博士論文

Temporal patterns in the dynamics of molecular markers for anti-malarial resistance in Myanmar

（ミャンマーにおける抗マラリア剤耐性分子マーカーの時間的ダイナミクス）

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ABBREVIATIONS

ACTs	Artemisinin based combination therapies
AMT	Artemisinin monotherapy
AL	Artemisinin-lumifantrine
dhfr	*Dihydrofollate reductase gene*
dhps	*Dihydropteroate synthase gene*
DP	Dihydroartemisinin-piperaquine
GPARC	Global Plan for Artemisinin Resistance Containment
GMS	Greater Mekhong Subregion
kelch 13	Kelch Protein 13
MARC	Myanmar Artemisinin Resistance Containment
pfmdr1	*Plasmodium falciparum multidrug resistance 1 gene*
pfcrt	*Plasmodium falciparum chloroquine resistant transporter gene*
RDT	Rapid diagnostic test
SP	Sulfadoxine-pyrimethamine
SNP	Single nucleotide polymorphism
WHO	World Health Organization

ABSTRACT

Understanding the dynamics of *Plasmodium falciparum* parasite population in terms of drug resistance is of paramount importance especially in Myanmar, a country with increasing prevalence of artemisinin resistance and the greatest burden of malaria in the Greater Mekhong Sub-region. Here, I attempt to clarify how change of drug treatment policy in Myanmar, particularly dramatic increase in artemisinin usage, affected population dynamics of *P. falciparum*.

Blood samples from patients infected with *P. falciparum* were collected from four areas in two periods; before (2002-5) and after (2013), the official implementation of artemisinin combination therapies (ACTs) in Myanmar. I determined variants of newly identified artemisinin-resistant gene (*kelch 13*) and well-known chloroquine and pyrimethamine/sulfadoxine resistant genes (*pfcrt*, *pfmdr1*, *dhfr*, and *dhps*). To clarify the evolutionary lineages of anti-malarial resistant parasites, microsatellite markers flanking *pfcrt*, *dhfr* and *dhps* were assessed and compared to those in the other endemic regions.

In artemisinin resistance, a significant increase in the prevalence of parasites harboring *kelch 13* mutation was observed from 8.6% in 2002-2005 to 24% in 2013 (p = 0.0031). One novel mutation (Y511H) was observed in the 14.8% of samples in 2013, suggesting potential selection after the ACTs initiation. In contrast, three SNPs (G449A, R561H and C580Y) that have been reported to be associated with artemisinin resistance in the Greater Mekhong Sub-region were not identified. Almost all fixation of *pfcrt* K76T and overwhelming of highly resistant types of *dhfr* and *dhps* mutant parasites persisted even after the withdrawal of official chloroquine and pyrimethamine/sulfadoxine usage. Lineages of chloroquine and pyrimethamine/sulfadoxine resistant parasites were shared between Myanmar and other endemic countries in Asia and Africa.

This study suggests that artemisinin resistance arose independently and was selected in Myanmar, in sharp contrast to chloroquine and pyrimethamine/sulfadoxine resistance. Change of malaria treatment regime has not induced any sign of reduction of chloroquine and SP resistant *P. falciparum*. These temporal changes in molecular markers of anti-malarial resistance under increasing artemisinin pressure in Myanmar will provide an insight into global prevention and control of artemisinin resistance.

Keywords

Malaria, *Plasmodium falciparum*, Myanmar, Drug resistance, Molecular epidemiology, artemisinin, chloroquine, pyrimethamine/sulfadoxine, molecular markers

1. INTRODUCTION

1.1 Global malaria burden and situation in Myanmar

Malaria is one of the life-threatening infectious-diseases caused by a hematoprotozoan parasite of the genus *Plasmodium*. Five different *Plasmodium* species, namely *Plasmodium falciparum* (*P. falciparum*), *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* are pathogenic to human [1]. Among these species, *P. falciparum* causes serious forms of malaria and accounts for the majority of morbidity and mortality in malaria endemic countries. It has been estimated that 3.4 billion people are at risk of malaria worldwide [2]. In 2013, about 198 million cases of malaria and 584 000 deaths were reported globally [2]. Currently, global malaria prevention and control greatly relies on the effectiveness of a multi-pronged strategy including vector control, preventive therapies, parasite based diagnostic testing and treatment with quality-assured artemisinin based combination therapies (ACTs) [2, 3]. Since the WHO recommendation of ACTs as a first-line treatment for uncomplicated malaria in 2006, expanding access to ACTs has been regarded as most contributing factor for the remarkable recent success in reducing the global malaria burden [3]. However, artemisinin resistance emerged from western Cambodia in 2006-7 [4] and now potential resistant parasites have been confirmed in the Lao People's Democratic Republic, Myanmar, Thailand and Vietnam [3].

Myanmar has the highest burden of malaria among Southeast Asian countries; it accounted for 78% of malaria cases and 75% of malaria deaths in the Greater Mekong Subregion (GMS) comprising Cambodia, the People`s Republic of China (PRC, specifically Yunnan Province and Guangxi Zhuang Autonomous Region), Lao People`s Democratic Republic (Lao PDR), Myanmar, Thailand, and Vietnam [5]. Myanmar is 676,578 square kilometers in area and composed of seven states and seven regions [6]. In Myanmar, malaria is the first priority public health problem which greatly affects the socioeconomic development of the country [6]. In 2010, WHO and Myanmar Ministry of Health described that more than 650,000 malaria cases were reported and 788 cases were fatal [6, 7]. Official report in Ministry of Health in Myanmar showed that morbidity and mortality rate reduced from 24.4% and 12.6% in 1990 to 11.7% and 1.2% in 2011 (Fig.1) [7]. Cumulative probability of malaria death for individuals of all ages in Myanmar has been estimated as 53.3 per 1000 population [8]. An estimated 69% of the population (over 40 million people) still reside in malaria endemic areas, and among them 24 million live in high-transmission areas (more than one case per 1000 population) in 2010 [2]. Malaria transmission is almost perennial and related to the forest (48%) and forest fringes (12%) [6]. Overall, 74% of malaria infection is caused by *P. falciparum* in Myanmar [6].

1.2 Life cycle and genome of *Plasmodium falciparum*

P. falciparum has a complex life cycle involving two hosts: humans and *Anopheles* mosquitoes (Fig.2). Infection begins with the inoculation of sporozoites into the human host by the bite of malaria-infected female *Anopheles* mosquito. Sporozoites travel to the host liver and infect hepatocytes where they mature into schizonts and start asexually (mitotically) replicating. After a period of 1-2 weeks, the mature schizonts rupture, producing thousands of merozoites into the blood stream and infect the red blood cells where they transform into trophozoites (intraerythrocytic stage). The trophozoites develop schizonts and multiply in the invaded red blood cells which rupture releasing merozoites within 48 hours of invasion. These merozoites invade new erythrocytes, continuing the intraerythrocytic stage. Some merozoites differentiate into gametocyte, a sexual erythrocytic stage of the parasite (gametocytegenesis). Male and female gametocytes are ingested by female *Anopheles* mosquito during their blood meal. Then, they fertilize to form the zygote in the midgut of mosquito and undergo meiosis. Ookinate penetrates the gut wall [9] and develops into an oocyst which produces and releases thousands of sporozoites into the body cavity of the mosquito. Then, these sporozoites migrate to the mosquito salivary gland.

P. falciparum has 23-megabase nuclear genome composed of 14 chromosomes which encodes about 5,300 genes with the (A+T) composition of 80% [10]. It also has the mitochondrion genome of 6-kilobase linear sequence and the apicoplast genome of

35-kilobase circular sequence. The mitochondrion genome is inherited in female gametocytes and does not undergo recombination process among lineages [11]. Geographic differentiation of *P. falciparum* has been achieved by determining sequence variation in mitochondrion genome [12]. Apicoplast is a plastid, homologous to chloroplasts of plants, that is found in most *Apicomplexa* including malaria parasites and believed to involve in the metabolism of fatty acids, isoprenoids and heme [13]. Provided that characteristic genetic composition, wide levels of transmission and endemicity of infection were found to produce diverse populations of *P. falciparum* [14]. These particular genetic characteristics and population diversity of *P. falciparum* are responsible for multiple effective immune evasion and acquirement of drug resistance of the parasite [15].

1.3 Anti-malarial resistance and molecular markers of *P. falciparum*

Anti-malarial resistance was initially defined as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject"[16]. This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action". ACTs can reduce parasite load by a factor of 10,000 per 48-hour asexual cycle and

clear most of the sensitive parasites within 2 days [17]. In case of artemisinin resistance, ring stage parasites that are shortly exposed to artemisinins have reduced susceptibility, resulting in delayed parasite clearance following treatment with ACTs [17]. The parasite load was found to be reduced only by a factor of 100 per cycle and it takes 3 or 4 days to empty resistant parasites after treatment with ACTs [17]. There have been four methods to monitor anti-malarial drug efficacy; namely, therapeutic efficacy studies, in vitro test/ex vivo test and assessment of drug resistant related molecular markers. Therapeutic efficacy study measures the clinical and parasitological responses to anti-malarial drugs by consistent monitoring over time. The test procedure is based on a 28 or 42 day follow-up (depending on drug) with clinical, parasitological, hematocrit and fever assessment [16]. Treatment outcomes are classified as early treatment failure, late clinical failure, late parasitological failure and adequate clinical and parasitological response. In vitro assays are used to assess the level of *P. falciparum* susceptibility to anti-malarial drugs by measuring inhibition of parasite growth or reduction of schizont maturation [16]. Results are expressed as the 50% and 90% inhibitory concentration (IC_{50} and IC_{90}) or the minimum inhibitory concentration (MIC). Ex vivo test uses malaria infected patients' blood for culturing the parasites to determine their sensitivity to anti-malarial drugs. However, in case of artemisinin resistance, significant fall in in vivo parasite clearance rates was not supported by conventional in vitro drug susceptibility tests [4]. In this regard, Witkoski, et al introduced ring-stage survival assay (RSA) which measures

the survival rate of young ring-stage parasites to dihydroartemisinin and its results showed strong correlation with the half-life parameter [18].

Mutations including SNPs and copy number polymorphisms that are associated with anti-malarial resistance are widely used as molecular markers for screening drug resistant parasites [19]. To date, some molecular markers for anti-malarial drug resistance have been identified: the genes encoding the chloroquine resistance transporter (*pfcrt*), the multidrug resistance 1 protein (*pfmdr1*), dihydrofolate reductase (*dhfr*), and dihydropteroate synthase (*dhps*), K13-propeller gene (*kelch 13*) [19-25].

Regarding molecular markers of chloroquine resistance, *pfcrt* is a gene encoding 48.6 kDa transmembrane proteins containing 424 amino acids and it is located on chromosome 7 [26]. Mutations in *pfcrt* particularly, K76T confer chloroquine resistance in *P. falciparum* by lowering the accumulation of chloroquine in the parasite food vacuole by increasing efflux of chloroquine [27, 28]. *pfmdr1* is a mammalian multidrug resistance gene homologue, encoding P-glycoprotein and it is located on chromosome 5 [29]. Mutations at codon 86, 184, 1034, 1042 and 1246 of *pfmdr1* and variations in its copy number were associated with sensitivity to chloroquine, quinine, mefloquine,lumefantrine, and artemisinin [23, 30]. Recent structural analysis of *pfmdr1* proposed two mechanisms: internal allosteric modulation by polymorphisms at codons 86/184/1246 and altering drug pocket by polymorphisms at codons 1034 and 1042 [31, 32]. In substrate specific model, wild type *pfmdr1* mediates transport of

chloroquine and quinine to parasite digestive vacuole whereas mutations in *pfmdr1*do not [31]. In sulfadoxine and pyrimethamine (SP) resistance, mutations in the parasite genes encoding two enzymes of folate synthesis; *dhfr* and *dhps* confer resistance [24]. Amino acid substitutions in *dhfr* gene at codons 50, 51, 59, 108, and 164 were found to be responsible in vivo and in vitro resistance to pyrimethamine by altering the shape of the DHFR enzyme active site [20, 21], inhibiting the reduction of dihydrofolate. Similarly, mutations at codon 436, 437, 540, 581, and 613 in *dhps* confer sulfadoxine resistance by decreasing enzyme's affinity for sulfadoxine [21, 24]. Moreover, both *dhfr* and *dhps* mutations occurred in a stepwise fashion, i.e. additional mutations accumulate only on the background of preceding mutations resulting in higher levels of resistance [33, 34]. Regarding recent emergence of artemisinin resistance, Takala, et al linked SNPs at *MAL13-1718319* and *MAL10-688956* with delayed parasite clearance [35]. *MAL10-688956* is located on chromosome 10 in the 3' untranslated region of the DNA polymerase delta catalytic subunit gene and *MAL13-1718319* is in or near a RAD5 homolog [36]. Very recently, mutations in the PF3D7_1343700 Kelch propeller domain (*kelch 13*) of *P. falciparum* have been suggested to play a critical role in the increasing in vitro parasite survival rates and in vivo parasite clearance rates [25].

