RESEARCH NOTE

DETECTION OF PUTATIVE ANTIMALARIAL-RESISTANT *PLASMODIUM VIVAX* IN *ANOPHELES* VECTORS AT THAILAND-CAMBODIA AND THAILAND-MYANMAR BORDERS

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Abstract. Monitoring of multidrug-resistant (MDR) *falciparum* and *vivax* malaria has recently been included in the Global Plan for Artemisinin Resistance Containment (GPARC) of the Greater Mekong Sub-region, particularly at the Thailand-Cambodia and Thailand-Myanmar borders. In parallel to GPARC, monitoring MDR malaria parasites in anopheline vectors is an ideal augment to entomological surveillance. Employing *Plasmodium*- and species-specific nested PCR techniques, only *P. vivax* was detected in 3/109 salivary gland DNA extracts of anopheline vectors collected during a rainy season between 24-26 August 2009 and 22-24 September 2009 and a dry season between 29-31 December 2009 and 16-18 January 2010. Indoor and outdoor resting mosquitoes were collected in Thong Pha Phum District, Kanchanaburi Province (border of Thailand-Myanmar) and Bo Rai District, Trat Province (border of Thailand-Cambodia): one sample from *Anopheles dirus* at the Thailand-Cambodia border and two samples from *An. aconitus* from Thailand-Myanmar border isolate. Nucleotide sequencing of dihydrofolate reductase gene revealed the presence in all three samples of four mutations known to cause high resistance to antifolate pyrimethamine, but no mutations were found in multidrug resistance transporter 1 gene that are associated with (*falciparum*) resistance to quinoline antimalarials. Such findings indicate the potential usefulness of this approach in monitoring the prevalence of drug-resistant malaria parasites in geographically regions prone to the development of drug resistance and where screening of human population at risk poses logistical and ethical problems.

Keywords: *Anopheles* spp, *Plasmodium vivax*, antimalarial resistance, Greater Mekong Sub-region, nested PCR, vector surveillance

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INTRODUCTION

Surveillance and monitoring systems of multidrug-resistant (MDR) *Plasmodium falciparum* are pivotal in endemic countries of the Greater Mekong Sub-region (GMS), and collaboration with concerted international partners is needed to cease emergence and spread of MDR *falciparum* malaria, especially of parasites with reduced sensitivity to artemisinin and its analogs (Alker *et al*, 2007; Wongsrichanalai and Meshnick 2008; Lim *et al*, 2009; Rogers *et al*, 2009; Anderson *et al*, 2010; Pyae *et al*, 2012). Surveillance and monitoring of MDR *falciparum* malaria is part of the Global Plan for Artemisinin Resistance Containment (GPARC) in GMS (WHO, 2008; Schapira, 2010; WHO 2011, 2012). In addition, monitoring *Plasmodium vivax* drug-resistant malaria has become increasingly important because the rise in incidence of chloroquine-resistant *P. vivax* (Baird, 2009; Price *et al*, 2009), and has been addressed in GMS, particularly in the Thailand-Myanmar, Thailand-Cambodia, Cambodia-Vietnam and Myanmar-China border regions (WHO, 2008; Schapira, 2010; WHO, 2011; USAID, 2012; WHO, 2012). Major factors attributing to the spread or emergence of drug-resistant *vivax* malaria transmission include ecological changes, human activities and vector population dynamics (Satitvipawee *et al*, 2012; Bhumiratana *et al*, 2013a,b).

According to GPARC, *in vitro* tests and molecular marker-based PCR methods constitute important tools for monitoring MDR malaria parasites in endemic areas or hotspots in GMS. In parallel, monitoring the presence of MDR *falciparum* and *vivax* parasites in anopheline vectors is an ideal augment for entomological surveillance (Snounou *et al*, 1993; Arez *et al*, 2000; Mahapatra *et al*, 2006; Temu *et al*, 2006; Mohanty *et al*, 2009; Imwong *et al*, 2011). In the present study, *Plasmodium*- and species-specific nested PCR methods were employed to detect malaria parasites in salivary gland DNA (SG DNA) extracts from wild-caught *Anopheles* vectors collected from Thailand-Myanmar and Thailand-Cambodia border areas. Nested PCR methods and DNA sequencing of *dhfr*, encoding dihydrofolate reductase, target of the antifolate pyrimethamine (Imwong *et al*, 2003; Vivain *et al*, 2007), and *mdr1*, encoding multidrug resistance transporter 1, associated with chloroquine resistance in *falciparum* malaria (Foote *et al*, 1990; Fidock *et al*, 2000), then were conducted on *P. vivax*-positive SG DNA samples.

