# รายงานวิจัยฉบับสมบูรณ์

## โครงการ

# วิวัฒนาการของตัวรับดัฟฟีแอนติเจนสำหรับคีโมไคส์ของลิงกังและลิงแสม และความสามารถในการติดเชื้อมาลาเรียชนิดพลาสโมเดียมโนวลิไซ และพลาสโมเดียมไซโนโมลจิที่ใกล้ชิดกับพลาสโมเดียมไวแวกซ์


## โดย

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**ตุลาคม 2557**

สัญญาเลขที่ RSA5480008

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**ผู้วิจัย**

**รองศาสตราจารย์ ดร. จตุรงค์ พุทธพรทิพย์**

**สังกัด**

**ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย**



**สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย**

**และ จุฬาลงกรณ์มหาวิทยาลัย**

# กิตติกรรมประกาศ

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

ผู้วิจัย

ตุลาคม 2557

# บทคัดย่อ

**รหัสโครงการ :** RSA5480008

**ชื่อโครงการ :** วิวัฒนาการของตัวรับดัฟฟีแอนติเจนสำหรับคีโมไคส์ของลิงกังและลิงแสมและความสามารถในการติดเชื้อมาลาเรียชนิดพลาสโมเดียมโนวลิไซและพลาสโมเดียมไซโนโมลจิที่ใกล้ชิดกับพลาสโมเดียมไวแวกซ์

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**ระยะเวลาโครงการ :** 3 ปี

ดัฟฟีแอนติเจนสำหรับคีโมไคส์หรือดีเออาร์ซีทำหน้าที่เป็นตัวรับบนผิวเม็ดเลือดแดงสำหรับเชื้อพลาสโมเดียมไวแวกซ์ในคน พลาสโมเดียมโนวลิไซในไพรเมตบางชนิด และเฮปปาโตซิสติสในลิงบาบูนเหลือง ยีนที่สร้างดีเออาร์ซีประกอบด้วยส่วนซิสเร็คกูราทอร์รี่ที่ควบคุมการแสดงออกของยีน เอ็กซอนหนึ่งอินตอน และเอ็กซอนสอง การศึกษานี้จึงมีวัตถุประสงค์เพื่อการวิเคราะห์ภาวะหลายรูปแบบในตำแหน่งดีเออาร์ซี โดยเฉพาะบริเวณซิสเร็คกูราทอร์รี่ที่อาจมีอิทธิพลต่อความสามารถในการติดเชื้อไซเมียนมาลาเรียชนิดอื่นๆ ดังนั้นในการศึกษาครั้งนี้จึงได้ทำการวิเคราะห์ภาวะดังกล่าวในลิงแมกคาคาฟาสสิคูลาลิส หรือลิงหางยาว จำนวน 43 ตัว และลิงแมกคาคานิเมสตินา หรือลิงกัง จำนวน 118 ตัวจากจังหวัดนราธิวาส ผลการศึกษาสามารถตรวจพบเชื้อมาลาเรียชนิดพลาสโมเดียมอินูไอได้มากที่สุดในลิงทั้งสองชนิด รองลงมาคือเชื้อเฮปปาโตซิสติส ส่วนไซเมียนมาลารียชนิดอื่นๆ ที่ตรวจพบได้แก่ พลาสโมเดียมไซโนมอลจิ พลาสโมเดียมโนวลิไซ พลาสโมเดียมโคทนิไอ พลาสโมเดียมเซมิโอวาเล่ พลาสโมเดียมฟิวดี้ พลาสโมเดียมไฮโลบาติ และพลาสโมเดียมกอนเดอรี่ ผลการวิคราะห์ยีนดีเออาร์ซี ตรวจพบสนิปส์ 31 ตำแหน่งในบริเวณซิสเร็คกูราทอร์รี่ และมีสนิปส์สองและสามตำแหน่งที่สัมพันธ์กับความสามารถในการติดเชื้อพลาสโมเดียมโนวลิไซ และพลาสโมเดียมไซโนมอลจิในลิงหางยาวและลิงกังตามลำดับ ดังนั้นผลการศึกษาครั้งนี้ทำให้เกิดองค์ความรู้ใหม่สำหรับปฏิสัมพันธ์ระหว่างไซเมียนมาลาเรียและภาวะหลายรูปแบบในตำแหน่งดีเออาร์ซีของไพรเมต

**คำหลัก :** สนิปส์, ดัฟฟีแอนติเจนสำหรับคีโมไคส์, ไซเมียนมาลาเรีย, ลิงหางยาว, ลิงกัง

**Abstract**

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**Project Code :** RSA5480008

**Project Title :** Evolution of Duffy antigen receptor for chemokines (DARC) of Macaca nemestrina and *M. fascicularis* and susceptibility to *Plasmodium vivax*-related malaria (*P. knowlesi* and *P. cynomolgi*)

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**Project Period :** 3 years

The Duffy antigen receptor for chemokines (DARC) serves as erythrocyte receptor for *Plasmodium vivax* in humans, *Plasmodium knowlesi* in some primates and *Hepatocystis* in yellow baboon. The gene encoding DARC contains *cis*-regulatory region that governs gene expression, two exons and an intron. To further determine whether polymorphism in the DARC locus, especially in the *cis*-regulatory region could influence susceptibility to other simian malaria infections, we recruited *Macaca fascicularis* or long-tailed macaques (n=43) and *Macaca nemestrina* or pig-tailed macaques (n=118) from Narathiwat Province for analysis. *Plasmodium inui* was the most prevalent simian malaria identified in both macaque species, followed by *Hepatocystis* spp. Other simian malarias identified in these macaques included *Plasmodium cynomolgi*, *Plasmodium knowlesi*, *Plasmodium coatneyi*, *Plasmodium simiovale, Plasmodium fieldi, Plasmodium hylobati* and *Plasmodium gonderi.* Sequence analysis of the DARC gene has shown 31 single nucleotide polymorphisms in the *cis*-regulatory region, 2 and 3 of which were significantly associated with susceptibility to *Plasmodium knowlesi* and to *Plasmodium cynomolgi* infections in long-tailed macaques and in pig-tailed macaques, respectively. Therefore, this study has expanded our knowledge on interactions between simian malaria and polymorphism in the DARC locus of nonhuman primates.

**Keywords :** single nucleotide polymorphism, Duffy antigen receptor for chemokines, simian malaria, long-tailed macaques, pig-tailed macaques

## INTRODUCTION

Macaque monkeys belong to Old World primates and are taxonomically placed within subfamily Cercopithecinae, occupying a large ecological niche from Asia to northern Africa (Rowe 1996). Although the main natural habitats of macaques are tropical forest areas, human activities such as forest encroachment, ecotourism and domestication of certain macaque species have posed a high-risk of some infectious diseases that cross-transmit between humans and macaques (Wolfe et al., 1998). Besides susceptibility to a number of intestinal protozoan and helminthes known to infect humans, macaques serve as natural hosts for at least 9 malaria species and three of these are capable of causing diseases in humans, i.e. *Plasmodium knowlesi*, *Plasmodium cynomolgi* and *Plasmodium inui*, either under naturally acquired infections or experimental conditions (Coatney 1968; Fooden 1994; Ta et al., 2014). In Thailand, 5 species of macaque monkeys have been identified, i.e. *Macaca fascicularis* or long-tailed macaque, *Macaca nemestrina* or pig-tailed macaque, *Macaca mulatta* or rhesus macaque, *Macaca arctoides* or stump-tailed macaque and *Macaca assamensis* or Assamese macaque. Of these, long-tailed and pig-tailed macaques inhabit a wide geographic range of the country, partly because they are highly adaptive to changing environments and feeding behaviors (Malaivijitnond et al., 2005). Importantly, a number of pig-tailed and long-tailed macaques have been domesticated and used for coconut picking in several southern communities of Thailand, leading to a potential risk for malaria transmission from these macaques to humans.

Despite over 4 decades since the discovery of several nonhuman primate malarias capable of causing overt infections with significant clinical symptoms in humans, it was not until very recently that a widespread occurrence of naturally acquired malaria in humans caused by *Plasmodium knowlesi* has been identified in several countries (Chin et al., 1965; Fong et al., 1971; Jongwutiwes et al., 2004; Singh et al., 2004; Cox-Singh et al., 2008; Putaporntip et al., 2009a). The distribution of *Plasmodium knowlesi* in humans has been confined to mainland Southeast Asia, Borneo, Malay Archipelago and The Philippines with variation in prevalence of infections (Ng et al., 2008; Luchavez et al., 2008). Our previous large-scale surveys in Thailand have shown that human infections with *Plasmodium knowlesi* were widely distributed especially along international borders of the country albeit at low prevalence and most of which were cryptic infections (Putaporntip et al., 2009a; Jongwutiwes et al., 2011). By contrary, a high prevalence of knowlesi malaria was identified in Malay Peninsula and Malaysian Borneo where potentially fatal infections were reported (Cox-Singh et al., 2008). A phylogenetic study has suggested that *Plasmodium knowlesi* isolates from infected Thai patients were mostly placed in a distinct clade, implying genetic difference among parasite strains from Thailand and Malaysia. Furthermore,

*Plasmodium knowlesi* isolated from the same endemic area of Thailand seems not to be originated from a single source because the infecting parasite isolates harbored different gene sequences of the merozoite surface protein-1 (msp-1) and the small subunit ribosomal RNA gene sequences. It is noteworthy that the majority of *Plasmodium knowlesi*-infected patients kept macaque monkeys as pets or had monkeys in their vicinities, suggesting zoonotic rather than anthroponotic transmission of this malaria species (Putaporntip et al., 2009a). On the other hand, recent analysis of the msp-1 sequences of *Plasmodium knowlesi* has revealed remarkably higher genetic diversity of human isolates than that of macaque origins, suggesting both zoonotic and anthroponotic transmission of this simian malaria (Putaporntip et al., 2013).

Nonhuman primate malarias capable of infecting humans are phylogenetically closely related to *Plasmodium vivax*, suggesting that they have evolved from a common ancestor and probably undergone radiation along with their respective host species. The emergence of *Plasmodium vivax* in humans could arise from a host switch from macaques (Mu et al., 2005). Time to the most recent common ancestor of *Plasmodium vivax* inferred from the mitochondrial genome sequences is independently estimated around 53,000 - 265,000 or 100,000 - 300,000 years ago (Mu et al., 2005; Jongwutiwes et al., 2005). Consistently, analysis of the genes encoding msp-1 of *Plasmodium vivax* and *Plasmodium knowlesi* has revealed that polymorphism at this locus has been maintained for around 4.5 to 5.6 million years ago, contemporary with the period of macaque radiation in Asia less than ~ 4.9 million years ago (Putaporntip et al., 2006). Despite an extremely long evolutionary history of the msp-1 locus that plausibly predates *Plasmodium* speciation, sequence analysis has shown that *Plasmodium vivax*, *Plasmodium knowlesi* and *Plasmodium cynomolgi* shared several sequence motifs at polymorphic regions of this gene, suggesting some functional or structural constraints at the protein level. Because msp-1 is known to involve in erythrocyte invasion by malarial merozoites, cross-species transmission of nonhuman primate malarias between macaques and humans could partially stem from certain remnants of shared functional characteristics of msp-1 between related *Plasmodium* species (Putaporntip et al., 2002; Putaporntip et al., 2006). Meanwhile, it has been well documented that both *Plasmodium vivax* and *Plasmodium knowlesi* require Duffy antigen receptor for chemokines (DARC) or Duffy blood group antigen as a receptor for host cell invasion (Miller et al., 1975). Consequently, Duffy-negative erythrocytes that are highly prevalent in negroid ethnic groups are completely refractory to invasion by *Plasmodium vivax* and *Plasmodium knowlesi* merozoites, resulting in global differential distribution of *Plasmodium vivax* and a low or almost absence of *Plasmodium vivax* infection among African and American blacks (Miller et al., 1975).

Specific contact region between DARC and binding region in *Plasmodium vivax* Duffy binding protein (PvDBP) has been mapped to polymorphic domain II that is subject to a strong balancing selection. The interaction between DARC and PvDBP has resulted in a complex selection of Duffy-negative phenotype to a near fixation in Africa and polymorphism driven by positive selection probably from host immune responses to parasite binding ligand (Carter and Mendis, 2002; Cole-Tobian and King, 2003). Interestingly, the expression of the DARC homologue in yellow baboons living in Kenya (*Papio cynocephalus*) seems to be governed by phenotypic variation in susceptibility to *Hepatocystis*, a hemoparasite closely related with malaria, suggesting that the evolution of baboon DARC expression is akin to the human one (Tung et al., 2009).

