ความหลากหลายทางพันธุกรรมและการดื้อยาของเชื้อมาลาเรียชนิดฟัลซิพารัม ในพื้นที่อำเภอไทรโยค จังหวัดกาญจนบุรี

นางสาวเทพนาฏ พุ่มไพบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE GENETIC DIVERSITY AND DRUG RESISTANCE OF *Plasmodium falciparum* POPULATION IN SAI YOK DISTRICT, KANCHANABURI

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เทพนาฏ พุ่มไพบูลย์ : ความหลากหลายทางพันธุกรรมและการดื้อยาของเชื้อมาลาเรียชนิดฟัลซิพารัม ในพื้นที่อำเภอไทรโยค จังหวัดกาญจนบุรี. (THE GENETIC DIVERSITY AND DRUG RESISTANCE OF *Plasmodium falciparum* POPULATION IN SAI YOK DISTRICT, KANCHANABURI) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ. นพ. จิตร สิทธิอมร 158 หน้า.

โครงสร้างประชากรของเชื้อมาลาเรียที่ก่อโรคในคน โดยเฉพาะเชื้อมาลาเรียชนิดฟัลซิพารัมนั้น ขึ้นอยู่กับสถานการณ์การแพร่ระบาด และประชากรในบริเวณนั้นๆ ซึ่งรวมถึง อุบัติการณ์ของโรค ความหนาแน่นของพาหะนำโรค และการเคลื่อนย้านถิ่นฐานของประชากร การศึกษานี้แสดงให้เห็นถึงการเปลี่ยนแปลงโครงสร้างประชากรเชื้อมาลาเรียในจังหวัดกาญจนบุรีระหว่างปี 2547 ถึง 2550 เมื่อวิเคราะห์ด้วยไมโครแซทเทลไลท์จำนวน 12 ตำแหน่ง พบว่าการเคลื่อนย้ายเข้าออกของประชาชนระหว่างชายแดนไทย-พม่าในช่วง 2 ปีหลัง มีผลต่อการเปลี่ยนแปลงประชากรเชื้อในพื้นที่นี้ นอกจากนี้การเปลี่ยนแปลงโครงสร้างประชากรยังพบได้จากการวิเคราะห์ด้วยไมโครแซทเทลไลท์จำนวน 4 ตำแหน่งซึ่งอยู่ภายในยีนและคร่อมยีน *pfmdr1* ซึ่งได้รับการเสนอว่าน่าจะเป็นยีนที่เกี่ยวข้องกับการดื้อต่อยาเมฟโฟลควิน การลดลงของเชื้อดื้อต่อยาเมฟโฟลควินในพื้นที่นี้ตั้งแต่ปี 2548 แสดงให้เห็นว่าการใช้ยาผสมระหว่างอาร์ทีซูเนตกับเมฟโฟลควินจะช่วยลดจำนวนเชื้อดื้อต่อยาเมฟ-โฟลควินที่เกิดขึ้นในบริเวณนี้และยังแสดงให้เห็นว่ายาผสมนี้ยังสามารถใช้เป็นยาขนานที่ 1 เพื่อรักษาผู้ป่วยมาลาเรียที่มีอาการไม่รุนแรงได้ จากการวิเคราะห์โครงสร้างประชากรของเชื้อมาลาเรียในพื้นที่อีก 6 จังหวัดที่มีการแพร่ระบาดพบว่ามีความแตกต่างทางพันธุกรรมระหว่างประชากรในที่ต่างๆ ถึงแม้ว่าประเทศไทยจะมีการระบาดของโรคมาลาเรียต่ำ แต่กลับพบว่ามีความหลากหลายทางพันธุกรรมสูงและไม่พบ linkage disequilibrium ในประชากร 5 จังหวัดจาก 7 จังหวัดที่ทำการศึกษา ยกเว้นประชากรเชื้อในจังหวัดยะลาและจังหวัดกาญจนบุรี ซึ่งในจังหวัดยะลาพบประชากรมีลักษณะพันธุกรรมแบบเดียวกันในขณะที่พบ linkage disequilibrium และความหลากหลายทางพันธุกรรมสูงในประชากรเชื้อในจังหวัดกาญจนบุรี สิ่งที่พบนี้สามารถช่วยให้เข้าใจถึงการเปลี่ยนแปลงของประชากรเชื้อมาลาเรียในแต่ละพื้นที่ที่มีรูปแบบการใช้ยาในการรักษาแตกต่างกัน เมื่อเปรียบเทียบความแตกต่างทางพันธุกรรมของประชากรเชื้อในประเทศไทยกับประชากรเชื้อจากประเทศเฟรนช์กายอานา คองโกและแคเมอรูน พบว่าประชากรในแต่ละกลุ่มมีความแตกต่างทางพันธุกรรมอย่างมีนัยสำคัญ ยกเว้นประชากรจาก 2 ประเทศในแอฟริกา ในขณะที่ความหลากหลายทางพันธุกรรมของเชื้อมาลาเรียชนิดฟัลซิพารัมระหว่างประเทศนั้นมีการกระจายที่เกี่ยวเนื่องกัน

ไม่พบความสัมพันธ์ระหว่างลำดับเบสในยีน *pfmdr1* จีโนไทปด์ของไมโครแซทเทลไลท์ และระดับการตอบสนองต่อยาเมฟโฟลควินของเชื้อที่เลือกมาจากประชากรในจังหวัดกาญจนบุรีซึ่งเก็บในช่วงเวลาต่างกัน จากผลการทดลองกล่าวได้ว่าไมโครแซทเทลไลท์ชุดนี้ให้ข้อมูลที่ไม่เพียงพอที่จะแยกเชื้อแต่ละสายพันธุ์ที่มีการตอบสนองต่อยาเมฟโฟลควินที่แตกต่างกันได้

สาขาวิชา ชีววิทยาศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

4689669320 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS: MALARIA / *Plasmodium falciparum* / MICROSATELLITES / DRUG RESISTANCE / *Plasmodium falciparum* DRUG RESISTANCE GENE

TEPANATA PUMPAIBOOL: THE GENETIC DIVERSITY AND DRUG RESISTANCE OF *Plasmodium falciparum* POPULATION IN SAI YOK DISTRICT, KANCHANABURI. ADVISOR: PROF. CHITR SITTHI-AMORN, M.D., Ph.D. 158 pp.

The population structure of the causative agents of human malaria, *Plasmodium* sp., especially *P. falciparum,* depends on the local epidemiological and demographic situations. Examples include the incidence of malaria, the vector transmission intensity and migration of inhabitants. This study revealed changes in population structure of parasite in Kanchanaburi between 2004 and 2007 using 12 microsatellite loci. It was found that migrations of people between the Thai-Myanmar borders in the last two years had an effect on *P. falciparum* population dynamics in this area. Furthermore, the changes in population structure were also observed by using four microsatellite loci within and flanking the *pfmdr1* gene previously proposed to be responsible for mefloquine resistance. The declining trend in the proportion of mefloquine resistance in this area since 2005 implies that the combining artesunate and mefloquine may help reduce mefloquine resistance in this area. It also implies that mefloquine-artesunate combination can continue to be the first-line drug regimen for uncomplicated malaria. The parasite structures in other six malaria endemic provinces of Thailand were analyzed. The analysis showed that *P. falciparum* shows a variation of genetically structured populations across local areas of Thailand. The analysis also showed that although Thailand is considered a low transmission area, a relatively high level of genetic diversity and the lack of linkage disequilibrium were found in five of the seven study areas. The two exceptions were Yala and Kanchanaburi. In Yala province, a clonal population structure was revealed, while some linkage disequilibrium and high genetic diversity was observed in Kanchanaburi. This finding could help in understanding the special dynamics of parasite populations in areas with different pattern of drug use. A comparison of the genetic structure of *P. falciparum* populations in Thailand with those in the French Guyana, Congo and Cameroon revealed a significant genetic differentiation between them, except the two African countries, whilst the genetic variability of *P. falciparum* amongst countries showed overlapping distributions.

No obvious relationship was found between the *pfmdr1* gene sequence, the microsatellite genotype and mefloquine response level of sampling parasite from Kanchanaburi populations collected at different time periods. The result suggested that this set of microsatellite loci was not informative enough to distinguish individual parasite possessed different response to mefloquine.

Field of Study: Biomedical Sciences Student's Signature Tepanata Pumpaibool

Academic Year : 2008 Advisor's Signature Chitr Sitthi-amorn

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LIST OF ABBREVIATIONS

A	Adenine
A	Alanine
Asn	Asparagine
Asp	Aspatate
ATP	Adenosine triphosphate
bp	base pair
C	Cytosine
C	Cystein
Cys	Cystein
D	Aspartate
DNA	Deoxyribonucleic acid
E	Glutamate
F	Phenylalanine
G	Guanine
IC_{50}	Half of maximal inhibitory concentration
K	Lysine
L	Leusine
LD	Linkage disequilibrium
m	meter
mg	milligram
ml	milliliter
mM	millimolar
N	Asparagine
ng	nanogram
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
Phe	Phenylalanine
pmole	picomole
RNA	Ribonucleic acid

S	Serine
Ser	Serine
T	Thymine
T	Threonine
TBE	Tris-borate-EDTA
Tyr	Tyrosine
UV	Ultraviolet
V	Valine
Y	Tyrosine
µl	microliter
µM	micromolar

CHAPTER I

INTRODUCTION

1.1 Rationale

Malaria remains one of the important infectious diseases in Thailand and worldwide. Even though there are many efforts to eradicate and control the disease, there has been resurgence in malaria in many countries. In Thailand, although the total number of malaria cases has decreased annually (Chareonviriyaphap, Bangs and Ratanatham, 2000), malaria remains most prevalent along the Thai borders with Myanmar, Cambodia and Malaysia (Konchom et al., 2003). Moreover resistance of malaria parasite, *Plasmodium falciparum*, against many antimalarial drugs has become a serious and growing problem in most malaria endemic areas (White, 1999; Cowman, 2001). At present *P. falciparum* strains become more resistant to mefloquine in some endemic areas especially the provinces located along Thai-Myanmar border; Tak, Ranong and Kanchanaburi and also along Thai-Cambodian border; Trat, Chantaburi and Sa Kaeo (Socheat et al., 2003). The efficacy of mefloquine alone declined to less than 50% in 2002. As a result, the drug policy has been changed to a drug combination between mefloquine and artesunate in those areas (Socheat et al., 2003).

Failure of malarial control has resulted from the changing of major epidemiological factors such as (i) the evolution of pesticide-resistant mosquitoes; (ii) changing human behavior such as population migration, job seeking practices and life styles; (iii) changing global environment; and (iv) the emergence and spread of drug-resistant parasites. The rapid evolution of the drug-resistant parasites to most of antimalarial drugs means that new effective drugs need to be developed. This can be an expensive process and retards malarial control.

Understanding of the genetic complexity of the malaria parasite population is a crucial aspect for the control of this disease. The malaria parasite is haploid for the most of its life, except for a short period in the mosquito vector where sexual recombination occurs. It is believed that parasite diversity in nature is generated if sexual recombination occurs between gametes from different clones of parasite or outcrossing. The transmission rate (Babiker et al., 1994; Paul et al., 1995) and

migration of human inhabitants (Lum, 2004), which are different in each endemic area, affect the genetic variation and population structure of the parasite. The basic information of structure of parasite populations would help us explain the dynamics of drug resistance and indicate which epidemiological process should be targeted for disease control.

Most of previous works on the molecular genetics of malaria parasites have used a limited number of surface antigen loci, which are under strong natural selection (Paul et al., 1995; Paul et al., 1998; Babiker et al., 1997; Mueller et al., 2002). It is not clear whether the patterns observed reflect population history or natural selection. After the *P. falciparum* genome project had been completed, the microsatellite loci that are present throughout the parasite genome (Su and Wellems, 1996) seems to be a sensitive tool for investigation the population genetics of *P. falciparum* (Anderson et al., 1999; Anderson et al., 2000; Conway et al., 2001; Wootton et al., 2002; Nair et al., 2003; Roper et al., 2003; Nash et al., 2005). These markers have been used to analyze and compare genetic structures of *P. falciparum* populations in different parts of the world. Data revealed dramatic differences in parasite population structure in different locations (Anderson et al., 2000). The population structure of *P. falciparum* depends on local factors related to parasite, vector and host biology (Babiker and Walliker, 1997). Besides, these markers can be used to not only for monitoring the dynamics of parasite population in a local area, but also for the study of patterns of parasite population changes and gene flow. The knowledge of the population genetics of *P. falciparum*, combined with appropriate screening strategies for detecting drug resistance, might prolong the effective lifetime of new drugs (Hartl, 2004). Therefore, the monitoring system for drug resistance of malaria parasite should be extended to all sentinel sites in order to elucidate the resistance situation in our country.

The study of molecular markers of drug resistance is particularly important in surveillance studies. Numerous studies have tried to identify correlations between sensitivity to different antimalarials such as chloroquine, arylaminoalcohols and endoperoxides and the possession of different alleles of *Plasmodium falciparum* multi-drug resistance gene 1 (*pfmdr1*) and its copy number (Wilson et al., 1993; Price et al., 1997; Duraisingh, Roper et al., 2000b; Duraisingh, Jone et al., 2000a; Price et al., 2004; Congpuong et al., 2005; Uhlemann et al., 2005; Nelson et al., 2005; Sidhu

et al., 2006). Mefloquine resistance is now widespread in Southeast Asia, especially in Thailand where mefloquine has been used to treat uncomplicated falciparum malaria in numerous endemic areas. So, a study of genetic diversity and drug resistance of *P. falciparum* population under drug selection is necessary.

This study is performed in Kanchanaburi province, one of ten provinces with highest malaria incidence of Thailand (Malaria Cluster, Department of Disease Control, Ministry of Public Health, 2006). *Plasmodium falciparum* field isolates collected from malaria clinics in Sai Yok district, Kanchanaburi province, where mefloquine and artesunate combination have been used for uncomplicated falciparum malaria treatment, will be analyzed for genetic diversity and magnitude of drug resistance at different time. A set of microsatellite markers will be used to reveal parasite's diversity in population. Also, another set of microsatellite markers will be used to track for *pfmdr1* genotypes which contribute to parasite gene pool in this population. Susceptibility to mefloquine will be performed to determine magnitude of drug resistance. Correlation between mefloquine susceptibility level and *pfmdr1* genotype will be tested. Results from this study will shed light on the diversity of *P. falciparum* and the extent of drug resistance in focal endemic area. Furthermore, Kanchanaburi parasite population will be compared with other Thai parasite populations and parasite populations of other countries in different continents.

1.2 Research questions

1. What is the population diversity of *P. falciparum* population in Sai Yok district, Kanchanaburi?

2. Are there differences in parasite population diversity and population structure among parasite populations collected at different time?

3. Do Thai parasite populations have different genetic structure across the country?

4. Are there the differences in parasite population structure among continents?

5. What is the magnitude of drug resistance in Kanchanaburi province?

6. Is there an association between microsatellite haplotype observed by microsatellite markers within and flanking the *pfmdr1* gene, sequence polymorphisms of *pfmdr1* gene and mefloquine susceptibility using parasites in Kanchanaburi population as a model?

1.3 Objectives

1. To investigate the population diversity and population structure of *P. falciparum* in Kanchanaburi province.

2. To document parasite population diversity and population structure among parasite populations collected at different time.

3. To investigate the population diversity and population structure of *P. falciparum* in Thailand and compare the genetic differentiation between provinces in Thailand and further compare parasite population of Thailand with other countries in different continent where malaria endemic are different.

4. To determine the magnitude of drug resistance in Kanchanaburi province.

5. To determine an association between microsatellite genotype pattern within and around *pfmdr1* gene and mefloquine response.

