–	–	–	*1(0.53)*
XVI (TD529)	..	..IDT..	.F..I.....	T...S	E	...	.S..	...T	I	–	–	–	1	–	–	–	*1(0.53)*
XVII (YL2823)	..	..IE ..	.....	....S	E	...	.S..	...T	I	–	–	–	–	–	1	–	*1(0.53)*
XVIII (NR367)	..	..IET..	.....	....	E	...	.S..	...T	I	–	–	–	–	–	–	1	*1(0.53)*
Exon	*1-*	*2------*	*3----------*	*4----*	*6*	*7--*	*9---*	*10--*	*11*	*84*	*27*	*6*	*36*	*13*	*16*	*7*	*189(100)*

* Positions found in experimental clones/lines induced by selective drug pressure.[21] Italicized amino acids denote mutations induced by drug pressures. Dots are identical residues with the 3D7 sequence (GenBank accession no. KM28867). Known isolates/strains carrying haplotypes I–V are shown in bold. Novel residues are highlighted. Representative isolates are in parentheses. Demarcations of exons are indicated by dashes.

TABLE 2

Frequencies of amino acid variants in *Pfcrt* of Thai isolates

Province-year (*n*)	N75D	K76I	F78L	H97R	H97L	A144F	G147D	L148I	F172L	I174M	I194T	I218F	A220P	A320T	T333S	T333I	I337N	I351V
Trat-1991 (*N* = 19)	–	–	–	–	15.8%	–	–	–	–	–	–	–	–	–	–	–	–	–
Trat-2003 (*N* = 13)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ranong-2003 (*N* = 1)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trat-2004 (*N* = 2)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ranong-2004 (*N* = 10)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trat-2005 (*N* = 2)	50%	–	–	–	–	50%	–	50%	–	–	50%	–	–	–	–	–	–	–
Tak-2006 (*N* = 14)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ranong-2006 (*N* = 2)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tak-2008 (*N* = 14)	–	–	–	–	–	–	–	–	–	7.1%	–	–	–	–	–	–	–	–
Ubon-2008 (5)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Yala-2008 (*N* = 3)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Narathiwat-2008 (*N* = 7)	–	–	–	–	–	–	–	–	–	–	–	–	14.3%	–	–	–	–	–
Tak-2009 (*N* = 3)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chantaburi-2009 (*N* = 6)	–	–	–	–	16.7%	–	–	–	–	–	–	–	–	–	–	16.7%	–	–
Tak-2010 (*N* = 21)	–	–	–	–	–	–	–	–	4.8%	–	–	–	–	–	–	–	4.8%	–
Tak-2011 (*N* = 22)	–	–	4.5%	–	–	–	–	–	–	–	–	–	–	4.5%	–	–	–	4.5%
Ubon-2012 (*N* = 1)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tak-2013 (*N* = 10)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ubon-2014 (*N*-10)	20%	–	–	10%	–	20%	–	20%	10%	–	20%	10%	–	–	20%	–	–	–
Ubon-2015 (*N* = 10)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Yala-2015 (*N* = 8)	–	12.5%	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Yala-2016 (*N* = 5)	20%	–	–	20%	–	20%	–	20%	20%	–	20%	20%	–	–	20%	–	–	–
Ubon-2016 (*N* = 1)	–	–	–	–	–	–	100%	–	–	–	–	–	–	–	–	–	–	–
Total (*N* = 189)	2.1%	0.5%	0.5%	1.1%	0.5%	2.1%	0.5%	2.1%	1.6%	0.5%	2.1%	1.1%	0.5%	0.5%	1.6%	0.5%	0.5%	0.5%

Pfcrt = *Plasmodium falciparum* chloroquine resistance transporter; Ubon = Ubon Ratchathani. Amino acid variants are those differed from the *Pfcrt* sequences of either the Dd2 or the 3D7 clones (GenBank accession nos. AF030694 and KM288867, respectively).

with the 2-day ACT group ($P < 0.05$, randomization test). By contrast, there was no significant difference in the number of haplotypes of *Pfdhfr*, *Pfmsp-1*, and *Pfmsp-2* between these groups (Supplemental Table 3). The distribution of *Pfcrt* haplotypes was more skewed toward few haplotypes in the pre-3-day ACT than in the 3-day ACT groups as viewed from haplotype diversity values of total samples. The haplotype diversity in *Pfdhfr*, *Pfmsp-1*, and *Pfmsp-2* of the corresponding populations was almost comparable (Supplemental Table 3). The number of mutations and nucleotide diversity of *Pfcrt* in the 3-day ACT group exceeded those in the pre-3-day ACT group. Nonsynonymous nucleotide diversity consistently outnumbered synonymous nucleotide diversity for all comparisons shown in Table 3.

Domain-specific selection in *Pfcrt*. A signature of positive selection in *Pfcrt* was evidenced by a significantly greater d_N (0.0036 ± 0.0009) than d_S (0.0010 ± 0.0006) ($P < 0.05$). The transmembrane domains of PfCRT reportedly dictate the transport of chloroquine and other charged molecules across parasite's acid vesicles, thereby affecting drug accumulation and heme detoxification process.[3] Hence, we analyzed nucleotide diversity for the putative transmembrane domain-encoding regions containing 199 codons and compared with that for the remainders of the coding regions spanning 225 codons. The mean nucleotide diversity for the transmembrane domains was 7-fold greater than that for the remaining regions and the difference was statistically significant ($P < 0.05$) (Table 4). The nucleotide diversity at nonsynonymous sites (π_N) of 189 isolates significantly outnumbered that at synonymous sites (π_S) for transmembrane domains ($P < 0.05$), but not for the remaining regions, and occurred only in the 3-day ACT group. There was no significant difference in π_S between these groups. Meanwhile, Tajima's *D* statistics yielded significantly negative values (Table 5). Importantly, significant negative Tajima's *D* values were observed at nonsynonymous sites but not at synonymous sites. Meanwhile, no evidence of recombination was discernible in *Pfcrt* by all tests analyzed.