MATERIALS AND METHODS

Study site and collection of salivary glands of *Anopheles* vectors

Anopheles mosquitoes were collected both indoors and outdoors at Raipa Village in Thong Pha Phum District, Kanchanaburi Province (Thailand-Myanmar border) and Bo Phloi Sub-district, Bo Rai District, Trat Province (Thailand-Cambodia border) (Bhumiratana *et al*, 2012b). The study took place in a rainy season between 24-26 August 2009 and 22-24 September 2009 and a dry season between 29-31 December 2009 and 16-18 January 2010. The environments of the two villages are similar, with mountainous forest, plenty of rubber and palm plantations and steams passing nearby the villages (Fig 1). Temperatures ranged from 21ºC to 26ºC. Catch collection of mosquitoes was done by separated indoor and outdoor teams from 6:00 PM to 12:00 PM. Mosquito species were identified and salivary glands of *Anopheles* spp preserved in 90% alcohol.

Anthropophagous *Anopheles* vectors collection and SG DNA extraction

In all, 109 *Anopheles* mosquitoes from

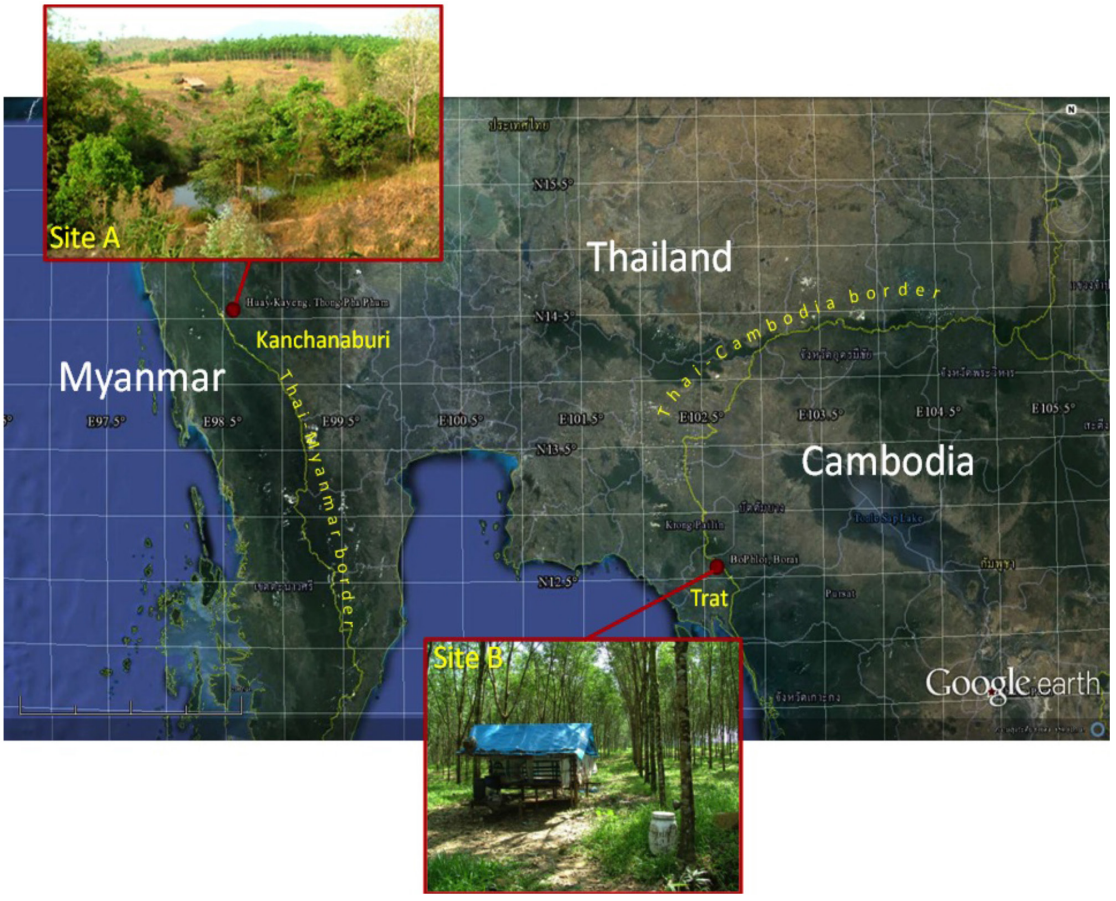


Fig 1–Mosquito collection sites. Site A, malaria endemic village close to rubber plantation in Huay Khayeng Sub-district, Thong Pha Phum District, Kanchanaburi Province, close to Thailand-Myanmar border (lat. 12.59°, long. 102.61°). Site B, malaria endemic village within rubber plantation in Bo Phloi Sub-district, Bo Rai District, Trat Province, close to the Thailand-Cambodia border (lat. 14.57°, long.98.59°).

endemic localities close to Thailand-Camboida border (n= 53) and Thailand-Myanmar border (n = 56) were examined and identified (Table 1). At the time of mosquito collection, each *Anopheles* mosquito was dissected under a stereomicroscope to recover salivary glands in the presence of sterilized 0.9% normal saline solution and kept in 90% alcohol. Extraction of SG DNA was carried out using Genomic DNA Mini Kit (Geneaid Biotech, New Taipei City, Taiwan). SG DNA solutions ($A260_{nm}/A280_{nm}$ > 1.7) were kept at -20°C until used.

Plasmodium species detection

Nested PCR assays for *Plasmodium* genus- and species-specific detection of each SG DNA sample was carried out as previously described (Snounou *et al*, 1993; Singh *et al*, 1999). In brief, first round PCR using universal primers amplified a 235 bp fragment of orthologous 18S rDNA of the four human malaria parasites (*P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*) and, in the second round PCR, internal sequences of the amplicons were amplified separately using species - specific primers

Table 1
Summary of *Anopheles* spp caught indoor and outdoor in Thong Pha Phum District, Kanchanaburi Province and Bo Rai District, Trat Province, Thailand during dry and rainy seasons.