6. To determine sequence polymorphism in *pfmdr 1* gene associated with mefloquine response.

1.4 Anticipated benefit

The results from this study will help us understand the dynamics of *P. falciparum* population as well as drug resistance in each study area. If sufficiently refined to monitor parasite resistance on a routine basis, microsatellite markers may be used as a valuable tool to assist decision making for resistance control priorities in specific area. An increase in drug resistant parasites and a decrease in parasite diversity can prompt health providers and drug policy makers to modify drug regimens for malarial treatment and to campaign for people to strictly adhere to treatment regimens. In addition, policy makers can take steps to delay drug resistance

by rendering specific attentions to fake drugs, rapid diagnosis and treatment of Thai and other migrant populations at the border areas.

CHAPTER II

LITERATURE REVIEW

2.1 Global situation of malaria

Malaria remains to be one of the important infectious diseases worldwide especially in tropical area (Figure 2.1). Even though there are many efforts to eradicate and control the disease, there has been resurgence in malaria in many countries. In 2004, 107 countries are reported as areas at risk of malaria transmission and around 350-500 million clinical malaria cases occur annually. Approximately 60% of the clinical cases and over 80% of the deaths occur in Africa. Most of the deaths (more than 1 million) are African children under 5 year old (World Health Organization, 2005). Most of the severe disease and deaths are caused by *Plasmodium falciparum*, one of the four species of malaria parasite – *P. falciparum, P. vivax, P. malariae* and *P. ovale* - that infect humans. Malaria has not only been the cause of health problem, but it has also been the economic and social burden problem. The disease is estimated to be responsible for an estimated average annual reduction of 1.3% in economic growth for countries with intensive malaria (Gallup and Sachs, 2001).

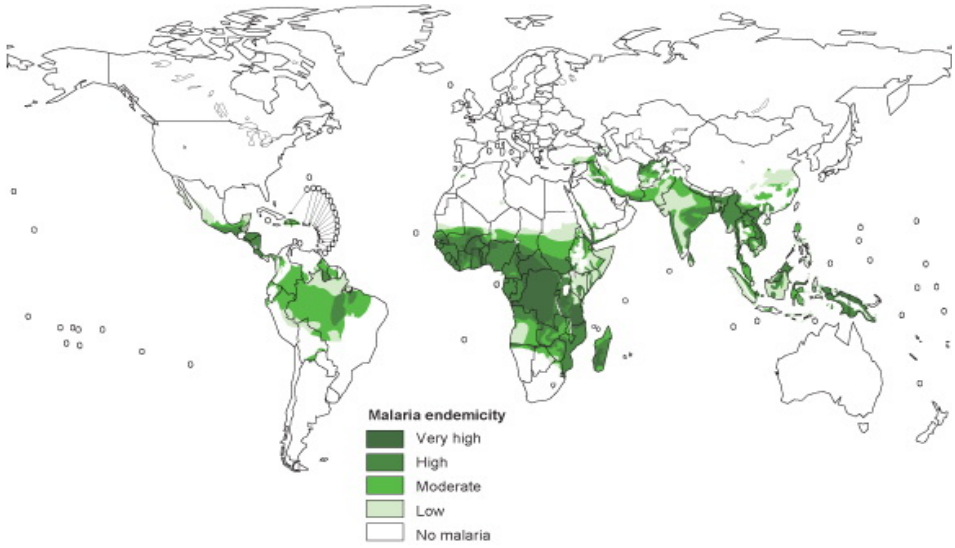


Figure 2.1 Global distribution of malaria. Very high malaria endemicity is distributed in the sub Sahara, Africa (World Health Organization, 2005).

The wide variation in the burden of malaria between different regions of the world is driven by several factors. First, there is great variation in parasite-vector-human transmission dynamics in each endemic area that enhance or limit the transmission of malaria infection and the associated risk of disease and death. The second factor contributing to variability in malaria burden in regional and local level is the differences in levels of socioeconomic development such as poverty, quality of life, access to health care and health education and the existence of active malaria control program for disease prevention and treatment.

In Thailand, although the total number of malaria cases has decreased annually (Chareonviriyaphap, Bangs and Ratanatham, 2000), malaria remains most prevalent along the Thai borders with Myanmar, Cambodia and Malaysia (Konchom et al., 2003). Malaria incidence in 2006, a total of 66,651 malaria cases (both Thai and foreigners) was reported. Tak province showed the highest number of cases in Thailand- 8,648 clinical cases – and the top ten provinces reported cases are Tak, Yala, Maehongson, Narathiwat, Kanchanaburi, Chumphon, Chanthaburi, Songkhla, Prachuapkhirikhan and Ranong (Figure 2.2) (Malaria Cluster, Department of Disease Control, Ministry of Public Health, 2006). Even though, the malaria cases in Thailand have decreased, resistance of malaria parasite, *P. falciparum*, against many antimalarial drugs has become a serious and growing problem in most malaria endemic areas (Chareonviriyaphap, Bangs and Ratanatham, 2000; Wongsrichanalai et al., 2002). At present *P. falciparum* strains become more resistant to mefloquine in some endemic areas especially the provinces located along Thai-Myanmar border; Tak, Ranong and Kanchanaburi and also along Thai-Cambodian border; Trat, Chantaburi and Sa Kaeo (Socheat et al., 2003). The efficacy of mefloquine alone declined to less than 50% in 2002. As a result, the drug policy has been changed to a drug combination between mefloquine and artesunate in those areas (Socheat et al., 2003).

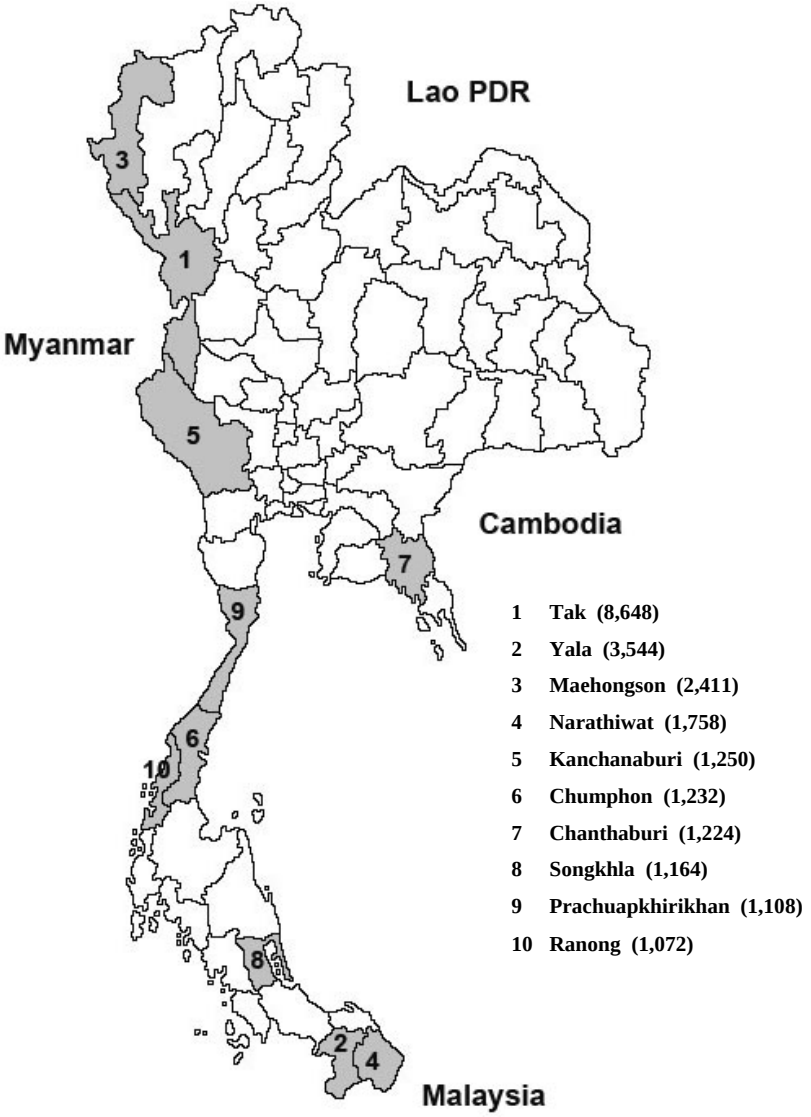


Figure 2.2 Ten provinces with highest malaria cases, Thailand in 2006

2.2 Biological characteristic of *Plasmodium falciparum*

Malaria is caused by infection of human being by parasite belonging to the genus *Plasmodium.* These parasites are introduced into the blood of human host by mosquito bites. Malaria parasites are unicellular organisms with a complex life cycle as shown by their ability to pass through a whole series of development stages in human and mosquito hosts (Figure 2.3).

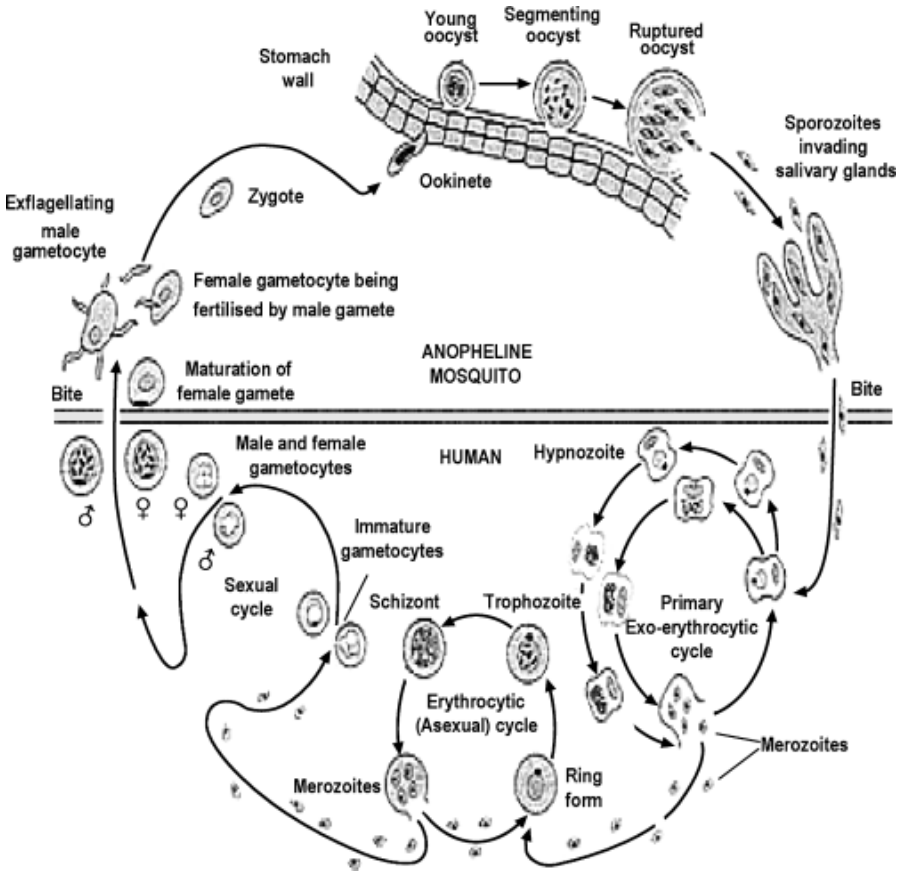


Figure 2.3 Life cycle of malaria parasite (Wipasa et al., 2002)

2.2.1 Life cycle of human malaria parasite

The life cycle of all species of human malaria parasites is essentially the same. It comprises an exogenous sexual phase (sporogony) with multiplication in Anopheles mosquitoes and an endogenous asexual phase (schizogony) with multiplication in the vertebrate host. The latter phase includes the development cycle in the red corpuscles in the blood (erythrocytic schizogony) and the phase taking place in the parenchyma cells in the liver (pre-erythrocytic schizogony).

2.2.1.1 The parasite in the mosquito host

A parasite development cycle in mosquito occurs when a female Anopheles mosquito takes the blood of an infected human host with malaria parasites, the asexual parasites in blood meal are digested together with the red blood cells in mosquito's stomach while the mature sexual cells (gametocytes) develop further. In male gametocyte, it undergoes a process called exflagellation, the nucleus divides into 4 to 8 nuclei, each of which then forms a long thread-like structure and then break free. This process takes place only a few minutes. A maturation process continues and finally forms a microgamete. The female gametocyte undergoes a maturation process and forms a female gamete or macrogamete. The male and female gametes complete the fertilization in mosquito's stomach and form a zygote which develops a cytoskeleton and becomes mobile, this is known as ookinete. The ookinete forces its way to the stomach wall and passes between epithelial cells to the outer surface of the stomach and settles down. It becomes rounded up into a small sphere and is now called an oocyst. The oocyst gradually increases in size and the nucleus divided repeatedly then form elongated sporozoites. They burst through the ruptured wall of the oocyst then invade the body cavity of the mosquito and reach the salivary glands of the female mosquito which now becomes infective.

2.2.1.2 The parasite in the vertebrate host

When the mosquito feeds on blood, the sporozoites are injected into the wound on host's skin and pass into the bloodstream of the vertebrate host. The sporozoites remain in the blood in a short period of about half an hour. Many are destroyed by phagocytes, but some enter the parenchymal cells of the liver

(hepatocytes) directly or via the Kupffer cells and undergo a process of development and multiplication known as pre-erythrocytic schizogony. In *P. falciparum*, the sporozoites develop directly into pre-erythrocytic schizonts and produce numerous merozoites. When liver cell ruptures, the merozoites liberate into the blood circulation. Most of them invade the red blood cells, but some are phagocytosed. Merozoites attach the erythrocyte surface by using their apex organelles (rhoptries and micronemes) cause an invagination of the erythrocyte surface so that the merozoite slips into it. After invasion, parasite differentiates in many steps from young parasite known as ring form to trophozoites and proceeds asexual dividing process. The nucleus divides 3-5 times into a variable number of nuclei following by the division of cytoplasm forming a schizont. The segmentation of the nucleus and the cytoplasm is complete and a number of small rounded forms (merozoites) are produced in this mature schizont. After the process is completed the red blood cell bursts and the merozoites are released into the blood stream. The merozoites invade fresh erythrocytes; some repeat the same process of erythrocytic schizogony again while some merozoites differentiate into sexual forms (gametocytes).

2.3 Epidemiology of malaria

2.3.1 Geographical distribution

Malaria distributes in the limits of latitude and altitude. Indigenous malaria has been recorded as far in the north at 64^{o}N latitude (Archangel in the former USSR) and as far in the south at 32^{o}S latitude (Cordoba in Argentina). It has occurred at 400 m below sea level (Londiani in Kenya) and at 2,600 m above sea level or at 2,800 m (Cochabamba in Bolivia) (Gilles, 1993).

The limitation of malaria distribution depends greatly on local environmental and other conditions which changes over a period of time. Moreover, differences in environmental conditions in endemic areas result in a geographical range of four human malaria species. Among four species, *Plasmodium vivax* has the widest range. It is widespread in many temperate zones, but also found in the subtropical and tropical zones. *Plasmodium falciparum,* the commonest species present throughout the tropical and subtropical zones, although it may occur in some temperate areas. *Plasmodium malariae* is present in the same range as *P. falciparum* but much less

common. For *Plasmodium ovale*, it is mainly found in tropical Africa, but occasionally found in the West Pacific.

2.3.2 Malaria epidemiology in Thailand

In Thailand, all four species of human malaria present, but the majority of malaria cases is caused by *P. falciparum* and *P. vivax* infections at the ratio of approximately 50:50 (Na-Bangchang and Congpuong, 2007; Zhou et al., 2005; Snounou and White, 2004). Malaria remains prevalent in 30 provinces bordering with Maynmar, Cambodia, Laos and Malaysia where are mostly forest-fringe foot hills (Konchom et al., 2003). A difference in type of forested habitat for Anopheles from the north to the south of country has a consequence to the distribution of Anopheles species (O'Loughlin, Somboon and Walton, 2007). Among a total of 73 species of *Anopheles* reported in Thailand, only *An. baimaii*, *An. dirus*, *An. minimus* and *An. maculatus* (each belonging to complex of species) are considered major vectors for malaria (Rattanarithikul et al., 2006). Anopheles feeding and host-seeking behavior are important in the epidemiology of malaria transmission as well. Mosquitoes that prefer to feed on animals are less efficient in transmitting human disease than those that prefer to feed on human (Rwegoshora et al., 2002). The high incidence of malaria transmission in bordering areas is also caused by movement of human population across the international border (Socheat et al., 2003). In addition, socioeconomic factors such as poverty and political instability force people more expose to malaria (Panvisavas, 2001).