DISCUSSION

The complete *Pfcrt* coding sequences of 198 isolates collected over two decades from seven endemic provinces contained 21 distinct genotypes, 13 of which were novel. Together with previous reports, at least 67 naturally occurring *Pfcrt* haplotypes have been documented.[7,8] Haplotypes I–III in this study were previously detected among Thai isolates collected over three decades ago, suggesting temporal stability of these haplotypes in this country. Likewise, identical sequences of isolate Cam734 from Cambodia collected a decade ago and haplotype IV, and isolate GB4 from Ghana and haplotype V could imply local and intercontinental spread of chloroquine-resistant *P. falciparum*. Spatiotemporal persistence of particular *Pfcrt* haplotypes has suggested adaptive evolutionary success of parasites carrying these variants in response to antimalarial drug pressure across endemic areas.

Twelve novel amino acid substitutions identified in this study have contributed to a total of 34 naturally occurring amino acid substitution sites in PfCRT so far identified. Most novel amino acid substitutions were found in single isolates and were not confined to particular endemic areas. Therefore, it is likely that mutations in *Pfcrt* could have arisen as independent events, probably as a result of the stochastic nature of a de novo point mutation. The significance of these novel mutations in PfCRT remains to be elucidated. However, a mutation at K76I in *Pfcrt* of two laboratory lines (J3D4 and $C5^{K76I}$) induced by in vitro chloroquine selective pressure has been observed in isolate YL2823 from Yala collected in 2015.[20] Although mutations in *Pfcrt* could have arisen initially from a stochastic mutational process, the shared mutations between a clinical isolate and drug-induced laboratory clones could suggest a common evolutionary pathway exerted by antimalarial selective pressure.

The interplay between antimalarial drug treatment regimens and genetics of malaria parasites has been documented. A timely withdrawal of chloroquine as a standard treatment of falciparum malaria has resulted in reemergence and expansion of chloroquine-sensitive parasites that still remain in the containment areas.[37] By contrast, an increase in drug pressure could enhance the emergence of drug resistance genotypes as nationwide modification of ACT from 2-day to 3-day regimens in Thailand gave rise to a remarkable increase in the prevalence of artemisinin resistance genotypes. Several lines of evidence have suggested that mutations in *Pfcrt* can modulate susceptibility of artemisinin and some partner drugs used in ACT such as piperaquine, mefloquine, lumefantrine, and amodiaquine.[6,21] Importantly, some of these compounds share with chloroquine a quinolone scaffold and affect heme detoxification process of the parasites.[3] This in turn suggests that some antimalarials other than chloroquine could mediate selective pressure on *Pfcrt* as evidenced by the emergence of *Pfcrt* mutations induced by stepwise selection with amantadine or halofantrine.[20] Therefore, cessation of chloroquine

TABLE 3

Nucleotide diversity in *Plasmodium falciparum* chloroquine resistance transporter of *Plasmodium falciparum* populations in relation to sample collection period

Population bordering	Year	*n*	M	*h*	$\pi \pm SE$ ($\times 10^{-3}$)	$\pi_S \pm SE$ ($\times 10^{-3}$)	$\pi_N \pm SE$ ($\times 10^{-3}$)
Myanmar	1991–2008	41	10	2	0.038 ± 0.037	0.000 ± 0.000	0.049 ± 0.045
	2009–2016	56	15	7	0.197 ± 0.074	0.131 ± 0.127	0.215 ± 0.084
Cambodia	1991–2008	41	15	4	0.340 ± 0.140	0.179 ± 0.166	0.384 ± 0.180
	2009–2016	28	20	9	1.488 ± 0.392##	0.000 ± 0.000	1.896 ± 0.571*
Malaysia	1991–2008	10	10	2	0.157 ± 0.154	0.000 ± 0.000	0.200 ± 0.190
	2009–2016	13	19	5	1.701 ± 0.409###	0.565 ± 0.537	2.013 ± 0.572***
Total	1991–2008	92	17	6	0.187 ± 0.071	0.080 ± 0.078	0.217 ± 0.087
	2009–2016	97	27	17¶	0.780 ± 0.228#	0.151 ± 0.106	0.951 ± 0.255**

h = number of haplotypes; M = number of mutations relative to the 3D7 coding regions; π = nucleotide diversity; π_S = nucleotide diversity at synonymous sites; π_N = nucleotide diversity at nonsynonymous sites. Test of significant difference between π for population during 1991 and 2008 and those during 2009 and 2016: #$P < 0.05$; ##$P < 0.01$; ###$P < 0.005$. Test of significant difference between π_N for population during 1991 and 2008 and those during 2009 and 2016: *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$. Randomization test of the hypothesis that the number of haplotypes for the population during 1991 and 2008 equals those during 2009 and 2016: ¶$P < 0.05$.

TABLE 4

Nucleotide diversity (π) at synonymous sites (π_S) and nonsynonymous sites (π_N) of the *Pfcrt* coding sequences of Thai isolates collected during 1991 and 2016

Collection period/*Pfcrt* domain	π ($\times 10^{-3}$)	π_S ($\times 10^{-3}$)	π_N ($\times 10^{-3}$)
1991–2008 ($N = 92$)			
All coding region	0.187 ± 0.071	0.080 ± 0.078	0.217 ± 0.084
Transmembrane domains	0.363 ± 0.154♦	0.162 ± 0.163	0.422 ± 0.187
Remaining regions	0.032 ± 0.032	0.000 ± 0.000	0.041 ± 0.039
2009–2016 ($N = 97$)			
All coding region	0.780 ± 0.228•	0.151 ± 0.102	0.951 ± 0.245##**
Transmembrane domains	1.427 ± 0.431♦	0.307 ± 0.210	1.753 ± 0.523#*
Remaining regions	0.211 ± 0.126	0.000 ± 0.000	0.265 ± 0.159
All			
All coding region	0.494 ± 0.128	0.117 ± 0.067	0.597 ± 0.160#
Transmembrane domains	0.915 ± 0.253♦	0.236 ± 0.134	1.113 ± 0.330#
Remaining regions	0.124 ± 0.076	0.000 ± 0.000	0.156 ± 0.094