One of the crucial steps during erythrocyte invasion that is shared by *Plasmodium vivax* and *Plasmodium knowlesi* is an interaction between human Duffy positive erythrocytes and an adhesion ligand in DBP on the surface of merozoites. PvDBP is a 140-kDa transmembrane protein comprising 1115 amino acids and is encoded by a single copy gene containing five exons (Wertheimer et al., 1989; Fang et al., 1991; Adams et al., 1992). The PvDBP can be partitioned into seven domains in which domain II (PvDBPII) and domain IV (PvDBPIV) contained cysteine-rich motifs. Several lines of evidence indicate that the erythrocyte receptor recognition by the PvDBP requires PvDBPII ligand spanning 330 amino acids with conserved 12 cysteines involving in the formation of disulfide bonds and a number of aromatic residues suggestive of a complex structure in the region (Adams et al., 1992). Like most other malarial surface proteins, analysis of clinical isolates from Papua New Guinea, Colombia and South Korea has shown that PvDBPII contains the highest sequence diversity comparing with other domains and the nucleotide substitution pattern is characterized by statistically significant higher rates of nonsynonymous substitutions than synonymous substitutions, a signature of positive natural selection (Cole-Tobian and King, 2003). It is noteworthy that three of five T cell epitopes and all four B cell epitopes possess polymorphic residues among wild isolates, an issue that should take into account for PvDBPII-based vaccine design because both cellular and humoral immune responses against these epitopes are variant-specific (Xainli et al., 2002; Xainli et al., 2003). Likewise, additional studies using clinical isolates from Thailand, Papua New Guinea and Iran have reaffirmed that polymorphism in PvDBPII has been maintained by natural selection while the frequency distribution of PvDBP variants exhibits geographic variation (King et al., 2008; Gosi et al., 2008; Babaeekho et al., 2009).

Fine mapping of regions in PvDBP responsible for erythrocyte Duffy antigen receptor has revealed a critical binding motif encompassing 170 amino acids located between amino acids

291-460 or cystienes 4 and 7 (Ranjan and Chitnis, 1999). In vitro assays have shown that rabbit antibodies specific to recombinant PvDBPII are capable of inhibiting invasion of human erythrocytes by *P. vivax* merozoites (Grimberg et al., 2007). Consistently, naturally acquired anti-PvDBPII from individuals in an area with unstable malaria transmission in Brazil and from repeated malaria exposure in a holoendemic area of Papua New Guinea elicit invasion-inhibitory antibodies (Grimberg et al., 2007; Ceravolo et al., 2008). Likewise, antibodies against equivalent region in the erythrocyte binding protein-α of *Plasmodium knowlesi* (PkEBP-α or subsequently referred to as PkDBP-α) confer inhibition of *P. knowlesi* merozoites invasion into human erythrocytes (Singh et al., 2002). PvDBP and PkDBP share approximately 70% sequence identity. Genome sequencing has revealed that PkDBP consists of 3 distinct gene members, designated PkDBP-α, -β, and -γ (Fang et al., 1991). Deletion of the gene encoding PkDBP-α precludes *P. knowlesi* knock-out parasites to form a junction with Duffy-positive human erythrocytes and thus prevent host cell invasion (Singh et al., 2005). Crystal structure analysis of the equivalent domain in PkDBP-α, also referred to as Pkα, has shown that the residues involving in binding Duffy blood group antigen and residues at polymorphic sites under selective pressure are mapped to opposite surfaces of the Duffy binding-like domain, implying that such changes could possibly be attributable to immune evasion mechanism (Singh et al., 2006). On the other hand, site-directed mutagenesis of surface-predicted polymorphic residues of PvDBPII has revealed that nearly all polymorphic residues substituted with alanine retain erythrocyte binding property in assays using transfected COS cells, suggesting functional conservation of receptor recognition (VanBuskirk et al., 2004). To date, little is known about the extent of variation and roles in erythrocyte entry of the remaining 2 members of PkDBP, i.e. PkDBP-β and –γ. Meanwhile, despite no available evidence to support the interaction of Duffy binding-like motif on *P. cynomolgi* merozoites and DARC on host erythrocytes, the structural similarity and evolutionary relatedness of erythrocyte binding protein or Duffy binding protein of *P. cynomolgi* (PcyEBP or PcyDBP) and PvDBP could suggest a shared functional property of these proteins (Michon et al., 2002). Genomic analysis has shown that PcyDBP comprises 2 highly related genes, designated PcyDBP1 and PcyDBP2 (Tachibana et al., 2012). The presence of conserved cysteine-rich motifs in Duffy-binding-like (DBL) domains in adhesive molecules of different malaria species such as DBP, EBP, *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) and apical membrane antifen-1 (AMA-1) further suggests that these proteins have ancient origins which probably predate *Plasmodium* speciation (Michon et al., 2002).

Although the invasion process of malaria parasites is mediated by a series of molecular interaction, invasion of human erythrocytes by *Plasmodium vivax* and *Plasmodium knowlesi*

essentially requires the presence of Duffy blood group antigen. The human Duffy blood group system is initially identified based on the pattern of anti-$Fy^a$ and anti-$Fy^b$ reactivity that uniquely recognizes glycine and aspartic acid substitution at residue 42 in the extracellular domain at the N-terminal part of the protein. Four phenotypes have been detected: Fy(a+b-), Fy(a-b+), Fy(a+b+) and Fy(a-b-). Subsequent studies have identified another phenotype, Fy(a-b+wk), that reacts weakly to anti-$Fy^b$ as a result of substitution from arginine to cysteine at residue 89 in the first cytoplasmic domain. Duffy-negative human erythrocytes are resistant to invasion by *Plasmodium vivax* and *Plasmodium knowlesi* while host cell invasion by *Plasmodium cynomolgi* requires further investigation (Miller et al., 1975; Miller et al., 1976). The domain on the Duffy blood group antigen for binding of *Plasmodium vivax* and *Plasmodium knowlesi* to erythrocytes has been mapped to a stretch of 35 amino acids from alanine (residue 8) to aspartic acid (residue 43). The Duffy-negative blood group is mediated by a single transition from the wild-type T to a C at nucleotide -33 in the Fy *cis*-regulatory region that abolishes expression of this gene in erythrocytes (Pogo and Chaudhuri, 2000). Importantly, a paradigm that invasion of *Plasmodium vivax* merozoites into erythrocytes requires Duffy blood group antigen may not be universally applicable because a recent analysis has revealed that Malagasy people who possess Duffy negative eryhtrocytes remain susceptible to *Plasmodium vivax* infection (Menard et al., 2010). The reasons underlying this phenomenon await further investigation. Nevertheless, a recent report on *Hepatocystis* (a *Plasmodium*-related hemoprotozoan transmitted by *Culicoides*) resistance gene among wild baboon populations in Kenya has defined two functional SNPs in the 5' upstream untranscribed region that control expression of the homologous gene to human Fy gene of baboon in a *cis*-regulatory manner. Interestingly, the expression profiles of the Fy gene in baboons are related with susceptibility to *Hepatocystis* infections (Tung et al., 2009).

Intriguingly, the ability of cross-species transmission from macaques to humans of *Plasmodium knowlesi* and *Plasmodium cynomolgi* probably arises from a close evolutionary relatedness of the adhesive molecules involving in erythrocyte binding such as DARC protein akin to those found in *Plasmodium vivax*. Likewise, the Duffy blood group antigen homologue of macaques might share similar characteristics including the *cis*-regulatory region that defines the expression of the protein on erythrocytes. Consequently, differential expression of the Duffy blood group antigen homologue may determine *Plasmodium vivax*-related malaria susceptibility in macaques. Taken together, understanding the patterns of variation in nonhuman primates and nonhuman primate malaria transmissible to humans should provide a fundamental basis for control of the widespread distribution of zoonotic malaria in humans including prevention.

## OBJECTIVES

1. To explore the diversity in the DARC locus of *Macaca nemestrina* and *Macaca fascicularis* populations in southern Thailand.
2. To determine which SNP at the promoter (*FY cis*-regulatory region) of the DARC gene governs Duffy antigen expression in macaques' erythrocytes.
3. To investigate the extent of sequence diversity and the pattern of selection at the DBP homologues in *Plasmodium knowlesi* and *Plasmodium cynomolgi*.
4. To correlate SNPs in the DARC homoloques of *Macaca nemestrina* and *Macaca fascicularis* in Thailand and susceptibility to *Plasmodium vivax*-related malaria infections.

## RESEARCH METHODOLOGY

### *Study Population*

Monkey blood samples were obtained from Narathiwat province in southern Thailand during September 2011. The areas of capture were located in forest areas of 4 districts (Chanae, Waeng, Su-Ngai Padi and Sukhirin) as indicated on the map in Figure 1. 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.

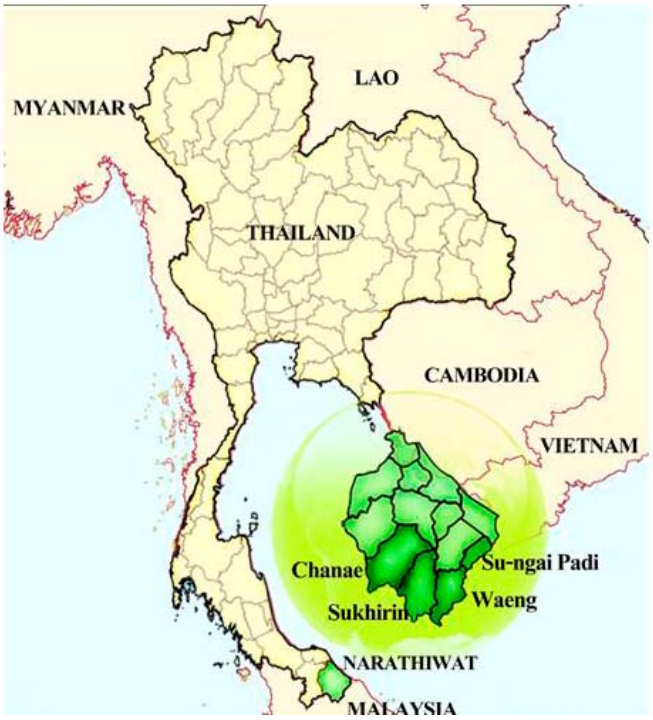


**Figure 1**. Map of Thailand showing monkey sampling sites in Narathiwat Province.

### *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 small subunit ribosomal RNA gene (SSU rRNA) 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 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.

***PCR, cloning and sequencing of the DARC genes of Macaca nemestrina and Macaca fascicularis***

To design the PCR primers for simultaneous amplification of the DARC genes including their upstream Fy *cis*-regulatory region of both *Macaca nemestrina* and *Macaca fascicularis*, sequences of related primates' DARC genes with their 5' non-coding regions available in the GenBank database were aligned by CLUSTAL X program. The sequences included or comparison were those from *Homo sapiens* (GenBank accession number X75875), *Hylobates* sp. (accession numbers AF303533), *Gorilla gorilla* (accession numbers AF311914), *Pan troglodytes* (accession numbers AF311914), *Macaca mulatta* (accession numbers AF311914) and *Papio cynocephalus* (accession number FJ95309). The resulting appropriate forward and reverse PCR primers were DARCFYF0: 5'-AATCTGCTTGGCAGAAAGGG-3' and DARCFY_R0: 5'-AGTTCTTGCCATTGTCTGGTTCT-3', respectively. The thermal cycling profile contained 35 cycles of denaturation at 96°C for 20 seconds, annealing at 62°C for 5 min with an initial pre-amplification denaturation at 94°C for 1 min and a final post-amplification extension at 72°C for 10 min. DNA amplification was performed by using a Gene-Amp 9700 PCR thermal cycler (Applied Biosystems,

Foster City, CA). To minimize error introduced in the sequences during PCR amplification, we used LaTaq DNA polymerase (Takara, Shiga, Japan), which has efficient 5' – 3' exonuclease activity to increase fidelity and shows no strand displacement. PCR products were analyzed by 1% agarose gel electrophoresis. The size of PCR product for DARC gene was expected to be ~2500 bp. The PCR products were then excised from agarose gel, purified by using a QIAquick PCR purification kit (Qiagen) and ligated into pGEM-T-Easy Vector (Promega, Madison WI). After incubation overnight at 4°C, the reaction mixture was precipitated, dissolved in 10 μL of double-distilled water, and transformed into *Escherichia coli* strain JM109. Recombinant DNA from positive clones was prepared by using the QIAGEN plasmid mini kit (Qiagen). The DNA sequences were determined from at least 5 plasmid subclones for each isolate. Sequences were determined from both directions for each template using the BigDye Terminator version 3.1 Cycle Sequencing Kit on an ABI3100 Genetic Analyzer (Applied Biosystems). Overlapping sequences were obtained by using sequencing primers. Each unique haplotype was verified by obtaining identical sequences from at least 2 different clones.