Suspected partial artemisinin resistance is defined as: ≥5% of patients carrying *kelch 13* resistance-associated mutations; or ≥ 10% of patients with persistent parasitemia by microscopy on day 3 after treatment with ACTs or artesunate monotherapy; or ≥ 10% of

patients with a parasite clearance half-life of ≥ 5 hours after treatment with ACTs or artesunate monotherapy. Confirmed partial artemisinin resistance is defined as: ≥ 5% of patients carrying *kelch 13* resistance-associated mutations, all of whom have been found, after treatment with ACTs or artesunate monotherapy, to have either persistent parasitaemia by microscopy on day 3, or a parasite clearance half-life of ≥ 5 hours [37] .

1.4 Epidemiology of anti-malarial resistance

To date, *P. falciparum*has been resistant or reduced sensitivity to all the conventional anti-malarial drugs including artemisinin derivatives currently used for treating uncomplicated *P. falciparum* malaria [16, 37, 38]. Independent evolution of chloroquine resistance has been documented in at least four different regions: Southeast Asia, two regions in South America, and New Guinea [39, 40]. Regarding pyrimethamine resistance, quadruple mutant *dhfr* (CIRNL) (where letters indicate amino acid positions 50, 51, 59, 108, and 164, and the altered residues are underlined) and triple mutant *dhfr* (CIRNI) have been the most predominant haplotype observed in Southeast Asia but not in Africa [39, 41-44]. All *dhfr* triple and quadruple mutant parasites in Southeast Asia were found to share same or nearly identical lineage and also its subsequent spread to Africa has been demonstrated [44-47]. In global spread of sulfadoxine resistance, six distinct lineages of *dhps* mutant parasites has been identified, of which four highly sulfadoxine resistant lineages (three mutations in the

dhps gene) stemmed from Southeast Asia and South America [36, 47-49]. Artemisinin resistance emerged from western Cambodia in 2006-7 [4] and now it has been confirmed in other countries of the GMS [38, 50].

1.5 Timeline of conventional anti-malarial drug usages in Thailand, Cambodia and Myanmar

Chloroquine was used as a first line drug from 1950s to 1972 in Thailand, until 1993 in Cambodia and 2002 in Myanmar [7, 51, 52]. Chloroquine-resistant parasites were first detected in Myanmar in 1969, about 10 years after the initial identification in Thai-Cambodia border in the end of 1950s [7, 51-53]. In vivo and in vitro resistance levels reached to the highest level (over 90%) around 1980 in Myanmar. After that, the gradual recovery of chloroquine susceptibility was observed (Fig.3). In contrast, persistence of high level chloroquine resistance has been observed in Thailand and Cambodia since 1990s [54-59]. Consistently, most of molecular studies described almost fixation of *pfcrt* K76T mutation in these countries [40, 43, 57-62].

After the spread of chloroquine resistance, SP was implemented as the first line drug in Thailand in the late 1960s while in Myanmar and Cambodia, it was used as the second line drug in 1980-2002 and 1982-1993, respectively [7, 63, 64]. However, in Myanmar, emergence of SP resistance had been already reported in 1971, 9 years before the official

introduction (Fig.4) [7]. An in vitro study conducted in middle Myanmar in 1990 revealed that 34% of isolates were resistant to SP [7]. In Thailand, in vivo and in vitro resistance to SP appeared earlier than Myanmar and Cambodia, which reached at 100% around 1980 [65].

Since the mid-1970s in Cambodia and 1990s in Thailand and Myanmar, before the official implementation of ACTs, unregulated artemisinin or artesunate monotherapy was used [5-7, 16, 38, 54]. In vivo surveys showed varying degrees of susceptibilities to artemisinin from 61 to 100% in Myanmar since the late 1980s (Fig.5) [7]. Accordingly, in vitro studies revealed various levels of IC_{50} ranging from 85.7 to 100% [7]. In Cambodia and Thailand, most in vivo studies conducted before 2000 showed that late treatment failure rate was less than 10% [54]. Clinical cases showing day 3 parasitemia after ACT treatment that is a clinical hallmark of reduced susceptibility to artemisinin was first identified in 2006 (Cambodia) and in 2008 (Thailand and Myanmar) [4, 7, 38, 66, 67]. Since then, prevalence of day 3 parasitemia has been increasing in these three countries (Fig.5) [56, 68].

The GMS has been regarded as an epicenter of drug resistance as chloroquine and SP resistance emerged from this region and spread into Africa. If artemisinin resistant parasites follow the similar pattern, it will undoubtedly threaten to the current strategy of malaria control [38]. In this scenario, Myanmar becomes a particular malaria endemic region with a global focus. However, previous documentation about molecular epidemiology and evolution for anti-malarial drug resistance is very limited in Myanmar [45, 69-76]. In

addition to the molecular epidemiology, understanding of parasite population dynamics under treatment policy change surely provides important implications for current global artemisinin containment effort as well as future prevention of emergence of resistance. Here, I attempt to clarify how change of anti-malarial treatment policy in Myanmar, particularly dramatic increase in artemisinin selecting pressure, affected population dynamics of *P. falciparum* using samples obtained before (2002-5) and after (2013) the official introduction of ACTs.

2. MATERIALS AND METHODS

2.1 Study site and collection of samples

Based on the previous research and surveys conducted and with the establishment of Myanmar Artemisinin Resistance Containment Program, Bago region and surrounding three areas (Mandalay region, Yangon region, and Mon state) were regarded as Tier I area by WHO, where "there is credible evidence of artemisinin resistance for which intensified and accelerated malaria control towards universal coverage is recommended"[77] (Fig.6). Bago region consists of 28 townships and is located in the south of Myanmar with 39,402.3 km^2 of forested land. With an annual rainfall of 3,291 mm and temperatures ranging from 20.9°C and 32.3°C, malaria is endemic all year round with a peak in transmission in June and July. According to 2014 Myanmar census, population sizes in Bago, Mandalay, Yangon regions and Mon state are 4,867,373, 6,165,723, 7,360,703 and 2,054,393 respectively.

DNA samples from 2002-5 period stored in Nagasaki University, Japan were kindly provided by my research collaborator. These samples were collected from patients of all ages and genders diagnosed as *P. falciparum* malaria by clinical features and blood film microscopy in Bago region and surrounding three areas. In 2013, patients of all ages and genders with clinical symptoms suspected of malaria attending township hospital and rural health centers in Bago region were recruited by convenient sampling method. Written informed consent was obtained from patients or from their parents in case of children.

Patients with *P. falciparum* infection confirmed by rapid diagnostic tests (RDT) were enrolled and provided with ACTs according to the national anti-malarial treatment guidelines. All the samples in both 2002-5 and 2015 were collected in rainy season.

Finger-prick blood (100 μL) was spotted onto Whatman ET31CHR chromatography filter paper. *P. falciparum* DNA was extracted from a quarter of spot using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) with some modifications as described elsewhere [78]. This study was approved by the ethical committees of Military Institute of Nursing and Paramedical Sciences in Myanmar, Juntendo University and The University of Tokyo, Japan.

2.2 Genotyping and sequencing of molecular markers for anti-malarial resistance

P. falciparum infection was confirmed by species-specific polymerase chain reaction (PCR) using small subunit ribosomal ribonucleic acid (ssrRNA) genes as previously described [79].

2.2.1 Artemisinin resistance

Mutations in the *kelch 13* propeller domain which locates on chromosome 13 were evidenced to confer delayed parasite clearance in vitro and in vivo [25, 80-82]. C580Y and other mutations (Y493H, R539T, I543T) in and near the *kelch 13* propeller region were also observed to be linked with artemisinin resistance. Recently, artemisinins are found to be

potent inhibitors of *Plasmodium falciparum* phosphatidylinositol-3-kinase (PfPI3K). Recently, C580Y, the most prevalent K13 mutation in GMS was evidenced to reduce polyubiquitination of *P. falciparum* phosphatidyl inositol-3-kinase (PfPI3K) and its binding to K13 and thereby raised the levels of the kinase by limiting proteolysis of PfP13K [82]. Propeller domain was amplified by nested PCR followed by direct sequencing as described by Ariey et al. [25]. Sequences showing mixed infections, as judged from overlapping peaks in an electrogram, were treated as follows. If height of minor peak was shorter than a half of major peak, the minor peak was ignored. If height of minor peak exceeded a half of major peak, two peaks were taken as a mixed infection.

2.2.2 Chloroquine resistance

Amino acid substitution at position 76 in *pfcrt* [28] and five amino acid substitutions (N86Y, Y184F, S1034C, N1042D and D1246Y) in *pfmdr1* [23, 29, 30, 83] were determined by a nested PCR, followed by restriction fragment length polymorphism (RFLP) as previously reported [84].

2.2.3 SP resistance

dhfr and *dhps* were amplified by nested PCR, followed by direct sequencing with Big Dye terminator version 1.1 cycle sequencing kit in ABI 3100 DNA Sequencer (Applied Biosystems) as described elsewhere [21]. All known mutations that were evidenced to be associated with SP resistance (C50R, N51I,C59R, S108N and I164L in *dhfr* and S436A,

A437G, K540E, A581G and A613T/S in *dhps* were determined [85-87]. Accumulation of these mutations was suggested to follow a unique stepwise trajectory to produce the most highly resistant form with four mutations in *dhfr* and three in *dhps* [33].

2.3 Microsatellite markers flanking *pfcrt, dhfr* and *dhps*

To find out the evolutionary history of chloroquine and SP resistance, microsatellite haplotypes were determined by measuring nucleotide length variation at microsatellite loci closely linked to *pfcrt* (-29.268 kb, -10.833 kb, -2.814 kb, 0.59 kb, 10.389 kb and 23.576 kb), *dhfr* (-4.49 kb, -3.87 kb, -0.1 kb, +0.52 kb, +1.48 kb and +5.87 kb) and *dhps* (-2.9 kb, -1.5 kb, -0.13 kb, +0.8 kb, +4.3kb, and +7.7 kb) as previously described [40, 42, 44, 45, 88].

2.4 Haplotype network

Based on polymorphisms at the six microsatellite loci flanking *pfcrt*, *dhfr* and *dhps* respectively, three haplotype networks of chloroquine and SP resistances were constructed using the median-joining network method implemented in NETWORK version 4.6 (http://www.fluxus-engineering.com/sharenet.htm) [89].

2.5 Statistical analysis

The Fisher`s exact test in SPSS version 22.0 was used to assess the statistical significance of differences between two groups accordingly. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Demographic distribution of the study population

Out of 76 patients in 2002-5 and 63 in 2013, the number of *P. falciparum* infected males and females were 66 (87%) and 10 (13%) in 2002-5 and 51(81%) and 12 (19%) in 2013, respectively. There is a male preponderance compared to female in the study group (Tab.1). Age distribution among the study population ranges from 5 to 54 years. Most of the patients were in the 21- 30 year age group (43.4 % in 2002-5 and 47.6% in 2013) followed by 11-20 year age group (21% in 2002-5 and 20.6% in 2013) (Tab.1). In 2002-5 samples, most of the patients were from Mandalay Yangon and Bago regions.

3.2 Artemisinin resistance

Species-specific PCR confirmed 139 samples (76 in 2002-2005 and 63 samples in 2013) as *P. falciparum* mono infection. Among a total of 11 SNPs (8 non-synonymous and 3 synonymous) observed in the *kelch 13* propeller domain (Tab.2), only three SNPs (P441L, F446I, and R561H) were previously reported [25]. The prevalence of parasites harboring variants in *kelch 13* significantly increased from 8.6% in 2002-2005 to 24% in 2013 (p = 0.0031) (Fig.7). No isolate harbored two or more mutations. Only one SNP (R561H) was observed in both 2002-2005 and 2013. All SNPs were observed as singletons except Y511H.

The Y511H mutation that has not been previously reported in Asia and Africa was observed in the 14.8% of samples in 2013, suggesting an independent emergence of this

mutation. We did not find five major mutations (M476I, Y493H, R539T, I543T and C580Y) that have been evidenced to confer artemisinin resistance [25, 90].