Natural transmission of malaria depends on the presence or absence of three basic epidemiological factors which are the host, the agent (parasites and its vectors) and the environment. The illustration of a relationship between these factors is shown in Figure 2.4.

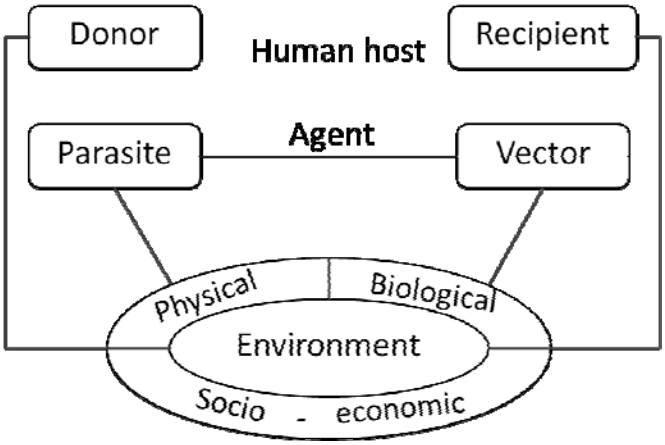


Figure 2.4 A relationship between host, agent and environment involved in the transmission of malaria (Gilles, 1993)

In summary, malaria is a focal disease which differs in its characteristics from area to area. These differences may be due to a variety of factors, including (i) the biological, anthropological, cultural and social characteristics of the population; (ii) the intensity and periodicity of malaria transmission; (iii) the species of malaria parasites and their sensitivity to antimalarial drugs; (iv) the species of the mosquito vector, their behavior and their susceptibility to insecticides; (v) the presence of social and ecological change; (vi) the characteristics of the existing health services (Phillips, 2001). Thus, no single strategy for malaria control and prevention is applicable for all situations.

2.4 Drug resistant malaria

2.4.1 Epidemiology of drug resistant malaria

To date, malaria parasites develop resistance to most of available antimalarial drugs. This is an obstacle for an effective control and management of this disease. The resistance to drug of malaria parasites commonly develops within 10-15 years after introducing (Wongsrichanalai et al., 2002). Therefore, the preparation for novel antimalarial is crucial.

There are several chemical groups of antimalarial compounds in general use. Some drugs are blood schizontocides effective only on the blood stages of the malaria parasite. Drugs in this group are, for example, **quinine**, **mefloquine**, **chloroquine**, **amodiaquine**. The antifolates such as **sulfadoxine, pyrimethamine**, **proguanil**, **chlorproguanil** and naphthoquinones, **atovaquone**, inhibit all growing stages of the malaria parasite. The 8-aminoquinolines such as **primaquine** are active against the non-growing stages (hypnozoites and gametocytes) in the human host (Warrell, 1993). **Artemisinin**, sesquiterpenelactone, and its derivatives inhibit all asexual stages including early gametocyte and the parasite can be killed rapidly (ter Kuile et al., 1993). In contrast, the drugs in this group do not affect parasites in the liver stages. Additionally, they also have the potential to interfere with mosquito transmission (Kumar and Zheng, 1990). Some of these drugs are now ineffective because of the development of resistant parasite.

Chloroquine resistance in *Plasmodium falciparum* appeared in the late 1950s on Thai-Cambodian border and in Colombia (Wernsdorfer and Payne, 1991). Initially spreading through South America and Southeast Asia, chloroquine resistance reached East Africa in 1978 and had crossed the African continent by 1985 (Björkman and Phillips-Howard, 1990). Nowadays, resistance to chloroquine in malaria caused by *P. falciparum* occurs everywhere except in Central America and in some regions of Southwestern Asia (Baird, 2005). Resistance to the antifolates developed more quickly after their introduction as a first-line drug against chloroquine resistant *P. falciparum.* Although, the combination of pyrimethamine with sulfa drugs delayed the spread of resistance, resistance to sulfadoxine-pyrimethamine was first noted on the Thai-Cambodian border in the mid 1960s (Björkman and Phillips-Howard, 1990). At present, high level resistance is found in Southeast Asia and Eastern Africa (Baird, 2005). Quinine was introduced for the treatment of malaria in the 17th century. It has had the longest period of effective use until the clinical resistance began to accumulate during the mid 1960s, especially from the Thai-Cambodian border (Wernsdorfer, 1994). Currently, clinical resistance to quinine monotherapy occurs sporadically in Southeast Asia and western Oceania. Widespread use of quinine in Thailand for therapy in the early 1980s in the situation of declining in sulfadoxine-pyrimethamine efficacy resulted in significant reduction

of its sensitivity. Therefore, to increase the effectiveness of treatment, quinine has been used in combination with antibiotic such as tetracycline or doxycycline. Quinine is now preserved as a second-line or third-line drug and is used in cases of severe malaria (Wongsrichanalai et al., 2002). Resistance to mefloquine was first observed near Thai-Cambodian border in the late 1980s within a few year of introduction routinely use (Wongsrichanalai et al., 2001). It is perhaps owing to widespread use of the chemically related drug quinine (Wernsdorfer, 1994). Mefloquine alone is no longer effective on the Thai-Myanmar and Thai-Cambodian borders, although it is still operationally useful in most other endemic areas in and around Thailand with field efficacy of more than 75% (Wongsrichanalai et al., 2002). High prevalence of mefloquine resistance in Chanthaburi and Trat provinces led to replacement by artesunate-mefloquine combination in Thailand in 1995 (Wongsrichanalai and Meshnick, 2008). The efficacy of this drug combination declined in the Thai-Cambodian border with the efficacy of more than 75%, this most likely resulted from mefloquine resistance rather than artemisinin resistance (Wongsrichanalai and Meshnick, 2008). At present, drug combination of artesunate with mefloquine (300 mg artesunate plus 1,250 mg mefloquine in split dose on the first day, and 300 mg artesunate plus 30 mg primaquine on the second day) is used as standard treatment for uncomplicated falciparum malaria (Na-Bangchang and Congpuong, 2007).

2.4.2 Mechanism of resistance

Currently, we have more knowledge of the mechanisms that malaria parasite uses to evade the lethal effect of many of the current antimalarial drugs used to treat and control malaria. This understanding is helpful for new drug development. Targets of many antimalarial drugs were identified. Broadly, there are two ways in which malaria parasites have become resistant to antimarial drugs. Firstly, by mutations in drug targets, those reduce their sensitivity as occurred in resistance against antifolates and atovaquone. Secondly, other drugs such as chloroquine and mefloquine may not have parasite protein targets that can mutate, thus, parasite become resistant through mutations in transporters involved in determining drug disposition within the intraerythrocytic parasite and its organelles (Woodrow and Krishna, 2006). The mechanisms of resistance in the antifolates such as pyrimethamine and the sulfa drugs are well known. Pyrimethamine and sulfadoxine

inhibit the main enzymes in folate synthesis pathway which are dihydrofolate reductase and dihydropteroate synthetase, respectively. Blockage of folate synthesis results in decreased synthesis of pyrimidines and consequently arrest of DNA replication also decreased few essential amino acid syntheses. The alteration of important amino acids in these enzymes confers resistance (Cowman et al., 1988; Peterson, Walliker and Wellems, 1988; Snewin et al., 1989; Zolg et al., 1989; Brooks et al., 1994; Wang et al., 1997). The antimalarial activity of chloroquine acts by inhibition of the detoxification of haematin inside the parasite food vacuole. It appears to inhibit the haem polymerization process causing a build-up of toxic haematin/chloroquine complex (Slater and Cerami, 1992; Dorn et al., 1995; Sullivan and Meshnick, 1996). The proteins involved in mediating chloroquine resistance may play a role in regulating pH of the food vacuole. To find out genes link to cholroquine resistant phenotype, analysis of a genetic cross between chloroquine-resistant and chloroquine-sensitive *P. falciparum* cloned line has mapped (Wellems et al., 1990; Su et al., 1997). A closely linked gene called *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and protein encoded by this gene plays an important role in chloroquine resistance (Fidock et al., 2000). Polymorphism in the *Plasmodium falciparum* multidrug resistance (*pfmdr1*) gene which encodes a protein that has a typical structure shared by members of the ATP binding cassette (ABC) family of transporters including the P-glycoproteins had an effect on resistance and sensitivity to mefloquine (Reed et al., 2000; Price et al., 2004), halofantrine and quinine (Reed et al., 2000). Nowadays, falciparum malaria had developed resistance to current antimalarials except artemisinin and its derivatives. There is no evidence for the existence of artemisinin resistance *in vivo* (Wongsrichanalai and Meshnick, 2008). However, artemisinin target, sarcoplasmic reticulum Ca^{2+}-transporting ATPase, which regulates the cytosolic free calcium concentration, had been proposed (Eckstein-Ludwig et al., 2003).

2.4.3 Determinants of antimalarial resistance

Identification of the molecular determinants of resistance allows population surveillance to be performed, ideally to inform policy on first-line and second-line drug to be recommended for use within a country or region. There are

several alternative methods for efficiently genotyping polymorphisms for drugs resistance genes.

The molecular basis of resistance to sulfadoxine-pyrimethamine is the best characterized. Specific mutations in *P. falciparum* that lead to resistance to both sulfadoxine and pyrimethamine have been identified. Point mutations in the five codons- 436, 437, 540, 581 and 623- of the dihydropteroate synthase gene and the four codons – 108, 51, 59 and 164- in the dihydrofolate reductase gene known to date are implicated in conferring resistance by decreasing the binding affinity between drugs and enzymes (e.g. Plowe et al., 1997; Wang et al., 1997). The genetic basis for chloroquine resistance has been previously linked to point mutations in the *Plasmodium falciparum* multidrug resistance (*pfmdr1*) gene encoding protein transporter in the parasite digestive vacuole (Foote et al., 1990; Reed et al., 2000). However, recent studies have provided strong evidence that chloroquine resistance is linked to mutation in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene (Fidock et al., 2000; Sidhu et al., 2002). Studies of the mechanism of chloroquine resistance in *P. falciparum* over the past 16 years have associated with point mutations at amino acid codons 184, 1034, 1042 and 1246 in *pfmdr1* and codon 76 in *pfcrt* genes (Plummer, Pereira and Carrington, 2004). Thus, resistance to chloroquine is clearly multifactorial and dependent on epistatic interactions with different genes in each parasite. For mefloquine resistance, copy number and polymorphism of the *pfmdr1* gene have been investigated as molecular markers. There were evidences that a higher copy numbers of this gene may be associated with more resistance to mefloquine (Wilson et al., 1993; Price et al., 1999; Price et al., 2004; Wongsrichanalai and Meshnick, 2008). A parasite transfection experiment showed that Ser1034, Asn1042 and Asp1246 mutation were a cause of resistance to mefloquine (Reed et al., 2000).

2.4.4 Multidrug resistance and the *pfmdr1* gene

Multidrug resistance occurs when cells selected for resistance to one agent, become resistant to a broad range of structurally unrelated drugs. The major protein mediating this in many mammalian cell-lines is the multidrug-resistance (mdr) transporter or P-glycoprotein (Pgp). *P. falciparum* sequences corresponding to

homologues of the mdr transporters that mediate multidrug resistance in mammalian cell-lines were identified (Foote et al., 1989)

Pfmdr1 is a typical member of the ATP-binding cassette (ABC) transporter superfamily. It codes for approximately 162 kDa polypeptide with a conserved structure of two domains consisting of six predicted transmembrane segments coupled to a nucleotide binding fold joined together with a linker region and has been termed P-glycoprotein homolog 1 (Pgh1) (Cowman et al., 1991; Higgins, 1992).

There are two ways in which gene polymorphisms may lead to drug resistance both through gene amplification and mutation. Mutations in *pfmdr1* gene were identified by sequencing a series of laboratory strains from different geographical areas (Foote et al., 1990). Polymorphism was observed at five positions; codon 86, 184, 1034, 1042 and 1246 as shown in Figure 2.5. Both mutation and amplification of this gene in field isolates were found to be widespread in numerous geographical areas. Polymorphism in the linker region had also been observed (Duraisingh et al., 2000b).

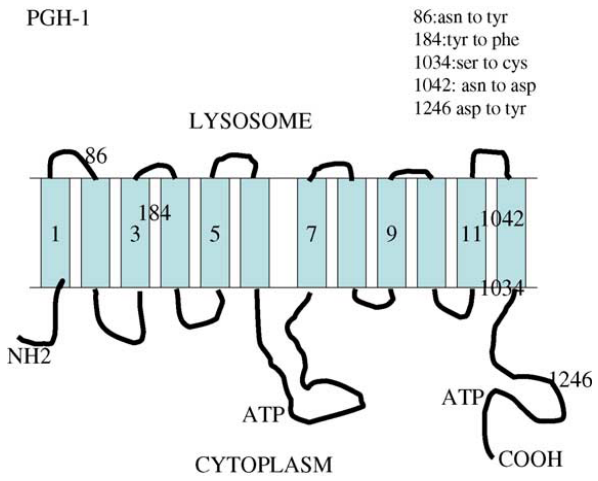


Figure 2.5 The Pgh1 protein of *P. falciparum* and its amino acid polymorphisms (Duraisingh and Cowman, 2005)

Polymorphism in *pfmdr1* was first found to be strongly linked to chloroquine resistance (Foote et al., 1990). Subsequently, in a genetic cross between a chloroquine-resistant and a sensitive-line, no association was found between chloroquine resistance and the *pfmdr1* locus (Wellem et al., 1990). However, polymorphism in *pfmdr1* was found to modulate chloroquine resistance (Ferdig et al., 2004). Resistance phenotypes of parasites to mefloquine and chloroquine were found to be related, although the relationship was reciprocal while associated with resistance to other arylaminoalcohols (halofantrine, lumefantrine and quinine) and the chemically unrelated artemisinins (peroxide). Several studies attempted to find the molecular basis of this phenotype (Wilson et al., 1993; Price et al., 1997, Duraisingh et al., 2000a; Duraisingh et al., 2000b; Price et al., 2004; Congpuong et al., 2005; Uhlemann et al., 2005; Nelson et al., 2005). In all five polymorphisms in *pfmdr1* gene (Asn86Tyr, Tyr184Phe, Ser1034Cys, Asn1042Asp and Asp1246Tyr), Tyr86 and Phe184 alleles (at the 5' end of *pfmdr1*) are found in isolates from all continents, while Cys1034, Asp1042 and Tyr1246 (at the 3' end of *pfmdr1*) are especially prevalent in South America (Woodrow and Krishna, 2006). The association between polymorphisms in *pfmdr1* and mefloquine resistance was controversial (Nelson et al., 2005; Congpuong et al., 2005) until Sidhu and colleagues (2005) used a reverse genetics approach to assess the importance of several commonly occurring alleles of the *pfmdr1* gene. The mutations at 3' end of *pfmdr1* (Cys1034, Asp1042 and Tyr1246) are shown to be associated with an increased resistance to quinine and increased sensitivity to mefloquine and the artemisinin drugs. The effect of these 3' polymorphisms in the field has been more difficult to assess since they are relatively rare in Southeast Asia and Africa. In a set of isolates from Thailand, the isolates containing Cys1034 and Asp1042 were combined, there was evidence that one or both 3'mutations were associated with a reduction in mefloquine IC_{50} (Pickard et al., 2003). The association of Asp1042 with lower IC_{50} for mefloquine was also found in a study of samples from the Thai-Myanmar border (Anderson et al., 2005). Mutation at 5' end of *pfmdr1*, Tyr86, also had an association with an increase in mefloquine susceptibility *in vitro* (Duraisingh et al., 2000a; Duraisingh et al., 2000b; Price et al., 2004). In summary, these laboratory and field data show remarkable agreement and suggest that some *pfmdr1* polymorphisms induce hypersensitivity to arylaminoalcohols. Not only the polymorphism of *pfmdr1* gene but an amplification

of this gene has been shown to be associated with mefloquine susceptibility. An increase in *pfmdr1* copy number was associated with an increase in median mefloquine IC_{50} (Wilson et al., 1993; Price et al., 1997; Price et al., 2004) and predicted clinical failure of mefloquine monotherapy (Price et al., 2004).