***PCR and sequencing of the DBP homologues of P. knowlesi and P. cynomolgi***

To obtain the genes encoding PkDBPs and PcyDBP, the forward and the reverse PCR primers were derived from interspecies conserved regions of PkDBP-α (accession numbers XM002261868, L14806, L14805, L14807, M68518, M68517 and M90466), PcyDBP sequences (accession numbers Y11936, AB617789, AB617788, JQ422036 and JQ422035). The primer sequences were obtained after alignment of these sequences by using the Clustal X program with the default option. The appropriate respective forward and reverse PCR primers for PkDBP-α were PKAP_F0 (5'-ATGTGTTTTACACCCCCCA-3') and PKABG_R0 (5'-AGCCAATAAAAGGAAATACAC-3'), PkDBP-β; PKBT_F0 (5'-GAGGTTTTAGAAATA GCAATAA-3') and PKABG_R0 (5'-AGCCAATAAAAGGAAATACAC-3'), PkDBP-γ; PKGM_F0 (5'-GGGGGTTTAGATGTAGTAATAA-3') and PKABG_R0 (5'-AGCCAATAAAAGGAAATACAC-3') and for PcyDBP; PcyDBP_F0 (5'-GTTTAGTTATATGTGTAGAACA-3'), PcyDBP_R0 (5'-TCATGAATAA TCCAGGGGGGT-3'), PcyDBP_F1 (5'-TTCCGTACACTTTCTGTTCTG-3') and PcyDBP_R1 (5'-AATGGTTGCATGT GTTCAATATC-3'). The PCR profiles for amplification of these genes were essentially the same, containing 35 cycles of denaturation at 96°C for 20 seconds, annealing at 62°C for 5 min with an initial pre-amplification denaturation at 94°C for 1 min and a final post-amplification extension at 72°C for 10 min. All positive DNA samples for *Plasmodium knowlesi* and *Plasmodium cynomolgi* were used as DNA template for amplification. The PCR products were then separated on 1% agarose gel; stained with ethidium bromide; and imaged under a UV transilluminator. After purification by using a QIAquick PCR purification kit (Qiagen), the resulting

PCR-amplified products, spanning ~3800 bp of both genes were used as templates for sequencing on an ABI3100 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Isolates with clonal mixtures were subcloned into plasmid vector and sequences were determined from at least 10 recombinants for each isolate. Singleton substitutions were re-determined using PCR products from two or more independent amplifications from the same DNA template.

***Semi-quantitative determination of macaques' Duffy blood group antigens***

Because human's and macaques' DARCs reportedly share remarkable structural similarity and the region that serves as an epitope for Fyb-antibody is perfectly conserved across primate species, DiaMed ID-MTS (BioRad, USA) that has been deployed for determination of DARC gene expression in human erythrocytes was applied for semi-quantitative determination of macaques' DARC gene expression. The procedure was as described in the manufacturer's instruction manual. In brief, DiaMed ID-Cards were allowed to equilibrate with room temperature before use. The microtubes were remove from covered aluminium foil while holding the tubes in the upright position. Fifty µl of 1% red cell suspension was applied to the appropriate microtube containing 50 µl of the corresponding ID test serum. The ID-Card was then incubated at 37 °C for 15 minutes. After centrifugation for 10 minutes, the results could be determined from the amount of agglutination reaction by visual comparison with the manufacturer's guidelines provided in the test kit. Positive results were the presence of agglutinated cells forming a red line on the surface of the gel or agglutinated dispersed in the gel. When compact button of cells occurred on the bottom of the microtubes, the reaction was considered to be negative.

***Data analysis***

Because macaques may harbour submicroscopic parasitemia, results from molecular detection of malaria were used for analysis. Alignment of the DARC nucleotide sequences was performed using the default option of the CLUSTAL_X program (Thompson et al., 1997) and manually edited. Insertions/deletions (indels) in coding regions were determined from multiple alignments of amino acid sequences to maintain the reading frame. The DARC gene sequences of *Macaca fascicularis* and *Macaca nemestrina* (GenBank accession numbers HQ285848 and HQ285850) were included as references (Oliveira et al., 2012). Haplotype (gene) diversity (*h*) and its sampling variance were estimated according to equations 8.4 and 8.12 (Nei, 1987) that has been implemented in the DnaSP version 5 (Librado and Rozas, 2009). Nucleotide diversity ($\pi$) or per-site heterozygosity was calculated from the average number of pairwise sequence differences in the sample and its standard deviation (or standard error) is the square root of the variance (Nei, 1987). Estimation of the number of synonymous substitutions per synonymous site

(dS) and nonsynonymous substitutions per nonsynonymous site (dN) was done by using Nei and Gojobori's method with Juke and Cantor correction (Jukes and Cantor, 1969; Nei and Gojobori, 1986). A statistical significance level of differences in dS and dN was set at 5%. Phylogenetic trees were constructed using the neighbor-joining method. Correlation between SNPs and expression profiles of DARC or susceptibility to malaria infections was performed by Chi-square test with 5% significant cut-off values. Tests of departure from neutrality at specific codons were performed based on estimation of the global ratio of the rate of non-synonymous to synonymous substitutions (dN/dS) across the gene. The single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), random effects likelihood (REL) and mixed effects model of evolution (MEME) methods implemented in the HyPhy package (Kasakovsky et al., 2005) were used for analysis. SLAC model is highly conservative based on the maximum likelihood reconstruction of the ancestral sequences and the counts of synonymous and nonsynonymous changes at each codon position in a phylogeny under the assumption of neutral evolution. FEL model compares the ratio of nonsynonymous to synonymous substitution on a site-by-site basis, without assuming an a priori 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. MEM algorithm detects codons under episodic positive selection unmasked by the abundance of purifying selection along the lineages [40]. Significance level settings for SLAC, FEL and MEME were p values < 0.1 and Bayes Factor > 1000 for REL followed the default values available on the Datamonkey Web Server (Kosakovsky and Frost, 2005).

## RESULTS

### 1. Monkeys and *Plasmodium* infections

In total, 244 monkeys were captured, comprising *Macaca fascicularis* or long-tailed macaques (n=57) and *Macaca nemestrina* or pig-tailed macaques (n=187). Of these, 11 long-tailed and 113 pig-tailed macaques harbored *Plasmodium* and/or *Hepatocystis* spp. in their circulations based on PCR diagnosis. Mixed species infections occurred in 5 long-tailed and 45 pig-tailed macaques, accounting for 45.5% and 39.8% 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 2 long-tailed and 18 pig-tailed macaques with almost comparable frequency as *Plasmodium coatneyi* (Table 1). Twenty-five blood samples (2 from long-tailed macaques and 23 from long-tailed macaques) gave positive primary PCR tests but negative nested PCR results, indicating that these samples

contained malaria species other than those detectable by nested PCR primers. Sequence analysis of the purified primary PCR products of these isolates has shown that these macaques were infected with *Plasmodium simiovale* (n = 3), *Plasmodium fieldi* (n = 14), *Plasmodium hylobati* (n = 5), *Plasmodium coatneyi* (n = 13) and *Plasmodium gonderi* (n = 3). Almost all of these malarias were found in macaques infected with more than one malaria species. It is noteworthy that microscopy could not efficiently determine malaria infections in macaques because only 4 long-tailed macaques and 58 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 1**. Distribution of malaria among macaque populations in Narathiwat Province.

| Monkey species | Malaria species* | No. Isolates | % positive** |
|---|---|---|---|
| *Macaca fascicularis* | *Plasmodium inui* | 10 | 52.62 |
| | *Plasmodium cynomolgi* | 0 | 0 |
| | *Plasmodium knowlesi* | 2 | 10.53 |
| | *Plasmodium coatneyi* | 2 | 10.53 |
| | Other primate malaria*** | 2 | 10.53 |
| | *Hepatocystis* spp. | 3 | 15.79 |
| *Macaca nemestrina* | *Plasmodium inui* | 67 | 32.21 |
| | *Plasmodium cynomolgi* | 28 | 13.46 |
| | *Plasmodium knowlesi* | 18 | 8.66 |
| | *Plasmodium coatneyi* | 14 | 6.73 |
| | Other primate malaria**** | 23 | 11.06 |
| | *Hepatocystis* spp. | 58 | 27.88 |

* Single and mixed species malaria infections were detected in 6 and 5 long-tailed macaques and in 53 and 45 pig-tailed macaques, respectively.
** Percent of all positive samples in each macaque species.
*** *Plasmodium simiovale* and *Plasmodium fieldi*
**** *Plasmodium hylobati*, *Plasmodium fieldi* and *Plasmodium gonderi*

**2. Nucleotide substitution pattern in the DARC locus**

The DARC gene contains (i) the *cis*-regulatory region spanning ~912 bp, (ii) a short exon I containing 21 nucleotides, (iii) an intron with ~480 nucleotides and (iv) an exon II comprising 990 nucleotides (Figure 2A). Analysis of all long-tailed and pig-tailed macaques recruited in this study has revealed 31 single nucleotide polymorphisms (SNPs) in the *cis*-regulatory region, 1 in exon I, 18 in intron and 33 in exon II. Of 31 SNPs in the *cis*-regulatory region in the DARC gene, 19 SNPs occurred in long-tailed macaques and 24 in pig-tailed macaques (Figure 2B). It is noteworthy that 12 of these SNPs were shared between these macaque species. Six SNPs were exclusively found in long-tailed macaques whereas 12 SNPs were unique for pig-tailed macaques (Figure 2B). Although a number of SNPs were identified, their frequency distributions were not even. There is no particular linkage of SNPs; thereby, 17 and 32 distinct haplotypes based on the *cis*-regulatory region were observed among long-tailed and pig-tailed macaques, respectively. Likewise, SNPs in the DARC intron among long-tailed macaques were slightly fewer than those among pig-tailed macaques, i.e. 11 and 13 SNPs, respectively. The lack of apparent linkage of SNPs in the intron region has generated 9 and 12 distinct haplotypes in long-tailed and pig-tailed macaque populations, respectively.

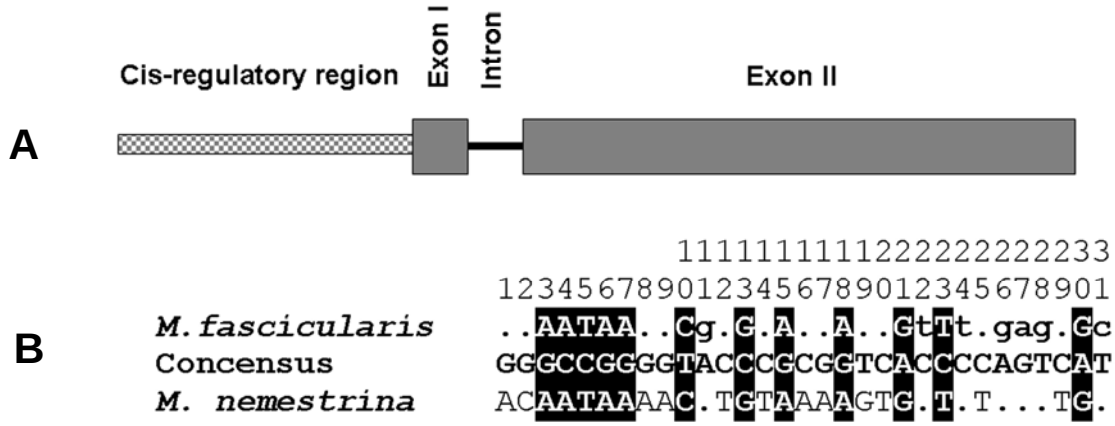


**Figure 2**. Structure of the DARC gene depicting *cis*-regulatory region (checkerboard box), exons (filled box) and intron (thick line)(A). SNPs in the *cis*-regulatory region in *Macaca fascicularis* and *Macaca nemestrina* are listed above and below the consensus residues, respectively (B). Numbers are listed from 5' to 3' direction. Shared SNPs between macaque species are highlighted. Unique SNPs for long-tailed and pig-tailed macaques are in lowercase and in regular font style, respectively. Dots are identical nucleotides with the consensus residues.