Setting	Bo Rai		Thong Pha Phum	
	Rainy season (n)	Dry season (n)	Rainy season (n)	Dry season (n)
Indoor	*An. dirus* (2)	*An. dirus* (3) *An. minimus* (5)	*An. minimus* (2) *An. aconitus* (1) *An. maculatus* (1)	*An. dirus* (1) *An. minimus* (2) *An. aconitus* (1) *An. maculatus* (1)
Outdoor	*An. dirus* (9)	*An. dirus* (22) *An. minimus* (12)	*An. dirus* (2) *An. minimus* (6) *An. aconitus* (2)	*An. dirus* (10) *An. minimus* (10) *An. aconitus* (3) *An. maculatus* (12)

yielding amplicon of 121, 145, 206, and 226 bp for *P. vivax*, *P. malariae*, *P. falciparum*, and *P. ovale*, respectively. All experiments were performed in duplicate and positive genomic (g)DNA isolated from blood of patients infected with *P. falciparum* and *P. vivax* and negative (g)DNA from blood of patients with other parasitic infections and nuclease-free deionized water controls were included in every set of experiments.

Pvdhfr and *Pvmdr1* amplification and nucleotide sequencing

P. vivax-positive SG DNA samples were subjected to nested PCR amplification of *Pvdhfr* and *Pvmdr1* as described previously (de Pecoulas *et al*, 1998b; Imwong *et al*, 2003; Brega *et al*, 2005). In short, for *Pvdhfr* amplification, first round PCR using *Pvdhfr*-targeted primers produced a 711-bp amplicon that was subjected to second round PCR employing newly designed primers Pvh- FW (5´-ATCTGCGCATGCTGCAAG-3´) and Pvh–RV (5´-GAAGAAGACGTCACACGGCTAG-3´) and the following thermocycling conditions to produce a 582-bp amplicon (covering I13 to F206): 95ºC for 5 minutes; 30 cycles of 98ºC for 20 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds; with a final step of 72ºC for 1 minute. For *Pvmdr1* amplification, first round PCR using *Pvmdr1*-specific primers produced a 784-bp amplicon that was subjected to second round PCR employing newly designed primers MCQF (5´-AGAGATGTTCATTTGTTAAAAACCG-3´) and MCQR (5´-AGGGATTTCATAAAGTCATCCACT-3´) and the following thermocycling condition to produce a 521-bp amplicon (covering R931 to S1103): 95ºC for 5 minutes; 30 cycles of 98ºC for 20 seconds, 56ºC for 1 minute and 72ºC for 30 seconds; with a final step of 72ºC for 1 minute. All amplicons were analyzed by 1.7-2.0% agarose gel-electrophoresis and staining with ethidium bromide. From three mosquito isolates (Pvdibbt-1, Pvachtk-1 and Pvachtk-2), the two amplicons were purified by NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Düren, Germany) and sequenced from both directions at Macrogen (Korea). Sequences were analyzed using BlastN and BlastP programs and compared with those deposited in GenBank genome