Pfcrt = *Plasmodium falciparum* chloroquine resistance transporter. Test of the hypothesis that mean π for transmembrane domains equals that for the remaining regions: ♦$P < 0.05$. Test of the hypothesis that mean π for all coding regions of population during 1991 and 2008 equals the corresponding value of population during 2009 and 2016: •$P < 0.05$. Test of the hypothesis that mean π_S equals the corresponding mean π_N: #$P < 0.05$; ##$P < 0.01$. Test of the hypothesis that mean π_N for population during 1991 and 2008 equals mean π_N for population during 2009 and 2016: *$P < 0.05$; **$P < 0.01$.

usage against falciparum malaria may not prevent ongoing selection in the *Pfcrt* locus because ACT may influence the evolution of this gene. A significant increase in the number of haplotypes and nonsynonymous mutations in *Pfcrt* across malaria-endemic areas in Thailand after nationwide change in ACT to 3-day regimen could be exerted by an increase in intensity of drug pressure from either increased duration of artesunate administration or long-term use and cumulative effect of mefloquine. This phenomenon occurred neither by chance nor by population process because no significant change in the number of haplotypes was observed in other unrelated genetic loci (*Pfdhfr*, *Pfmsp-1*, and *Pfmsp-2*), whereas gene flow was limited across endemic areas. Furthermore, intragenic recombination seems not to play an important role in generating sequence variation in *Pfcrt* among samples analyzed.

Natural selection exerted by antimalarial drugs could have influenced sequence variation in genes conferring drug resistance.[12,38] Our study has shown that positive Darwinian selection favoring amino acid variants in *Pfcrt* was evidenced in the 3-day ACT group but not in isolates collected previously, suggesting that selective pressure has been triggered by an increase in duration of treatment regimen. Evidence of positive selection occurred exclusively in the transmembrane domains of PfCRT for the 3-day ACT group (Table 3). Tajima's *D* test for nonsynonymous sites in *Pfcrt* yielded significant deviation from zero in negative direction, whereas a nonsignificant *D* value was found at synonymous sites (Table 4). The absence of recombination event along with the low level of gene (haplotype) diversity in the *Pfcrt* locus and the excess of low-frequency variants at nonsynonymous sites (strong negative Tajima's *D* value) are consistent with the presence of slightly deleterious mutations in coding regions of this gene. In fact, variation in the growth rate of *Pfcrt* mutants has been documented among different haplotypes.[5] Therefore, slightly deleterious mutations in *Pfcrt* will be subject to ongoing purifying selection which will either eliminate the change from the population or drive them to low frequencies.[38] On the other hand, some compensatory mutations seem to be required to restore the fitness of some mutants. For example, the Cam734 haplotype (haplotype IV in this study) reportedly acquired the mutations A144F, I148L, and S333T with dual chloroquine resistance phenotype and compensatory restoration of fitness.[6] A prolonged bottleneck effect or selective sweeps on the *Pfcrt* locus could occur during several decades of chloroquine selective pressure in Thailand as evidenced by analysis of flanking microsatellite loci.[39] Several lines of evidence have supported the prediction that slightly deleterious variants will accumulate in a species that has undergone a severe bottleneck or in cases where recombination is reduced or absent.[40,41] A number of singleton substitutions and the presence of synonymous polymorphism in *Pfcrt* of Thai isolates could have arisen from genetic drift, whereas the emergence of slightly deleterious mutations is a transient feature of evolution and these nonsynonymous mutations are subject to be eliminated by ongoing purifying selection. Importantly, integrated malaria control strategies in Thailand other than antimalarial treatment policy during the sample collection period were unchanged. Taken together, it is likely that an increase in duration of artesunate as a 3-day ACT regimen could provoke

TABLE 5

Genetic diversity in *Plasmodium falciparum* chloroquine resistance transporter of *Plasmodium falciparum* populations in Thailand

	Sampling period		Endemic areas bordering			
Population	1991–2008	2009–2016	Myanmar	Cambodia	Malaysia	Total
N	92	97	97	69	23	189
No. haplotypes	6	17	8	11	6	21
No. polymorphic sites	9	22	8	16	15	25
No. segregating sites	9	20	8	14	15	23
Tajima's *D*	–2.187###	–2.264###	–2.178###	–2.026#	–2.423###	–2.376###
D (synonymous sites)	–1.037	–1.382	–1.031	–1.069	–1.161	–1.516
D (nonsynonymous sites)	–2.119#	–2.198###	–2.105#	–1.972#	–2.403###	–2.302###

#, ##, and ### denote significant values at $P < 0.05$, $P < 0.02$, and $P < 0.01$, respectively.

further selective pressure on the *Pfcrt* locus among *P. falciparum* isolates in Thailand. The impact of these novel haplotypes on parasite growth rate and drug susceptibility status would require further elucidation such as by transfection-based approaches.

Received May 11, 2018. Accepted for publication July 5, 2018.

Published online August 20, 2018.

Note: Supplemental materials, figures, and tables appear at www.ajtmh.org.

Acknowledgments: We are grateful to all patients who donated their blood samples for this study. We thank the staff of the Bureau of Vector Borne Disease, Department of Disease Control, Ministry of Public Health, Thailand, for assistance in field work.

Financial support: The Thai Government Research Budgets (GRB-APS-12593011 and GBA-600093004) and Chulalongkorn University Research Grants (Fiscal Years 2006–2008) to S. J., C. P., and S. S.; The Thailand Research Fund (RSA5980054) to C. P.; and Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University (RA60/109) to C. P., S. J., and P. B.

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Tropical Biomedicine 35(4): 861–871 (2018)

Episodic positive selection in the Cam734 haplotype and low prevalence of the A144F mutation in *Plasmodium falciparum* chloroquine resistance transporter gene among Thai isolates

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Received 8 June 2018; received in revised form 18 July 2018; accepted 26 July 2018