3.3 Chloroquine resistance

In *pfcrt*, nearly all isolates had the K76T mutation in 2002-2005 (97%) and in 2013 (100%) even 12 years after the cessation of chloroquine official usage (Fig.8). Results of six microsatellite loci flanking *pfcrt* revealed that a combination of alleles of 152-180-182-150-203-191 at the microsatellite loci of -29.268 kb, -10.833 kb, -2.814 kb, 0.59 kb, 10.389 kb and 23.576 kb, respectively, was the most prevalent haplotype (Tab.3). This haplotype is an ancestral lineage of chloroquine-resistant isolates that has been widely distributed in GMS and Africa [40, 91]. In this analysis, all parasites harboring K76T allele had the ancestral or similar haplotype, indicating that chloroquine-resistant *P. falciparum* isolates in Myanmar shared a common origin with those in GMS. Parasites harboring chloroquine-sensitive allele (K76) had distinct microsatellite haplotypes from resistant parasites. This observation is further supported by haplotype network which was constructed using the median-joining network method based on six microsatellite alleles (Fig.9). Three major lineages (Indochina, Melanesian and Philippine) regarding independent evolution of chloroquine resistance have been reported so far [40]. Almost all of the Myanmar isolates were included in or closely related to Indochina lineage.

In *pfmdr1*, the N86Y mutation was detected in 23% of parasites in 2002-2005, including 6 of mixed allele combination with N86 (Fig.10). Notably, parasite harboring the N86Y disappeared in 2013. Significant changes of allele prevalence between 2002-2005 and 2013 were observed only in the codon 86. Parasites harboring mutant were extremely rare at all the other positions (1034, 1042 and 1246).

3.4 SP resistance

A total of five *dhfr* haplotypes were observed; CNCSI, CNRNI, CIRNI, CNRNL and CIRNL (where letters indicate amino acid positions at 50, 51, 59, 108 and 164, substitutions are underlined). In 2002-2005, 80% of parasites harbored either triple or quadruple mutants (Tab.4, Tab.5). The most prevalent haplotype was CIRNL (40%) which was evidenced to show the highest pyrimethamine resistance [34, 92]. Haplotype distribution in 2013 was similar to that in 2002-2005 (Fig.11). Only one difference was detection of wild type (4.7%) in 2013. No mutation was detected other than five known polymorphic sites. Six microsatellite markers flanking *dhfr* revealed that almost all parasites harbored a haplotype that is identical to the most prevalent one observed in the GMS and Africa (Tab.6)[47]. This finding was clearly demonstrated by haplotye network (Fig.12).

In *dhps*, among a total of 9 *dhps* haplotypes, the AGEAA (where the letters indicate amino acid positions 436, 437, 548, 581 and 613, and the altered residues are underlined) was

the most frequently observed in 2002-2005 (54.2%), but the prevalence of this haplotype significantly reduced to 15% in 2013 ($p< 0.0001$) (Fig.13). Instead, both SGEGA and SGKGA were increased in 2013 from 21% to 34% and 10% to 20%, respectively.

Haplotype network revealed that three resistant lineages were observed in *dhps* mutant parasites in Myanmar (Fig.14). Most of SGEGA alleles belong to the SEA/EAFR-1 lineage; one of the previously documented three highly sulfadoxine resistant lineages of Asia [36, 49](Table.7). Accordingly, most of AGEAA haplotypes had the microsatellite patterns similar to the remaining two lineages; SEA/PAC and SEA/EAFR-2. Lineages of SEA/EAFR-1 and SEA/EAFR-2 were found to have stemmed from Asia and migrated to East African countries.

4. DISCUSSION

4.1 Has the cessation of chloroquine usage reinduced its sensitivity?

The return of chloroquine sensitivity after its withdrawal was first reported in Malawi [93-96]. In Asia and Latin America, however, no sign of chloroquine sensitivity restoration has been evidenced except in Hainan, China [97]. Fitness disadvantage of *pfcrt* mutant parasites in the absence of chloroquine selecting pressure might play some role in the recovery of chloroquine sensitivity in some African countries. Some epidemiological findings indirectly supported this hypothesis [98, 99]. In Gambia, reduction of chloroquine selecting pressure in dry season due to considerable decrease of malaria patients was reported to induce a significant decline in the prevalence of mutant allele in both *pfcrt* and *pfmdr1* [100]. However, there has been no report that showed direct evidence of fitness cost of *pfcrt* mutant parasite so far. Further laboratory examination is required to clarify the possible fitness loss in chloroquine resistant parasites.

Previous in vivo studies in Myanmar showed that frequency of treatment failure by chloroquine reached a peak in the period from early 1980s to mid-1990s [7]. After that, the frequency of resistance decreased to about 25-30% in the early 2000s [7]. However, decrease trend of chloroquine resistance was not supported from the present analysis of *pfcrt* genotyping. Resistant mutation (K76T) was observed in almost all parasites in 2002-2005 and it became fixed in 2013.

In contrast, disappearance of *pfmdr1* N86Y mutation which is also associated with chloroquine resistance was observed in 2013 from 23.7% in 2002-2005. The introduction of artemisinin+lumifantrine may have played some roles in this observation. Previous in vivo studies in Uganda and Tanzania revealed the selection of *pfmdr1* N86 after treatment with artemether+lumefantrine [101, 102]. Additionally, parasites harboring *pfmdr1*N86 showed significantly higher lumefantrine IC_{50} than that carrying N86Y allele [103, 104]. These results support the idea that the N86 allele may be advantageous in the presence of lumefantrine pressure. Therefore, wide-spread usage of lumefantrine as an ACT regimen in Myanmar would have induced the significant decrease of *pfmdr1* N86Y mutation. Selection of the K76 allele in *pfcrt* was also reported after exposure to artemether +lumefantrine in Africa [105, 106], suggesting the possibility that *pfcrt* K76 allele might also be selected in Myanmar in the future. If that is the case, chloroquine sensitivity could be recovered.

4.2. Has the reduction of SP selecting pressure triggered SP sensitivity?

Similar to chloroquine resistance, predominance of highly mutant *dhfr* and *dhps* alleles in 2002-5 as well as in 2013 suggests that there is no sign of recovery of SP sensitivity. Previous molecular epidemiological studies in Myanmar and neighboring countries showed similar findings; triple and quadruple mutant in *dhfr* (CIRNI and CIRNL) and triple mutant in *dhps* (AGEAA and SGEGA) continued to be predominant after SP

withdrawal [36, 37, 43, 45, 49, 75, 107]. In contrast, in Peru, the removal of SP has led to a decline in the frequency of *dhfr* and *dhps* triple mutant from 47% to 16.9% and 0% respectively [107, 108]. However, this observation seems to be exclusive for Peru [16, 36, 49].

One contributing factor for maintaining highly *dhfr*/*dhps* mutant parasites in Myanmar would be low but continuous drug pressure. Even after the official switch to ACTs for first line treatment regime, SP is still used as preventive treatment and self-treatment, particularly for the military population due to its low cost and easy availability [7]. Widespread usage of Septrim (Co-trimoxazole) for treating respiratory tract infections might also play a role in the maintenances of *dhfr*/*dhps* quadruple and triple mutant parasites [7]. Laboratory studies using yeast expression system showed cross-resistance between Co-trimoxazole and SP by demonstrating significant differences in IC_{50} of *P. falciparum* parasites harboring *dhfr*, wild and mutant, to both pyrimethamine and trimethoprim [24, 109].

In this present study, a significant shift of the predominant triple mutant *dhps* haplotype from AGEAA to SGEGA was found after the implementation of ACTs. Similar findings were reported after the drug policy change from SP to ACTs in Tanzania and Kenya [110, 111]. So far, no clear mechanism that can explain this finding has been described. However, SGEGA may be more fit than the other *dhps* AGEAA in the presence of artemisinin, which also might play a role in the lack of SP sensitivity recovery.

4.3. Distinct evolutionary pattern of artemisinin resistance

Evolutionary patterns of drug resistance in Myanmar parasites have been quite predictable for both chloroquine and SP. Haplotyping of six microsatellite markers flanking *pfcrt* in the present analysis revealed that chloroquine-resistant isolates in Myanmar share a common resistant lineage with resistant isolates in the other GMS countries. Similar findings were obtained for SP resistance. By contrast, a different evolutionary pattern is probable in artemisinin resistance. In the initial report of *kelch 13*, the C580Y mutation was characterized as the responsible mutation for artemisinin resistance in the natural parasite population [25]. Shortly thereafter, various alleles in *kelch 13* have been evidenced to confer artemisinin resistance, suggesting that *kelch 13* mutants independently arose multiple times within GMS [74, 80]. In the present study however, no parasite was found to harbor the known artemisinin-resistance related mutations in both 2002-2005 and 2013 (Tab.6). Rather, in 2013, the most predominant allele was a novel Y511H mutation with a prevalence of 14.8%. Accumulated evidence have shown that nearly all *kelch 13* mutations observed at significant frequencies in the endemic areas were associated with delayed parasite clearance after artemisinin treatment [25, 74, 80, 112]. Therefore, the observed high prevalence of Y511H mutation is most likely due to selection under increasing artemisinin pressure. Very recently, in a report published this year, country-wide molecular analysis for *kelch 13* using 940 *P. falciparum* isolates in Myanmar has shown that 11 SNPs were major alleles with significant

frequencies and the most predominant SNPs were found to differ among different geographic regions [76]. Surprisingly, the Y511H mutation was not found in that analysis. This further suggests the multiple indigenous evolution of artemisinin resistance in GMS.

Haemoglobinopathies play a major role in immunity against malaria, and therefore also in selection of anti-malarial drugs, and in that regard, the prevalence of HbE in malaria endemic areas of Myanmar has been estimated to be 11.4% [113]. There is a paucity of data on HbS, HbC, and thalassemia countrywide, especially in relation to malaria. However, it would generally be difficult to draw an inference since many other confounding factors are present in resistance to anti-malarials.

4.4 Treatment Policy implementation and anti-malarial drug resistance

In Malawi, unacceptably high treatment failure rates of CQ led to a change of national anti-malarial treatment policy from using CQ to SP as the first-line drug in 1993 [114]. Simultaneous implementation of strict restriction of CQ usage and allowing SP to be sold without prescription resulted in abrupt and near-total removal of CQ drug pressure [115]. In the case of Myanmar, such kind of ban has never been implemented and almost all kinds previous anti-malarial drugs are still being used in rural areas due to their easy availability and low cost [7]. This low but persistent drug pressure might be responsible for the absence of the recovery of CQ and SP sensitivity in this present study.

Because of early remission of clinical symptoms of malaria produced by artemisinin, poor adherence to the full 7-day treatment regimen is common [116]. Since incomplete treatment courses induce emergence and spread of artemisinin resistance, WHO called for a ban on artemisinin monotherapy (AMT) and replacement with ACTs (commonly 3-day treatment regimen) in 2005 [16]. In Myanmar, public health care providers are treating patients of all ages by using RDT (Rapid diagnostic test) and ACTs free of charge according to WHO guidelines [7]. However, more than 70% of malaria treatments are directly accessed from the private sector and more than 80% of all malaria cases are still treated with AMT in Myanmar [7, 117]. About 2.4 million packages of 12 artesunate tablets are currently distributed by the informal sector every year [7].

To further compound the problem of anti-malarial resistance, malarial drugs are prescribed and given by health assistants, midwives, nurses and quacks – notably betel shop owners and small private "pharmacies" or street corner betel shops where laypersons with no knowledge of malaria and its prescribed treatment have a roaring business especially in the districts and villages in rural areas [7, 117]. Furthermore, these drugs may be sub-standard or counterfeit, exacerbating the failure of treatment [7, 38]. Another important contributing factor is the patient who stops treatment when the initial malaria episode has subsided or poor compliance due to many socio-economic reasons and low educational status [7, 38].

4.5 Migrant population and anti-malarial drug resistance

Migrant/mobile populations are considered to play a major role in the potential spread of anti-malarial drug resistant malaria within countries and across borders [38]. By their nature, these populations live and work in areas with high malaria transmission, high human–vector contact and limited access to health services including effective malaria prevention tools, accurate diagnosis and treatment with ACTs [38]. Migrant populations are thus likely to practice self-medication with anti-malarial drugs purchased from unregulated, private vendors, which may increase their risk of exposure to oral artemisinin monotherapy (AMT) [7, 38]. Once artemisinin resistance has widely spread among the rural population and migrants, it will in turn, spread to new areas.