The DARC protein encoded by both exons can be partitioned into 4 regions, i.e. region 1, the extracellular binding region of *Plasmodium vivax* and *Plasmodium knowlesi* spanning the first 60 amino acids; region 2, the seven transmembrane regions; region 3, the four cytoplasmic

regions; and region 4, the extracellular regions other than region 1. Of 34 SNPs identified in the coding region, 27 create nonsynonymous codon changes as shown in Figure 3. Of these nonsynonymous codons, 9 residues were shared between long-tailed and pig-tailed macaques whereas 10 and 7 were unique for each species, respectively. Five amino acid substitutions occurred in region 1 whereas 11 nonsynonymous codon changes were located in transmembrane domians. Triple amino acid substitutions that were also shared between macaque species have been identified at residue 28, containing tyrosine, serine or phenylalanine. Of 27 nonsynonymous codon changes, the majority (22 or 81.5%) preserved their amino acid properties in terms of charge or polarity profiles. Based on the coding sequences, 34 and 40 haplotypes were observed in pig-tailed and long-tailed macaques, respectively. The haplotype distribution in long-tailed macaques seems to be more even than that of pig-tailed macaques as shown by the higher value of haplotype diversity in the former than the latter (Table 2). Analysis of pattern of nucleotide substitutions in the coding region has shown that nucleotide diversity in long-tailed macaques significantly exceeds that of pig-tailed macaques, *albeit* the former having smaller sample size. However, the number of synonymous nucleotide substitutions per synonymous site or dS and the number of nonsynonymous nucleotide substitutions per nonsynonymous site or dN were not statistically different ($p>0.05$).

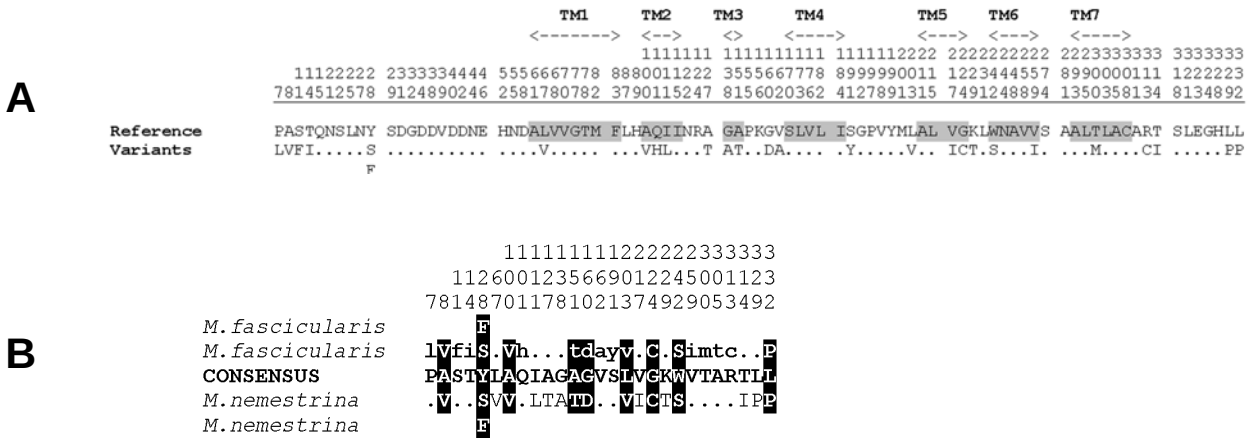


**Figure 3**. Amino acid substitutions in coding region of the DARC of macaques. Amino acid numbers are based on coding region of a reference sequence and listed below the sequence. Transmembrane domains (TM1-TM7) are shaded (A). Amino acid substitutions in *Macaca fascicularis* and *Macaca nemestrina* are listed above and below the consensus residues, respectively (B). Shared residues between macaque species are highlighted. Unique residues for long-tailed and pig-tailed macaques are in lowercase and regular font style, respectively. Dots represent identical residues.

**Table 2** Diversity in the DARC genes of *Macaca fascicularis* and *Macaca nemestrina*.

| Category | *Macaca fascicularis* | *Macaca nemestrina* |
|---|---|---|
| Number of sequences | 86 | 236 |
| Number of mutations | 27 | 32 |
| Number of haplotypes | 40 | 34 |
| Haplotype diversity (*h*) ± S.D. | 0.963 ± 0.010** | 0.731 ± 0.030 |
| Nucleotide diversity (¶) ± S.D. | 0.00418 ± 0.00017** | 0.00167 ± 0.00011 |
| $d_S$ ± S.E. | 0.00480 ± 0.00235 | 0.00461 ± 0.00235 |
| $d_N$ ± S.E. | 0.00420 ± 0.00134 | 0.00344 ± 0.00113 |

$d_S$ = the number of synonymous substitutions per synonymous site; $d_N$ = the number of nonsynonymous substitutions per nonsynonymous. Test of the hypothesis that *h* of *Macaca fascicularis* equals *h* of *Macaca nemestrina*: **$p$<0.0001. Test of the hypothesis that ¶ of *M. fascicularis* equals ¶ of *M. nemestrina*: **$p$<0.0001. Test of the hypothesis that $d_S$ of *Macaca fascicularis* equals $d_S$ of *Macaca nemestrina*: $p$>0.05. Test of the hypothesis that $d_N$ of *Macaca fascicularis* equals $d_N$ of *Macaca nemestrina*: $p$>0.05. Test of the hypothesis that $d_S$ of *Macaca fascicularis* equals $d_N$ of *Macaca fascicularis*: $p$>0.05. Test of the hypothesis that $d_S$ of *Macaca nemestrina* equals $d_N$ of *Macaca nemestrina*: $p$>0.05.

### 3. Analysis of single nucleotide polymorphisms in the *cis*-regulatory region and malaria infections

Although a number of SNPs with minor allele frequency in the *cis*-regulatory region of the DARC gene were identified, association test was performed for only SNPs with minor allele frequency of more than 0.05. Because the number of individuals infected with *Plasmodium hylobati, Plasmodium simiovale* or *Plasmodium gonderi* was not enough for the association analysis, the associations of SNPs with negative, *Plasmodium knowlesi, Plasmodium inui, Plasmodium cynomolgi, Plasmodium coatneyi, Plasmodium fieldi*, or *Hepatocystis* were calculated for long-tailed macaques and for pig-tailed macaques. Although the associations were assessed by Chi-square or Fisher exact tests, assuming four genetic models: recessive, dominant, genotype, and allelic models, results from allelic model (i.e., allele frequencies are compared between cases and controls) were considered.

Of 19 SNPs occurred in long-tailed macaques, 7 SNPs displayed allele frequencies that can be used for analysis. For long-tailed macaques, significant association between *Plasmodium knowlesi* infection and SNP281 (p=0.0405) was detected. Similar finding was observed for SNP690 (p=0.0405). The remaining SNPs did not yield any statistically meaningful results (Table

3). All SNPs in the *cis*-regulatory region in long-tailed macaques did not give significant association with *Hepatocystis* infections.

**Table 3** Analysis of single nucleotide polymorphism in the *cis*-regulatory region in long-tailed macaques and *Plasmodium knowlesi* infection.

| SNP# | major allele | frequency | minor allele | frequency | direction | Chi-square | p-value |
|---|---|---|---|---|---|---|---|
| 281 | A | 0.5 | G | 0.5 | protective | 4.1951 | 0.0405 |
| 308 | T | 0.8256 | C | 0.1744 | protective | 0.8863 | 0.3465 |
| 467 | G | 0.6977 | A | 0.3023 | susceptible | 0.0545 | 0.8155 |
| 690 | A | 0.5 | G | 0.5 | protective | 4.1951 | 0.0405 |
| 773 | C | 0.8256 | T | 0.1744 | protective | 0.8863 | 0.3465 |
| 796 | A | 0.9186 | G | 0.0814 | protective | 0.3717 | 0.5421 |
| 807 | T | 0.8488 | G | 0.1512 | protective | 0.7471 | 0.3874 |

Meanwhile, 8 of 24 SNPs in the *cis*-regulatory region in pig-tailed macaques had allele frequencies that are suitable for analysis. Despite a considerable number of malaria infections in these pig-tailed macaque population, a significant association was found between *Plasmodium cynomolgi* infections and SNP281 (p=0.017), SNP467 (p=0.0162) and SNP690 (p=0.0208) (Table 4). The remaining 5 SNPs does not show any significant association with any malaria infections. No significant association was observed between *Hepatocystis* infections and SNPs in the *cis*-regulatory region in pig-tailed macaques.

**Table 4** Analysis of single nucleotide polymorphism in the *cis*-regulatory region in pig-tailed macaques and *Plasmodium cynomolgi* infection.

| SNP# | major allele | frequency | minor allele | frequency | direction | Chi-square | p-value |
|---|---|---|---|---|---|---|---|
| 149 | G | 0.9195 | A | 0.0805 | susceptible | 0.7197 | 0.3962 |
| 179 | G | 0.911 | C | 0.089 | susceptible | 1.1358 | 0.2866 |
| 225 | C | 0.9492 | A | 0.0508 | susceptible | 0.0113 | 0.9154 |
| 281 | G | 0.928 | A | 0.072 | protective | 5.6994 | 0.017 |
| 285 | G | 0.9407 | A | 0.0593 | protective | 3.0089 | 0.0828 |
| 382 | C | 0.928 | G | 0.072 | susceptible | 1.4489 | 0.2287 |
| 467 | G | 0.7924 | A | 0.2076 | susceptible | 5.7798 | 0.0162 |
| 690 | A | 0.9322 | G | 0.0678 | protective | 5.3398 | 0.0208 |

## 4. Analysis of the Duffy-binding protein of *Plasmodium cynomolgi*

### 4.1 Sequence diversity

The complete PcyDBP gene spans ~3.8 kilobases containing 5 exons. The number of codons in exons 1 to 5 were 19, 961-969, 26, 25 and 14, respectively. Of 28 *Plasmodium cynomolgi* isolates diagnosed by species-specific PCR in *Macaca nemnestrina*, 17 isolates were successfully amplified by PCR for the complete PcyDBP gene. The relatively low efficiency of PCR amplification of the PcyDBP locus could stem from the low number of parasites in these samples because most of these samples harbored submicroscopic parasitemia. Six of the PCR-amplified PcyDBP samples contained mixed clone infections as evidenced by multiple superimposed signals on electropherogram from direct sequencing; therefore, these isolates were excluded from further analysis. Of 11 isolates with single clone infections, sequences were completely determined from the PCR purified templates. There were 1045-1053 codons among foeld isolates. Exon 1 had 4 amino acid substitutions. Exon 2 had 961-969 codons with insertion or deletion of sequence. Exon 3 had 5 codon changes. Exon 4 had 2 codon changes. Two codon changes were found in exon 5. The mean nucleotide diversity of the PcyDBP of Thai isolates was 0.021 ± 0.011 while the 7 sequences from the GenBank database (JQ422035, JQ422036, Y11396, AB617788, AB617789, XM004220981 and XM004221494) was about thrice higher (Table 5). The difference was statistically difference ($p<0.001$). In total, the average nucleotide diversity of the PcyDBP locus was 0.052 ± 0.008. It is of note that none of the 11 Thai isolates and 7 sequences from the GenBank database shared identical sequences resulting in high haplotype diversity values.

**Table 5** Molecular diversity at the DBP locus of *Plasmodium cynomolgi*

| Source | n | N | S | H | *h* ± S.D. | $\pi$ ± S.D. |
|---|---|---|---|---|---|---|
| Wild Isolates | 11 | 318 | 311 | 11 | 1 ± 0.039 | 0.021 ± 0.011 |
| GenBank sequences | 7 | 510 | 485 | 7 | 1 ± 0.076 | 0.071 ± 0.008 |
| Wild isolates + GenBank sequences | 18 | 588 | 550 | 18 | 1 ± 0.019 | 0.052 ± 0.008 |

n = number of sequences; N = number of mutations; S = number of segregating sites; H = number of haplotypes; $\pi$ = nucleotide diversity.