Table 2
P. vivax-positive detection of salivary gland DNA samples isolated from wild-caught anthropophagous *Anopheles* vectors determined by genus-specific nested PCR assay.

PCR assay	Thailand-Cambodia border					Thailand-Myanmar border				
	An. dirus	*An. minimus*	*An. maculatus*	*An. aconitus*	Total	*An. dirus*	*An. minimus*	*An. maculatus*	*An. aconitus*	Total
Positive	1	0	0	0	1	0	0	0	2	2
Negative	35	17	0	0	52	13	20	16	5	54
Total	36	17	0	0	53	13	20	16	7	56

database. ClustalW2 program was employed to compare protein sequence homologies with those already published (de Pecoulas *et al*, 1998a; Imwong *et al*, 2003; Hasting, 2004; Brega *et al*, 2005; Na *et al*, 2005; Sa *et al*, 2005; Auliff *et al*, 2006; Lu *et al*, 2010). All DNA sequences were deposited at GenBank, accession nos. KC121333 - KC121335 (for *Pvdhfr*) and KC121336 - KC121338 (for *Pvmdr1*).

RESULTS

There was one *Plasmodium*-positive SG DNA sample obtained from *An. dirus* among the 53 *Anopheles* samples at the Thai-Cambodia border and two were detected from *An. aconitus* among the 56 *Anopheles* specimens at Thailand-Myanmar border (Table 2).

PCR-based assay using *Plasmodium* species-specific primers revealed all three SG DNA samples to be *P. vivax* (Fig 2B).

Sequence alignment of *Pvdhfr* 582-bp fragments (Fig 2C) showed that samples Pvdibbt-1 (from Thailand-Cambodia border) and Pvachtk-2 (from Thailand-Myanmar border) shared identical amino acid sequences, but Pvachtk-2 differed at two positions, namely, D105 instead of N and V145 instead of L (Fig 3A). Multiple sequence alignment of 25 deduced amino acid sequences of *P. vivax dhfr* homologs, consisting of 19 geographically prone haplotypes revealed that 2 haplotypes from Pvdibbt-1, Pvachtk-1 and Pvachtk-2, one haplotype (CAA05830) from one Comoros patient isolate and 16 haplotypes from 21 patients' isolates in the Asian region (Fig 3A). However, three sequences that share the same haplotype were originally isolated from different geographic areas, namely, The Philippines (ABC02003), Vanuatu (ABC02005) and Papua New Guinea (ABC02010). Similarly, two sequences, ABC02002 (from