Abstract. Chloroquine resistance transporter of *Plasmodium falciparum* (PfCRT) is a food vacuolar transmembrane protein that mediates susceptibility of the parasite to chloroquine. A mutation at K76T of the *Pfcrt* gene is a key determinant for chloroquine resistance phenotype. In the absence of drug pressure, *in vitro* growth rate of chloroquine-resistance parasites was outcompeted by wild-type parasites unless intragenic compensatory mutations occurred. Chloroquine-resistant *P. falciparum* bearing the Cam734 haplotype known to circulate in endemic areas of Cambodia bordering Thailand contains 9 mutations in *Pfcrt* and exhibits both chloroquine resistance and comparable growth rate to the chloroquine-sensitive 3D7 strain. To analyze the evolution of the Cam734 haplotype, codon-based analysis was performed by using the mixed effects model of evolution (MEME), branch-site random effects likelihood (BR-REL) and other related methods. Results revealed that the Cam734 haplotype has evolved distinctively from other known mutant haplotypes including the most common Dd2 haplotype in Southeast Asia. Evidence of episodic positive selection was detected at codon 144, characterized by c.[430G>T; 431C>T] (p.A144F), known to be indispensable for both chloroquine resistance and restoration of growth rate of the parasites. To survey the prevalence of mutations at codons 76 and 144 in *Pfcrt* among Thai isolates, restriction fragment analysis of 548 *P. falciparum* isolates collected from six endemic provinces of Thailand during 1991 and 2016 was performed. The 144F *Pfcrt* mutant was detected in 7 (1.28%) isolates. All Thai isolates analyzed herein harbored a mutation at codon 76 whilst the wild-type parasite was not found. The low prevalence of isolates bearing the mutation 144F in PfCRT could imply little or lack of survival advantage of this mutant in endemic areas of Thailand where the wild-type parasites seem to be absent or extremely rare.

INTRODUCTION

Chloroquine is a 4-aminoquinoline compound first introduced as an antimalarial agent active against asexual blood stages of malaria parasites in 1945 and was served as the frontline of treatment for all human malaria species for several decades (Wernsdorfer, 1991). It was not until the late 1950s and early 1960s that chloroquine-resistant *Plasmodium falciparum* was reported independently at Thailand-Cambodia border and a few scattered foci in Central America. Recent genetic analysis has suggested at least four distinct geographic origins for the emergence of drug resistant parasites (Awasthi & Das, 2013; Mita *et al.*, 2016). To date, chloroquine-resistance *P. falciparum* occupies the majority of malaria endemic areas.

Chloroquine resistance phenotype of *P. falciparum* is characterized by a higher rate of chloroquine efflux from the parasite's digestive vacuoles than the chloroquine-susceptible one, leading to a suboptimal level of chloroquine remaining inside the acidic vacuoles where heme detoxification takes place (Ecker *et al*., 2012; Summers *et al*., 2012). The leading candidate of protein conferring chloroquine resistance in *P. falciparum* is a 48.6 kDa transmembrane protein located on the digestive vacuolar membrane, designated chloroquine resistance transporter (PfCRT)(Fidock *et al*., 2000). PfCRT consists of 424 amino acids, encoded by a single copy 13-exon gene known as *Pfcrt*. A point mutation at codon 76 (K76T) is considered to be a key determinant of chloroquine resistance that has been ascertained in a number of laboratory and field studies (Ecker *et al*., 2012). Therefore, most of the molecular epidemiological reports on *Pfcrt* genotypes have been confined to sequence variation in exon 2. Importantly, recent evidences have suggested that the hitherto mutation (K76T) *per se* is not the sole predictor of drug resistance while specific substitutions beyond codon 76 could modulate susceptibility status of the parasites to other antimalarials believed to interfere with heme detoxification (Ecker *et al*., 2012).

Pfcrt has evolved not without fitness cost. In the absence of drug pressure, the chloroquine-resistance parasites were outcompeted in terms of growth rate by the wild-type parasites upon *in vitro* co-cultivation (Rosenthal, 2012). Consistently, withdrawal of chloroquine as a standard treatment regimen for falciparum malaria in endemic areas of Kenya (Kiarie, 2015) and Malawi (Kublin, 2003) has resulted in reemergence and expansion of the remaining chloroquine-sensitive parasites in the containment areas. It is noteworthy that *P. falciparum* bearing different mutant *Pfcrt* haplotypes seem to display differential growth disadvantage in the absence of drug pressure (Sa & Twu, 2010). Interestingly, the Cam734 haplotype, characterized by M74I, N75D, K76T, A144F, L148I, I194T, A220S, Q271E and T333S (Durrand *et al*., 2004), conferred chloroquine resistance status while the growth rate of parasites bearing these mutations was comparable to the wild-type parasites, e.g. the 3D7 strain (Petersen *et al*., 2015).

Meanwhile, the Cam734 haplotype displayed comparatively higher number of nucleotide substitutions than other *Pfcrt* mutants. Whether antimalarial drug pressure on individual mutant codons in this haplotype occurred constantly (pervasive positive selection) or instantaneously and changed over time (episodic or past positive selection) remains to be elucidated. The objectives of this study are (i) to explore the prevalence of mutation at codon 76 in *Pfcrt* among *P. falciparum* isolates collected from diverse malaria endemic areas of Thailand during 1991 and 2016, (ii) to identify specific codons in the Cam734 *Pfcrt* haplotype that have evolved under episodic positive selection and (iii) to determine the prevalence and geographic distribution of *P. falciparum* isolates bearing the Cam734 haplotype in Thailand.

MATERIALS AND METHODS

Ethics Statement

Th ethical issues of this study were reviewed and approved by the Institutional Review Board on Human Research of Faculty of Medicine, Chulalongkorn University (IRB No.257/57). Inform consent was obtained from all participatants or their guardians prior to blood sample collection.

Parasite populations

Blood samples (~1 ml) were obtained from falciparum malaria patients who attended malaria clinics or district hospitals in endemic areas. Diagnosis of *P. falciparum* infections was done by Giemsa-stained thin and thick blood smears. In total, 548 *P. falciparum*-infected blood samples were collected from 5 endemic provinces of Thailand including Tak (n = 200), Ubon Ratchathani (n = 160), Chantaburi (n = 48), Yala (n = 81), Narathiwat (n = 34) and Trat (n = 25) collected during 1991 and 2016. Fresh blood sample from each patient was spotted onto chromatography-grade filter paper

(ET31CHR; Whatman, Madison, UK) and the remaining volume was preserved in EDTA anticoagulant.

DNA extraction and PCR-based detection of Plasmodium species

Genomic DNA was extracted from 200 mL of each blood sample by using QIAamp DNA mini kit (Qiagen, Hilden, Germany) per the manufacturer's recommendation and kept at -40°C until use. *P. falciparum* was reaffirmed by species-specific PCR targeting the mitochondrial cytochrome *b* gene as described previously (Putaporntip *et al.*, 2010). Genomic DNA of known species of human malaria parasites including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* were used as positive control.