At the same time, it is still not possible to estimate the number of Myanmar migrants who cross the border to work and then return home, as in the case along the Thai Myanmar border where drug resistant malaria is rampant, some with undiagnosed malaria and some with no access to treatment [5, 7, 38, 54, 118]. However, in some small hospitals and clinics in border towns on the Thai side, patients are mostly from Myanmar ethnic tribes and they are receiving anti-malaria treatment of some kind [7, 38, 54, 118].

Another highly malaria endemic region in Myanmar is on the border with Bangladesh where an influx of Bengalis have migrated and settled in Myanmar, most with no legal status and therefore with no access to proper healthcare [7, 119]. This has also created a nidus for

resistance to anti-malarial drugs since undiagnosed cases and intermittent treatment compounded by conflict in the region has been a deterrent to the malaria project [7, 120]. Some have returned of their own volition and some have been returned having been proven to be from Bangladesh but some still come in, resulting in crossovers of malaria cases back and forth [7, 121].

Even within Myanmar itself, there are still pockets of conflict with certain ethnic groups and insurgencies and government health policies cannot be properly implemented in those regions. The villagers and rural population do not receive anti-malarial drugs according to the prescribed drug policy and malaria cannot be controlled [7]. Currently, there are people from all over rural Myanmar who migrate to big cities like Yangon in Lower Myanmar and Mandalay in Upper Myanmar, the economic and social hubs, to look for jobs and a better life but with low or no educational status [7]. This type of migration sometimes brings in drug resistant malaria cases which can be diagnosed but due to lack of awareness and lack of finances, they do not seek medical health [7, 117]. However, the longer they have settled, the more knowledgeable they become and malaria is less of a problem in the big cities compared to tuberculosis and HIV/AIDS.

5. CONCLUSION

Observing the novel *kelch 13* alleles of Y511H and others, this study suggests the indigenous evolution of artemisinin resistance in Myanmar in contrast to the previously used conventional anti-malarial drugs. A significant rise in frequency of the parasites harboring mutations in *kelch 13* implies that they may have been selected by the increasing artemisinin pressure. Almost fixation of *pfcrt* K76T and considerably high prevalence of highly resistant type of *dhfr* and *dhps* alleles in both 2002-5 and 2013 reflects no sign of reduction of chloroquine and SP resistant *P. falciparum* even a decade after the withdrawal of the drug. The shift of the predominant triple *dhps* haplotype from AGEAA to SGEGA highlights the potential fitness advantage of this haplotype in the presence of artemisinin drug pressure. These molecular epidemiological observations regarding the dynamics of molecular markers for anti-malarial resistance after the introduction of ACTs in Myanmar will provide an insight into future policy making for implementing a strategy to prevent and control artemisinin resistance, initially in the Mekong subregion, expanding worldwide and utilising multi-drug regimens with different ACTs.

However, it is beyond the scope of this study to provide an accurate pattern of the dynamics of molecular markers for anti-malarial resistance in Myanmar since the whole country cannot be represented - not only in the molecular epidemiology aspect but also in the relevant socio-demographic aspects. It would be worthwhile, however, to conduct a bigger

project to monitor the known molecular markers and the novel ones which have emerged especially in the endemic regions of Myanmar, which at the moment, includes hard-to-reach areas and conflict zones.

This study has been conducted to contribute to the existing but scanty information on malaria molecular epidemiology in selected areas of Myanmar so as to provide baseline data on which many more studies can evolve in the future.

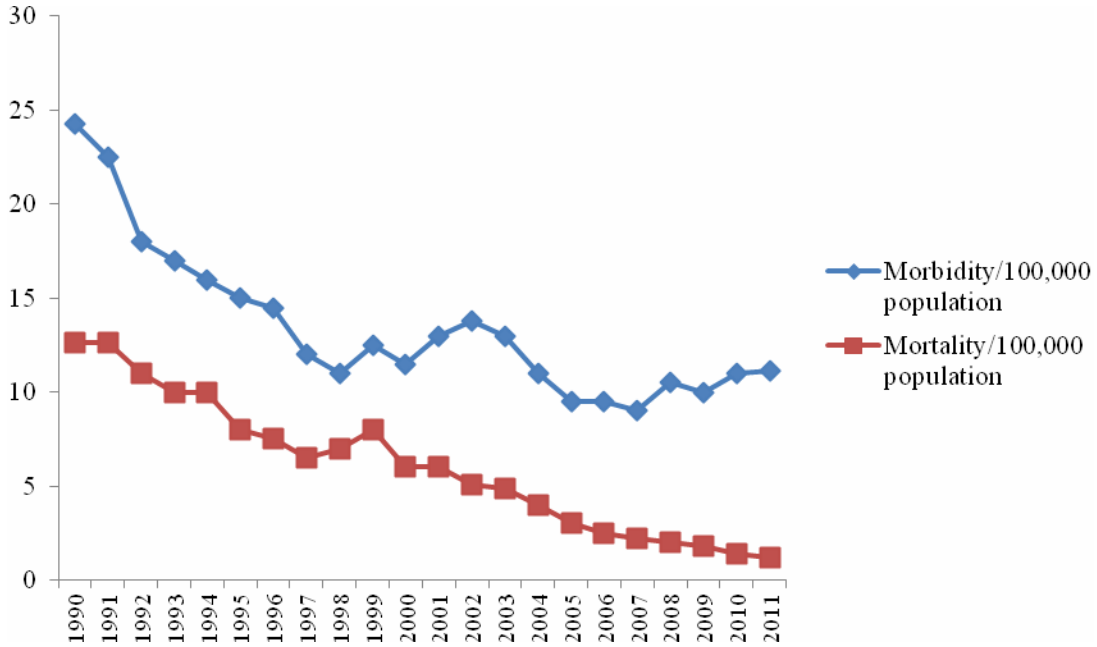

Figure 1. Malaria morbidity and mortality rates in Myanmar (1988-20011)
(Department of Health/Ministry of Health, Myanmar., Health in Myanmar 2012, Unpublished data)

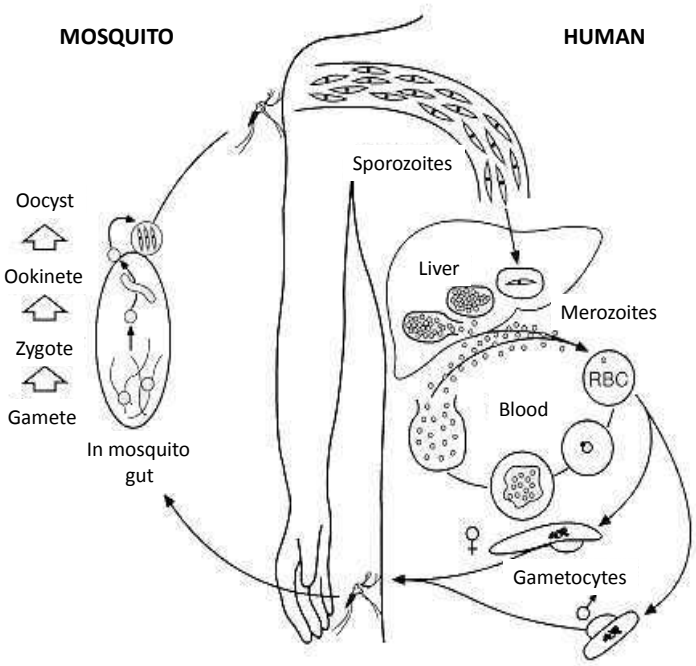

Figure 2. Life cycle of *Plasmodium falciparum* in two hosts: mosquito and human [9]

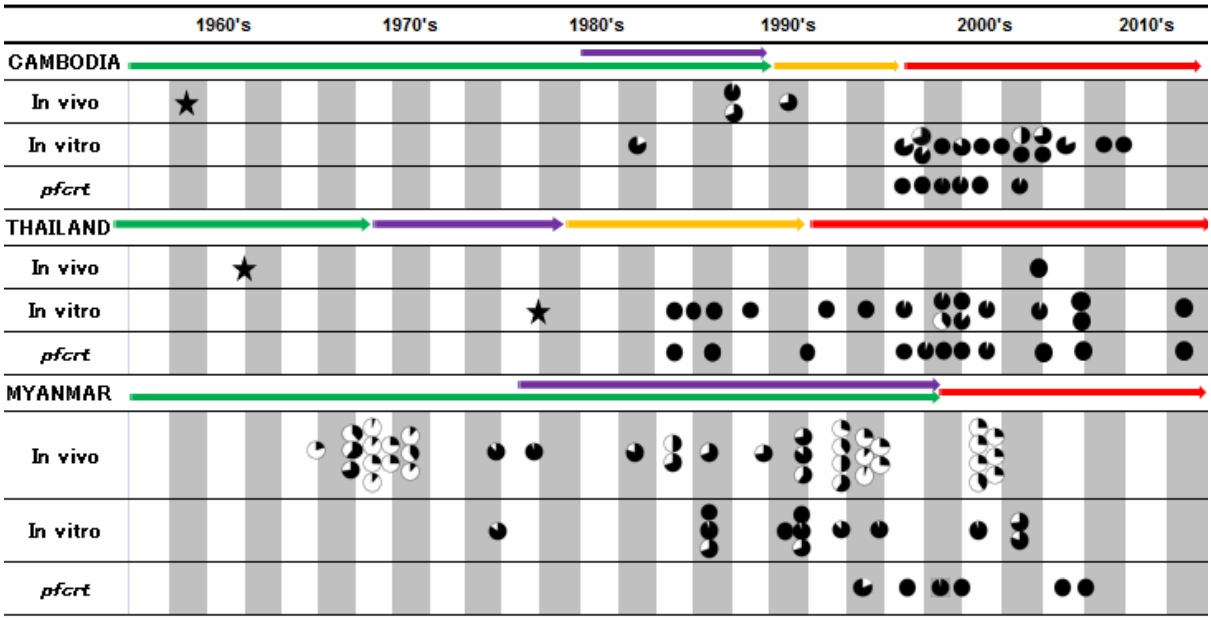

Figure 3. Timeline of chloroquine usage and prevalence of resistance in Cambodia, Thailand and Myanmar. Green, purple and red lines show periods of chloroquine, sulfadoxine -pyrimethamine and ACTs usage respectively. Yellow line represents deployment of other anti-malarial drugs such as quinine, mefloquine, etc. White and black portions of circle express percentage of susceptibility and resistance invivo and invitro, respectively. In *pfcrt*, white and black portions of circle represent frequency percentage of wild (K76) and mutant type (K76T) alleles.

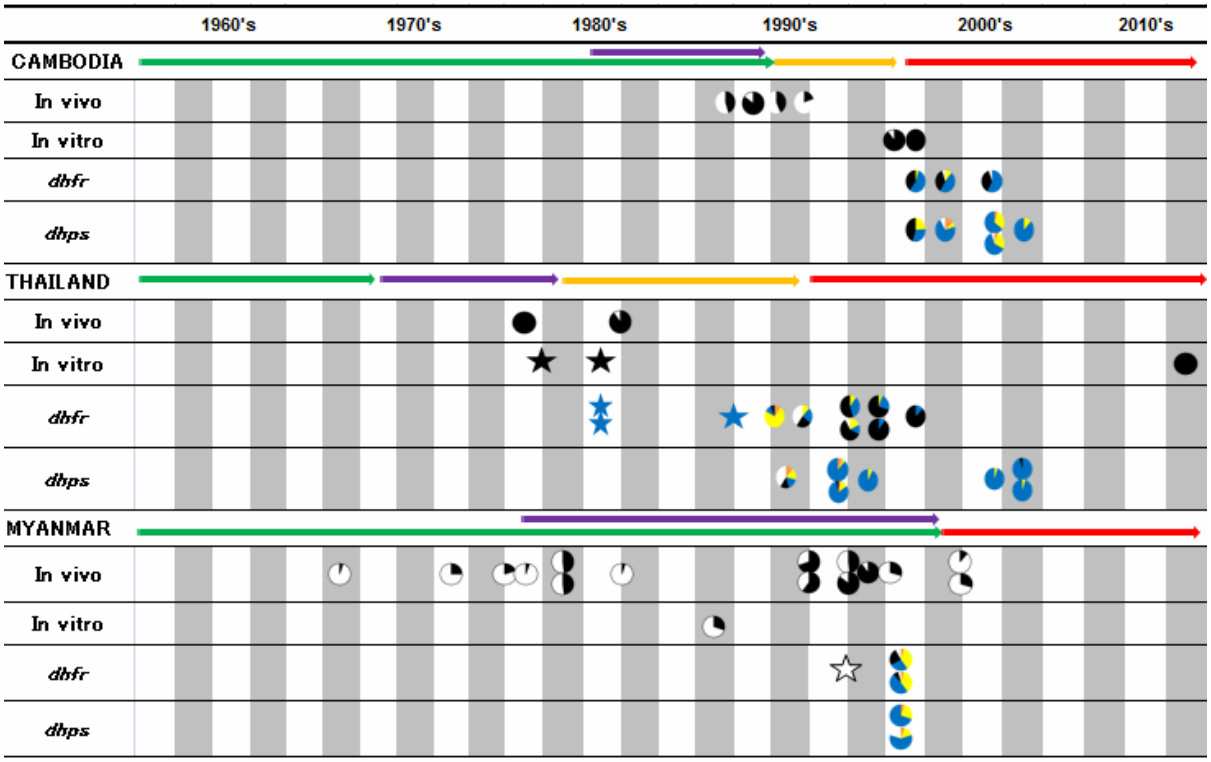

Figure 4. Timeline of sulfadoxine-pyrimethamine usage and prevalence of resistance in Cambodia, Thailand and Myanmar. Periods of chloroquine, sulfadoxine-pyrimethamine and ACTs usage were shown by green, purple and red lines respectively. Yellow line indicates deployment of other anti-malarial drugs such as quinine, mefloquine, etc. White and black portions of circle represent percentage of susceptibility and resistance in vivo and in vitro, respectively. In *dhfr* and *dhps*, black, blue, yellow, brown and white portions of circle stand for frequency of quadruple, triple, double, single mutant and wild type alleles.