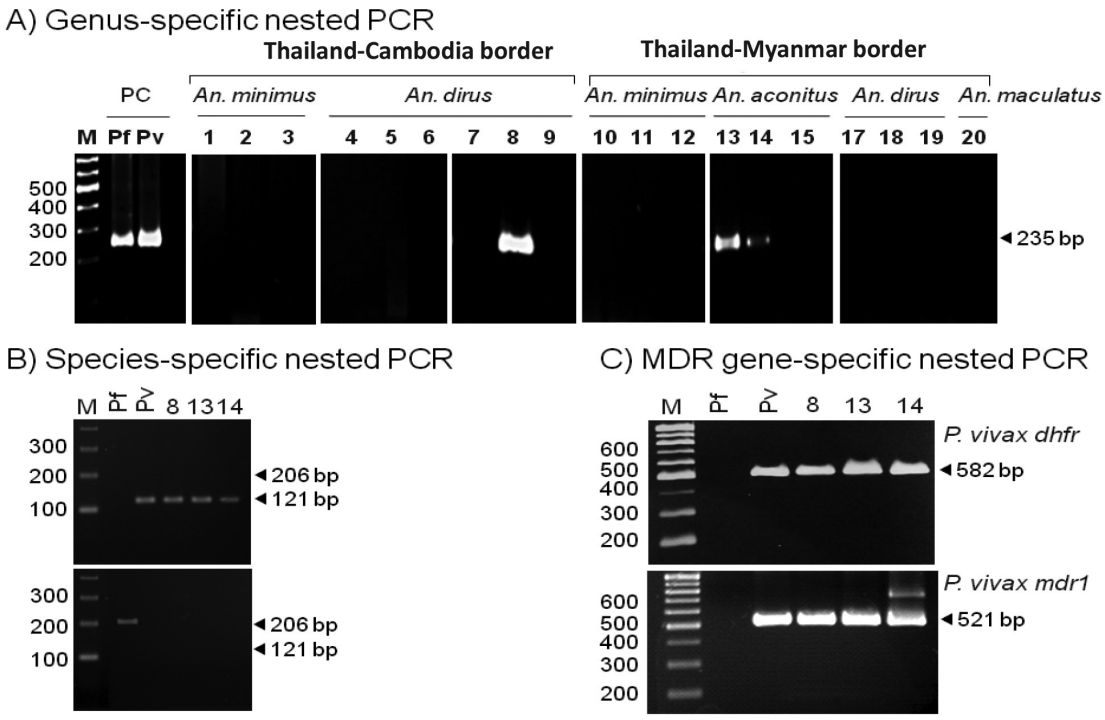


Fig 2–Electrophoresis of amplicons from mosquito salivary gland DNA. Amplicons were generated by nested PCR as described in Materials and Methods. (A) *Plasmodium* genus-specific amplicons of representative samples from *Anopheles* sp. PC, positive control; M, 100-bp DNA size markers; *Pf, P. falciparum*; *Pv*, *P. vivax*. (B) *Plasmodium* species-specific amplicons. (C) Gene-specific amplicons. MDR, malaria drug resistance.

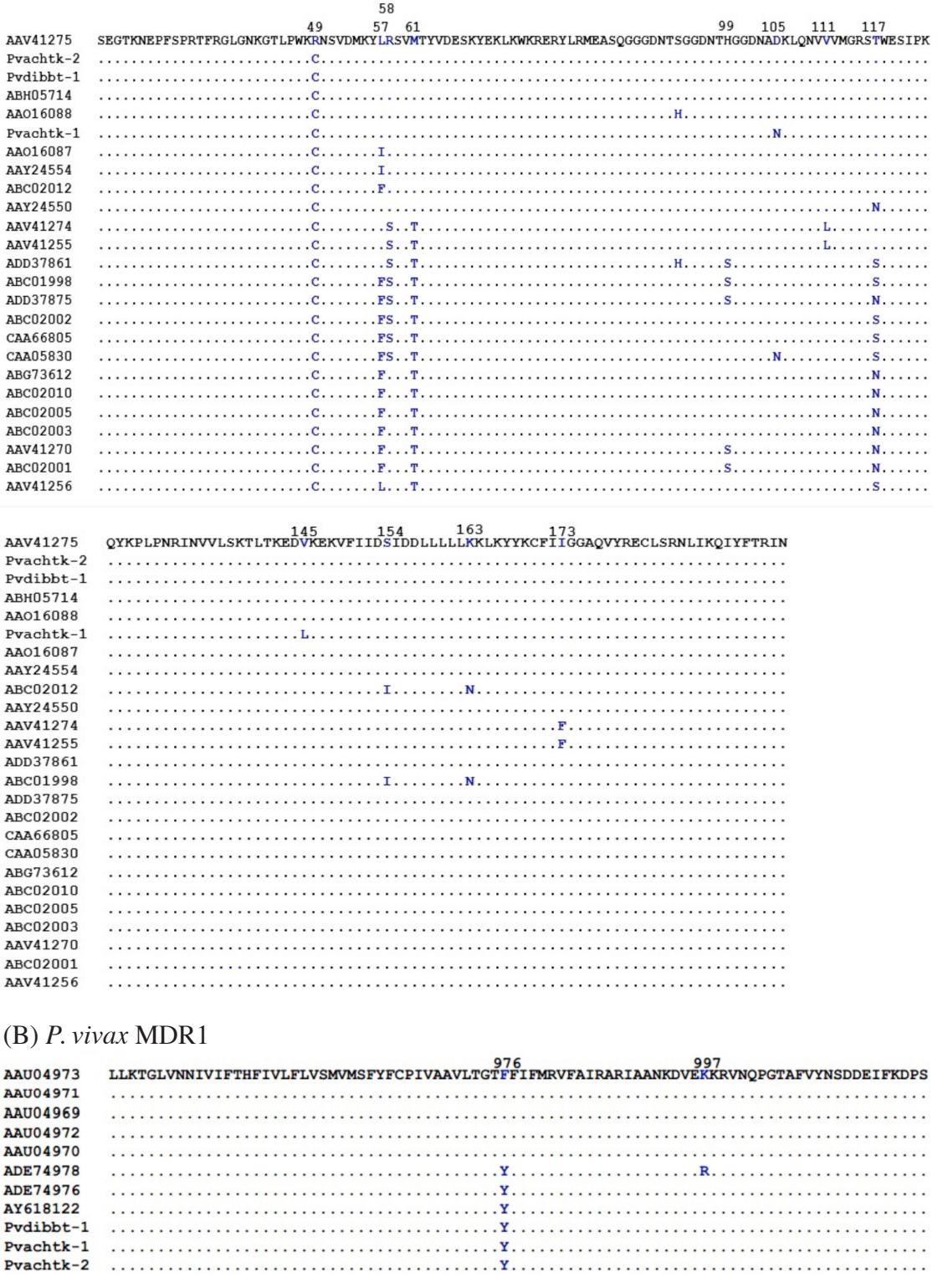
The Philippines) and CAA66805 (from laboratory strain of *P. vivax* asexual blood stages) also share amino acid variations.