In some parts of Southeast Asia the main drug for malaria treatment for over a decade has been mefloquine and parasites with wild-type *pfmdr1* and mutant *pfcrt* are selected for in these areas. In the mean time, in Africa, the main drug of use is chloroquine rather than mefloquine and therefore parasites with mutant *pfmdr1* and mutant *pfcrt* genes are selected for in this continent (Duraisingh and Refour, 2005). In an area of high mefloquine resistance, the wild-type alleles and amplified *pfmdr1* prevalent that is associated with resistance to mefloquine (Price et al., 2004). This is a clear demonstration of how drug resistance genotypes are influenced by their selection histories. This identification of the determinants of drug resistance will provide markers for transmission studies.

2.5 Molecular approaches for study structure of malaria parasite population

Since the development of genetic and molecular studies of pathogen with a use of the polymerase chain reaction, many efforts have been made to establish links between basic research (evolutionary biology) and applied studies (molecular epidemiology, vaccine and drug development). *P. falciparum*, the malaria agent with the most severe form, has received much attention. Various types of genetic variation are available and used as markers for exploring the *P. falciparum* population structure with advantages and drawbacks. The examples are shown in Table 2.1. Majority of studies utilize antigenic and microsatellite loci for population structure.

Table 2.1 Types of genetic variation used for population genetic study (Hartl, 2004)

Type of genetic variation	Main advantages	Main disadvantages
Antigenic variation	Abundant	Strong selection for diversity to evade the host immune system
Microsatellites	Abundant	High mutation rate; unknown pattern of mutation
Synonymous nucleotide sites	Low mutation rate; weak selective constraints	Possible selection against variation due to biased codon usage
Intron sites	Low mutation rate; weak sequence constraint	High A/T content; many microsatellite; possible selection against variation due to unrecognized selective forces
Upstream and downstream noncoding regions	Low mutation rate	High A/T content; many microsatellite; possible selection for diversity due to effects on gene expression

Extensive surveys of the genetic diversity of *P. falciparum* have now been carried out in countries with a wide range of transmission intensities in Africa, South America and Southeast Asia. An attention has been focused on understanding what the underlying pattern of mating could be to maintain a high level of variation. The first studies attempted to test for linkage equilibrium, i.e. the degree of association between unlinked genes. If linkage disequilibrium exists, it could result from frequent self-fertilization or from population substructuring.

2.5.1 Antigenic loci

After early work on variant genes in *Plasmodium* made use of electrophoresis forms of enzymes. Other proteins revealed by two-dimensional polyacrylamide gel electrophoresis, and serotypes of many antigens, detected by monoclonal antibodies, proved to be stable genetic characters of *P. falciparum*. Furthermore, molecular techniques had revealed polymorphisms in the genes

encoding these antigens. Many studies on antigenic gene (i.e. merozoite surface protein (*msp*), glutamate-rich protein (*glurp*), circumsporozoite protein (*csp*)) gave contradictory results of population structure of this parasite ranging from panmixia (Babiker et al., 1994; Conway et al., 1999) to important levels of linkage disequilibrium (LD) and indications for clonality (Rich, Hudson and Ayala, 1997). These surface antigens are under strong natural selection.

Babiker and colleagues (1994) examined the genetic structure of a population of the malaria parasite *P. falciparum* in a highly endemic (holoendemic) village of Tanzania. With utility the polymerase chain reaction for detection alleles of the merozoite surface protein 1 and 2 (*msp-1* and *msp-2*) genes both in blood stage samples and oocysts from wild-caught mosquitoes, the alleles found were the same as those in both forms. Sixty-four percent of oocysts examined were heterozygous for one or both genes, showing that cross mating between clones was taking place frequently after mixtures of gametocytes in blood meal were taken up by mosquitoes. Furthermore, the frequency of heterozygous forms showed that random mating events probably occurred within mosquito blood meals between gametes belonging to different parasite clones.

Babiker and colleagues (1997) also extended their work to mesoendemic area in Sudan. They compared allelic polymorphism of *msp-1* and *msp-2* and the parasite load in infected individuals in two different transmission intensity areas, holoendemic area (in Tanzania) and mesoendemic area (Sudan). They found that the numbers of alleles of both genes and the mean number of parasite clones per patient were much greater in holoendemic than in mesoendemic area. Besides, in higher malaria intense area, more parasite clones exhibiting higher allelic polymorphisms of the gene studied were carried by infected inhabitants. These evidences were associated with a very high frequency of out-crossing in the higher intensity.

Conway and others (1999) examined a rate of decline in linkage disequilibrium with increasing nucleotide distance in the *msp1* gene in six populations in Africa (Gambia, Nigeria, Gabon, Sudan, Tanzania and South Africa). The rate of this decline should be proportional to the effective meiotic recombination rate in the population. They found the much lower rate of this decline in Sudan compared with

other areas. This was consistent with the much lower endemicity of malaria in the area of Sudan. These results showed the effective recombination rate expected in natural populations of *P. falciparum*.

Paul and colleagues (1995) measured oocyst heterozygosity and patterns of genetic LD in *P. falciparum* in Papua New Guinea where intense transmission of malaria occurs. Allelic diversity was observed in parasite populations from both human blood and mosquitoes in polymorphic loci (*msp-1*, *msp-2* and *glurp*). A high degree of inbreeding occurred in the absence of detectable linkage disequilibrium. This observation means that there was sufficient outbreeding to disrupt any linkage disequilibrium. Thus, the genetic structure of malaria parasite populations is neither clonal nor panmictic.

Paul and others (1998) observed a high number of genotypes per infected person with antigenic loci, *msp-1*, *msp-2* and *glurp*, and the absence of LD between *msp-1* and *glurp* in Thai *P. falciparum* population on Northwestern border, an area of very low transmission intensity.

Tanabe and colleagues (2000) analyzed the polymorphism in loci at 5'- and 3' - regions of the *msp-1* in parasite populations from Brazilian Amazon (hypoendemic) and Vietnam (mesoendemic), then compared the results with that of Thai population. LD was found in all populations with different linked clusters between the 5'- and 3'- regions. However, the overall strength of LD was stronger in Brazil than in Vietnam and Thailand. This suggested that linkage disequilibrium in *msp-1* inversely correlated with the intensity of transmission.

Rich, Hudson and Ayala (1997) examined 25 DNA sequences of the gene coding for the highly polymorphic antigenic CSP from different global origins. Analysis of the amino acid polymorphisms observed in nonrepeat regions indicated that: (i) the incidence of recombination events did not increase with nucleotide distance; (ii) the strength of LD between nucleotide was also independent of distance. This was contrary to what is expected with meiosis crossing-over. According to these results, they proposed two hypotheses: (i) variation in the highly polymorphic central repeat region arose by mitotic intragenic recombination, and (ii) the population structure of *P. falciparum* is clonal.

Utilization of antigenic loci which are under strong selection or exhibit little diversity made it difficult to assess population structure. Inferences from population genetic parameters estimated using data such as Wright's F_{ST}, LD, which are assumed to be driven by neutral processes, could be misleading (Awadalla et al. 2001).

2.5.2 Microsatellites

Eukaryotic chromosome contain regions in which short sequence of DNA are tandem repeated. Each cluster of repeats is considered a locus and the number of repeats at a particular locus is variable, thus are called variable number of tandem repeats (VNTR). There are two kinds of VNTR loci, minisatellite and microsatellite, depending on the length of the repeat sequences. These repeats localized near the centromeres and telomeres of chromosome (Halliburton, 2004). Microsatellite markers have been found in every eukaryote studied, although their density varies among species. In *P. falciparum* genome, after the project of the whole genome sequencing had been completed, the microsatellite loci those are present throughout the parasite genome, mostly $(TA)_n$ or $(T \text{ or } A)_n$ (the number of repeats in a cluster is typically between 10 and 30), occurring about one per kilobase genome-wide (Su and Wellems, 1996; Gardner et al., 1998; Bowman et al., 1999). Most microsatellite loci do not code for proteins, and thus variation is often assumed to be neutral with respect to natural selection. However, microsatellite loci flanking protein coding genes are used in positional cloning and gene identification. Not only general ubiquitousness of microsatellites but the ease with which they can be scored using PCR base typing. According to the advantages of microsatellites, utility of these markers are very informative in some population genetic studies, such as gene mapping or estimating levels of gene flow.

The first analysis of *P. falciparum* population structure at global scale with a set of microsatellite loci was established by Anderson and colleagues (2000). The analysis covered nine worldwide populations with different transmission intensities. Only three populations of nine with higher transmission intensities met the criteria of panmixia. The six other populations with low levels of transmission

exhibited a highly significant level of LD. The LD results observed suggesting high levels of self-fertilization exhibited in populations in low transmission areas.

Durand and colleagues (2003) used 28 microsatellite loci to examine *P.falciparum* population in Congo where high endemicity for malaria occurred. This parasite population exhibited very high genetic diversity but significant LD was observed. This result could be explained by uniparental propagation and a Wahlund effect. The observed significant LD was inconsistent with other population genetic analyses conducted in other hyperendemic areas.

Razakandrainibe and others (2005) investigated population genetic structure of *P. falciparum* in the midgut oocysts of the mosquito vector, *Anopheles gambiae*- the most important vector in Africa, in eleven endemic areas of Kenya with seven microsatellite markers. They also observed significant LD, inbreeding caused by selfing and nonrandom genotype distribution of oocysts among mosquito guts even a high allelic variation per locus was considered. These observations showed strong deviation from panmixia and the consequent reproduction of genetic clonality occurred in areas studied.

Bogreau and colleagues (2006) examined population genetic structure of *P. falciparum* in three hypoendemic and one holoendemic areas of Africa with microsatellite markers. They found many more differences in genetic diversity among parasite population in the areas of hypoendemicity. Significant LD was also found in one of hypoendemic area. Furthermore, *P. falciparum* populations were structured in Africa. These findings suggested that malaria epidemiology in any endemic area depends on local transmission.

Machado and others (2004) performed *P. falciparum* genetics study in five populations of the Brazilian Amazon region. There was significant multilocus LD, particularly within population with low proportions of mixed genotype infections. However, most multilocus genotypes in different isolates were distinct; this is thus minimal gene flow. There was no evidence of recent epidemic expansion of particular clones in this region. Genetic divergence between populations was considerable but did not fit a model of isolation by distance. Thus, different foci of *P. falciparum* in

Brazil are quite independent with distinct population structures and minimal gene flow.

Anthony and others (2005) conducted population study of *P. falciparum* in Malaysian Borneo where malaria endemicity has been declining. They revealed that the genetic of parasites were structured with high level of differentiation among eight separated population on this island. The population with the highest proportion of multiclonal infection had the highest allelic diversity and no significant LD. On the contrary, several populations showed evidence of clonal expansion with one population with a high level of LD. Genetic differentiation was strongly associated with the geographical distance between foci.

According to studies mentioned above, there is a broad spectrum of *P. falciparum* population structures in different areas, dependent on the levels of transmission and endemicity of infection. Different strategies for prevention and control may be optimal for its diverse endemic locations.

2.6 Genetic analyses at the level of populations

2.6.1 Genetic variation

Heterozygosity

An indicator to quantify the amount of genetic variation within a population is the average heterozygosity. Individuals in diploid species can be heterozygous or homozygous at a locus, the proportion of heterozygotes at a locus is considered a more sensitive indicator of variability. In general, heterozygosity value ranges from 0 to 1. High value of heterozygosity means high genetic variability, and vice versa, low heterozygosity means low genetic variability.

The expected heterozygosity under Hardy-Weinberg conditions can be calculated as

$$H_{exp} = 1 - \Sigma p_i^2$$

which is one minus the expected frequencies of all of the homozygotes.

Expected heterozygosity is sometimes called gene diversity (Nei, 1987). It can be interpreted as the probability that two randomly chosen copies of gene will be different alleles, and can be applied to haploid organisms such as bacteria. For small sample sizes, Nei (1987) suggests corrections to adjust for sample bias. An unbiased estimate of heterozygosity is

$$\hat{H}_{exp} = \frac{2N}{2N-1}(1 - \Sigma \hat{p}_i^2)$$

where N is the number of individuals sampled. For haploid organisms, mitochondrial DNA or Y-chromosome, the estimate is

$$\hat{H}_{exp} = \frac{n}{n-1}(1 - \Sigma \hat{p}_i^2)$$

where n is the number of individuals sampled.

2.6.2 Linkage disequilibrium

Under Hardy-Weinberg assumptions, the alleles present at a locus are randomly associated with each other in genotypes (Harlt, 2000). In a same population, two genes may each be under Hardy-Weinberg equilibrium individually, the alleles of these two genes can remain in nonrandom association in the gametes that form each generation. A parameter (designated D) measures the nonrandom association between loci, called linkage disequilibrium or LD. The D is the deviation of the observed frequency from the expected and can be calculated with the simplest equation as

$$D = x_{11} - p_1 q_1$$

If the two loci (A and B) and the alleles (A_1, A_2, B_1 and B_2) are independent from each other, x_{11} is the frequency of A_1B_1, p_1 and q_1 are the frequencies of A_1 and B_1, respectively.

In order to compare LD quantities among different pairs of loci with differing allele frequencies, standardization of D is provided by dividing it by its maximum value given the allele frequencies as the following equation

$$D' = \frac{D}{D_{\max}}$$

D' has a range of -1 to +1 and dependent of allele frequencies. The significance testing of association between genotypes at pairs of loci in each sample can be employed: a chi-square test, a likelihood ratio test or Fisher's exact test (Mueller, 2004).

In population genetics study, LD has been used to describe demographic and evolutionary processes such as admixture or migration among populations. Furthermore, LD is used in mapping genes relevant for specific traits of interest mainly in humans and useful in animals, plants and pathogens.

2.6.3 Population genetic structure

The populations of most living organisms show some levels of genetic structuring. Environmental barrier, historical processes and life histories (e.g. mating system) may shape the gene structure of populations. In addition, populations are often genetically differentiated through isolation by distance (Balloux and Lugon-Moulin, 2002). If two populations are geographically isolated from each other, they will tend to diverge. Mutation, natural selection and genetic drift will act independently in each population, and after period of time, they will be genetically different from each other. Gene flow impedes this divergence by moving alleles from one population to the other (Halliburton, 2004).

Several approaches have been used to investigate the amount of genetic differentiation the subdivisions of a population (Balloux and Lugon-Moulin, 2002). The "*F*-statistics" which originally introduced by Wright (1951) are widely used to assess levels of structuring in samples of natural population. These statistics partition the heterozygote deficit relative to its expectation under Hardy-Weinberg equilibrium into a within and an among population component. They measure the heterozygote deficit at three levels of hierarchical population structure: within subpopulations, between subpopulations and total population. There are three *F* coefficients that describe these deviations; $F_{IS,}$ F_{ST} and F_{IT} (where *I* stands for individuals, *S* for subpopulations and *T* for the total population). F_{IS} measures the deficit within populations, F_{ST} among populations (a measure of the Wahlund effect), and F_{IT} the global deficit of heterozygotes. The three statistics are related as followed

$$(1 - F_{IT}) = (1 - F_{IS})\,(1 - F_{ST})$$

F_{ST} is very commonly used to describe population differentiation at various levels of genetic structuring, either directly as differentiation estimators or through their link with the effective number of migrants. The value of F_{ST} is range from zero to one. A value of zero means that studied samples are within a panmictic unit. A value of one means that there is no diversity within subpopulations and that at least two of the sampled subpopulations are fixed for different alleles. For a value of F_{ST} between zero and one, the interpretation is as follows: little genetic differentiation if the value is between 0-0.05; a value between 0.05 and 0.15 is moderate differentiation; a value between 0.15 and 0.25 is great differentiation; values above 0.25 are very great genetic differentiation (Balloux and Lugon-Moulin, 2002).