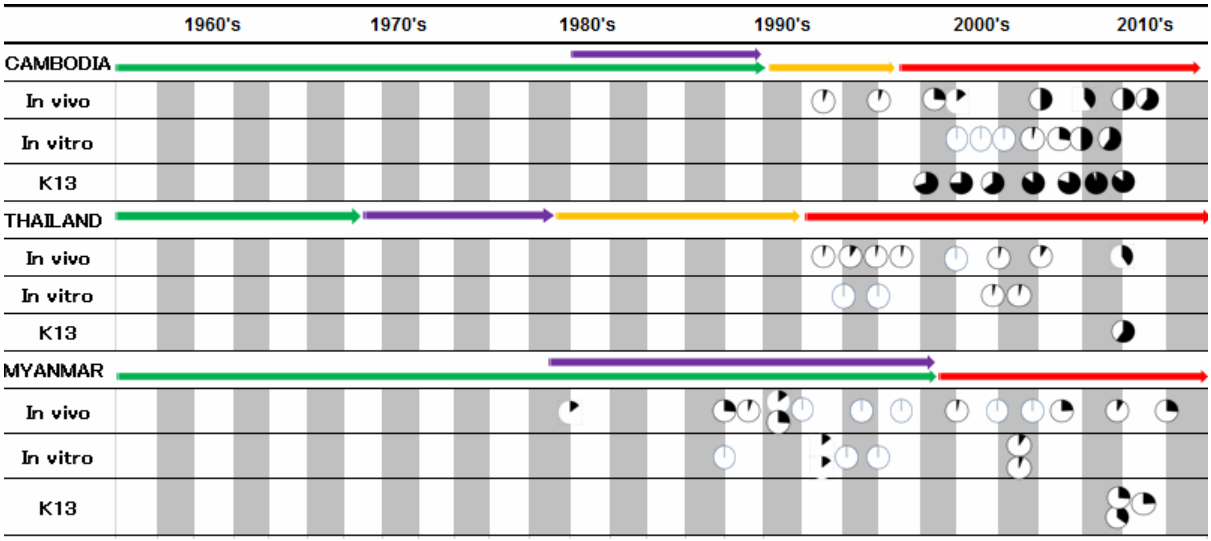

Figure 5. Timeline of artemisinin usage and prevalence of resistance in Cambodia, Thailand and Myanmar. Green, purple and red line reveal periods of chloroquine, sulfadoxine -pyrimethamine and ACTs usage respectively. Yellow line express deployment of other anti-malarial drugs such as quinine, mefloquine, etc. White and black portions of circle stand for percentage of susceptibility and resistance in vivo and in vitro, respectively. In K13-propellar gene, white and black portions of circle represent frequency of wild and mutant type alleles.

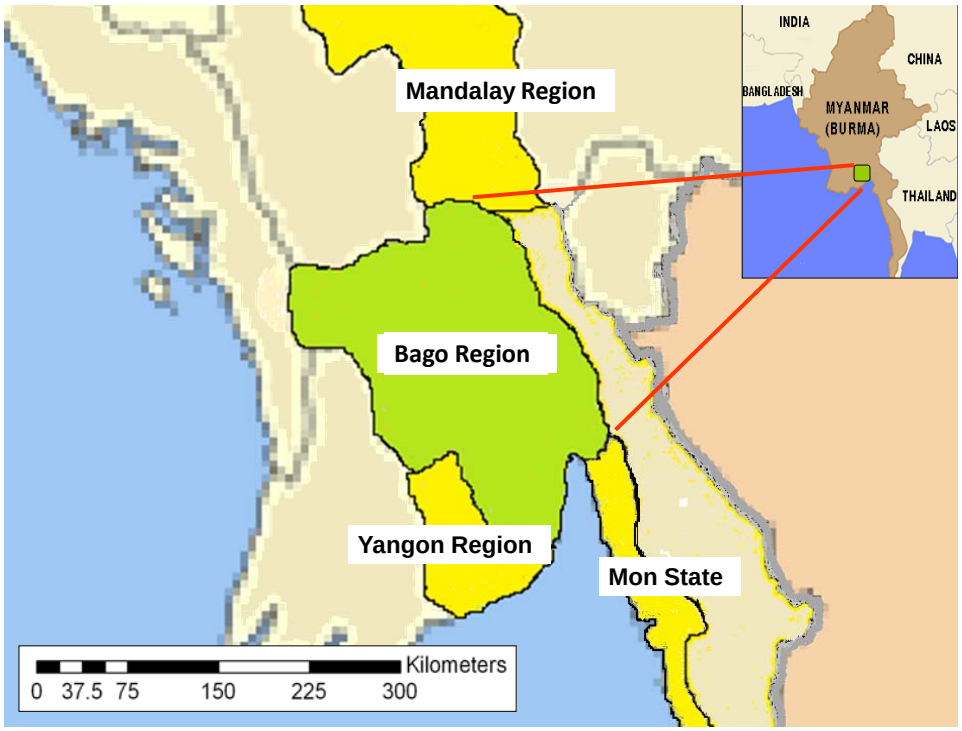

Figure 6. Map of study area in Myanmar. Green area represents Bago region and yellow ones show the surrounding three areas; Yangon region, Mandalay region and Mon state. All study areas were regarded as Tier1 areas. (http://www.mapsofworld.com/myanmar/)

Table 1.

Demographic characteristics of study population		
	2002-5	2013
Age (years)		
<10 years	5 (6.6%)	3 (4.7%)
11-20 years	16 (21%)	13 (20.6%)
21-30 years	33 (43.4%)	30 (47.6%)
31-40 years	15 (19.7%)	12 (19%)
41-50 years	5 (6.6%)	4 (6.3%)
>50 years	2 (2.6%)	1 (1.6%)
Gender		
Male	66 (87%)	51 (81%)
Female	10 (13%)	12 (19%)
Regions		
Bago region	18 (23.7%)	63 (100%)
Mandalay region	31 (40.8%)	0
Yangon region	22 (28.9%)	0
Mon state	5 (6.6%)	0

Table 2. Distribution of K13-propellar variants in 2002-5 and 2013 in Myanmar

Sampling period	P441	P443S	F446I	510V	Y511H	W518R	P553L	R561H	T685
2002-5	-	-	1	1	-	-	1	1	-
2013	1	1	-	-	8	1	-	1	1

Letters and numbers indicate amino acids and their location in K13-propellar gene

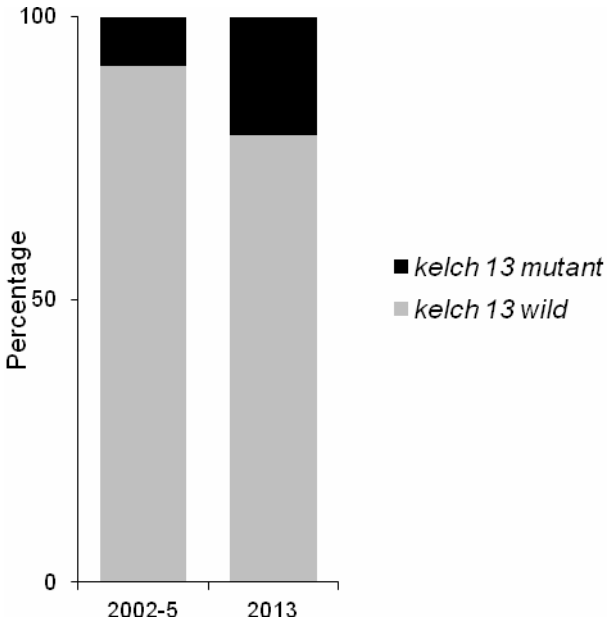

Figure 7. Prevalence of *kelch 13* variants in 2002-5 and 2013 in Myanmar. Black and grey portions of the graph stand for percentage of *kelch 13* mutant and wild type parasites, respectively.

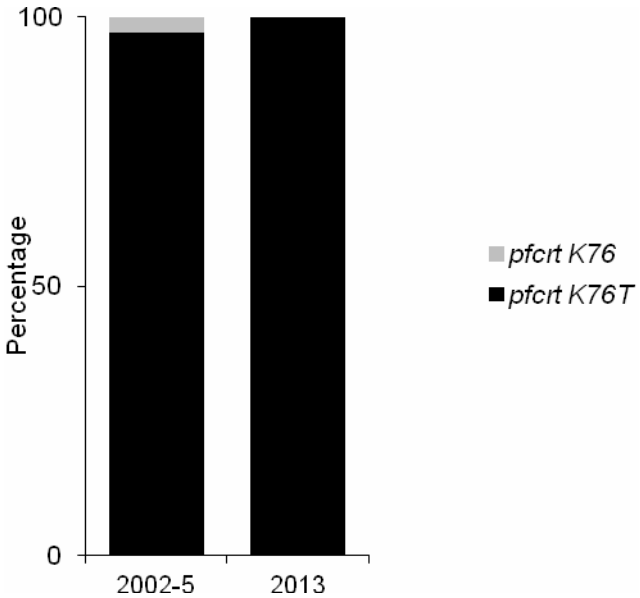

Figure 8. Prevalence of *pfcrt* K76T mutation in 2002-5and 2013 in Myanmar. Black and grey portions of the graph represent percentage of *pfcrt* K76 mutant and wild type parasites, respectively.

Table 3. Microsatellite haplotypes of *pfcrt*

No.	29.268kb	10.833kb	2.814kb	*pfcrt* codon 76	0.59kb	10.389kb	23.576kb	Frequency
M1	152	180	182	K76T	150	203	191	43
M2	152	180	182	K76T	150	203	189	10
M3	152	180	182	K76T	150	201	191	12
M4	152	180	182	K76T	150	207	189	9
M5	152	185	182	K76T	150	210	191	1
M6	152	180	182	K76T	150	201	179	1
M7	152	173	182	K76T	150	203	191	2
M8	152	172	182	K76T	150	206	187	2
M9	152	174	182	K76T	153	203	171	1
M10	152	180	182	K76T	150	203	199	1
M11	152	180	182	K76T	205	203	191	1
M12	152	180	182	K76T	150	210	191	2
M13	152	182	182	K76T	150	201	191	1
M14	152	171	194	K76	150	201	189	1
M15	152	171	187	K76	159	198	195	1

Microsatellite haplotypes in yellow area represent previously documented Southeast Asian lineage [40].