### 4.2 Phylogenetic relationship

The neighbor-joining tree topology of the coding regions of the PcyDBP gene of Thai isolates and those from elsewhere (from GenBank database) did not show distinct clades. Therefore, it seems that no distinct members as a subfamily occur within the PcyDBP locus. However, most of the Thai isolates displayed close genetic relatedness because the tree branches that represent 10 Thai sequences were clustered together with short branch length. Although the PcyDBP sequence under the accession number JQ422035 seems to be most related with these 10 Thai isolates, the divergence of tree topology received 100% bootstrap support, indicating that they were not truly clustered together. Interestingly, a Thai isolate, designated HB530, was more distantly related with most of the Thai isolates than 2 isolates elsewhere, i.e. AB617788 and XM004221494 (Figure 4).

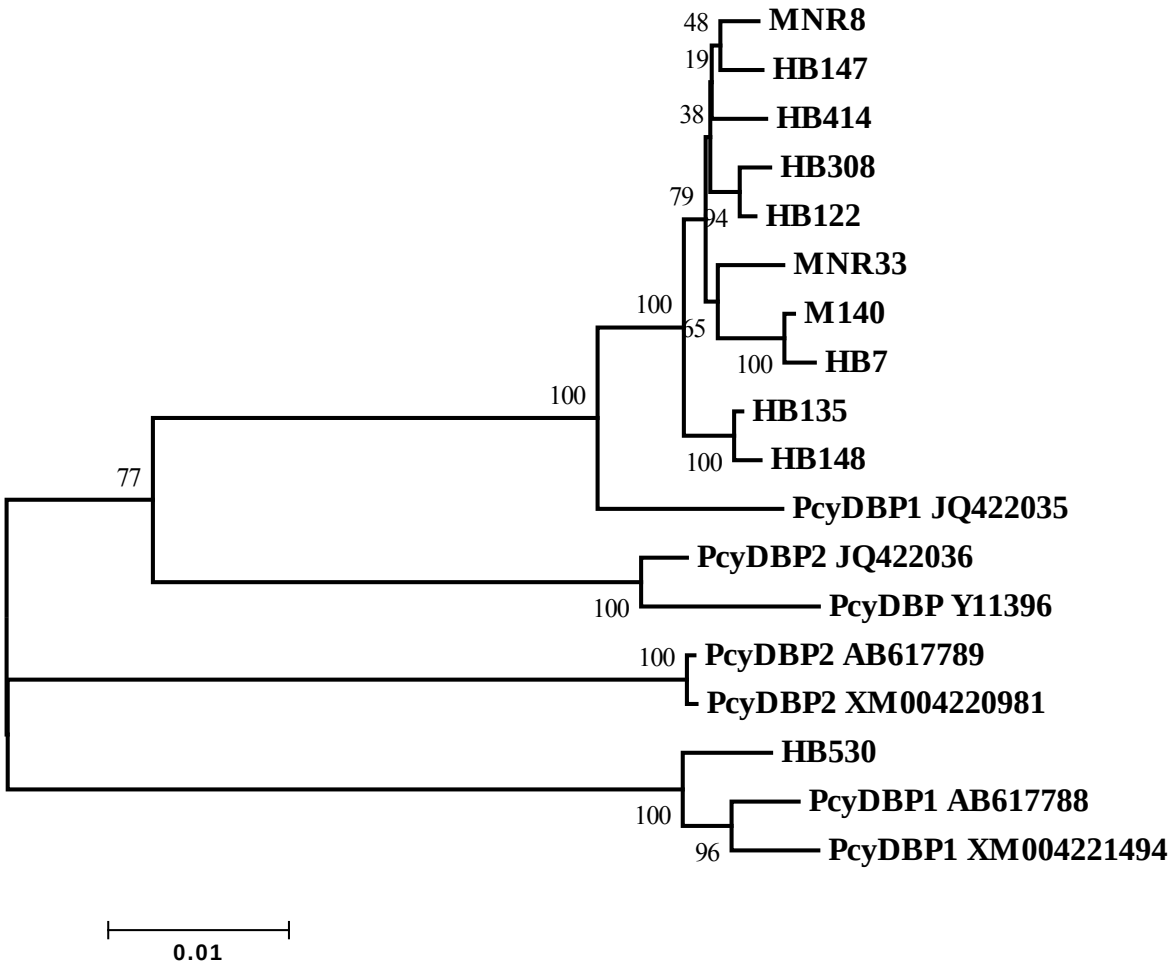


**Figure 4 Neigbor-joining tree inferred from Duffy-binding gene sequences of** *Plasmodium cynomolgi* sampling from long-tailed and pig-tailed macaques in Narathiwat Province, Southern Thailand. Sequence with accession numbers are from Genbank Database. Scale indicates 0.01 substitution per nucleotide site.

### 4.3 Neutrality tests

To determine whether the extent and pattern of nucleotide substitutions in the PcyDBP locus has been influenced by selective pressure, neutrality tests was performed by Tajima's *D*, Fu and Li's *D** and *F**, and the rates of synonymous and nonsynonymous substitutions per sites (dS and dN). Tajima's *D* statistic measures the difference between the average number of nucleotide differences and an estimate of $\theta = 4Ne\mu$ from the number of segregating sites (Tajima, 1983). Fu and Li's *D** test is based on the differences between the number of singletons, and the total number of mutations while their *F** test statistic takes into account the differences between the number of singletons and the average number of nucleotide differences between pairs of sequences (Fu and Li, 1993). Results revealed that Tajima's *D* of the PcyDBP locus of Thai isolates gave significant value in negative direction ($p < 0.05$). When Tajima's *D* statistic was applied for either synonymous or nonsynonymous sites, concordant negative values of the test were observed albeit at non-significant levels. Likewise, Fu & Li's *D** and *F** statistics yielded significant negative values for Thai isolates ($P < 0.05$). Significant values of these tests in negative direction were due to the presence of excess of low frequency polymorphism, implying population expansion after bottleneck effects or purifying selection (Table 6).

**Table 6** Neutrality tests for the DBP locus of *Plasmodium cynomolgi*

| Parameter | Wild isolates (n=11) | Wild isolates + GenBank sequences (n=18) |
|---|---|---|
| Tajima's *D* | | |
| All sites | -1.8425# | 0.1097 |
| Synonymous sites | -1.5407 | 0.0769 |
| Nonsynonymous sites | -1.8774 | 0.0765 |
| Fu & Li's statistics | | |
| *D** | -2.1343# | 0.7534 |
| *F** | -2.3394# | 0.5574 |
| *d* ± S.E. | 0.0224 ± 0.0017 | 0.0556 ± 0.0045 |
| *dS* ± S.E. | 0.0266 ± 0.0032 | 0.0210 ± 0.0014 |
| *dN* ± S.E. | 0.0348 ± 0.0059 | 0.0338 ± 0.0027 |

*d* = number of nucleotide substitutions per site; *dS* = number of synonymous substitutions per synonymous site; *dN* = number of nonsynonymous substitutions per nonsynonymous site; # = statistically significant departure from neutral expectation ($p < 0.05$).

Comparison between the rate of synonymous substitutions per synonymous site or dS and the rate of nonsynonymous substitutions per nonsynonymous site or dN has revealed that dN exceeds dS but not significant difference when either Thai isolates or all sequences were considered. Tests of departure from neutrality at specific codons were performed based on estimation of the global ratio of the rate of non-synonymous to synonymous substitutions (dN/dS) across the PcyDBP gene. Consensus results from SLAC, FEL, REL and MEME methods implemented in the HyPhy package have identified 12 negatively selected codons as shown in Table 7. These results suggest that purifying selection has predominantly operated on the *PcyDBP* locus.

**Table 7** Negatively selected sites in PcyDBP

| Codon | dS | dN | dN/dS | Normalized dN-dS | p-value |
|---|---|---|---|---|---|
| 56 | 85.1953 | 5.00E-09 | 0 | -738.296 | 0.0104 |
| 90 | 88.9803 | 5.00E-09 | 0 | -771.097 | 0.0345 |
| 127 | 277.238 | 5.00E-09 | 0 | -2402.52 | 0.0069 |
| 191 | 23.3979 | 5.00E-09 | 0 | -202.764 | 0.0443 |
| 224 | 61.0917 | 5.00E-09 | 0 | -529.417 | 0.0098 |
| 296 | 23.3979 | 5.00E-09 | 0 | -202.764 | 0.0443 |
| 320 | 507.798 | 3.5952 | 0.007 | -4369.39 | 0.0009 |
| 457 | 88.6754 | 5.48662 | 0.062 | -720.908 | 0.0434 |
| 458 | 97.4015 | 5.00E-09 | 0 | -844.074 | 0.0010 |
| 490 | 40.3330 | 5.00E-09 | 0 | -349.523 | 0.0088 |
| 707 | 23.3979 | 5.00E-09 | 0 | -202.764 | 0.0443 |
| 1036 | 26.1937 | 5.00E-09 | 0 | -226.992 | 0.0474 |

## 5. Analysis of the Duffy-binding protein of *Plasmodium knowlesi*

### 5.1 Sequence diversity

The Duffy-binding protein of *Plasmodium knowlesi* is encoded by 3 closely related but distinct genes, designated PkDBP-α, PkDBP-β and PkDBP-γ. Twelve macaques' blood samples were successfully amplified to obtained all these 3 members of PkDBP locus. Of these, 9 isolates were from *Macaca nemestrina* and the remaining 3 isolates were from *Macaca fascicularis*. The complete coding sequences of PkDBP contain 5 exons with structural organization similar to that of PcyDBP. Size variation was observed in the complete coding region of the PkDBP gene, varying from 1074 to 1214 codons. Exon 1 of PkDBP possesses signal sequence containing 20 codons. Most of the codons in exon 1 encode amino acids with positive charge and hydrophobic amino acids. Exon 2 occupies 988 to 1126 codons containing erythrocyte binding domain equivalent to a molecular weight of 135 kDa. A transmembrane domain is encoded by exon 3 comprising 26 to 28 codons. The cytoplasmic domain of PkDBP is encoded by both exons 4 and 5, containing 40 codons. Of 12 isolates examined, all three genes in the PkDBP locus had comparable nucleotide diversity although PkDBP-α contains slightly more nucleotide substitutions than PkDBP-β and PkDBP-γ, resulting in a slightly higher mean nucleotide diversity (0.0200 ± 0.0035). However, when all gene sequences were considered together, a remarkable higher mean nucleotide diversity was observed, being almost 5-fold increase from that of individual gene sequences (Table 8). Most isolates in this study had different PkDBP sequences. Therefore, the haplotype diversity of each gene was very high, being 1 or approaching 1.

**Table 8** Molecular diversity at the DBP orthologues of *Plasmodium knowlesi*

| Locus | n | N | S | H | *h* ± S.D. | π ± S.D. |
|---|---|---|---|---|---|---|
| PkDBP-α | 12 | 199 | 196 | 10 | 0.955 ± 0.057 | 0.0200 ± 0.0035 |
| PkDBP-β | 12 | 186 | 180 | 12 | 1.000 ± 0.034 | 0.0180 ± 0.0015 |
| PkDBP-γ | 12 | 152 | 151 | 11 | 1.000 ± 0.002 | 0.0184 ± 0.0019 |
| All | 35 | 899 | 839 | 33 | 0.995 ± 0.009 | 0.1058 ± 0.0064 |

n = number of sequences; N = number of mutations; S = number of segregating sites; H = number of haplotypes; π = nucleotide diversity.

### 5.2 Phylogenetic relationship

The topology of phylogenetic tree inferred from the coding regions of the PkDBP gene of Thai isolates and those from elsewhere (from GenBank database) has shown 3 distinct clades for PkDBP-α, PkDBP-β and PkDBP-γ with 100% bootstrap supports. It is noteworthy that PkDBP-α and PkDBP-γ were closely related while PkDBP-β formed a separate cluster (Figure 5). Three isolates had identical PkDBP-α sequences while sequences of the other 2 loci, PkDBP-β and PkDBP-γ differed in all isolates examined. It is, therefore, likely that each gene for PkDBP could have segregated independently during sexual reproduction of malaria parasite.