Analysis of mutations at codons 76 and 144 of *Pfcrt* by PCR-RFLP

The *Pfcrt* gene fragment spanning exons 2 and 3 was amplified by PCR using the forward primer CRT1F (5'-TTGTCGACC TTAACAGATGGCTCAC-3')(Djimde *et al.*, 2001) and the reverse primer CRT0R (5'-TCCGAGATAATTGTATAAGTGATATC-3'). PCR amplification was done in a total volume of 30 µL containing PCR buffer, 200 µM dNTP, 0.2 µM of each primer, nuclease free water, 2 µL of template DNA and 1.25 units of *ExTaq* DNA polymerase (Takara, Seta, Japan). The thermal cycler profiles consisted of a preamplification denaturation at 94°C for 1 min, followed by 35 cycles of 94ºC for 40 s, 56ºC for 30 s, and 72ºC for 1.5 min. Two microlitres of the PCR products were used as template for subsequent PCR amplification of exon 2 using the same amplification conditions except that the primers were replaced with CRT2F (5'-CTTGTCTTGGT AAATGTGCTC-3') and CRT2R (5'-GAACA TAATCATACAAATAAAGT-3') as described previously (Djimde *et al.*, 2001). Likewise, amplification of exon 3 was carried out with primers PFCRTE3-F (5'-TATTTA TTTCTTATGACCTTTTTAGGAACG-3') and PFCRTE3-R (5'-GTAATTTAAAATAGTATA CTTACCTATATC-3') using the same PCR condition. After purification with QIAquick PCR purification kit (Qiagen, Hilden, Germany), the PCR products spanning exon 2 were digested with *Apo* I for detection of the wild-type codon 76 (AAA for lysine) of *Pfcrt*; therefore, indigestible products indicated chloroquine-resistance mutants. Meanwhile, the recognition site of *Hae* III restriction endonuclease (GG|CC) occurred at codon 144 in which digestible PCR products encompassing exon 3 indicated the presence of wild-type codon (GCC for alanine); therefore, uncut products indicated mutation at this codon. The digested PCR products were analyzed by using 1.5% agarose gel electrophoresis.

RNA extraction, RT-PCR amplification and sequencing of Pfcrt

Dry blood spot was excised from the filter paper and cut into small pieces with sterile scissors that had been flamed to eliminate RNase prior to use. Total RNA was extracted from the blood sample using QIAamp RNA blood minikit (Qiagen, Hilden, Germany) following the recommendations provided by the manufacturer. Synthesis of cDNA was obtained from 2 mL of RNA sample using TaKARa RNA PCR (AMV) version 3.0 kit (TaKaRa, Japan) in a total volume of 10 mL. Two microlitres of cDNA product were used as template for nested PCR amplification of the complete coding region of *Pfcrt*. The outer PCR primers were PFCRTF (5'-CATATAACAAAATGAAATTCGC-3') and PFCRTR (5'-TTATTGTGTAATAATTGAAT CGACG-3') and the inner primers were PFCRTF1 (5'-TCAAGCAAAAATGACGA GCG-3') and PFCRTR1 (5'-ACGTTGGTTAA TTCTCCTTC-3'). PCR amplification was performed in a total volume of 30 µL containing PCR buffer, 200 µM dNTP, 0.2 µM of each primer, nuclease free water, 2 µL of template DNA and 1.25 units of ExTaq DNA polymerase (Takara, Seta, Japan). The amplification profiles for both primary and nested PCR were essentially the same, consisting of pre-amplification denaturation at 94ºC, 60 s and 30 cycles of 94ºC, 30 s; 50ºC, 30 s and 72ºC, 2 min; followed by 72ºC, 5 min. DNA amplification was performed by using a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). The PCR products were analyzed on 1% agarose gel electrophoresis, stained with

ethidium bromide and visualized under UV transillumination. After purification with QIAquick PCR purification kit (Qiagen, Hilden, Germany), the PCR product was used as template for direct sequencing from both directions using ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems) and sequencing primers.

Statistical analysis

Despite a number of reports on mutations in PfCRT, complete or near complete nucleotide sequences of this protein were limited in the GenBank database. In this study, we retrieved 13 distinct *Pfcrt* sequences of natural *P. falciparum* isolates and 4 distinct sequences from the Cambodian isolates reported previously (Durrand *et al.*, 2004). The CLUSTAL_X program was deployed for alignment of the complete or near complete *Pfcrt* sequences. Geographic origins, GenBank accession numbers and amino acid substitutions of *Pfcrt* used in this study were listed in Table 1. Phylogenetic tree was constructed by the Maximum Likelihood method based on the Tamura 3-parameter model that gave the lowest Bayesian Information Criterion (BIC) score. The reliability of the tree was determined by the bootstrap method implemented the MEGA 6.0 software (Tamura *et al.*, 2013). Detection of selection on specific codons was performed by estimation of the global ratio of the rate of non-synonymous to synonymous substitutions (dN/dS or ω value) across the *Pfcrt* gene using various models. The fixed effects likelihood (FEL), random effects likelihood (REL), fast unconstrained Bayesian approximation (FUBAR), branch site REL analysis (BR-REL) and mixed effects model of evolution (MEME) methods were used for analysis (Pond & Frost, 2005). FEL model compares the ratio of nonsynonymous to synonymous substitution on a site-by-site basis, without assuming an *a priori*