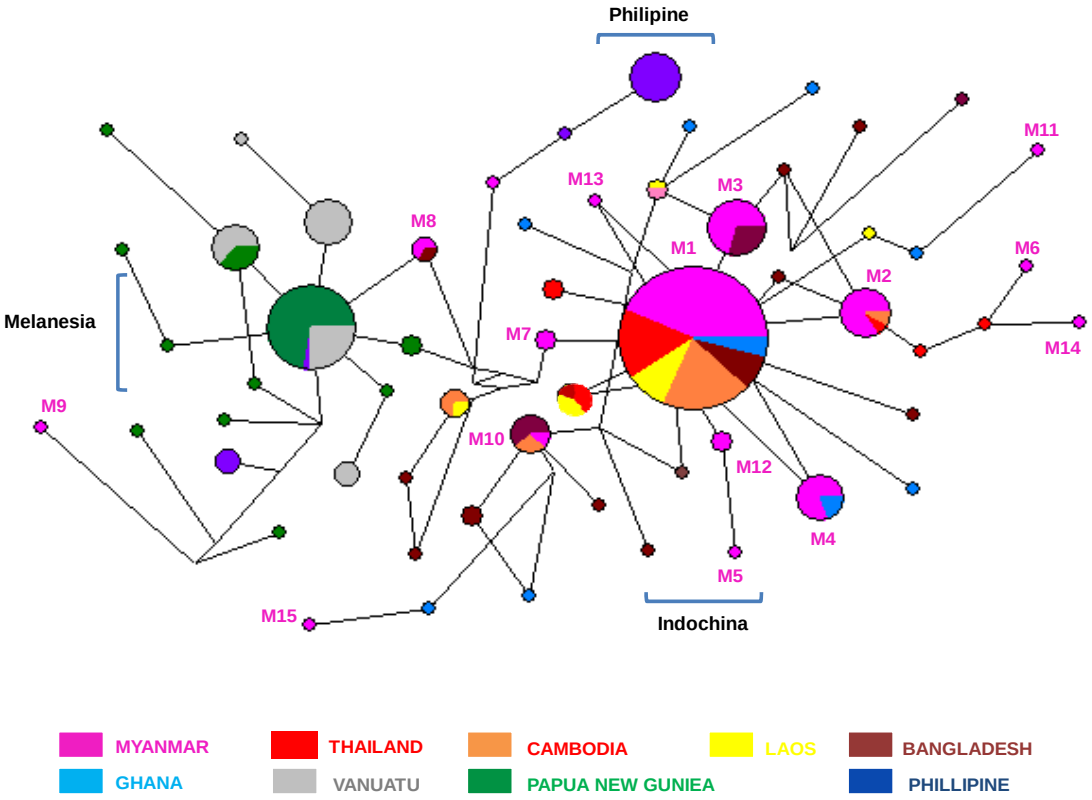

Figure 9. Medium-joining network diagram indicating three major lineages (Indochina, Melanesian and Philippine) regarding independent evolution of chloroquine resistance [40]. Network tree is shown according to countries where samples were taken. The haplotype network was constructed for *P. falciparum* isolates harbouring *pfcrt* mutation, based on allelic variations in six microsatellite loci flanking the *pfcrt* locus. The size of each circle corresponds to the number of samples sharing the same haplotype, and the length of an edge is proportional to a variation in repeat number between two haplotypes.

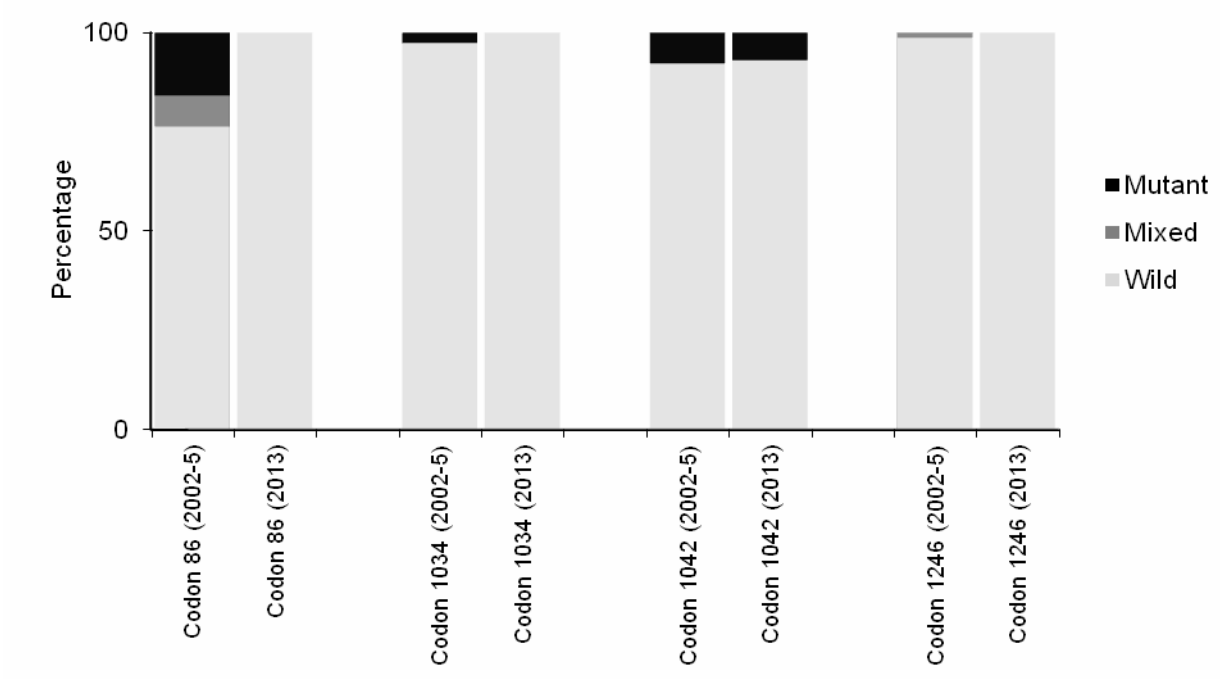

Figure 10. Prevalence of *pfmdr1* mutations in 2002-5 and 2013 in Myanmar. Grey, dark grey and black blocks stand for proportion of *pfmdr1* wild, mixed and mutant alleles at codons 86, 1034, 1042 and 1246 respectively.

Table 4. Prevalence of *dhfr* haplotypes in 2002-5 and 2013 in Myanmar

dhfr haplotypes	2002-5	2013
CIRNL	23 (39.6%)	16 (37.2%)
CIRNI	9 (15.5%)	7 (16.3%)
CNRNL	14 (24.1%)	9 (20.9%)
CNRNI	12 (20.7%)	9 (20.9%)
CNCSI	0	2 (4.7%)

CIRNL, CNRNL, CIRNI and CNRNI stand for haplotypes of *dhfr* (where letters indicate amino acid positions 50, 51, 59, 108, and 164, and the altered residues are underlined).

Table 5. Prevalence of *dhfr* haplotypes in 2002-5 and 2013 in Myanmar

dhfr haplotypes	2002-5	2013
Quadruple mutant	23 (39.6%)	16 (37.2%)
Triple mutant	23 (39.6%)	16 (37.2%)
Double mutant	12 (20.7%)	9 (20.9%)
Wild type	0	2 (4.7%)

Quadruple mutant (CIRNL), triple mutant (CNRNL, CIRNI), double mutant (CNRNI) and wild type (CNCSI) stand for haplotypes of *dhfr* (where letters indicate amino acid positions 50, 51, 59, 108, and 164, and the altered residues are underlined).

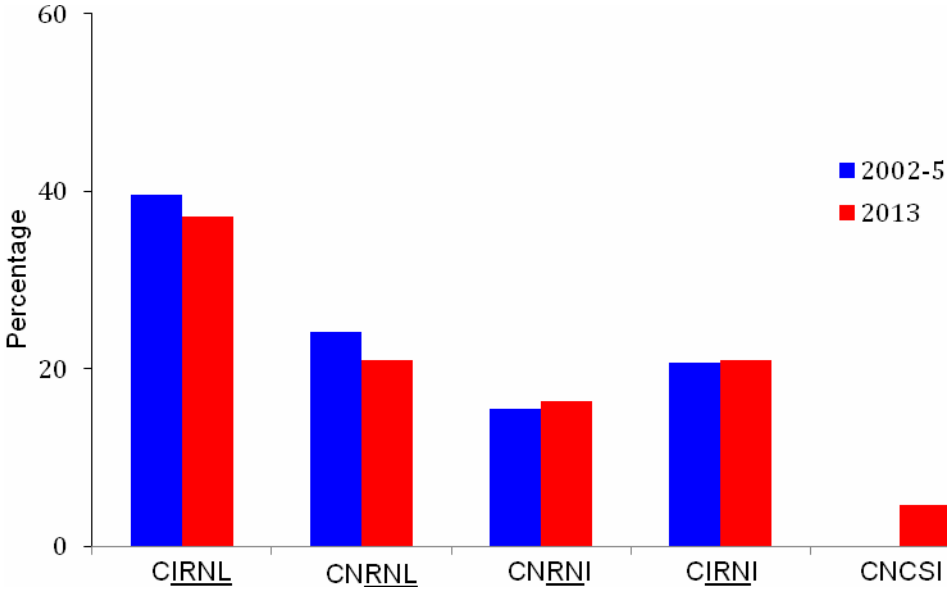

Figure 11. Prevalence of *dhfr* haplotypes in 2002-5 and in 2013 in Myanmar. Blue and red blocks express proportion of five *dhfr* haplotypes; CIRNL, CNRNL, CNRNI, CIRNI, CNCSI with mutations at codons 50, 51, 59, 108 and 164 in 2002-5 and 2013 respectively.

Table 6. Microsatellite haplotypes of *dhfr*

No.	4.49 kb	3.87 kb	0.1 kb	*dhfr* haplotypes	0.52 kb	1.48 kb	5.87 kb	No. of isolates
1	201	197	179	CIRNL	108	204	112	31
2	201	197	179	CNRNL	108	204	112	19
3	201	197	179	CIRNI	108	204	112	14
4	201	197	179	CNRNI	108	204	112	19

CIRNL, CNRNL, CIRNI and CNRNI stand for haplotypes of *dhfr* (where letters indicate amino acid positions 50, 51, 59, 108, and 164, and the altered residues are underlined). Microsatellite haplotypes in red area represent previously documented Southeast Asian lineage [45].

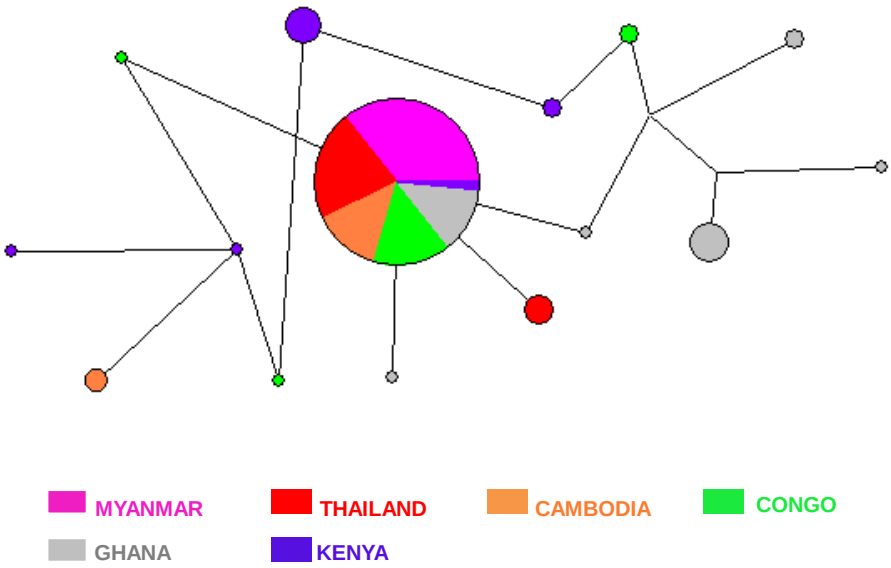

Figure 12. Medium-joining network diagram indicating the presence of single lineage of pyrimethamine resistant *Plasmodium falciparum* [45]. Network tree was drawn according to countries where samples were taken. The haplotype network for *P. falciparum* isolates harbouring *dhfr* mutation was constructed based on allelic variations in six microsatellite loci flanking the *dhfr* locus. The size of each circle corresponds to the number of samples having the same haplotype, and the length of an edge is proportional to a variation in repeat number between two haplotypes.