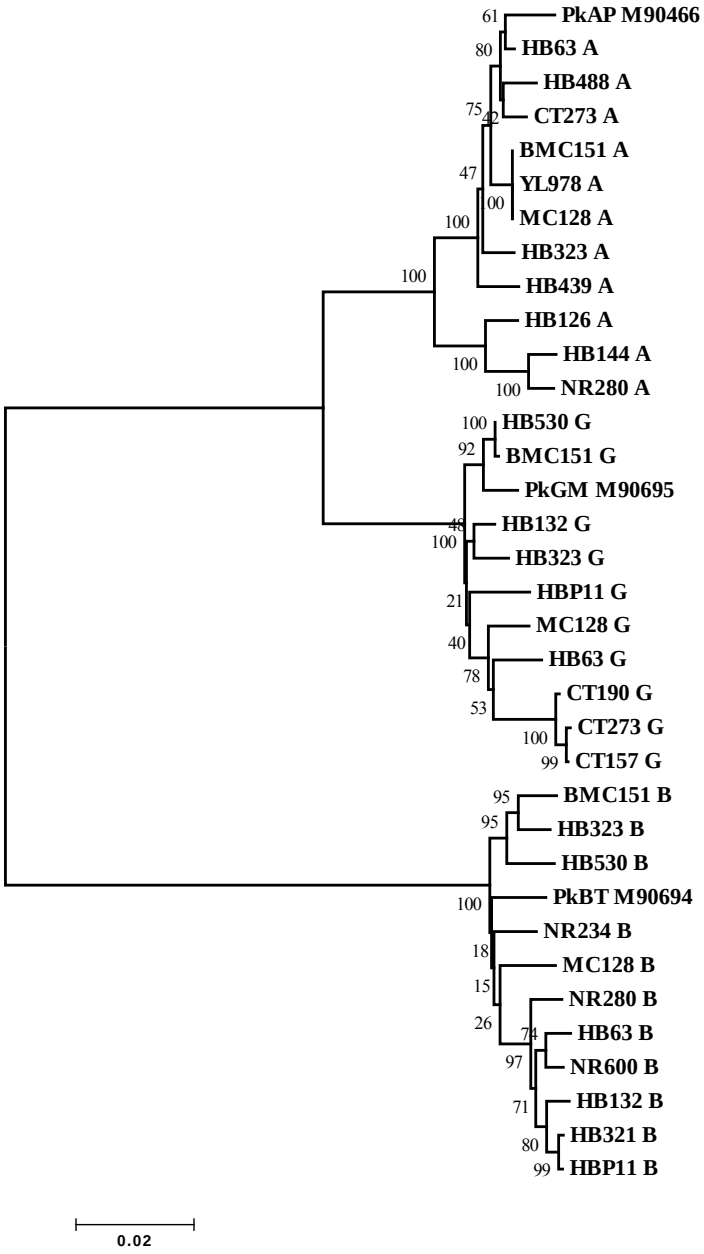


**Figure 5** Phylogenetic tree of PkDBP-α, PkDBP-β และ PkDBP-γ of *Plasmodium knowlesi* from macaques in Southern Thailand. Sequences with accession numbers are from the Genbank Database. Scale on the left lower corner represents 0.02 substitutions per site

### 5.3 Neutrality tests

Tests for departure from neutral evolution of the PkDBP genes was performed using similar tests as those for PcyDBP. When individual gene of the PkDBP family was considered separately, Tajima's D statistic did not detect any departure from neutral expectation at all nucleotide substitutions. Likewise, no significant Tajima's D value was obtained when either synonymous or nonsynonymous sites were tested separately. However, when PkDBP-α, PkDBP-β and PkDBP-γ were compiled into a single group, a significant Tajima's D value was observed at synonymous sites ($p < 0.05$), suggesting diversifying selection predating divergence of these genes or the presence of population bottleneck. On the other hand, Fu & Li's *F** and *D** tests did not show deviation from neutrality at these loci. Importantly, the rate of synonymous substitutions per synonymous site significantly outnumbered the rate of nonsynonymous substitutions per nonsynonymous site in PkDBP-α and PkDBP-γ, suggesting that these genes were under negative selection (Table 9). Codon-based analysis of pattern of dS and dN has revealed that 27 codons in PkDBP-α, 29 codons in PkDBP-β and 25 codons in PkDBP-γ had *dS* exceeded *dN* significantly ($p < 0.05$). It is noteworthy that most of the codons under selective pressure were located in exon 2. However, codon 672 in PkDBP-β had *dN* greater than *dS* with p value less than 0.05, indicating that positive selection has operated at certain amino acid residues (Tables 10-12).

**Table 9** Neutrality tests for the DBP orthologues of *Plasmodium knowlesi*

| Parameter | PkDBP-α | PkDBP-β | PkDBP-γ | All |
|---|---|---|---|---|
| Tajima's *D* | | | | |
| All sites | -0.2098 | -0.0994 | 0.4873 | 1.8379 |
| Synonymous sites | -0.0215 | -0.1715 | 0.5236 | 2.1107# |
| Nonsynonymous sites | -0.3082 | 0.0553 | 0.4650 | 1.7962 |
| Fu & Li's statistics | | | | |
| *D** | 0.1146 | -0.3714 | 0.2952 | 0.9834 |
| *F** | 0.0331 | -0.3418 | 0.3916 | 1.5270 |
| *d* ± S.E. | 0.0209 ± 0.0017 | 0.0196 ± 0.0021 | 0.0195 ± 0.0015 | 0.1203 ± 0.0068 |
| *dS* ± S.E. | 0.0370 ± 0.0045§ | 0.0182 ± 0.0017 | 0.0293 ± 0.0043§ | 0.1658 ± 0.0120§ |
| *dN* ± S.E. | 0.0167 ± 0.0015 | 0.0224 ± 0.0017 | 0.0169 ± 0.0017 | 0.1077 ± 0.0056 |

*d* = number of nucleotide substitutions per site; *dS* = number of synonymous substitutions per synonymous site; *dN* = number of nonsynonymous substitutions per nonsynonymous site; # = statistically significant departure from neutral expectation ($p < 0.05$). § = test of hypotheses that *dS* = *dN* ($p < 0.01$).

**Table 10** Negatively selected sites in PkDBP-α

| Codon | dS | dN | dN/dS | Normalized dN-dS | p-value |
|---|---|---|---|---|---|
| 59 | 26.79 | 5.00E-09 | 0 | -301.774 | 0.0282 |
| 95 | 28.7693 | 5.00E-09 | 0 | -324.07 | 0.0373 |
| 154 | 14.8707 | 5.00E-09 | 0 | -167.51 | 0.0253 |
| 199 | 25.3645 | 5.00E-09 | 0 | -285.717 | 0.0117 |
| 507 | 31.3644 | 5.00E-09 | 0 | -353.303 | 0.0087 |
| 523 | 40.479 | 5.00E-09 | 0 | -455.973 | 0.0312 |
| 555 | 30.4938 | 5.00E-09 | 0 | -343.495 | 0.0111 |
| 733 | 45.6796 | 5.00E-09 | 0 | -514.555 | 0.0006 |
| 758 | 30.5545 | 5.00E-09 | 0 | -344.179 | 0.0127 |
| 769 | 39.8372 | 5.00E-09 | 0 | -448.744 | 0.0085 |
| 772 | 85.461 | 5.00E-09 | 0 | -962.671 | 0.0076 |
| 782 | 135.411 | 5.00E-09 | 0 | -1525.34 | 0.0007 |
| 838 | 41.9577 | 5.00E-09 | 0 | -472.63 | 0.0260 |
| 852 | 39.1289 | 5.00E-09 | 0 | -440.766 | 0.0326 |
| 925 | 25.2971 | 5.00E-09 | 0 | -284.958 | 0.0231 |
| 926 | 23.4595 | 5.00E-09 | 0 | -264.258 | 0.0137 |
| 930 | 26.9469 | 5.00E-09 | 0 | -303.542 | 0.0195 |
| 936 | 30.5142 | 5.00E-09 | 0 | -343.725 | 0.0094 |
| 946 | 55.3183 | 5.00E-09 | 0 | -623.13 | 0.0408 |
| 965 | 101.131 | 5.00E-09 | 0 | -1139.18 | 0.0111 |
| 966 | 90.6705 | 5.00E-09 | 0 | -1021.35 | 0.0326 |
| 1004 | 19.195 | 5.00E-09 | 0 | -216.221 | 0.0492 |
| 1063 | 26.9127 | 5.00E-09 | 0 | -303.157 | 0.0137 |
| 1119 | 25.0194 | 5.00E-09 | 0 | -281.83 | 0.0091 |
| 1125 | 39.1289 | 5.00E-09 | 0 | -440.766 | 0.0438 |
| 1163 | 64.8046 | 5.00E-09 | 0 | -729.988 | 0.0056 |
| 1188 | 51.7248 | 5.00E-09 | 0 | -582.652 | 0.0093 |

**Table 11** Positively (bold) and negatively selected sites in PkDBP-β

| Codon | dS | dN | dN/dS | Normalized dN-dS | p-value |
|---|---|---|---|---|---|
| 261 | 37.1557 | 5.00E-09 | 0 | -391.118 | 0.0283 |
| 300 | 37.7347 | 5.00E-09 | 0 | -397.214 | 0.0063 |
| 409 | 346.919 | 5.00E-09 | 0 | -3651.84 | 0.0001 |
| 412 | 274.293 | 5.00E-09 | 0 | -2887.34 | 0.0001 |
| 425 | 16.5583 | 5.00E-09 | 0 | -174.301 | 0.0305 |
| 603 | 88.1156 | 5.00E-09 | 0 | -927.547 | 0.0001 |
| 640 | 17.0196 | 5.00E-09 | 0 | -179.157 | 0.0295 |
| **672** | **5e-09** | **17.5653** | **4E+09** | **184.901** | **0.0484** |
| 698 | 254.129 | 5.00E-09 | 0 | -2675.08 | 0.0185 |
| 701 | 35.2963 | 5.00E-09 | 0 | -371.546 | 0.0303 |
| 753 | 19.8224 | 5.00E-09 | 0 | -208.66 | 0.0240 |
| 823 | 39.21 | 5.00E-09 | 0 | -412.743 | 0.0269 |
| 840 | 14.1657 | 5.00E-09 | 0 | -149.115 | 0.0364 |
| 887 | 29.0959 | 5.00E-09 | 0 | -306.277 | 0.0101 |
| 889 | 40.2706 | 5.00E-09 | 0 | -423.908 | 0.0014 |
| 904 | 95.1578 | 5.00E-09 | 0 | -1001.68 | 0.0004 |
| 920 | 44.0173 | 5.00E-09 | 0 | -463.347 | 0.0042 |
| 921 | 51.6206 | 5.00E-09 | 0 | -543.383 | 0.0217 |
| 925 | 26.4562 | 5.00E-09 | 0 | -278.491 | 0.0198 |
| 930 | 65.8978 | 5.00E-09 | 0 | -693.671 | 0.0036 |
| 935 | 59.9531 | 5.00E-09 | 0 | -631.095 | 0.0019 |
| 936 | 53.9544 | 5.00E-09 | 0 | -567.95 | 0.0223 |
| 940 | 22.2908 | 5.00E-09 | 0 | -234.643 | 0.0363 |
| 945 | 24.3346 | 5.00E-09 | 0 | -256.158 | 0.0190 |
| 946 | 41.4716 | 5.00E-09 | 0 | -436.55 | 0.0257 |
| 950 | 19.7382 | 5.00E-09 | 0 | -207.774 | 0.0405 |
| 966 | 57.0111 | 3.15668 | 0.055 | -566.897 | 0.0049 |
| 970 | 34.0003 | 5.00E-09 | 0 | -357.903 | 0.0126 |
| 1021 | 431.771 | 5.00E-09 | 0 | -4545.03 | 0.0004 |
| 1038 | 33.8634 | 5.00E-09 | 0 | -356.462 | 0.0086 |