CHAPTER III

MATERIALS AND METHODS

3.1 Sampling

3.1.1 Study sites

The samples obtained for the study were collected mainly from patients in Sai Yok district, Kanchanaburi province. Other samples from different parts of Thailand were also analyzed and compared to the parasite population in Kanchanaburi. At the time of data collection in study area in Kanchanaburi, there were two malaria clinics, located in Loomsoom and Bongtee sub-districts, responsible for the diagnosis and treatment for malaria patient. The two clinics covered four sub-districts: Loomsoom, Simongkon, Bongtee and Singha in Sai Yok district (Figure 3.1 and 3.2). Parasite samples were collected over a period of four years since 2004. Moreover, parasite blood samples collected from provinces in the north, the west, the east and the south of Thailand, preserved and cultivated in the laboratory of Malaria Research Program, College of Public Health Sciences, Chulalongkorn University were also studied. These samples obtained from malaria patients living along Thai-Myanmar border in Maehongson, Tak and Ranong; along Thai-Loas and Cambodia border in Ubonratchathani and Trat respectively; and along Thai-Malaysia border in Yala (Figure 3.3).

Figure 3.1 Frontier territory of Kanchanaburi was shown in white color. This province is divided into 13 districts. The studied site is located in Sai Yok sub-district which shown in red color. The dark brown area is Myanmar.

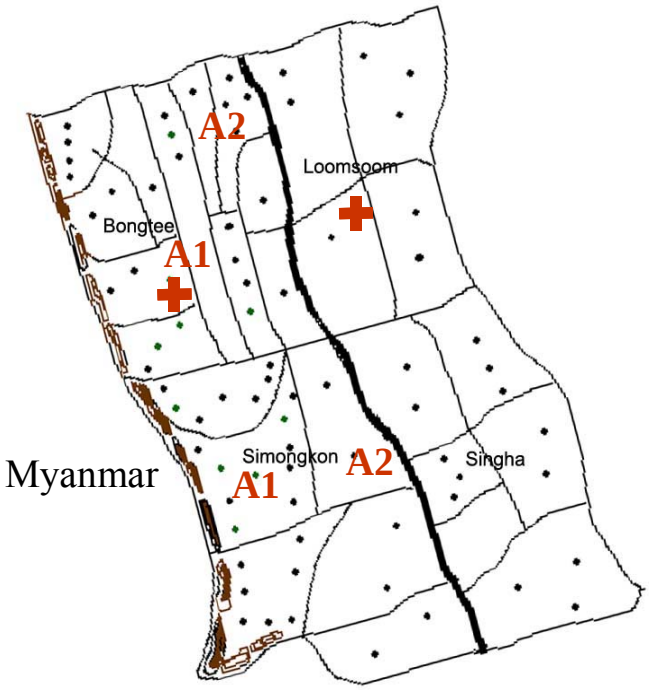


Figure 3.2 Localization of malaria clinics in studied area. These two malaria clinics were responsible for inhabitants in four sub-districts. All villages in Bongtee and some villages in Simongkon and Loomsoom were classified into the parennial transmission areas or A1 where transmission occurs throughout the year. Some villages in Simongkon and Loomsoom were considered periodic transmission areas or A2 where transmission occurs less than 6 months per year. The remaining were non-transmission areas. Each red cross in the picture stands for a malaria clinic and dots represent villages in this area.

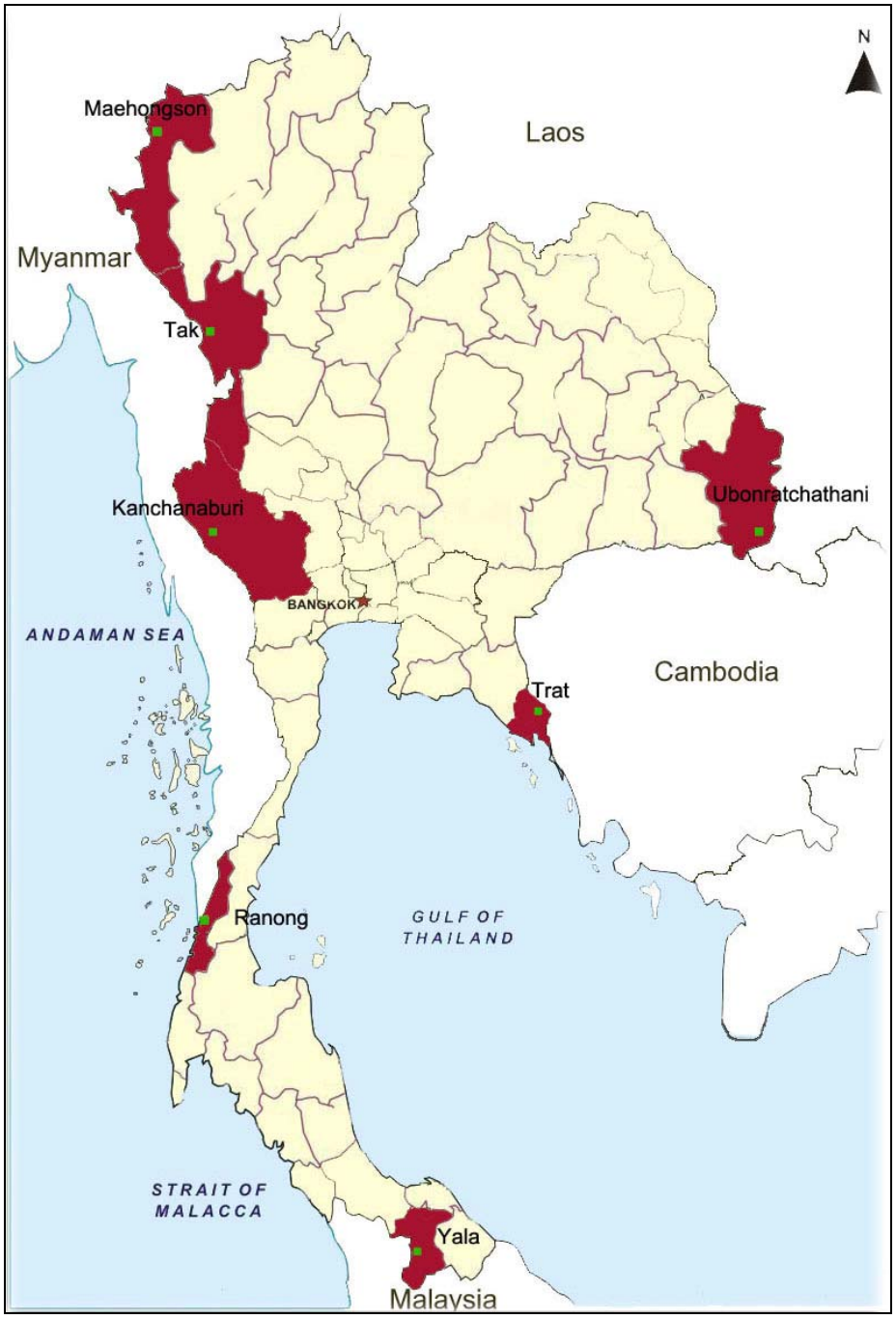


Figure 3.3 Localization of the 7 sites studied in Thailand. Maehongson, Tak, Kanchanaburi and Ranong sites are located on the border with Myanmar. Ubonratchathani and Trat are located in the east along the border with Laos and Cambodia, respectively. Yala is a province near Malaysia. A green square showed the sampling place of *P. falciparum* in the different sites.

3.1.2 Sample size

A sample size was calculated as following equation:

$$\text{Sample size} = Z_{\alpha}^{2}\, PQ/(\text{P-lower limit})^{2}$$

When, Z_{α} = confidence level at 95% (standard value of 1.96)

P = estimated heterozygosity or prevalence of mefloquine resistance in the study area

Q = 1-P

The expected heterozygosity in the study area is estimated at 0.5 and the lower limit is estimated at 0.4. Sample size is equal 96 cases collected in each year.

If prevalence of mefloquine resistance is 80% and lower limit is 70%, then the calculated sample size is 61 cases.

3.1.3 Parasite collection

Blood samples were collected from patients who attended malarial diagnosis at malaria clinics. Patients with microscopy-confirmed *Plasmodium falciparum* infection and fulfill the study inclusion criteria; age 5 or above and no history of treatment for malaria infection within 28 days were informed about the details and the scope of a study. Blood samples were obtained from patients who agreed to participate and gave informed consent. This was done by pricking fingers with a sterile lancet. A drop of blood sample was stored into 2 different protocols based on the purposes of study: i.e., for molecular analysis and susceptibility test. For molecular analysis, the blood sample was absorbed on a piece of 3 MM Whatman filter paper. This sample was then air-dried and stored in a container filled with silica gel at room temperature. For drug susceptibility test, blood sample was preserved into a transporting medium and carried in a cold condition to the laboratory for cultivation and test for drug susceptibility. All blood samples were obtained from patients before they took any anti-malarial pills.

3.2 Slide confirmation and determination of parasitemia

Thick blood smears of individual patient were examined under microscopy by a senior scientist, an expert in malaria species characterization, in the Malaria Research Program, College of Public Health Sciences. The second examination by the senior scientist was to confirm the diagnosis result done by malaria clinic staffs in Kanchanaburi. This is particularly useful in the case of mixed infections when the clinic staffs reported questionable or doubtful result. The numbers of parasites observed in fields per 200 white blood cells were recorded. Parasitemia was expressed as numbers/µl of blood following multiplication by 40 if it is assumed that a normal level of white blood cell is 8,000 cells/µl.

3.3 Drug susceptibility test

The parasites in transporting medium were cultured according to the protocol of Trager and Jensen (Trager and Jensen, 1976). The well-growing parasites were tested for drug susceptibility (Thaithong et al., 1983) with mefloquine. Morphology of parasites and minimum inhibitory concentration value were observed. Laboratory parasite clone, T9/94RC17 was use as a control. The drug susceptibility tests were done by the specialist staff of the Malaria Research Program, College of Public Health Sciences. Differences in the geographic means of the *in vitro* mefloquine sensitivity among different year of collection were tested by using *t*-test after normalized data by logarithmic transformation. Statistical significant was assumed if the *P*-value was <0.05.

3.4 DNA sample extraction

DNA samples in the dried blood spots were extracted following 2 protocols.

3.4.1 Chelex extraction protocol (Kain and Lanar, 1991)

Samples collected in the Whatman filter papers were incubated in PBS solution overnight at 4^{o}C. After that the PBS solutions were replaced with 0.5% saponin in PBS and incubated at 4^{o}C for 10 minutes. Treated filter papers were

transferred to hot 5% chelex-100 resin solutions (Bio-Rad Laboratories, CA) and incubated at 95^{o}C for 10 minutes, vortex briefly between incubations. Supernatants were kept to be used for parasite genotyping after centrifugation.

3.4.2 Protocol adapted from DNeasy Tissue kit (Qaigen)

Dried blood samples on Whatman filter papers were excised and washed in 500 µl sterile distilled water in 1.5 ml microcentrifuge tubes by briefly vortexing for several times. The washed paper was transferred to a 600 µl microcentrifuge tube containing 120 µl of sterile water. The washed filter paper was heated at 95^{o}C for 30 minutes in a thermocycler, and then vortexed for several times before the filter paper was discarded. The supernatant was kept at -20^{o}C for genotyping.

3.5 Identification of human malaria parasite species

Small subunit ribosomal RNA genes were amplified with species specific primers specify to four species of malaria parasite according to protocol of Snounou (1993). These genes consist of regions whose sequence is conserved amongst the different species, interspersed with regions containing sequences specific to each of the species. Nested PCR strategy was used for amplification. The outer primer pair annealed at genus-specific site and amplified a large PCR product fragment. Other four inner primer pairs were used to amplify species-specific for four species; *P. falciparum, P. vivax, P. malariae* and *P. ovale* located inside the first PCR product. The primer name, its sequence and the expected size of the amplification products were shown in Table 3.1. The first amplification was performed in a 20 µl reaction volume containing 1 µl of DNA template, 125 µM of each deoxynucleoside triphosphate, 5 pmole of each primer, 2 mM $MgCl_2$ (Finnzymes, Finland) and 0.4 units of *Taq* DNA polymerase (DyNAzyme™ II, Finnzyme, Finland) in the buffer supplied by the manufacturer. Thermocycling was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, CA) with an initial denaturation at 95^{o}C for 5 minutes, 25 cycles at 94^{o}C for 1 minute, 58^{o}C for 2 minutes and 72^{o}C for 2 minutes, and a final elongation step at 72^{o}C for 5 minutes. The secondary reaction containing 1 µl of the product from the first amplification was performed in the same condition.

The DNA products were sized on 2% w/v total of a 3:1 mixture of agarose (USB™, Ohio): NuSieve agarose (FMC BioProducts, ME) in TBE buffer. The PCR products were visualized by using UV transluminator after staining with Ethidium Bromide. The scheme of the amplification of *Plasmodium* small subunit ribosomal RNA genes was shown in Figure 3.4.

Table 3.1 The primer for amplification of *Plasmodium* small subunit ribosomal RNA genes.

Primer name	Sequence of primer (5'→3')	Amplicon length (bp)
rPLU 5	CCTGTTGTTGCCTTAAACTTC	1,200
rPLU 6	TTAAAATTGTTGCAGTTAAAACG	
rFAL 1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
rFAL 2	ACACAATGAACTCAATCATGACTACCCGTC	
rVIV 1	CGCTTCTAGCTTAATCCACATAACTGATAC	120
rVIV 2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	
rMAL 1	ATAACATAGTTGTACGTTAAGAATAACCGC	144
rMAL 2	AAAATTCCCATGCATAAAAAATTATACAAA	
rOVA 1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	800
rOVA 2	GGAAAAGGACACATTAATTGTATCCTAGTG	

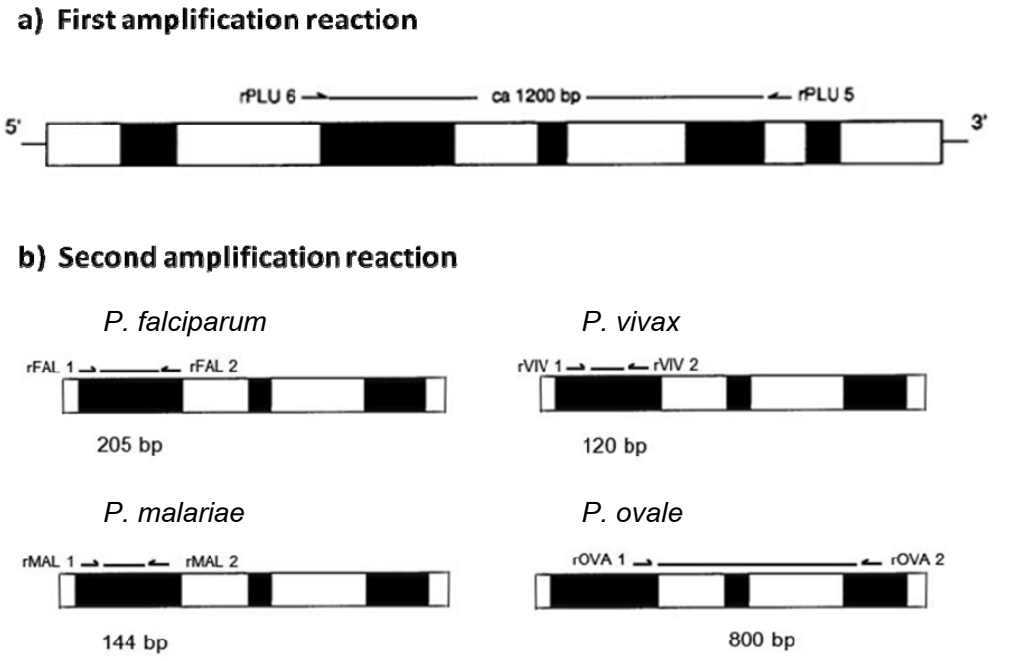


Figure 3.4 Schematic representation of *Plasmodium* ssrRNA genes; a) the first reaction for genus specific amplification, b) the second reaction for species specific amplification. The black boxes represent variable sequences unique to each species (Modified from Snounou et al., 1993).