Based on amino acid substitution and short tandem repeat variation, two groups of geographically prone MDR *vivax* malaria parasites are observed (Fig 3B). There are two short tandem repeats, a wild type (NTHGGD) and the other with a mutation H99S (arrow) juxtaposed to the wild type. Reference sequences used for alignment of *P. vivax* mdr1 are AAU04973 from India and AAU04971 from Papua New Guinea (Sa *et al*, 2005), ADE74976 and ADE74978 from Korea (Lu *et al*, 2011), and AAT38886 from strain Sal-1 (Brega *et al*, 2005). The sequences of all three *Pvmdr1* 521-bp fragments (Fig 2C) were identical and had high homology with *P. vivax* sequences from other geographical regions (Fig 3B).

DISCUSSION

Our study showed the potential use of PCR-based techniques to identify *Plasmodium* species present in *Anopheles* SG DNA and to characterize parasite genes that are linked to antimalarial resistance, in this case, *dhfr* and *mdr1*. Although only *P. vivax* was detected (3%) in *Anopheles* samples from the Thailand-Cambodia and Thailand-Myanmar border regions, the

(A) *P. vivax* DHFR



(B) *P. vivax* MDR1

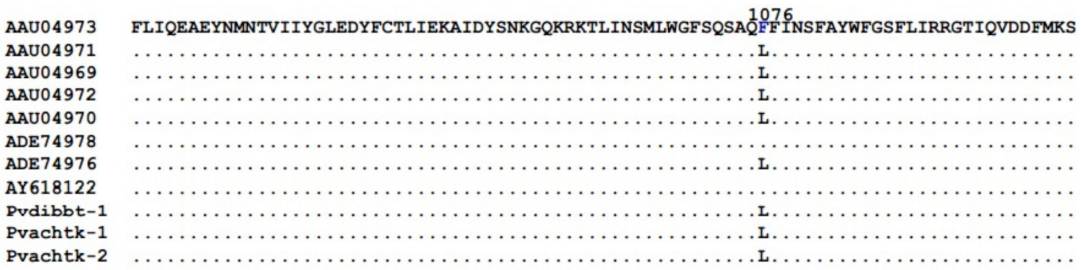


Fig 3–Sequence alignment of (A) *P. vivax* DHFR and (B) *P. vivax* MDR1 from South and southeast Asian regions. Dot designates same amino acid as indicated by the top uppermost sequence. *P. vivax* dhfr, Pvdibbt-1 (KC121333), Pvachtk-1 (KC121334), and Pvachtk-2 (KC121335) and *P. vivax* mdr 1, Pvmdr1-1 (KC121336), Pvmdr1-2 (KC121337) Pvmdr1-3 (KC121338) are from this study (submitted to GenBank by Sorosjinda-Nunthawarasilp *et al*, 1 Nov 2012). The other representative sequences are retrieved from GenBank: AAV41255 and AAV41270 from Indonesia (ID), AAV41256 from Papua New Guinea (PG), AAV41275 from ID/PG (Hasting *et al*, 2005); AAO016087 and AAO016088 from Thailand (Imwong *et al*, 2003); AAY24550 and AAY24554 from Myanmar (Na *et al*, 2005); ABC02001 from East Timor, ABC02002 and ABC02003 from The Philippines, ABC02005 from PG, ABC 02010 and ABC02012 from Vanuatu (Auliff *et al*, 2006); ABG73612 from India (submitted by Prajapati and Jhoshi, 9 Jun 2006); ABH05714 from Iran (submitted by Alam and Sharma, 21 Jun 2006); ADD37861 from South Korea (Lu *et al*, 2010); CAA05830 from Comoros Island (de Pecoulas *et al*, 1998a); CAA66805 from Yemen (de Pecoulas *et al*, 1998b).