**Table 12** Negatively selected sites in PkDBP-γ

| Codon | dS | dN | dN/dS | Normalized dN-dS | p-value |
|---|---|---|---|---|---|
| 45 | 49.6373 | 5.00E-09 | 0 | -647.698 | 0.0071 |
| 547 | 32.6589 | 5.00E-09 | 0 | -426.153 | 0.0137 |
| 555 | 29.4842 | 5.00E-09 | 0 | -384.729 | 0.0155 |
| 564 | 27.6052 | 5.00E-09 | 0 | -360.21 | 0.0136 |
| 620 | 100.265 | 5.00E-09 | 0 | -1308.32 | 0.0007 |
| 648 | 15.4118 | 5.00E-09 | 0 | -201.103 | 0.0381 |
| 655 | 27.6052 | 5.00E-09 | 0 | -360.21 | 0.0305 |
| 690 | 30.1751 | 5.00E-09 | 0 | -393.744 | 0.0081 |
| 696 | 16.5542 | 5.00E-09 | 0 | -216.009 | 0.0342 |
| 782 | 22.3259 | 5.00E-09 | 0 | -291.323 | 0.0187 |
| 880 | 53.6468 | 3.04901 | 0.057 | -660.231 | 0.0278 |
| 887 | 49.9069 | 3.64091 | 0.073 | -603.707 | 0.0451 |
| 889 | 30.276 | 5.00E-09 | 0 | -395.061 | 0.0162 |
| 904 | 65.2876 | 5.00E-09 | 0 | -851.914 | 0.0026 |
| 906 | 56.5271 | 5.00E-09 | 0 | -737.601 | 0.0016 |
| 930 | 36.3415 | 5.00E-09 | 0 | -474.206 | 0.0148 |
| 936 | 30.1359 | 5.00E-09 | 0 | -393.232 | 0.0189 |
| 946 | 57.7341 | 5.00E-09 | 0 | -753.351 | 0.0029 |
| 950 | 151.955 | 5.00E-09 | 0 | -1982.8 | 0.0114 |
| 1000 | 9999.88 | 5.00E-09 | 0 | -130485 | 0.0225 |
| 1004 | 26.6937 | 5.00E-09 | 0 | -348.316 | 0.0417 |
| 1038 | 49.0804 | 0 | 0 | -640.432 | 0.0065 |
| 1063 | 19.154 | 5.00E-09 | 0 | -249.934 | 0.0289 |
| 1125 | 79.6842 | 5.00E-09 | 0 | -1039.77 | 0.0026 |
| 1212 | 188.774 | 5.00E-09 | 0 | -2463.25 | 0.0023 |

**6. Genetic recombination**

To detect evidence of intragenic recombination, Genetic Algorithm Recombination Detection method was applied to parsimony informative sites in PkDBP-α, PkDBP-β and PkDBP-γ separately. The GARD method implemented in the HyPhy package has identified evidence of 1 recombination breakpoint with significant topological incongruence between AICc score of the best fitting GARD model ($p < 0.05$) for PkDBP-α at nucleotide position 1973. Meanwhile, recombination breakpoints were found at 4 and 2 nucleotide sites in PkDBP-β and PkDBP-γ, respectively (Table 13).

**Table 13** Recombination sites in PcyDBP and PkDBP orthologues

| Locus | No. recombination sites | Recombination breakpoint at nucleotides |
|---|---|---|
| PcyDBP | 2 | 1263, 1399 |
| PkDBP-alpha | 1 | 1973 |
| PkDBP-beta | 4 | 900, 1322, 1625, 1838 |
| PkDBP-gamma | 2 | 482, 1307 |

**7. Determination of DARC phenotypes**

Eighty-six fresh blood samples from *Macaca nemestrina* (n = 69), *Macaca fascicularis* (n = 14), hybrid between *Macaca nemestrina* and *Macaca fascicularis* (n = 2) and Semnipithecus spp. (n = 1) were deployed to determine DARC phenotypes on erythrocytes using DIAMED ID Card test. Results reveal that all 86 blood samples were non-reactive to anti-Fya antibody (Figure 6A). By contrast, anti-Fyb antibody yielded reactivity with 4 blood samples from Macaca fascicularis while the remaining 10 samples were non-reactive. The degree of reactivity to anti-Fyb antibody was found to be +1 and weakly +1 in 3 and 1 monkeys, respectively, suggesting that the DARC gene did not express in the majority of long-tailed macaques in the study population. Meanwhile, erythrocytes from both hybrid monkeys had DARC expression showing reactivity of 1+ and 2+. On the other hand, erythrocytes from the majority of pig-tailed macaques (n = 65) were reactive to anti-Fyb antibody at 2+ while 3 monkeys were non-reactive to this antibody and one monkey showed weakly reactive (Figure 6B).

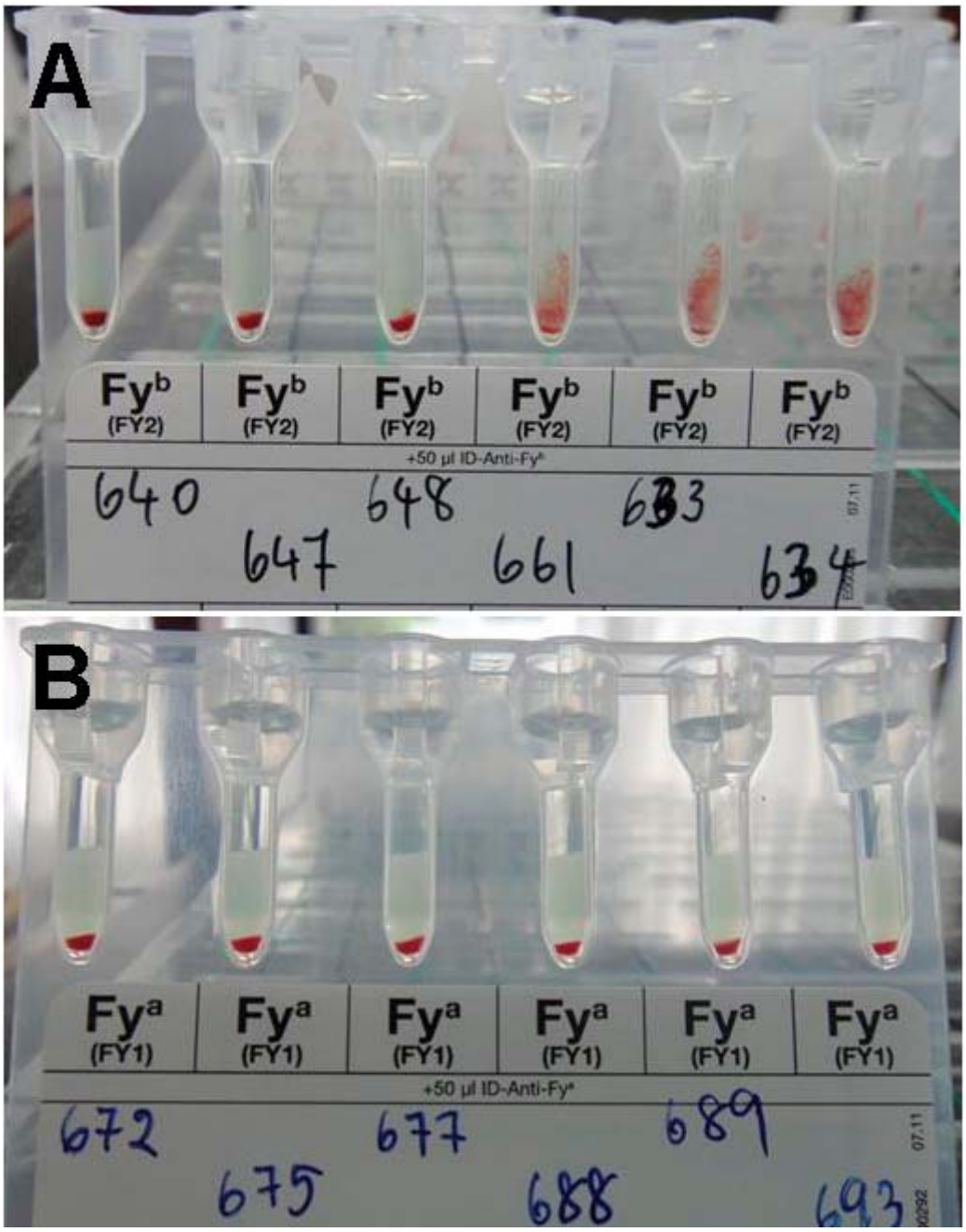


**Figure 6 (A)** Reactivity of anti-Fya antibody with macaques' erythrocytes. (**B)** Reactivity of anti-Fyb antibody with macaques' erythrocytes.

**8. DARC phenotype and susceptibility to nonhuman primate malaria**

Association analysis of DARC polymorphisms and DARC expression was performed using 14 individuals of *Macaca fascicularis* (28 chromosomes) and 69 individuals of *Macaca nemestrina* (138 chromosomes) whose erythrocyte Duffy blood group antigen was determined by using Anti-Fyb antibody. In total, 53 (51 bi-allelic and 2 tri-allelic) and 70 (70 bi-allelic) SNPs were identified in *Macaca fascicularis* and *Macaca nemestrina*, respectively. SNPs in each population were analyzed in the association test, where genotype at each SNP, rather than

haplotype, was considered. Results have shown that 2 and 8 SNPs were found to be deviated from Hardy-Weinberg equilibrium (HWE) test in *Macaca fascicularis* and *Macaca nemestrina*, respectively. At these SNP sites, the excess of homozygotes was observed. This implies that our samples are not entirely unrelated, i.e. some subjects could shared common ancestors. However, this issue does not influence our analysis on DARC phenotype and susceptibility to nonhuman primate malaria.

DARC negative erythrocytes of macaques were compared with their DARC positive counterparts that included a range of positive reactivity from weak positive, 1+, 2+, 3+, and mixed field as determined following the manufacturer's guideline. Various analytical models were performed including four genetic models (i.e, recessive, dominant, genotype, and allele), Among these four models, the results from allele model displayed relevant results pertaining to the data for association analysis. Results show that no strong evidence of association between DARC polymorphisms and DARC expression in these macaque species

Of interest, most *Macaca fascicularis* erythrocytes were DARC negative whereas most *Macaca nemestrina* erythrocytes were DARC positive. Among 14 *Macaca fascicularis*, only two were infected with *Plasmodium inui*. On the other hand, among 69 *Macaca nemestrina*, 34 were infected with any malaria. There was a significant difference in the proportion of infected subjects between two macaque species (Fisher's exact test $p = 0.019$). The lower infection rate in *Macaca fascicularis* may reflect the higher rate of DARC deficiency. Because *Plasmodium inui* infected *Macaca fascicularis* showing no DARC expression, Duffy antigen may not be used by *Plasmodium inui* as a receptor for RBC invasion in this macaque species. Most *Macaca fascicularis* erythrocytes were DARC negative; thereby, association of DARC polymorphisms with malaria infection could not be analyzed with good statistical power.

## DISCUSSION

Interactions between infectious agents and their hosts have led to adaptive evolutionary process that can shape genetics and phenotypes of both pathogens and hosts. On one hand, unsuccessful pathogens can be eliminated from hosts without establishment of reproductive cycle. On the other hand, competent pathogens can be highly adaptive to hostile efforts from host defense mechanisms such as host immune responses. Meanwhile, successful pathogens may cause a range of pathological spectrum from asymptomatic to fatal infections; thereby, selective pressure caused by pathogens may have significant influence on hosts at individuals or even at population level. Highly adapted hosts may evolve to reach refractoriness to certain infectious agents whereas poorly adapted ones may be eliminated from certain ecological niche (Hughes, 2000). In the case of *Plasmodium vivax* and humans, one of the major targets for such

interactive evolutionary process occurs during malarial merozoite invasion into human erythrocytes. It has been well recognized that human erythrocytes that lack DARC expression are resistant to *Plasmodium vivax* (Miller et al., 1975). A recent study has also supported that DARC expression on the surface of yellow African baboon's erythrocytes is essential for *Hepatocystis* infection (Tung et al., 2009). The genetic basis of such resistance is rather similar because certain mutations in the *cis*-regulatory region of both human and yellow baboon can control the expression of the DARC gene on erythrocyte surface (Tung et al., 2009).