3.6 Microsatellite amplification in neutral loci

3.6.1 Characteristic of microsatellite marker

All samples were screened for mono-infection using the first set of 7 microsatellite markers named P20, P21, P22, P23, P24, P25 and P26. After that, only mono-infected samples were genotyped further with 5 microsatellite markers named P04, P17, P18, P27 and P33. Both a one-step PCR and two-step hemi-nested PCR strategy were used for DNA microsatellite repeats amplification as described in Razakandrainibe and colleagues (2005), following development by Anderson (1999 and 2000). The characteristics of microsatellite loci of *P. falciparum* and their sequences were shown in Tables 3.2 and 3.3. All primers were listed 5' to 3', for the one-step PCR, forward primers were end-labeled with fluorescent dye. For two-step

hemi-nested PCR for each locus, the first and the second primers were used in the primary reaction and the second and third primers were used in the secondary reaction. Primers listed third were internal to the other 2 and were end-labeled with fluorescent dye.

Table 3.2 Characteristic of 12 microsatellite loci

Code	Chromosome	GENBANK access no.	Microsatellite marker	Type of repeats
P04	10	G38857	TA80	$(TAA)_8$
P17	13	G37793	ARP2	$(TAA)_{10}$
P18	6	AF010507	TA1	$(TAA)_6$ $(TGA)_4$ $(TAA)_9$
P20	4	L18785	Poly α	$(TAA)_6$ CAA $(TAA)_5$ CAA $(TAA)_6$ CAA $(TAA)_{11}$
P21	13	AF010556	TA60	$(TAA)_9$
P22	11	X17484	ARA2	$(TAA)_9$
P23	12	L04161	Pfg377	$(TAA)_8$
P24	12	X63648	PfPK2	$(ATT)_4$ GTT $(ATT)_2$ $(GTT)_2$ $(ATT)_2$ GTT ATT $(GTT)_3$ $(ATT)_5$ GTT ATT ACT $(ATT)_{10}$
P25	6	AF010571	TA87	$(CAA)_{11}$ $(TAA)_{10}$
P26	6	AF010508	TA109	$(ACT)_7$
P27	5	AF010510	TAA81	$(TAA)_{10}$
P33	1	G38013	C1M8	$(ATT)_{18}$ GTA $(ATT)_6$ GTA $(ATT)_8$ GTA $(ATT)_3$

Table 3.3 Sequence of primers and fluorescence tag-primers

Code	Sequence of primers	Fluorescent dye
P04	F: CTACTTATGTATCATCCAAT R: TAATTGATGTTTGTAATTGT	Ned
P17	F: TATGAACATTAACGAAGA R: ATTTTTATCCTGAGAGCC	Vic
P18	F1:CTACATGCCTAATGAGCA R:TTTTATCTTCATCCCCAC F2:CCGTCATAAGTGCAGAGC	Vic
P20	R1 : ATCAGATAATTGTTGGTA F : AAAATATAGACGAACAGA R2 : GAAATTATAACTCTACCA	6-Fam
P21	F1 : CTCAAAGAAAAATAATTCA R : AAAAAGGAGGATAAATACAT F2 : TAGTAACGATGTTGACAA	Ned
P22	F1 : GTACATATGAATCACCAA R : GCTTTGAGTATTATTAATA F2 : GAATAAACAAAGTATTGCT	6-Fam
P23	R1 : TTATGTTGGTACCGTGTA F : GATCTCAACGGAAATTAT R2 : TTATCCCTACGATTAACA	Pet
P24	R1 : CCTCAGACTGAAATGCAT F : CTTTCATCGATACTACGA R2 : AAAGAAGGAACAAGCAGA	Vic
P25	F1 : ATGGGTTAAATGAGGTACA R : ACATGTTCATATTACTCAC F2 : AATGGCAACACCATTCAAC	Vic
P26	F1 : TGGGAACATCATAAGGAT R : CCTATACCAAACATGCTAAA F2 : GGTTAAATCAGGACAACAT	Ned
P27	F1:GAAGAAATAAGGGAAGGT R:TTTCACACAACACAGGATT F2:TGGACAAATGGGAAAGGATA	Ned
P33	F:AGTATTATTATTACCACTACT R:GAAGGCTAATACAATTGGTA	6-Fam

3.6.2 Amplification reactions and conditions

Amplification reactions and conditions were divided into three groups of microsatellite loci. The first group of markers, P20, P21, P22, P23, P24, P25 and P26, were amplified by hemi-nested PCR strategy. The first amplification was performed in a 15 µl reaction volume containing 1.5 µl of DNA template, 80 µM of each deoxynucleoside triphosphate, 0.75 pmole of each primer, 5 mM $MgCl_2$ (Promega, Madison, WI) and 0.2 units of *Taq* DNA polymerase (Promega) in the buffer supplied by the manufacturer. Thermocycling was performed in a Mastercycler gradient thermocycler (Eppendorf) with an initial denaturation at 94^{o}C for 2 minutes, 25 cycles at 94^{o}C for 30 seconds, 42^{o}C for 30 seconds, 40^{o}C for 30 seconds and 65^{o}C for 40 seconds, and a final elongation step at 65^{o}C for 2 minutes. The secondary reaction contained 1 µl of product from the first amplification, 80 µM of each deoxynucleoside triphospate, 2 pmole of each primer (one was labeled with fluorescence dye), 4 mM $MgCl_2$ and 0.2 units of *Taq* DNA polymerase. Cycling condition was as follow, denaturation at 94^{o}C for 2 minutes, 30 cycles at 94^{o}C for 20 seconds, 45^{o}C for 20 seconds and 65^{o}C for 30 seconds and final extension step at 65^{o}C for 2 minutes.

For the second group of microsatellite loci P04, P17 and P33 were amplified using one-step PCR performed in a 15 µl reaction volume containing 1.5 µl of DNA template, 80 µM of each deoxynucleoside triphosphate, 2 pmole of each primer, 4 mM $MgCl_2$ and 0.2 units of *Taq* DNA polymerase. Cycling condition was as follow, denaturation at 94^{o}C for 2 minutes, 40 cycles at 94^{o}C for 20 seconds, 45^{o}C for 20 seconds and 65^{o}C for 30 seconds and final extension step at 65^{o}C for 2 minutes.

For the last group of loci, P18 and P27, amplification was performed as the same condition as those of the second group except using hemi-nested PCR strategy. In the reaction, 1.5 µl and 1 µl of DNA template was added in primary and secondary amplification reaction, respectively.

3.6.3 Genotyping

After amplification, 1 µl of the amplified product was added into formamide as manufacturer instruction (Applied Biosystems, Foster City, CA) to denature double-stranded DNA before injecting into the automated sequencer. For size estimation, Genescan, a size marker labeled with fluorescent dye, was added into this mixture. This mixture was heated at 95^{o}C for 10 minutes, then immediately cooled on ice. The microsatellite PCR products were sized relative to a Genescan500LIZ internal size standard and resolved by using GENESCAN software version 3.1 on an ABI Prism310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

3.7 Amplification of microsatellite loci flanking *pfmdr 1* gene

3.7.1 Characteristic of microsatellite markers

Mono-infected samples screening by using neutral microsatellite loci were genotype with microsatellite markers flanking *pfmdr 1* gene, responsible for mefloquine resistant phenotype of *P. falciparum*. We examined 7 loci of microsatellite repeats on chromosome 5, of which the position and primer sequences were described by Nair (2007). Three of the seven loci (L01, L02 and L03) locate on the upstream of this gene, while the other 3 loci (L05, L06 and L07) downstream. One marker (L04) locates within the linker region of *pfmdr 1* gene. The markers all contain di-nucleotide repeats except one marker in the linker region of gene has tri-nucleotide repeats. The sequences of primer pairs were shown in Table 3.4.

Table 3.4 Sequence of primers and fluorescence tag-primers utilization for amplification of microsatellite loci surrounding *pfmdr 1* gene

Code	Position on chromosome 5	Sequence of primers	Fluorescent dye*
L01	953768	F: TACAATAAAATGATAACATCG R: AGAAAAATGATAATTAAGCATAC	6-Fam
L02	956686	F: AAAATGCGCTGACTTTAT R: AGGTGCAAAATGTAATATAGA	Hex
L03	957861	F: TTTTTGTCATTGTGTAAATA R: CAACACAAAATCAAATAAAA	6-Fam
L04	959894	F: TTGAAAGGAAATGAAAATAG R: CATGTGTACCTTGTTCAATA	Tet
L05	962445	F: ACTCTTGTCCGTTATATTGA R: AAAAAGGAAGAAGGAAAAA	Tet
L06	966096	F: AATGTAAATATTTTTAGGGTAG R: TTTTTCCTTTTGTAATATGG	Hex
L07	971251	F: AATTTTGATGTATTCCAAAC R: ACGCATACAGAAGAATGTAA	6-Fam

* Forward primers were tagged with fluorescent dye.

3.7.2 Optimization of polymerase chain reaction for microsatellite amplification

Amplifications of microsatellite loci were performed using chromosomal DNA of laboratory adapted clone, T9/94RC17, as a template for PCR optimization. A human white blood cell DNA was used as a negative control.

A PCR was performed in a 15 µl reaction volume containing 1 µl of DNA template, 80 µM of each deoxynucleoside triphosphate, 2 pmole of each primer, 4 mM $MgCl_2$ (Promega, Madison, WI) and 0.2 units of *Taq* DNA polymerase (Promega). Cycling condition was as follow, denaturation at 94^{o}C for 2 minutes, 40 cycles at 94^{o}C for 20 seconds, 45^{o}C for 20 seconds and 65^{o}C for 30 seconds and final extension step at 65^{o}C for 2 minutes. These PCR parameters were used as the initial condition. Some parameters could be varied; 1) a concentration of DNA template, 2) a concentration of $MgCl_2$ in PCR reaction, 3) an annealing temperature for each primer pair. The DNA templates may be undiluted (~ 300 ng) or diluted to 100-fold dilution

(~3 ng) prior to add into the reaction. The concentration of $MgCl_2$ was varied from 1.5 to 4 mM. The annealing temperature in thermal cycling was varied from 42°C to 50°C. The DNA products were sized on 2% w/v total of a 3:1 mixture of agarose: NuSieve agarose in TBE buffer. The PCR products were visualized by using UV transluminator after staining with Ethidium Bromide.

3.7.3 The amplification of microsatellite loci with an optimal reaction and condition in field samples

The mono-infected parasites of Kanchanaburi samples tested for mefloquine susceptibilily were genotyped with 4 microsatellite markers which were successful optimization for amplification from previous experiment. All Microsatellite loci were amplified with different conditions as shown in Table 3.5. The amplification was performed in a 15 µl reaction volume containing 2 µl of DNA template, 80 µM of each deoxynucleoside triphosphate, 2 pmole of each primer, 0.2 units of *Taq* DNA polymerase and optimal concentration of $MgCl_2$ for each primer pair. The cycling condition was as follow, denaturation at 94°C for 2 minutes, 40 cycles at 94°C for 20 seconds, annealing at optimal temperature for each primer for 20 seconds and 65°C for 30 seconds and final extension step at 65°C for 2 minutes.

Table 3.5 Amplification parameters for 4 microsatellite loci

Locus	$MgCl_2$ concentration (mM)	Annealing temperature (°C)
L03	1.5	50
L04	3	45
L05	3	50
L07	3	45

3.7.4 Fragment determination

After amplification, purification of amplified product was needed. One and a half microlitres of 7.5 M ammonium acetate and 38.5 µl of absolute ethanol were added into each reaction to adjust the final concentration of ethanol to 70%. The tubes were centrifuged at 12,000 rpm for 15 minutes and the supernatant was removed. After that, the DNA pellets were washed with 70% ethanol. The supernatant was removed and DNA pellet was air-dried for 2-5 minutes. A genotyping loading solution containing MegaBACE™ ET550-R size standard was added into dried-pellet according to a recommendation by manufacturer (Amersham Biosciences). Before injecting into the automated sequencer, this solution was heated at 95^{o}C for 1 minute and immediately cooled on ice. The microsatellite PCR products were sized relatively to an internal size standard and resolved by using MegaBACE fragment profiler software version 1.2 on a MegaBACE™ 500 DNA Analysis System Instrument (Amersham Biosciences).

3.8 Sequence analysis of multidrug resistant gene of *Plasmodium falciparum*

3.8.1 Primer design

We used Primer 3 Plus software to design sequencing primers. A part of complete sequence of *Plasmodium falciparum* 3D7 chromosome 5 (access number: NC_004326) covering *pfmdr 1* gene was used to design these primers. Two of eighteen primers, mdr_1F and mdr_5F, were manually designed. The nucleotide sequence of primer, position on the gene and melting temperature (T_m) were shown in Table 3.6.

Table 3.6 Primers for *pfmdr 1* gene sequencing

Name	Sequence of primer	Position (bp)	Tm
mdr_1F	TGATATATGTGTACATAGCTTATTTCA	-116	54.41
mdr_2F	AAAGAACATGAATTTAGGTGATGATA	246	54.11
mdr_3F	TTTTAAAAGCTAATTTTGTAGAAGCA	803	52.53
mdr_4F	ATGATCCAACCGAAGGAGATATT	1289	56.60
mdr_5F	AGGTTCCAATGCATCCAAAT	1807	56.00
mdr_6F	AAGAAAGCACCAAACAATTTACG	2305	54.82
mdr_7F	CAATTGTTGCAGCTGTATTAACTTTT	2789	55.68
mdr_8F	TGATGTAAGAGATGATGGTGGAA	3321	56.60
mdr_9F	AATTTGGAAGAGAAGATGCAACA	3809	54.82
mdr_1R	AATTTTCTATGTTGTGCAGGTAAA	167	53.43
mdr_2R	TTCTTATTACATATGACACCACAAACA	644	55.93
mdr_3R	TTTGGTAATGTTTCTCCATCATC	1121	54.82
mdr_4R	TTTTTGGACACATCAACAACATC	1613	54.82
mdr_5R	CGCTACTTTTGTTATCCGATCC	2146	58.21
mdr_6R	CACCTGGGGTATTTTTATCTTGA	2662	56.60
mdr_7R	GATCCAAACCAATAGGCAAAACT	3149	56.60
mdr_8R	CAGATCCAGATTGGTTTGAAAAT	3658	54.82
mdr_9R	ACCACAATTTTGTCTGATCGTTT	4148	54.82

3.8.2 Optimization of polymerase chain reaction for *pfmdr 1* gene amplification

According to a length of *pfmdr 1* gene is approximately 4,216 base pairs; we had to amplify this gene divided into 3 fragments. The amplifications were performed using 3 primer pairs which are 1) mdr_1F and mdr_4R, 2) mdr_4F and mdr_7R and 3) mdr_7F and mdr_9R. The amplified fragments were around 1,800, 1,900 and 1,500 bp in length, respectively.