Table 1. The *Pfcrt* sequences of *P. falciparum* isolates used in this study

Isolate/ strain	Origin	Amino acid substitutions 1111122233333 777792446902723357 245673480450163461	GenBank accession no./references
3D7	Africa?	CMNKHHALLITAQNTSIR	KM288867
Dd2	Thailand	.IET.......SES..TI	AF030694
TM6	Thailand	.IET.R....ASES...I	AF468006
1061	Sudan	.IE........SES...I	AF233065
GM4	Ghana	.IET.......SE....I	HM854027
7G8	Brazil	S..T.......S.D..L.	AF233067
DIV30	Brazil	S..T.......S.D..L.	AF233064
TA6182	Colombia	..ETQ......S..S..I	DQ156109
TA7519	Colombia	..ETQ......S.....T	DQ156107
TU741	Colombia	...T.......S.D.NL.	DQ156108
PH1	Philippines	...T..T.Y....D....	AY254700
FVO	Vietnam	.IET.......SES...I	DQ533840
2300	Indonesia	.IKT.......SES...I	AY651315
Cam734	Cambodia	.IDT..FI.T.SE.S...	Durrand *et al.*, 20
Cam738	Cambodia	.IDT...I.T.SE.S...	Durrand *et al.*, 20
Cam742	Cambodia	.IET.......SE....I	Durrand *et al.*, 20
Cam783	Cambodia	.IET.......SE...TI	Durrand *et al.*, 20

Dots are identical residues.

distribution of rates across sites whereas REL model first fits a distribution of rates across sites and then infers the substitution rate for individual sites. MEME algorithm detects codons under episodic positive selection unmasked by the abundance of purifying selection along the lineages. Significance level settings for FEL, iFEL, REL and MEME were p values < 0.1 and Bayes Factor > 1000 for REL followed the default values available on the Datamonkey Web Server (Pond & Frost, 2005). Searching for natural selection based on the measurement of selective influences on 31 structural and biochemical amino acid properties during cladogenesis was performed by using the TreeSAAP program. Amino acid under positive selection was analyzed by performing goodness-of-fit and categorical statistical tests (Woolley *et al.*, 2003).

RESULTS

Phylogeny and codon-specific selection in Cam734 and other Pfcrt haplotypes

Alignment of 17 *Pfcrt* sequences has revealed 19 nucleotide substitutions, resulting in 18 amino acid changes. Codon-based analysis of departure from neutrality has identified 13 positively selected sites based on positive results by at least one method. Most of these codons (77%) were located in the transmembrane domains (Table 2). Test of positive selection implemented in the TreeSAAP program has detected 10 amino acid substitutions with significant alteration in physicochemical properties (p<0.001). Taken together, 11 codons were under positive selection based on a consensus of concordant results from ≥ 2 tests. Meanwhile, only 9 positively selected codons gave concordant positive results from ≥ 3 tests (Table 2).

The fingerprint of episodic or past positive selection was found in the Cam734 haplotype by both the mixed effects model of evolution (MEME) and the branch-site random effects likelihood (BR-REL) methods in which substitution at codon 144 yielded statistically significant tests (p<0.05). The Cam734 lineage could have experienced a short burst of adaptive evolution, resulting in a mutation A144F (Figure 1).

P. falciparum populations

Of 548 *P. falciparum*-infected individuals diagnosed by microscopy in this study, 353 (64.4%) were males and 195 (35.6%) females. The population distribution by age ranged from 4 to 67 years old (mean = 25 years). No apparent severe manifestation of malaria was observed during blood sample collection. The duration of self-reported febrile illness prior to seeking medical diagnosis ranged from 1 to 7 days, of which

Table 2. Positive selection on amino acid substitutions in *Pfcrt* of worldwide isolates

Codon	Test method					Consensus	
	FEL	iFEL	REL	FUBAR	TreeSAAP	≥2 tests	≥3 tests
74*			✓				
75*			✓	✓	✓	✓	✓
76*			✓	✓	✓	✓	✓
97*			✓	✓	✓	✓	✓
144*		✓	✓	✓	✓	✓	✓
148			✓		✓	✓	
194*			✓		✓	✓	
220*			✓	✓	✓	✓	✓
271			✓				
326*		✓	✓	✓		✓	✓
333*			✓	✓	✓	✓	✓
356*			✓	✓	✓	✓	✓
371			✓	✓	✓	✓	✓

* Residues in transmembrane domains.

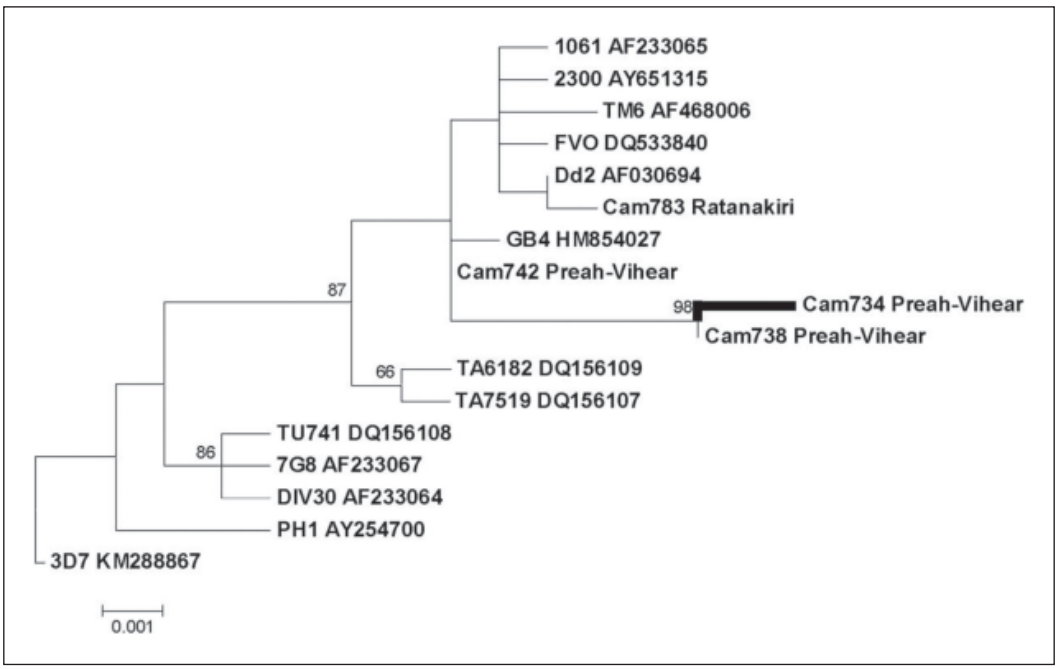

Figure 1. Maximum likelihood tree of the *Pfcrt* sequences among Thai isolates. Thick line indicates branch under episodic positive selection detected by branch-site random effects likelihood (BR-REL) analysis. The tree is rooted by using the 3D7 haplotype. Scale bar represents nucleotide substitutions per site.