Southeast Asian macaques have been known to be natural hosts for a number of nonhuman primate malaria. Of these, *Plasmodium knowlesi* have recently been identified as an important zoonotic malaria in humans. A high prevalence of this simian malaria in humans has been reported from Malaysian Borneo, Sabah and Malay Peninsula where low prevalent endemic areas have been widespread among Southeast Asian countries including Nicobar Island (Putaporntip et al., 2009a; Jongwutiwes et al., 2010; Singh and Daneshvar, 2013; Tyagi et al., 2013). It is noteworthy that establishment of *Plasmodium knowlesi* infection in humans requires the presence of susceptible monkey reservoirs, infected human hosts and appropriate anopheline vectors. Recently, a case of naturally acquired *Plasmodium cynomolgi* infection in human has been identified in Malay peninsular (Ta et al., 2014). Therefore, sporadic human infections with other nonhuman primate malaria reamin to be elucidated. Our cross-sectional surveys of malaria in long-tailed and pig-tailed macaques have reaffirmed the presence of these nonhuman primate malaria in southern Thailand, suggesting that transmission of these simian malaria to humans would be at higher risk than in western border of the country (e.g. Kanchanaburi Province) where none of these malaria were detected in native macaques (Seethamchai et al., 2008; Putaporntip et al., 2010). *Plasmodium knowlesi* was found in both long-tailed and pig-tailed macaques' blood samples while only *Plasmodium cynomolgi* was diagnosed from pig-tailed macaques' blood. Differential occurrence of *Plasmodium cynomolgi* in these macaques would have arisen from smaller number of sample size in long-tailed macaque group because this simian malaria is known to circulate in this monkey species (Coatney et al., 1966). Meanwhile, *Plasmodium inui* was the most prevalent malaria species in this survey that was in line with previous reports (Seethamchai et al., 2008; Putaporntip et al., 2010). It remains unknown why *Plasmodium inui* outnumbered other malaria in these macaques. Several possibilities could contribute to this finding such as high transmissibility of this simian malaria in mosquito vectors, high adaptability of this malaria in macaques' blood without causing severe pathological outcomes or other factors from host-parasite interaction. Although *Plasmodium inui* has been reported to cause overt malaria symptoms in experimental transmission from

macaques to humans, no evidence of natural transmission via mosquito vectors has been identified (Das Gupta, 1938; Coatney et al., 1966). Besides malaria, a *Plasmodium*-related parasite of the genus *Hepatocystis* has co-circulated among macaques in Narathiwat Province. Biting midges in the genus *Culicoides* serve as vectors for *Hepatocystis.* Only sexual stages of this malaria-related parasite develop in macaque's erythrocytes. The morphological features of these sexual stages could not be used for species identification (Seethamchai et al., 2008). By contrast, merocysts in liver are useful for speciation of *Hepatocystis*. At least four species of *Hepatocystis*, i.e. *Hepatocystis kochi, Hepatocystis simiae, Hepatocystis bouillezi,* and *Hepatocystis cercopitheci,* have reportedly infected African monkeys whereas *Hepatocystis semnopitheci* and *Hepatocystis taiwanensis* circulate among Oriental monkeys. Importantly, *Macaca fascicularis* is known to be a natural host for *Hepatocystis semnopitheci* and *Hepatocystis taiwanensis* (Garnham, 1966). However, this study detects a higher prevalence of *Hepatocystis* spp. in pig-tailed macaques than in long-tailed macaques, suggesting higher susceptibility of this hemoparasite in the former macaque species or from sampling artifact.

Our study is the first to explore the extent of sequence variation in the DARC gene including its regulatory region in long-tailed and pig-tailed macaque populations. It is noteworthy that the DARC locus of both macaque species exhibits more sequence diversity than those observed in humans. Meanwhile, differential sequence diversity occurred at each region of the gene, i.e. more SNPs in the *cis*-regulatory region in pig-tailed macaques than long-tailed macaques whereas the reverse was true for the coding region. Although it remains unknown how SNPs in the DARC locus have evolved, certain mutations in the *cis*-regulatory region could have been under adaptive evolution against malaria infections because significant difference in allele frequencies of SNP281 and of SNP690 in long-tailed macaques and susceptibility to *Plasmodium knowlesi* infection was observed in this study. This is also reaffirmed by the findings that SNP281, SNP467 and SNP690 in the same region of the DARC gene in pig-tailed macaques display significant difference in allele frequencies among those susceptible or resistance to *Plasmodium cynomolgi* infections.

Analysis of pattern of nucleotide substitutions in the coding region of the DARC locus has shown that long-tailed macaques possess more haplotype diversity than pig-tailed macaques. The haplotype diversity value is a measure of species evenness, i.e. a low haplotype diversity indicates that the distribution of haplotypes is skewed toward a few predominant haplotypes and a high haplotype diversity indicates the distribution is more even (Nei, 1987). Therefore, despite smaller sample size in long-tailed macaque population, difference in the number of samples included in this study would not drastically mislead our analysis. It is noteworthy that nucleotide

diversity in the coding region of long-tailed macaques significantly outnumbered that of pig-tailed macaques. However, the rates of synonymous substitutions and nonsynonymous substitutions per site were not significantly different, implying that the coding region of these macaques' DARC genes have been under neutral evolutionary process. Therefore, it is unlikely that the coding region has apparent interactive effect as a consequence of malaria infections. It is worth mentioning that DARC acts as a widely expressed promiscuous chemokine receptor besides being erythrocyte receptor for *Plasmodium vivax* and *Plasmodium knowlesi*. The expression of DARC is not only confined to erythrocyte surface but also in diverse tissues of the body such as endothelium cells and brain (Hadley and Peiper, 1997). Therefore, evolutionary pressure on the DARC locus can also arise from elsewhere other than from erythrocytes. Alternatively, the DARC locus in these macaque species may have different mutation rates or may under different structural or functional constraints. Although the results from this analysis do not support previous studies in humans and baboons that specific mutation in the *cis*-regulatory region of the DARC gene determines the phenotypes of corresponding erythrocyte receptors (Hadley and Peiper, 1997; Tung et al., 2009), our analysis has revealed for the first time that certain mutations in the 5' upstream sequence to the coding region of macaques' DARC genes have significant effect on susceptibility to or protection against some simian malaria in Southeast Asia.

The DARC locus of *PcyDBP* did not show distinct clusters among isolates. By contrast, *PkDBP-*$\alpha$*, PkDBP-*$\beta$ and *PkDBP-*$\gamma$ were placed in separate clusters, suggesting that divergence of malarial *DBP* seems to occur after speciation. Based on the topology of *PkDBP* family-derived phylogenetic tree, *PkDBP-*$\alpha$ and *PkDBP-*$\gamma$ diverged after the generation of *PkDBP-*$\beta$. Therefore, it is expected that nucleotide diversity in *PkDBP-*$\beta$ would be greater than those of *PkDBP-*$\alpha$ and *PkDBP-*$\gamma$ because a longer evolutionary period would increase the chance of accumulation of nucleotide substitutions. However, our analysis showed almost comparable levels of nucleotide diversity among these genes. It is likely that other genetic mechanisms would have influenced diversity at these loci. Analysis of the rates of synonymous and nonsynonymous substitutions per sites has shown significant departure from neutrality favoring synonymous changes in *PkDBP-*$\alpha$ and *PkDBP-*$\gamma$ while *PkDBP-*$\beta$ seems to evolve neutrally. Therefore, purifying selection would contributed to diversity in *PkDBP-*$\alpha$ and *PkDBP-*$\gamma$ loci, probably from some functional constraints on the encoded proteins. Consistently, the majority of nucleotide substitutions in these three loci were under negative selection. On the other hand, Tajima's *D* statistic reveals significant departure from neutrality at synonymous sites when *PkDBP-*$\alpha$*, PkDBP-*$\beta$ and *PkDBP-*$\gamma$ were compiled into a single group, suggesting diversifying selection predating divergence of these genes. Meanwhile, evidence of purifying selection was detected in the *PcyDBP* locus based on

Tajima's *D* and related statistics (Fu & Li's *D** and *F** tests) that showed significant negative values for Thai isolates. Alternatively, significant negative values of these tests may suggest that *Plasmodium cynomolgi* was under population expansion.

Despite selective pressure, intragenic recombination has been identified in a number of malarial genes (Putaporntip et al., 2001; 2002; 2009b; 2010). Genetic recombination exerts evolutionary advantages by generating and spreading of advantageous alleles and removal of deleterious variants. When back-mutation does not occur, harmful mutation or slightly deleterious mutation may be removed by recombination, thereby a higher level of nonsynonymous polymorphism will accumulate in recombining regions than those without recombination (Hughes, 2008). However, such finding was not observed in *PcyDBP* and *PkDBP* loci in this analysis, suggesting that elimination of harmful mutation or slightly deleterious mutation would be partly generated by other mechanisms. Nevertheless, the presence of recombination breakpoints in both *PcyDBP* and each member of *PkDBP* family would contribute to diversity at these genes. Therefore, sexual stages in mosquito vectors would be important for maintaining and enhancing diversity at these loci.

The gene encoding the Duffy antigen in humans contains two major polymorphisms as determined by anti-Fya and anti-Fyb antibodies (Yazdanbakhsh et al., 2000). An amino acid at codon 42 in the N-terminal part of this surface protein is associated with Duffy blood group phenotypes, being aspartic acid and glycine for Fyb and Fya blood-group antigens, respectively (Tournamille et al., 1995a). A single nucleotide substitution from T to C at nucleotide -33 in the cis-regulatory region of the Duffy gene abolishes Duffy expression on erythrocytes (Tournamille et al., 1995b). Sequence analysis of the DARC gene including its upstream sequence of *Macaca nemestrina* and *Macaca fascicularis* in this study has shown that all macaques had codon at position 42 coding for aspartic acid. Therefore, macaques erythrocytes could react with anti-Fyb antibody but not with anti-Fya antibody. However, the magnitude of reactivity seems to be variable, ranging from no reactivity to positive 3+. Unlike humans and baboons, association analysis fails to detect SNPs in the *cis*-regulatory region that control the expression of Duffy blood group on macaque erythrocytes, suggesting that genetic control of protein express at this locus may be complex. Importantly, substitution at codon 89 from ariginine to cysteine in human DARC results in very low expression caused by instability of RNA transcript; thereby, such change influences the level of Duffy blood group expression (Tournamille et al., 1998). The small number of blood samples included in Duffy blood group analysis in this study may preclude possible cryptic association in macaque populations. Further study is required to address this issue.

Duffy antigen on erythrocyte surface plays a crucial role in *Plasmodium vivax* erythrocyte invasion (Adams et al., 1992). The Duffy binding protein of *Plasmodium vivax* or PvDBP serves as the corresponding ligand on merozoite surface in which the binding domain has been located in a 330 amino acid cysteine-rich region referred to as region II, designated PvDBPII (Chitnis et al., 1996; King et al., 2008). Although a small number of *Macaca fascicularis* and *Macaca nemestrina* had Duffy negative blood group, this study reveals that DARC is not absolutely required for erythrocyte invasion by *Plasmodium fieldi, Plasmodium inui* and Southeast Asian species of *Hepatocystis*. Despite this finding, a particular SNP (V217I) in DARC of *Macaca nemestrina* has a significant influence on susceptibility to infections with these parasites. Unlike human erythrocytes, SNPs in the *cis*-regulatory region (as well as other SNPs in the coding region) of this locus did not determine the expression of DARC in both *Macaca fascicularis* and *Macaca nemestrina*. Undoubtedly, the control of DARC expression in long-tailed and pig-tailed macaques would be complicated than those found in humans and baboons.

## Conclusions

This study reveals polymorphisms at the DARC locus of *Macaca fascicularis* and *Macaca nemestrina* among wild populations in southern Thailand that are likely under neutral evolution. Genetic mechanism that controls the expression of pig-tailed and long-tailed macaques' DARC genes seems to differ from those of humans and baboons. However, certain SNPs in the DARC gene of *Macaca nemestrina and Macaca fascicularis* determine susceptibility to *Plasmodium knowlesi* and *Plasmodium cynomolgi* infections, respectively. Analysis of genes encoding Duffy-binding protein of *Plasmodium cynomolgi* and *Plasmodium knowlesi* has shown considerable sequence diversity that seems to be influenced by purifying selection. Understanding host-parasite interaction regarding diversity in the proteins that are known to play crucial roles to establish infection is important for subsequent implication in control measures or other related issues.

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## **Output** จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 **Putaporntip C**, Thongaree S, Jongwutiwes S. Differential sequence diversity at merozoite surface protein-1 locus of *Plasmodium knowlesi* from humans and macaques in Thailand. Infect Genet Evol. 2013;18:213-9.

1.2 **Putaporntip C**, Hughes AL, Jongwutiwes S. Low level of sequence diversity at merozoite surface protein-1 locus of *Plasmodium ovale curtisi* and *P. ovale wallikeri* from Thai isolates. PLoS One. 2013;8:e58962.

2. การนำผลงานวิจัยไปใช้ประโยชน์

เชิงวิชาการ

พัฒนาการเรียนการสอนการเรียนการสอน รายวิชา Clinical Hematology & Systemic Infection หลักสูตรแพทยศาสตร์บัณฑิต และ Advanced Medical Parasitology หลักสูตรปริญญาโท สาขาปรสิตวิทยาทางการแพทย์

3. การเสนอผลงานในที่ประชุมวิชาการ

ได้รับเชิญเป็นวิทยากรบรรยายเรื่อง Zoonotic and Novel Malaria Species in AEC ในงานประชุมวิชาการ The 52nd Annual Scientific Meeting 2013 - Healthcare Beyond Boundaries: Asean Initiative จัดโดย คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย วันที่ 18-21 มิถุนายน 2556