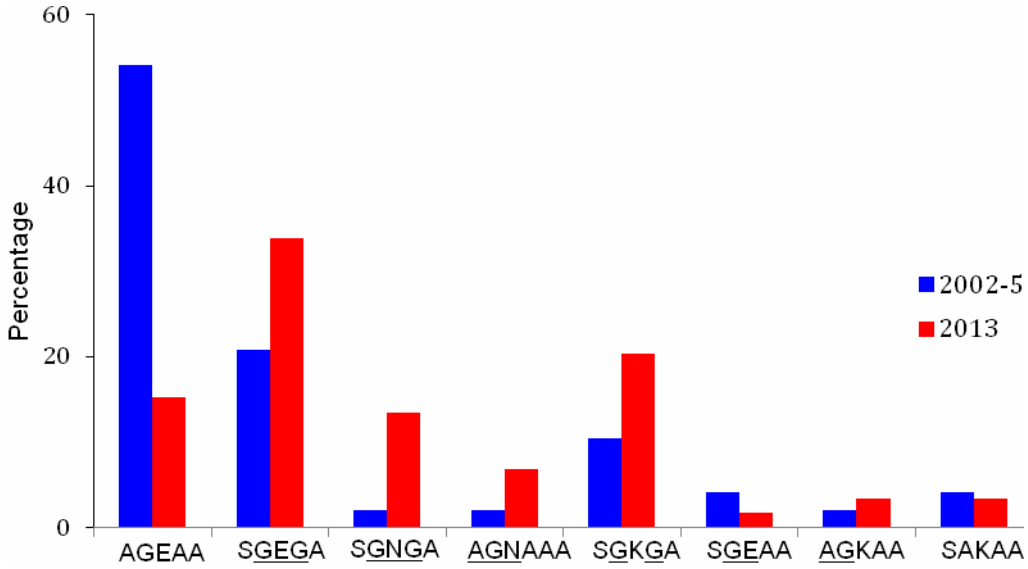

Figure 13. Distribution of *dhps* haplotypes in 2002-5 and 2013 in Myanmar. Blue and red blocks stand for proportion of six *dhps* haplotypes; AGEAA, SGEGA, SGNGA, AGNAA, SGKGA, SGEAA, AGKAA and SAKAA in 2002-5 and 2013 respectively (where letters indicate amino acid positions 50, 51, 59, 108, and 164, and the altered residues are underlined).

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Table 7. Microsatellite haplotypes of *dhps*

No.	2.9 kb	1.5 kb	0.13 kb	haplotypes	0.8 kb	4.3 kb	7.7 kb	Frequency
M1	191	172	134	SGEGA	131	104	111	9
M2	191	172	134	SGEGA	144	104	111	2
M3	191	172	134	SGEGA	131	114	119	3
M4	191	172	134	SGEGA	272	114	119	1
M5	191	172	134	SGEGA	119	108	107	1
M6	191	172	134	SGEGA	131	108	107	1
M7	191	172	134	SGEGA	62	104	119	1
M8	191	172	134	SGEGA	119	114	119	1
M9	183	172	134	AGEAA	117	114	119	12
M10	189	174	132	AGEAA	119	108	107	3
M11	189	174	132	AGEAA	119	104	107	2
M12	183	172	134	AGEAA	119	104	107	1
M13	183	172	134	AGEAA	131	104	107	1
M14	183	172	132	AGEAA	117	114	107	1
M15	183	172	132	AGEAA	119	114	107	1
M16	189	174	134	SGKGA	131	104	107	5
M17	189	174	134	SGKGA	59	104	107	3
M18	189	174	134	SGKGA	62	104	107	1
	189	174	134	SGNGA	62	104	107	1
M19	191	172	134	SGEAA	131	104	107	1
M20	191	172	134	SGEAA	119	104	107	1
M21	189	174	134	AGKAA	119	104	107	1
	189	174	134	SGNGA	119	104	107	1
M22	189	174	134	SGNGA	131	104	107	2
M23	189	174	134	AGNAA	119	108	107	1

AGEAA, SGEGA, SGKGA, AGNAA and SGNGA represent haplotypes of *dhps* alleles at codon (where letters indicate amino acid positions 50, 51, 59, 108, and 164, and the altered residues are underlined). Microsatellite haplotypes in red, yellow and blue areas represent SEA/EAFR1, SEA/EAFR2 and SEA/PAC lineages [49].

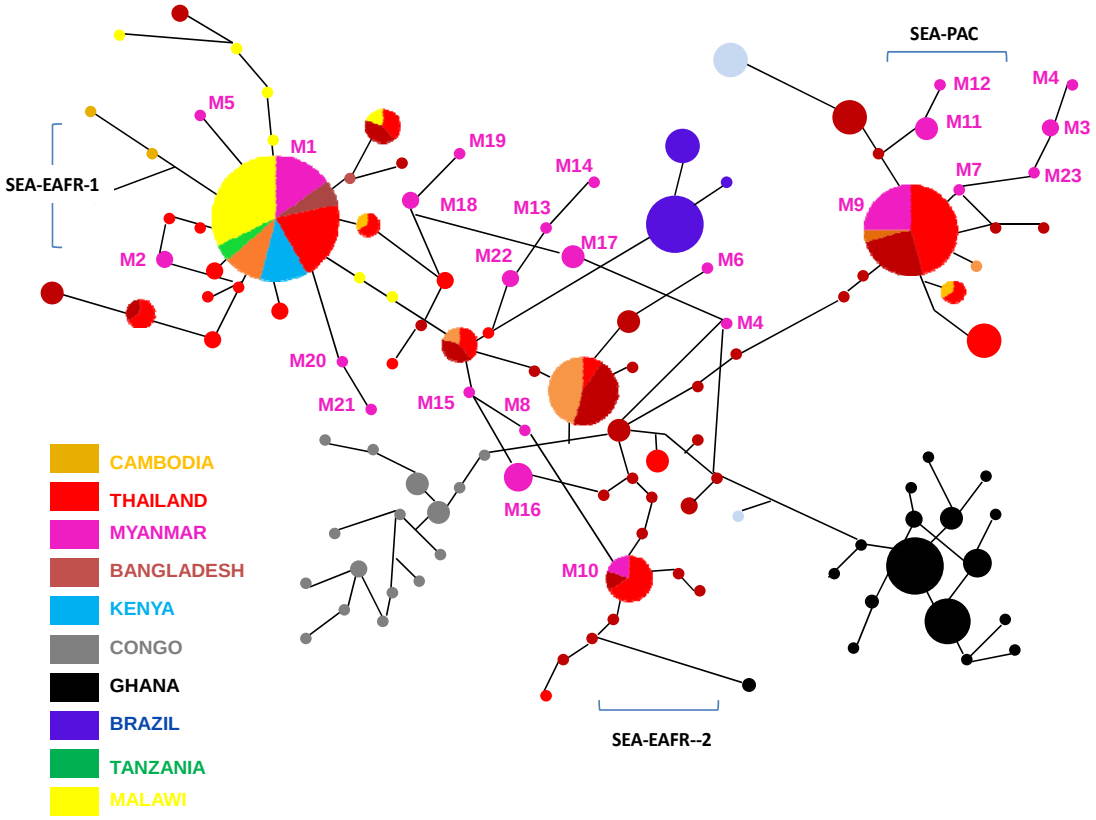

Figure 14. Medium-joining network diagram indicating the presence of 6 lineages of sulfadoxine resistant *Plasmodium falciparum*; two from Southeast Asia and East Africa; (SEA/EAFR-1 and SEA/EAFR-2), one from Southeast Asia and Pacific Islands (SEA/PAC) and the remaining three from Republic of the Congo (CON), Ghana (GHA), and Brazil (BRA) [49]. Network tree shows the countries where samples were taken. The haplotype network was drawn for *P. falciparum* isolates harbouring *dhps* mutation, based on allelic variations in six microsatellite loci flanking the *dhps* locus. The size of each circle corresponds to the number of samples sharing the same haplotype, and the length of an edge is proportional to a variation in repeat number between two haplotypes.

Table 8. Global distribution of Kelch 13 mutations

Kelch 13 mutation	SEA	Myanmar	China	Africa	My study	Significance
E252Q		+				
C439				+		
P441L	+	+				
P441					+	
M442V				+		
P443S						
F446I	+	+			+	
G449A	+	+				
G449D				+		
N458Y	+	+				
N458I	+	+				
S459				+		
I465T				+		
Q467				+		
E468				+		
C469				+		
W470X				+		
T474I	+	+				
R471						
M476I	+	+				Selected in in-vitro
S477				+		
T478				+		
A481V	+	+				
V487			+			
N489				+		
Y493H	+	+				Associated with delay parasite clearance
Y493				+		
V494I				+		
G496				+		
Y500				+		
K503				+		

T508N	+	+				
E509				+		
V510					+	
Y511H					+	Major one in my study
R513				+		
W518R					+	
V520A				+		
S522C	+	+		+		
S522				+		
N525D	+	+				
N531				+		
G533S	+	+				
T535				+		
N537I	+	+				
G538V	+	+		+		
G538G				+		
R539T	+	+	+			Associated with delay parasite clearance
C542Y				+		
I543T	+	+				
G544R				+		
G545E				+		
P553L	+	+		+	+	
P553				+		
N554S				+		
A557S				+		
Y558H				+		
R561H	+	+			+	
R561C	+	+		+		
V566I				+		
E567				+		
V568G	+	+				
A569T				+		
A569S				+		
P574L	+	+				
R575				+		
R575T			+			
S576L				+		

A578S	+	+		+		
C580Y	+	+	+			Associated with delay parasite clearance
C580F			+			
V581				+		
D584V	+	+	+			
L589I				+		
L589V				+		
G592				+		
K610K				+		
Q613E	+	+				
Q613T	+	+				
F614L	+	+				
A617T				+		
A617V				+		
L619S				+		
S623C	+	+				
A627			+			
Y630F				+		
V637A				+		
V637D				+		
G638R				+		
Q654				+		
A675V	+	+				
A676D		+				
T685					+	
V692L			+			
H719N	+	+				

Letters and numbers indicate amino acids and their location in K13-propellar gene. M476I was selected in in vitro under artemisinin drug pressure while Y493H, R539T and C580Y were evidenced to be associated with delay parasite clearance [25]. Y511H is the most prevalent mutation in my study.

Appendix I

Genotyping and sequencing of K13 propeller gene

PCR	Primers (5'-3')	PCR conditions		
1st	F1 CGGAGTGACCAAATCTGGGA	94°C	5 min	1
	R1 GGGAATCTGGTGGTAACAGC	94°C	30 sec	
		58°C	90 sec	40
		72°C	90 sec	
		72°C	10 mins	1
2nd	F2 GCCAAGCTGCCATTCATTTG	94°C	5 mins	1
	R2 GCCTTGTTGAAAGAAGCAGA	95°C	30 sec	
		58°C	45 sec	35
		72°C	1 min	
		72°C	10 mins	1

Appendix II

Genotyping of *pfcrt* codon 76

PCR	Primers (5'-3')	PCR conditions		
1st	F1 CCGTTAATAATAAATACACGCAG	94°C	3 mins	1
	R1 CGCATGTTACAAAACTATAGTTACC	94°C	30 sec	
		56°C	30 sec	45
		60°C	1 min	
		60°C	3 mins	1
2nd	F1 CCGTTAATAATAAATACACGCAG	95°C	5 mins	1
	R2 CGCATGTTACAAAACTATAGTTACC	92°C	30 sec	
		48°C	30 sec	25
		65°C	30 sec	
		65°C	3 mins	1

Appendix III

Genotyping of *pfmdr1* codon 86, 1034, 1042, 1246

Following images are bad quality. You have to modify these as good one!