Table 3. Chronological and geographic distribution of *P. falciparum*-infected blood samples

Province	Year of sample collection (n)	Total
Tak	1995 (13), 1996 (26), 1997 (8), 2003 (14), 2004 (11), 2005 (5), 2006 (14), 2008 (13), 2009 (3), 2010 (20), 2011 (21), 2012 (16), 2013 (36)	200
Ubon Ratchathani	2008 (10), 2014 (123), 2015 (27)	160
Chanthaburi	2007 (24), 2009 (21), 2010 (3)	48
Yala	2007 (19), 2008 (26), 2015 (17), 2016 (19)	81
Narathiwat	2008 (34)	34
Trat	1991 (25)	25
Total	1991-2016	548

308 (56.2%) patients had fever for 1 to 3 days. No previous self-administration of antimalarial drugs was reported among the study populations. The chronological distribution of samples by geographic origins is listed in Table 3. PCR analysis reaffirmed that all blood samples were infected with *P. falciparum*. However, co-infections of *P. falciparum* and *P. vivax* were detected in 15 patients (2.74%).

Analysis of mutation at codon 76 in *Pfcrt*

The *Pfcrt* fragment encompassing exon 2 could be amplified from genomic DNA of all isolates examined. The resulting PCR products contained 200 bp without size variation among isolates. For *Apo* I restriction endonuclease analysis, the recognition site is 5'-R|AATT|Y-3' where R represents A or G and Y can be either C or T. The wild-type codon 76 coding for lysine

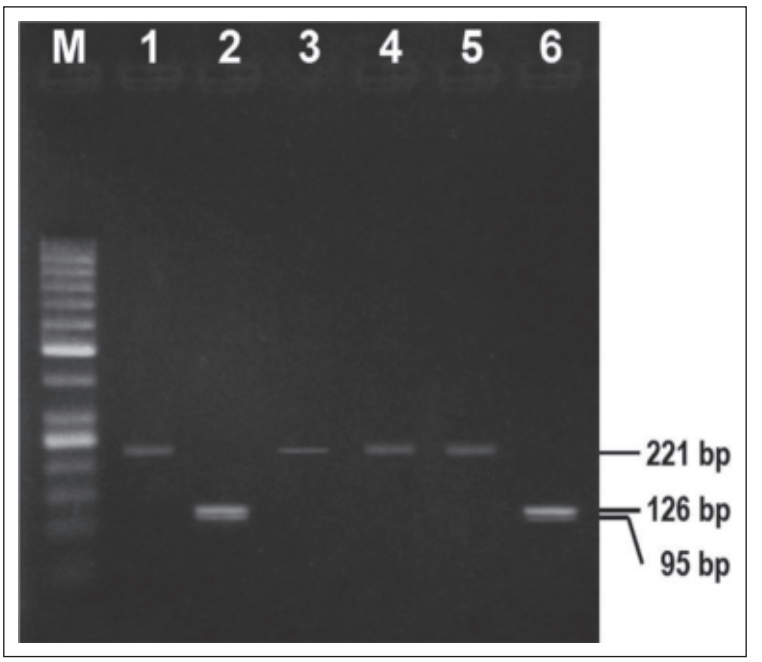

Figure 2. Agarose gel electrophoresis of representative PCR products spanning exon 3 of *Pfcrt* digested with *Hae* III. M denotes 50-bp ladder marker. Sample numbers 2 and 6 contained *Hae* III restriction site.

(codon AAA) and its adjacent codons 75 and 77 provide a restriction site for this enzyme. Therefore, the PCR amplified fragments that were digestible with *Apo* I, producing 2 DNA fragments of 112 and 88 bp, indicate the presence of codon for lysine at position 76, the key residue for chloroquine-sensitive parasites. On the other hand, if mutation(s) occurs at codon 76, the restriction site for *Apo* I would be disrupted; thereby the PCR products remained uncut with this restriction enzyme. Although the intact PCR fragments after *Apo* I digestion indicate nonspecific nucleotide substitution(s) at codon 76, previous sequencing analysis has shown that almost all nucleotide substitutions at this codon were due to A → C change, resulting in a change in amino acid from lysine to threonine. The PCR products from all 548 isolates in this study were indigestible by *Apo* I. Hence, no evidence of chloroquine-sensitive parasites was detected in the study populations.

Analysis of mutation at codon 144 in Pfcrt

The 221-bp PCR product spanning exon 3 of *Pfcrt* could be amplified from all 548 isolates. The wild-type codon 144 could be digestible with *Hae* III generating 95 bp and 126 bp fragments (Figure 2). Analysis of mutation at codon 144 has revealed that 7 of 548 (1.28%) isolates were indigestible with *Hae* III, indicating the absence of GGCC at nucleotides 429-431. These isolates were collected from Trat province in 1991 (n = 1), Tak province in 1995 (n = 2), Chanthaburi province in 2007 (n = 1) and Ubon Ratchathani province in 2014 (n = 3). The complete *Pfcrt* sequences of these isolates lacking *Hae*III resistriction site revealed perfect sequence identity with the Cam734 haplotype, characterized by mutations at c.222G>T (p.M74I), c.223A>G (p.N75D), c.227A>C (K76T), c430G>T;431C>T (A144F), c.442C>A (p.L148I), c.581T>C (p.I194T), c.658G>T(p.A220S), c.811C>G (p.Q271E) and c.998C>G (p.T333S).