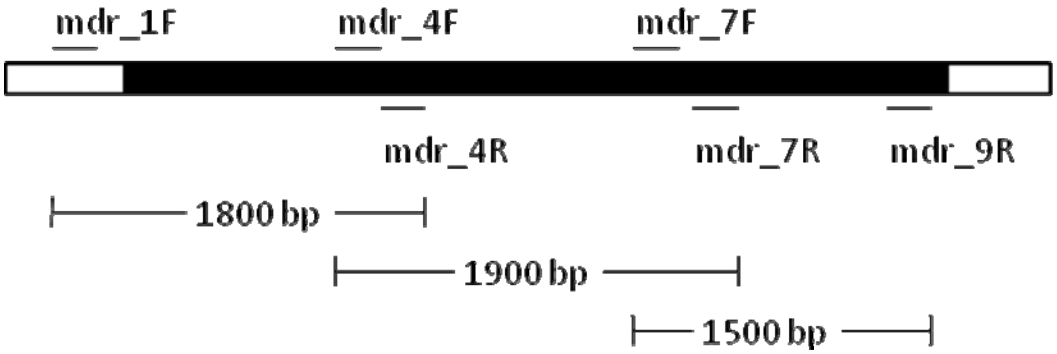


Figure 3.5 Schematic representation of *Plasmodium falciparum* multidrug resistance genes (black box). The relative position of all primers was marked with short lines above or under the gene. The three PCR products and their sizes also specified below the gene.

For optimization of PCR condition, an amplification reaction comprise of 2 µl of DNA template (T9/94RC17), 125 µM of each deoxynucleoside triphosphate, 5 pmole of each primer, and 0.4 units of *Taq* DNA polymerase in 20 µl reaction volume. The concentration of $MgCl_2$ was varied from 1.5 to 3 mM with increment of 0.5. A thermal cycling condition was as following, denaturation at 94^{o}C for 3 minutes, 30 cycles at 94^{o}C for 45 seconds, 50^{o}C for 30 seconds and 68^{o}C for 2 minutes and final extension step at 68^{o}C for 5 minutes. The DNA products were sized on 1.5% w/v of agarose in TBE buffer. The PCR products were visualized by using UV transluminator after staining with Ethidium Bromide.

3.8.3 Amplification *pfmdr 1* gene with an optimal reaction and condition in field samples

The mono-infected parasite samples which had different haplotypes examined by using microsatellite markers flanking *pfmdr 1* gene were selected for *pfmdr1* amplification analysis. Each sample was subjected to two rounds of PCR. The first round amplification was performed in a 20 µl reaction volume containing 2 µl of DNA template, 125 µM of each deoxynucleoside triphosphate, 2.5 pmole of each primer, 1.5 mM $MgCl_2$ and 0.4 units of *Taq* DNA polymerase. Cycling condition was as followed, denaturation at 94^{o}C for 3 minutes, 30 cycles at 94^{o}C for 45 seconds, 50^{o}C for 30 seconds and 68^{o}C for 2 minutes and final extension step at 68^{o}C for 5 minutes. The second round amplification was performed in a 20 µl reaction volume containing 2 µl of DNA template from the first round PCR, 125 µM of each deoxynucleoside triphosphate, 5 pmole of each primer, 1.5 mM $MgCl_2$ and 0.4 units of *Taq* DNA polymerase with the same thermal cycling condition. The DNA products were sized on 1.5% w/v of agarose in TBE buffer. The PCR products were visualized by using UV transluminator after staining with Ethidium Bromide.

3.8.4 Sequencing

The PCR products were purified by using spin column PCR products purification kit (Bio Basic Inc, Markham Ontario, Canada). The purified product was sequenced in both directions using Fluorescent dye-terminator sequencing technique and sequencing products were run on an ABI Prism 3730 DNA sequencer (Applied Biosystem).

3.9 Data Analysis

3.9.1 Microsatellite analysis

Genetic diversity was measured from allelic frequencies and assessed by the number of alleles per locus (A) from haploid data using FSTAT software version 2.9.4 (Goudet, 2003). Because of the observed number of alleles in a sample was highly dependent on sample size, allelic richness per locus and population based on

minimal sample size was estimated using FSTAT. Allelic richness is a measure of the number of alleles independent of sample size, hence allowed comparing this quantity between different sample sizes. After that, comparisons between populations for each locus were performed using non-parametric tests on samples (Wilcoxon signed-rank test) using the SYSTAT software.

Genotypic linkage disequilibrium (LD) analyses were performed with FSTAT software version 2.9.4. When two or more loci are present, it is of interest to verify whether alleles at the different loci assort independently. Linkage disequilibrium between pairs of loci was measured; the statistical significance of linkage disequilibrium was assessed with a randomization test. The genotypes at two loci are associated at random several times and the log likelihood G-test statistic was recalculated on the randomized data set. Because this procedure was repeated on all pairs of loci, we have applied a Bonferroni correction to the *P*-values. The null hypothesis is that genotypes at one locus are independent from genotypes at the other locus.

Genetic differentiation (F_{ST}) was estimated using FSTAT software. A permutation test is applied (n = 10,000) permuting alleles among samples for all loci to test whether F_{ST} significantly differ from zero. We used canonical correspondence analysis (CCA) (ter Braak, 1987) to determine the relative contribution of the isolates to the global genetic structure of populations.

3.9.2 Multi drug resistance gene analysis

Sequences of *pfmdr 1* gene were edited and aligned using Bioedit software, free available software. All 13 *pfmdr 1* sequence of Kanchanaburi samples and the laboratory clone, T9/94RC17 were compared with a complete sequence of 3D7 clone (access number: NC_004326) for examining the point mutation occurring in this gene. The detected point mutations were investigated whether they had an effect on alteration of amino acid at that position.

CHAPTER IV

RESULTS

4.1 Demography of collected samples from 7 locations in Thailand

4.1.1 Samples collected from Kanchanaburi province

Patients attended the two malaria clinics were Thai and non-Thai nationality. In three years of sample collection (2004-2006), two hundred and one cases were enrolled in this study. All these samples were characterized for species specificity using the PCR technique to confirm a microscopic diagnostic result of malaria staffs at malaria clinics. In addition, these samples were also cultivated and tested for drug susceptibility. An additional sample collected in 2007, fifty cases, were not characterized for species using the PCR and were not tested for drug susceptibility.

Some information of the 251 patients enrolled was summarized in Table 4.1. Majority of infected cases were male and most of patient were in age group of 15 year old or above. In this area there were three main nationalities of infected residents which are Karen, Thai and Myanmar, respectively. Most of patients lived in Bongtee and Simongkon sub-districts where transmission occurs throughout the year. More than 35% of patients had an experience in malaria infection. An important information, there were 37 patients who came back from Myanmar after doing job in forest industry and had infection. Prevalence of imported cases was observed since 2006 according to the Thai government allowed people pass through the border in Bongtee sub-district in that year.

Table 4.1 Information of enrolled patients in Kanchanburi from 2004 to 2007

Category		**Number of patients (cases)**
Genders	male	192
	female	59
Age	Less than 15 year old	64
	15 year old or above	187
Nationality	Karen	132
	Thai	93
	Myanmar	24
	Mon	1
	Bangladesh	1
History of previous infection	Never	147
	Once	34
	Twice	14
	more than twice	44
	no data	12
Address (sub-district)	Bongtee	181
	Loomsoom	14
	Simongkon	55
	Singha	1
Place of infection	Thailand	214
	Myanmar	37
Parasite density	≥ 10,000	110
(parasite/μl)	< 10,000	80
	no data	61

4.1.2 Samples collected from other six provinces; Maehongson, Tak, Ranong, Ubonratchathani, Trat and Yala

Thirty-two parasites samples from Maehongson were obtained between 2003 and 2007. There were only 2 cases infected from Myanmar. The samples from Tak province were collected in 2003, 2004 and 2007. The majority of the patients came from Myanmar (72%, 51 of 71 cases). The collected samples from Ubonratchathani province were obtained between 2003 and 2006, 3 of 25 samples were infected from Laos. In Trat province adjacent to Cambodia, 7 of the 34 cases were infected in Cambodia. These samples were obtained between 2003 and 2007. The parasite samples from Ranong were collected since 2003. In the four years of data collection, 43 of 59 (73%) samples were infected from Myanmar. In one of the deep south provinces, Yala, all 36 samples were collected in 2007 from patients infected in Thailand. The provincial origins and year of collection of the infected blood samples, and the number of samples originally infected outside Thailand, are summarized in Table 4.2.

Table 4.2 *P. falciparum* infected blood samples from each province in each year and the number of samples originally infected outside Thailand.

	Number of samples collected in the year						Total number	
Province	2002	2003	2004	2005	2006	2007	Total	Outside Thailand
Maehongson	--	2	2	16	7	5	32	2 (6.3%)[a]
Tak	--	4	10	--	--	57	71	51 (72%)[a]
Kanchanaburi	15	--	63	61	77	50	266	37 (14%)[a]
Ubonratchathani	--	5	3	6	11	--	25	3 (12%)[b]
Trat	--	9	5	4	12	4	34	7 (21%)[c]
Ranong	--	10	10	9	13	17	59	43 (73%)[a]
Yala	--	--	--	--	--	36	36	0
Total	15	30	93	96	120	169	523	143 (27%)

[a], [b] and [c] represent Myanmar, P.D.R. of Laos and Cambodia, respectively.

4.2 Mefloquine response of *P. falciparum* sample collected in 2004 to 2006

All 201 parasitized blood samples were cultivated. One hundred and sixty-five samples (82%) were successfully grown, 52, 53 and 60 samples from 2004, 2005 and 2006 collections, respectively. The majority of parasites, 52%, 62% and 77%, obtained from 2004, 2005 and 2006 periods respectively had a minimum inhibitory concentration (MIC) at $2x10^{-7}$ M (Figure 4.1). Furthermore, drug susceptibility data showed a decreasing in proportion of higher resistant parasites against mefloquine (44%, 26% and 17%) observed during 2004 to 2006.

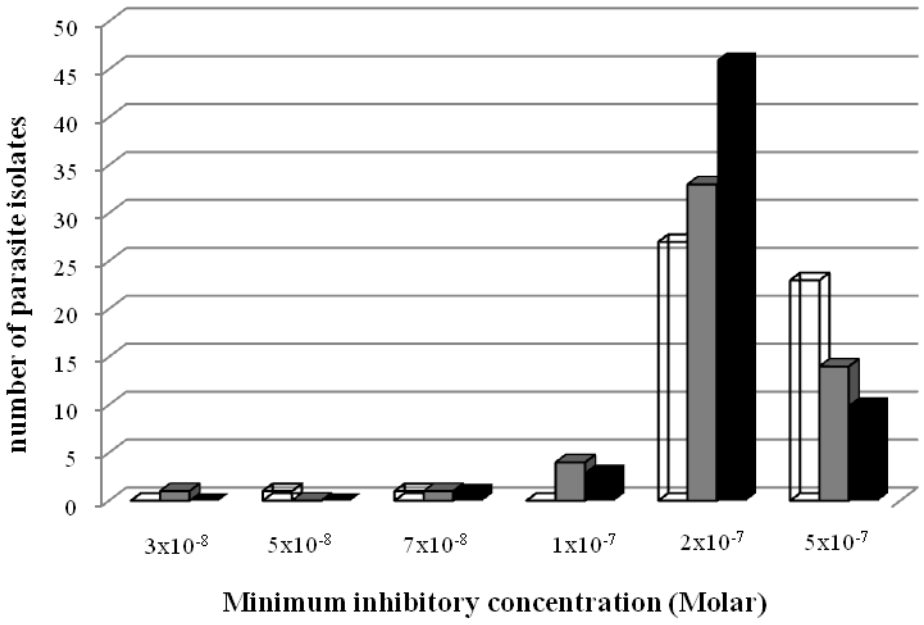


Figure 4.1 Mefloquine response of *P. falciparum* sampling in 2004-2006. A white column stand for parasite population in 2004; gray column stand for parasite population in 2005 and black column stand for parasite population in 2006.

The means of MIC value of parasite collected in 2004, 2005 and 2006 were $2.86x10^{-7}$, $2.29x10^{-7}$ and $2.21x10^{-7}$, respectively. The pairwise comparison of means MICs between each year, we found significant different in means MICs except those between year 2005 and 2006 as shown in Table 4.3.

Table 4.3 Means MIC of mefloquine response and their comparison

Year	n	Means	95% CI for mean		Year comparison	95% CI of the difference	
			Lower	Upper		Lower	Upper
2004	52	$2.86x10^{-7}$	$2.45x10^{-7}$	$3.34x10^{-7}$	2004-2005	0.0049	0.4437
2005	53	$2.29x10^{-7}$	$1.95x10^{-7}$	$2.68x10^{-7}$	2004-2006	0.0714	0.4443
2006	60	$2.21x10^{-7}$	$1.98x10^{-7}$	$2.46x10^{-7}$	2005-2006	-0.1573	0.2244

4.3 Identification of human malaria parasite species of collected samples

All of 201 samples were amplified with the species specific primers. Only 3 cases were identified as *Plasmodium vivax* infection and were excluded from further analysis. Eleven cases were mixed infection between *P. falciparum* and *P. vivax* (Table 4.4). No mixed infection between *P. falciparum* and two other species, *P. malariae* and *P. ovale* was found. The results from PCR were similar to those reported by the malaria clinics.

Table 4.4 Comparison of PCR and microscopy result for *P. falciparum* diagnosis

Microscopy result	Number of samples with PCR result (% of total cases examined)					
	P. falciparum	*P. vivax*	*P. malariae*	*P. ovale*	Mixed	negative
201	190 (95%)	3 (0.5%)	0 (0%)	0 (0%)	11 (5.5%)	0 (0%)

4.4 Genetic analysis of microsatellite loci

Because we analyzed the haploid stage of the parasites, multi-infection for each isolate was analyzed locus by locus, and was estimated from the locus that exhibited the highest number of alleles in a given isolate. Mono infection corresponds thus to an isolate exhibiting only one allele at each locus investigated. The proportions of multiple infections in a sample group were assessed for each locus and between loci in a sample group. In the case of multiple infections, it is impossible to match alleles of distinct locus to reconstruct a genotype. The analyses of population structure must thus be conducted on mono-infected isolates only. In genetic analysis, we examined

only mono-infected individual which had data in allele length at least ten loci. The data were tested at 4 levels: 1) a comparison of Kanchanaburi populations at different years of collection; 2) a comparison of parasite population structure in 7 provinces; 3) an analysis of data in the level of three regional of Thailand i.e. the west, the east and the south; 4) a comparison of Thailand parasite populations with those of other countries in different continents;

4.4.1 Population structure of *Plasmodium falciparum* in Kanchanaburi in different years of collection

Two hundred and sixty-six blood samples from Kanchanaburi province obtained between 2004 and 2007 (63, 61, 77 and 50 samples, respectively) and 15 additional samples from laboratory collection preserved in liquid nitrogen were genotyped at 12 microsatellite loci. From 266 samples, 24 samples were unsuccessfully genotyped, 67 samples were multi-infected (28%) and 175 were mono-infected (72%). We only carried out genetic analysis on mono-infected samples. The number of alleles per locus varied from 4 (locus TA80 and Pfg377) to 18 alleles (locus C1M8) as shown in Figure 4.2. Gene diversity (H_S) per locus in Kanchanaburi population varied from 0.315 (locus TAA109) to 0.826 (locus C1M8). Decreasing gene diversity was obviously observed in locus ARP2 in 2005 and 2007 populations (0.117 and 0.171, respectively) while in other populations the diversity in this locus were 0.593, 0.502 and 0.360 (2002, 2004 and 2006 populations). Moreover, decreasing gene diversity in populations was observed in 2006 and 2007 (Table 4.5). Genetic differentiation between years of collection was tested; a low but significant differentiation was detected between 2004 population versus 2007 population and 2005 population versus 2007 population as shown in Table 4.6. Although, most individuals in each population had the same major allele, some significant genetic differentiations among populations were observed as evidenced from the different distribution proportion in minor allele. For example, eleven and six samples in 2004 and 2005 populations, respectively, possessed the allele 1 at locus PfPK2 which was a minor allele while none of sample in 2007 population carried this allele (Figure 4.2). Significant LD was found in 2007 population between loci Polyα and C1M8 and loci TA60 and C1M8.