Codon	PCR	Primers (5'-3')	PCR conditions		
86	1st	F1 TGTTGAAAGATGGGTAAAGAGCAGAAAGA	94°C	3 mins	1
		R1 TACTTTCTTATTACATATGACACCACAAACA	94°C	30 sec	
			68°C	1 min	40
			72°C	1 min	
			72°C	5 mins	1
	2nd	F2 AAAGATGGTAACCTCAGTATCAAAGAAGAG	94°C	5 mins	1
		R2 GTCAAACGTGCATTTTTTATTAATGACCATTTA	94°C	30 sec	
			65°C	30 sec	25
			72°C	30 sec	
			72°C	3 mins	1

Codon	PCR	Primers (5'-3')	PCR conditions		
1034	1st	F1 AGAAGATTATTTCTGTAATTTGATACAAAAAGC	94°C	3 mins	1
		R1 ATGATTCGATAAATTCATCTATAGCAGCAA	94°C	30 sec	
			68°C	1 min	40
			72°C	1 min	
			72°C	5 mins	1
	2nd	F2 AGAATTATTGTAAATGCAGCTTTATGGGGAcTC	94°C	3 mins	1
		R2 AATGGATAATATTTCTCAAATGATAAcTTaGCA	94°C	30 sec	
			65°C	30 sec	25
			72°C	30 sec	
			72°C	5 mins	1

Codon	PCR	Primers (5'-3')	PCR conditions		
1042	1st	F1 AGAAGATTATTTCTGTAATTTGATACAAAAAGC	94°C	3 mins	1
		R1 ATGATTCGATAAATTCATCTATAGCAGCAA	94°C	30 sec	
			68°C	1 min	40
			72°C	1 min	
			72°C	5 mins	1
	2nd	F2 AGAATTATTGTAAATGCAGCTTTATGGGGAcTC	94°C	3 mins	1
		R2 AATGGATAATATTTCTCAAATGATAAcTTaGCA	94°C	30 sec	
			65°C	1 min	25
			72°C	1 min	
			72°C	5 mins	1

Codon	PCR	Primers (5'-3')	PCR conditions		
1246	1st	F1 AGAAGATTATTTCTGTAATTTGATACAAAAAGC	94°C	3 mins	1
		R1 ATGATTCGATAAATTCATCTATAGCAGCAA	94°C	30 sec	
			68°C	1 min	40
			72°C	1 min	
			72°C	5 mins	1
	2nd	F2 ATGATCACATTATATTAAAAAATGATATGACAAAT	94°C	3 mins	1
		R1 ATGATTCGATAAATTCATCTATAGCAGCAA	94°C	30 sec	
			65°C	1 min	25
			72°C	1 mn	
			72°C	5 mins	1

Appendix IV

Genotyping and sequencing of *dhfr*

PCR	Primers (5'-3')	PCR conditions		
1st	F1 CCAACATTCAAGATGATACATAAAG	95°C	10 mins	1
	R1 CATCGCTAACAGAAATAATTGATACTC	94°C	1 min	
		55°C	1 min	40
		72°C	1 min	
		72°C	5 mins	1
2nd	F2 CTCCTTTTTATGATGGAACAAGTC	95°C	10 mins	1
	R2 CATCACATTCATATGTACTATTTATTCTAGT	94°C	1 min	
		55°C	1 min	30
		72°C	1 min	
		72°C	5 mins	1

Appendix V

Genotyping and sequencing of *dhps*

PCR	Primers (5'-3')	PCR conditions		
1st	F1 GTGAGAAGCTTGAAAGAACAATAT	95°C	3 mins	1
	R1 ATATAGAATTCTTACTTTTGTATA	94°C	1 min	
		46°C	1 min	40
		72°C	1 min	
		72°C	5 mins	1
2nd	F2 GGTATTTTTGTTGAACCTAAACG	95°C	3 mins	1
	R2 ATCCAATTGTGTGATTTGTCCAC	94°C	1 min	
		48°C	1 min	30
		72°C	1 min	
		72°C	5 mins	1

Appendix VI

Microsatellite markers flanking *pfcrt*

Loci	PCR	Primers (5'-3')	PCR conditions		
-29.26kb	1st	F1 TAGGCATATTCCTTTTTATT	94°C	3 mins	1
		R1 ATTTATTCATTCCTTTTTGT	94°C	30 sec	
			53°C	40 sec	35
			72°C	30 sec	
			72°C	2 mins	1
	2nd	F2 **D4**TATGTACCCTCAAGTAGACC	94°C	3 mins	1
		R1 ATTTATTCATTCCTTTTTGT	94°C	20 sec	
			56°C	30 sec	25
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
-10.834kb	1st	F1 TGCGTAAATTTTGATGTAAT	94°C	3 mins	1
		R1 CCCCTTCAAAAAGGAAATAACAC	94°C	30 sec	
			63°C	40 sec	35
			72°C	30 sec	
			72°C	2 mins	1
	2nd	F2 D2AAGAATGAAAGTATTTTTAGC	94°C	3 mins	1
		R1 CCCCTTCAAAAAGGAAATAACAC	94°C	20 sec	
			59°C	30 sec	25
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
-2.814kb	1st	F1 AATTTCTACTAGTATCATAAACAA	94°C	3 mins	1
		R1 AAATCGAATTTATTTTATCG	94°C	30 sec	
			56°C	40 sec	35
			72°C	30 sec	
			72°C	2 mins	1
	2nd	F2 D3TTAAAAGCACCTTATTCATT	94°C	3 mins	1
		R1 AAATCGAATTTATTTTATCG	94°C	20 sec	
			58°C	30 sec	25
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
0.59kb	1st	F1 TAGAAATGAGAAGAAGCAAA	94°C	3 mins	1
		R1ACCTATTTATCAAAACACCA	94°C	30 sec	
			62°C	40 sec	35
			72°C	30 sec	
			72°C	2 mins	1
	2nd	F2 D4AATTCATACTGTGTCAAAGG	94°C	3 mins	1
		R1 ACCTATTTATCAAAACACCA	94°C	20 sec	
			62°C	30 sec	25
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
10.38kb	1st	F1 GCTCACATCATTTCTAACAT	94°C	3 mins	1
		R1 ACATTTAAGAAAAACCCATT	94°C	30 sec	
			62°C	40 sec	35
			72°C	30 sec	
			72°C	2 mins	1
	2nd	F2 D2CGGTATGATTATAATTTGAGA	94°C	3 mins	1
		R1 ACATTTAAGAAAAACCCATT	94°C	20 sec	
			62°C	30 sec	25
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
23.57kb	1st	F1 TTAATCCATACTGCAAAAAT	94°C	3 mins	1
		R1 TAAATGGAAAGGAGTTTGTA	94°C	30 sec	
			54°C	40 sec	35
			72°C	30 sec	
			72°C	2 mins	1
	2nd	F2 D3ATCCATACTGCAAAAATAAA	94°C	3 mins	1
		R1 TAAATGGAAAGGAGTTTGTA	94°C	20 sec	
			60°C	30 sec	25
			72°C	20 sec	

Appendix VII

Microsatellite markers flanking *dhfr*

Loci	PCR	Primers (5'-3')	PCR conditions		
-4.49kb	1st	F1 TTCTACGAATTATTTTTCCA	94°C	3 mins	1
		R1 TGAGCGTGCATATTTTATTACTATGTC	94°C	1 min	
			54°C	1 min	35
			72°C	1 min	
			72°C	5 min	1
	2nd	F2 D3TTCTACGAATTATTTTTCCA	94°C	3 mins	1
		R1 ACAAGTTAAAAGACGAAACA	94°C	20 sec	
			60°C	30 sec	25
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
-3.87kb	1st	F1 GTAATAAAATATGCACGCTCA	94°C	3 mins	1
		R1 ACTGATGAAATTGTAAATGA	94°C	1 min	
			60°C	1 min	30
			72°C	1 min	
			72°C	5 min	1
	2nd	F2 D4ACAGTTATAAGATTTAATGCAA	94°C	3 mins	1
		R1 ACTGATGAAATTGTAAATGA	94°C	20 sec	
			60°C	30 sec	25
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
-0.10kb	1st	F1 AAGTGAGTAAATATGAATGTG	93°C	1 min	1
		R1 ATTTTTGCTTTCAACCTTAC	93°C	20 sec	37
			62°C	5 mins	
			72°C	10 min	1
	2nd	F2 GGTATTTTTGTTGAACCTAAACG	94°C	2 mins	1
		R2 ATCCAATTGTGTGATTTGTCCAC	94°C	20 sec	
			52°C	30 sec	22
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
0.52kb	1st	F1 ATTTTACAATTTCGGATTTTAC	94°C	3 mins	1
		R1 CATTGAGATAAATAAGTGTTCA	94°C	1 min	
			58°C	1 min	35
			72°C	1 min	
			72°C	5 min	1
	2nd	F2 D3 TAAAGAAGGCATAATTTTCA	94°C	3 mins	1
		R1 CATTGAGATAAATAAGTGTTCA	94°C	1 min	
			52°C	1 min	20
			72°C	1 min	

Loci	PCR	Primers (5'-3')	PCR conditions		
1.48kb	1st	F1 ATTTTACAATTTCGGATTTTAC	94°C	1 min	1
		R1 AGACGAAGACGATATACACAAT	94°C	20 sec	35
			62°C	5 mins	
			72°C	10 min	1
	2nd	F2 D3TGGGACATATTTTTGATTAG	94°C	3 mins	1
		R2 ACTTAAAATTGCCTTTACCT	94°C	20 sec	
			60°C	30 sec	20
			72°C	20 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
5.87kb	1st	F1 TGATCTTATTAACCATGCGGATT	94°C	3 mins	1
		R1 GAATATGACACAAATTAGTAGG	94°C	1 min	
			60°C	1 min	35
			72°C	1 min	
			72°C	5 min	1
	2nd	F2 D4TTGTTTTTGTTAAGTTGTTT	94°C	3 mins	1
		R1 GAATATGACACAAATTAGTAGG	94°C	20 sec	
			58°C	30 sec	25
			72°C	20 sec	

Appendix VIII

Loci	PCR	Primers (5'-3')	PCR conditions		
0.8kb	1st	F1 GACCAAGTGTAATTTAC	94°C	2 mins	1
		R1 GACATATAATGAGCATG	94°C	30 sec	
			42°C	30 sec	25
			40°C	30 sec	
			65°C	40 sec	1
			65°C	2 mins	
	2nd	F2 Fam GGAAAGTGCAAACATGT	94°C	2 mins	1
		R1 GACATATAATGAGCATG	94°C	20 sec	
			45°C	20 sec	25
			65°C	30 sec	
			65°C	2 mins	

Loci	PCR	Primers (5'-3')	PCR conditions		
4.3kb	1st	F1 AACTTTTCGTGGGTAAAG	94°C	2 mins	1
		R1 GTTCGATATATGCACACA	94°C	30 sec	
			42°C	30 sec	25
			40°C	30 sec	
			65°C	40 sec	1
			65°C	2 mins	
	2nd	F2 NedGTATGAATAATATTACCCTT	94°C	2 mins	1
		R1 GTTCGATATATGCACACA	94°C	20 sec	
			45°C	20 sec	25
			65°C	30 sec	
			65°C	2 mins	

Loci	PCR	Primers (5'-3')	PCR conditions		
7.7kb	1st	F1GTGTCCTATAAGTATTGA	94°C	2 mins	1
		R1 TGATAGTACATTATGTAG	94°C	30 sec	
			42°C	30 sec	25
			40°C	30 sec	
			65°C	40 sec	1
			65°C	2 mins	
	2nd	F2 VicGGTTATCAATATGTACAT	94°C	2 mins	1
		R1 GTTCGATATATGCACACA	94°C	20 sec	
			45°C	20 sec	25
			65°C	30 sec	
			65°C	2 mins	

Loci	PCR	Primers (5'-3')	PCR conditions		
-2.9kb	1st	F1 ATGTTTGAACCCCTTAATTTA	94°C	1 min	1
		R1 AGGAGGTTTCCCTTCACTCCATCT	94°C	20 sec	
			62°C	10 mins	25
			72°C	10 mins	
	2nd	F1 ATGTTTGAACCCCTTAATTTA	94°C	2 mins	1
		R2 CACATGTAAATGCATATTTATG	94°C	30 sec	
			50°C	30 sec	5
			60°C	30 sec	
			94°C	30 sec	
			45°C	30 sec	25
			60°C	30 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
-1.5kb	1st	F1 ATGTTTGAACCCCTTAATTTA	94°C	1 min	1
		R1 AGGAGGTTTCCCTTCACTCCATCT	94°C	20 sec	
			62°C	10 mins	25
			72°C	10 mins	
	2nd	F1 TGTCTTGAAGGACAACACATAGATG	94°C	2 mins	1
		R2 CATAATATGAAGAGACTGAAAGTT	94°C	30 sec	
			50°C	30 sec	5
			60°C	30 sec	
			94°C	30 sec	
			45°C	30 sec	25
			60°C	30 sec	

Loci	PCR	Primers (5'-3')	PCR conditions		
-0.13kb	1st	F1 ATGTTTGAACCCCTTAATTTA	94°C	1 min	1
		R1 AGGAGGTTTCCCTTCACTCCATCT	94°C	20 sec	
			62°C	10 mins	35
			72°C	10 mins	
	2nd	F1 AAATATTTGCGCCAAACTTT	94°C	2 mins	1
		R2 TAGATTTCTTTACGCAAAAT	94°C	30 sec	
			50°C	30 sec	5
			60°C	30 sec	
			94°C	30 sec	
			45°C	30 sec	25
			60°C	30 sec	

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