limited number of mosquitoes collected from a single site at the two border areas did not allow any conclusion to be reached regarding prevalence of *Plasmodium* species or vector capacity. Mixed *P. falciparum* and *P. vivax* malaria infections occur at both Thailand-Myanmar and Thailand-Cambodia border provinces. The overall prevalence of both species are approximately equal (Luxemburger *et al*, 1999) and thus mixed infections of *P. falciparum* and *P. vivax* are not uncommon.

Uncomplicated malaria (*P. falciparum* and *P. vivax*) had been treated with chloroquine as the first-line drug with sulfadoxine/pyrimethamine used as the second-line drug in these geographical areas (Reyburn, 2010). A large majority of patients had been treated on the basis of presumptive diagnosis. *P. falciparum mdr1* and *dhfr,* linked to chloroquine or sulfadoxine-pyrimethamine chemoresistance respectively, have been widely reported (Djaman *et al,* 2001; Gama *et al*, 2011; Jovel *et al*, 2011). Chloroquine resistance of *P. vivax* was noted in infected patients in Thailand in 1999 (Looareesuwan *et al*, 1999). Evidences of *P. vivax*-chloroquine resistance have been reported in Myanmar since 1995 (Marlar-Than *et al*, 1995) and the emergence of *P. vivax* chloroquine resistance has spread across the country (Suwannarusk *et al*, 2007; Satitvipawee *et al*, 2012). However, evidence of *P. vivax*-chloroquine resistance had not been found in Thailand and Cambodia where chloroquine is still highly effective against *P. vivax* (Satitvipawee *et al*, 2012). Nevertheless, three cases of parasite re-appearance on days 28 and 42 of follow-up has been found in Pailin, Cambodia, which may be indicative of recrudescence or may indi-

cate *P. vivax* has developed chloroquine resistance in this region (WHO, 2010).

Interestingly, all three *Pvdfhr* sequences found in this study contain 57L, 58R, 61M and 117T, amino acids known to cause *P. vivax* dihydrofolate reductase resistance to pyrimethamine (the pyrimethamine-sensitive enzyme contains 57F, 58S, 61T and 117S) (Lee *et al*, 2010). In 2001, *P. vivax dhfr* in Cambodian blood samples was found to have mutations of S58R and S117N (Imwong, *et al*, 2001, 2003) and by 2005, *P. vivax dhfr* in Myanmar and Thailand-Myanmar border region had mutations of S58R, S117N and I173L, as well as F57L, S58R, T61M and S117T (Na *et al*, 2005; Auliff *et al*, 2006). Because chloroquine was the first-line and sulfadoxine/pyrimethamine was the second-line drug for malarial treatment, this may imply that *P. vivax* had been under sulfadoxine/pyrimethamine selective pressure due to mixed infection or treatments based on misdiagnosis (Mayxay *et al*, 2004).

The three *P. vivax* mdr1 sequences in this study contained 1076L, a variant not related to quinoline resistance, suggesting that *P. vivax* in the Thai-Cambodia and Thailand-Myanmar border regions are still sensitive to chloroquine and primaquine.

In summary, this study, although limited in scope, demonstrates that in areas with malaria transmission, monitoring anopheline vectors using PCR-based methods coupled with nucleotide sequencing of amplicons not only allows identification of *Plasmodium* spp but may also detect existence of parasite drug resistance. Monitoring parasites in vector populations is suitable in tracking migration of geographically prone MDR *P. falciparum* and *P. vivax* and can be performed together with entomological surveillance. This approach is advantageous in malaria transmission areas where there is a highly mobile human population who are not amenable for screening due to logistical and ethical reasons.

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