DISCUSSION

In Thailand, *P. falciparum* isolates collected during the past two decades exhibited chloroquine resistance phenotype while previous analysis of the key mutation at codon 76 of the *Pfcrt* gene failed to detect the wild-type codon (Chaicharoenkul *et al.*, 2011; Parker *et al.*, 2012; Takahashi *et al.*, 2012). Our analysis of mutation at codon 76 of *Pfcrt* among 548 isolates collected during 1991 and 2016 from six endemic provinces of the country was in line with these previous studies. Therefore, nationwide withdrawal of chloroquine for the treatment of falciparum malaria during the past 4 decades has no discernible impact on drug resistance status of *P. falciparum* in this country (Wongsrichanalai *et al.*, 2002). It is likely that *P. falciparum* bearing drug resistance phenotypes, which could have arisen as a new allelic variant or which could have circulated in the population at low prevalence, became populated and eventually reached fixation because of survival advantage following substantial chloroquine pressure. Intriguingly, about 10.8% of microscopically diagnosed *P. vivax* infections were co-infected with submicroscopic *P. falciparum* based on PCR detection and were treated with chloroquine (Jongwutiwes *et al.*, 2011). Therefore, selective pressure from chloroquine on subpopulations of *P. falciparum* could remain in Thailand, especially along Myanmar border where mixed species infections were prevalent (~23% to ~24%)(Putaporntip *et al.*, 2009). Importantly, coexistence of *P. falciparum* and *P. vivax* has been identified in all malaria endemic areas of Thailand. Therefore, it is likely that *P. falciparum* in infected mosquito vectors could have the possibility of exposure to chloroquine upon taking blood meals from individuals infected with *P. vivax* who were treated with chloroquine. Furthermore, the long half-life of chloroquine and its metabolites, lasting for 45 to 55 days and 59 to 67 days, respectively (Gustafsson *et al.*, 1987), in the circulation of chloroquine-treated patients would exert selective pressure on *P. falciparum* upon subsequent infections.

The emergence of chloroquine resistance phenotype in *P. falciparum* occurred with associated fitness cost as exemplified by the inferior growth rate of parasites bearing the Dd2 *Pfcrt* haplotype in comparison with the wild-type strains. On the other hand, intragenic compensatory mutations in the Cam734 haplotype has enhanced growth rate of *P. falciparum* carrying these mutations. Importantly, gene editing approach has shown that mutations at N75D, A144F, L148I and T333S in the Cam734 haplotype contributed directly to both chloroquine resistance and restoration of parasite growth rate whereas a mutation at I194T enhanced parasite growth without conferring drug resistance (Gabryszewski *et al.*, 2016). Although the Cam734 haplotype contained 9 amino acid substitutions in comparison with the chloroquine-sensitive haplotype in the 3D7 strain, reversal of A144F to F144A by gene editing approach has led to complete to near-complete sensitivity to chloroquine albeit the presence of K76T and other associated mutations in this parasite. Therefore, mutation at codon 144 seems to be crucial for chloroquine resistance in the Cam734 haplotype (Gabryszewski *et al.*, 2016).

Analysis of departure from neutrality has shown that all mutations in the Cam734 haplotype have evolved under positive selection as determined by codon-based methods and alteration in physicochemical properties of amino acid substitutions. Importantly, both MEME and BR-REL methods gave a concordant result for episodic positive selection at codon A144F in the Cam734 haplotype, suggesting a short burst of selective pressure on this codon. Phylogenetic analysis has suggested that the Cam734 haplotype could have arisen from step-wise mutation from the Dd2 lineage. The Cam734 haplotype was closely related with the Cam738 haplotype whose sequence was almost identical except the absence of mutation at codon 144 in the latter haplotype (Durrand *et al.*, 2004). Interestingly, mutations in the Cam738 haplotype did not confer growth advantage in the absence of drug pressure when compared with the Cam734 haplotype (Gabryszewski *et al.*,

2016). A strikingly lower prevalence of the Cam738 haplotype (2.5%) in comparison with the Cam734 haplotype (22.5%) in Cambodia in 2004 (Durrand *et al.*, 2004) and the absence of this haplotype in a more recent study (Gabryszewski *et al.*, 2016) could suggest survival advantage of additional mutation at codon 144 in the Cam734 haplotype. Although our screening of the Cam734 haplotype was performed by analysis of a mutation at codon 144, subsequent sequence analysis of the complete coding region of *Pfcrt* of isolates carrying the mutant codon at this position has revealed identical sequences with the Cam734 haplotype. It is noteworthy that the Cam734 haplotype could be detected almost across the sampling period and circulated at low frequency (1.28%) in diverse endemic provinces of Thailand bordering Myanmar and Cambodia.

Despite evidence for growth advantage while maintaining chloroquine resistance status, the Cam734 haplotype was less abundant than the Dd2 haplotype in Southeast Asia (Durrand *et al.*, 2004; Gabryszewski *et al.*, 2016). A recent genome-wide survey revealed a comparable prevalence of this haplotype in Laos and Vietnam, accounting for 34.12% and 32.99%, respectively, while a lower prevalence was observed in Cambodia, ranging from 18.42% to 22.50% (Durrand *et al.*, 2004; Gabryszewski *et al.*, 2016). The low prevalence of the Cam734 haplotype in Thailand in this study was in line with the previous survey in which only one of 148 (0.68%) isolates was found to carry this haplotype (Gabryszewski *et al.*, 2016). Differential prevalence of the Cam734 haplotype in this region could stem from differences in national antimalarial drug policies of these countries whilst specific mutations in *Pfcrt* could mediate alteration in susceptibility of *P. falciparum* to other antimalarials such as artemisinin, piperquine, mefloquine and amodiaquine. Intriguingly, other antimalarial treatment regimens that target heme detoxification process could affect evolution of the *Pfcrt* locus (Conrad *et al.*, 2014; Agrawal *et al.*, 2017). Meanwhile, chloroquine resistance associated with intragenic compensatory mutations *per se* may not be sufficient for *P. falciparum* to outcompete other variants because additional selective forces from parasite genetic background, mosquito-human transmission and selection within anopheline mosquito vector could have influenced the survival and competitiveness of the parasites (Rosenthal, 2013).

CONCLUSION

The low prevalence of *P. falciparum* populations bearing the mutant 144F in *Pfcrt* known to confer compensatory mutation in terms of growth rate may suggest little or lack of survival advantage of these mutants in the absence of wild-type parasites circulating in Thailand.

COMPETEING INTEREST

The authors declare no conflicts of interest.

Acknowledgements. We are grateful to all patients who donated their blood samples for this study. We thank the staff of the Bureau of Vector Borne Disease, Department of Disease Control, Ministry of Public Health, Thailand, for assistance in field work.

FUNDING

This study received financial supports from The Thai Government Research Budgets (GRB-APS-12593011 and GBA-600093004) to SJ, CP and SS; The Thailand Research Fund (RSA5980054) to CP; and Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University (RA60/109) to CP, SJ and PB.

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