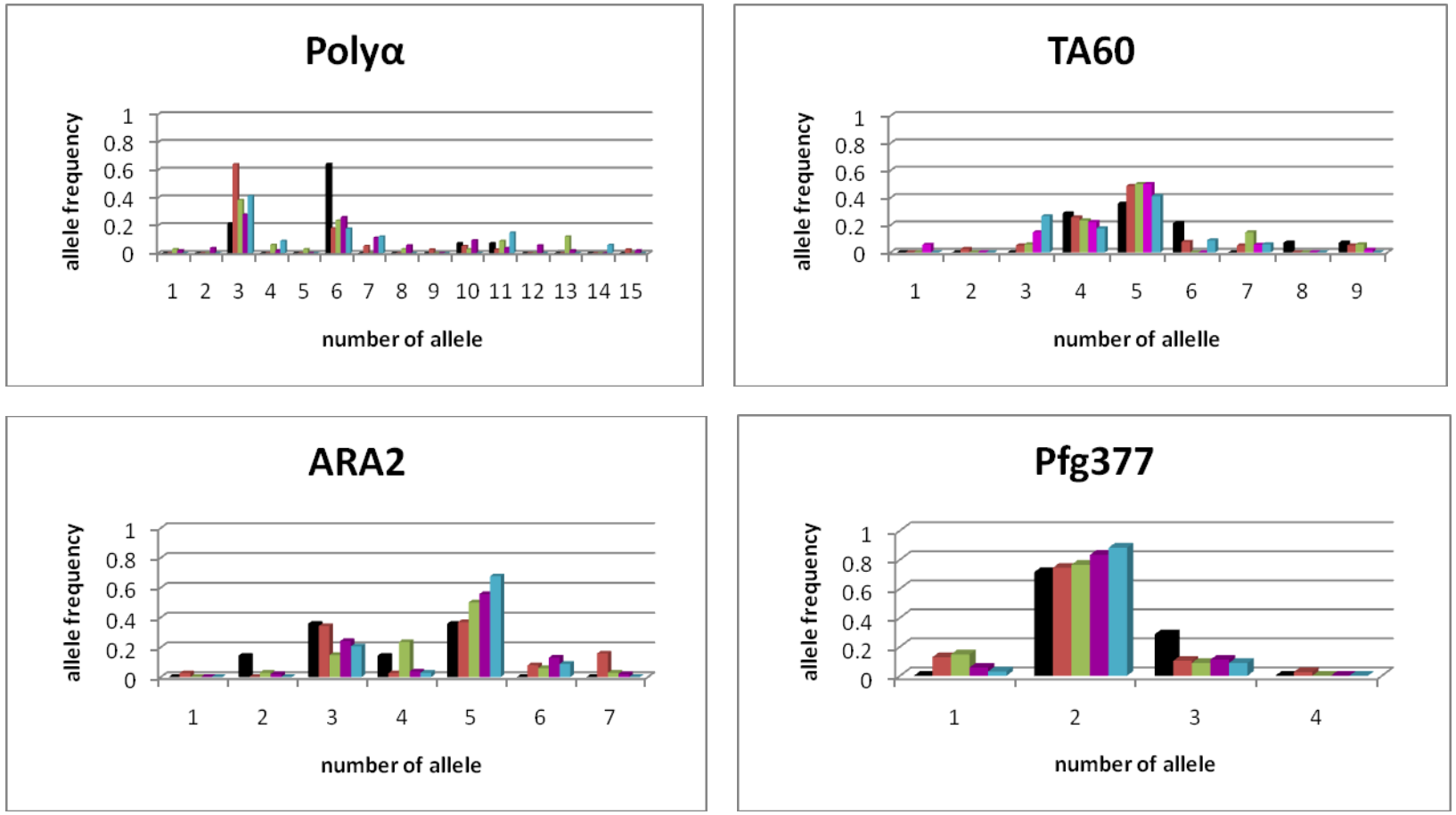


Figure 4.2 Number of alleles and allele frequencies observed for *P. falciparum* at each microsatellite locus in the different 5 year sampling. 2002 2004 2005 2006 2007

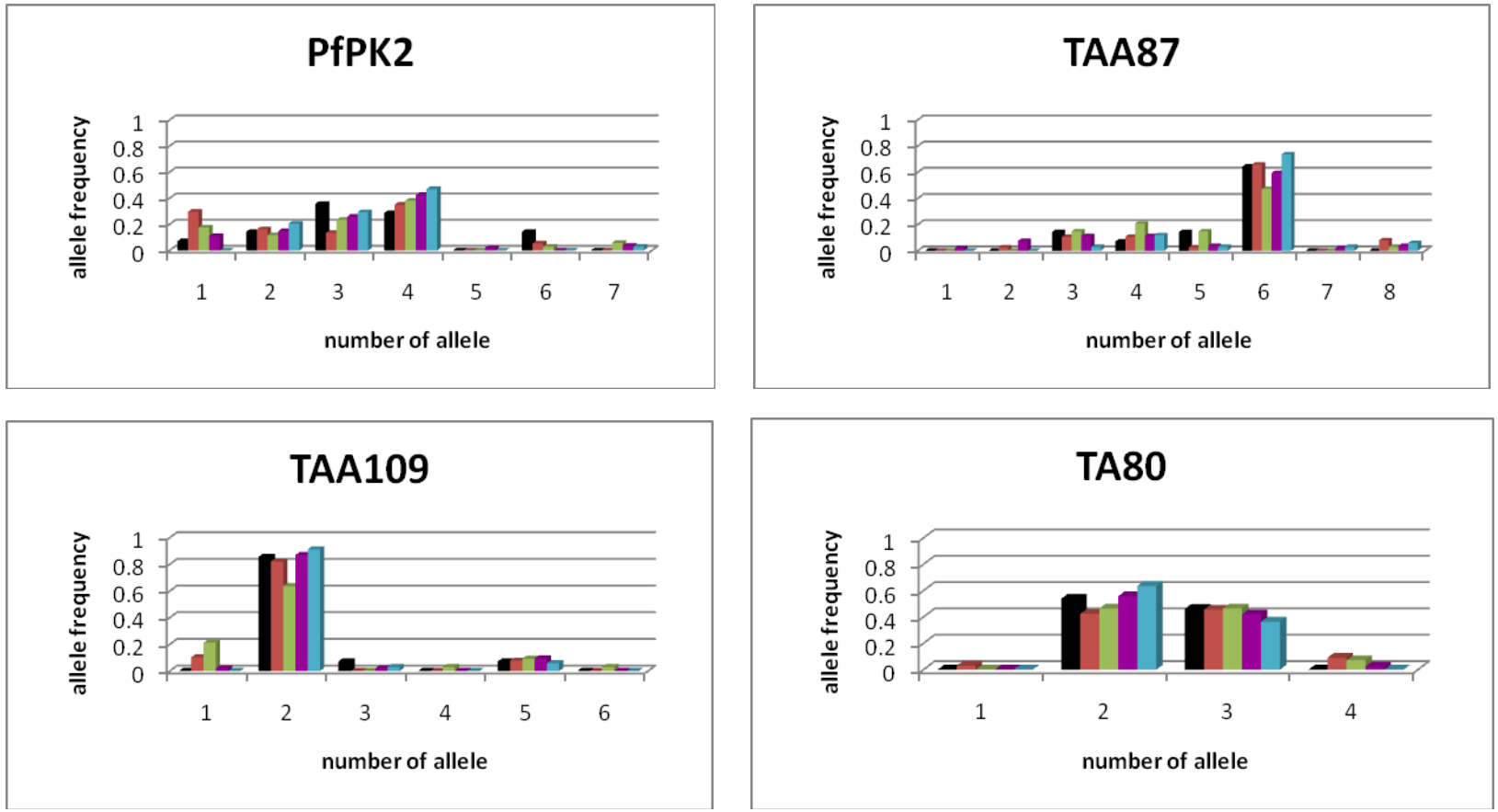


Figure 4.2 (continue) Number of alleles and allele frequencies observed for *P. falciparum* at each microsatellite locus in the different 5 year sampling. 2002 2004 2005 2006 2007

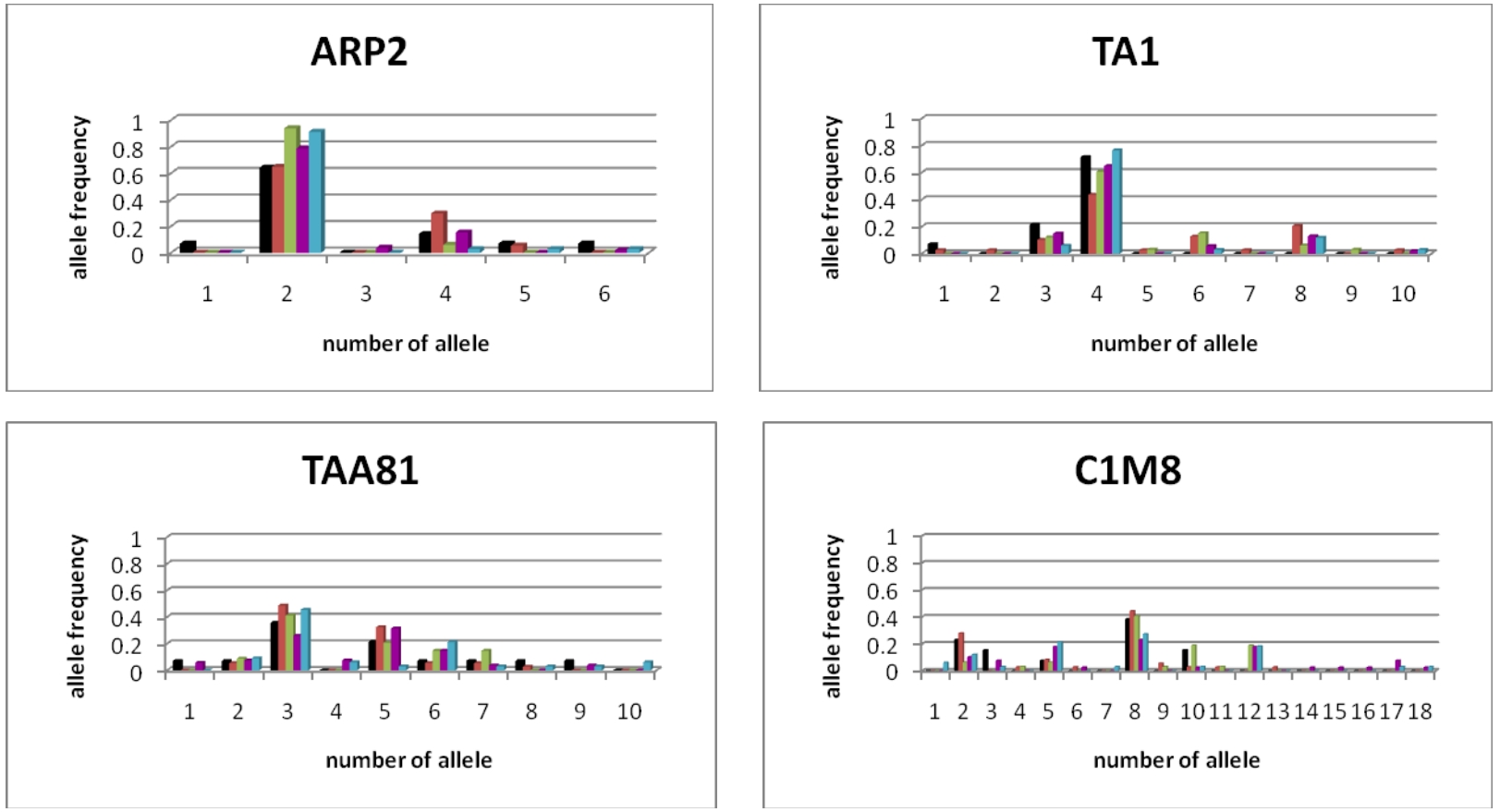


Figure 4.2 (continue) Number of alleles and allele frequencies observed for *P. falciparum* at each microsatellite locus in the different 5 year sampling. 2002 2004 2005 2006 2007

Table 4.5 Gene diversity of parasite populations in different years

	2002	2004	2005	2006	2007	All
N	14	39	34	54	34	175
TA80	0.538	0.623	0.585	0.525	0.477	0.543
ARP2	0.593	0.502	0.117	0.360	0.171	0.341
TA1	0.473	0.757	0.608	0.548	0.408	0.582
Poly α	0.571	0.564	0.793	0.84	0.775	0.766
TA60	0.791	0.700	0.686	0.685	0.740	0.709
ARA2	0.758	0.734	0.688	0.626	0.506	0.665
Pfg377	0.440	0.430	0.398	0.296	0.219	0.342
PfPK2	0.802	0.761	0.772	0.729	0.668	0.741
TAA87	0.582	0.552	0.713	0.627	0.453	0.591
TAA109	0.275	0.318	0.557	0.238	0.169	0.315
TAA81	0.857	0.667	0.759	0.810	0.752	0.773
C1M8	0.808	0.732	0.778	0.879	0.850	0.826
H_S±SD	0.62±0.18	0.61±0.14	0.62±0.20	0.60±0.21	0.52±0.24	0.60±0.18

Table 4.6 Genetic differentiation (F_{ST}) among years in Kanchanaburi samples

F_{ST}	n	2002	2004	2005	2006	2007
2002	14	-	0.0195	0.0133	0.0045	0.0324
2004	39		-	0.0204	0.0230	0.0474*
2005	34			-	0.0093	0.0240*
2006	54				-	0.0037
2007	34					-

* significant at p-value=0.005 after Bonferroni correction for multi tests.

4.4.2 Population structure of *Plasmodium falciparum* in 7 populations of Thailand

Five hundred and twenty-three blood samples from seven provinces of Thailand were genotyped at 12 microsatellite loci. Among them, 42 samples (8%) were unsuccessfully genotyped, 135 samples were multi-infected (28%) and 346 were mono-infected (72%) (Table 4.7). We found multiple infected samples in all six provinces ranging from 44% in Maehongson, 36% in Trat, 32% in Ranong, 31% in Tak, 27% in Kanchanaburi and 22% in Ubonratchathani population. An important result showed that, in the Yala population, all 31 isolates analyzed displayed an identical haplotype.

All twelve loci were polymorphic when we examined in these 346 mono-infected samples, the number of alleles per locus varied from 4 (locus TA80 and Pfg377) to 20 alleles (locus C1M8) shown in Table 4.8. Genetic diversity (H_S) per microsatellite locus varied from 0.391 (locus TAA109) to 0.841 (locus C1M8) (Table 4.8). The number of alleles per locus and allelic richness per locus in each population was shown in Table 4.9. Because the observed number of alleles in a sample is highly dependent on sample size, we measured and compared allelic richness per locus which is a measure of the number of alleles independent of sample size, among all population instead. Trat and Yala population had a significant lower allelic richness among 7 populations (Table 4.10)

Table 4.7 Mono- and multi-infected samples detected by 12 microsatellite genotype

Site	n	Number of unsuccessful-genotyped sample	n*	Multi-infected isolates	Mono-infected isolates	% Multi-infection
Maehongson	32	0	32	14	18	44%
Tak	71	1	70	22	48	31%
Kanchanaburi	266	25	241	66	175	27%
Ubonratchathani	25	2	23	5	18	22%
Trat	34	6	28	10	18	36%
Ranong	59	3	56	18	38	32%
Yala	36	5	31	0	31	0%
Total	523	42	481	135	346	28%

n = number of collected isolates; n*= number of isolates genotyped at 12 microsatellite loci;

% Multi-infection = proportion of isolates that exhibit at least more than one allele at one locus.

Table 4.8 Number of alleles, size rage of alleles and genetic diversity at each microsatellite locus.

Microsatellite marker	Chromosome	Number of alleles	Size range (base pairs)	H_S
Polyα	4	17	131-189	0.837
TA60	13	11	64-94	0.722
ARA2	11	10	51-81	0.731
Pfg377	12	4	95-104	0.450
PfPK2	12	9	160-193	0.750
TAA87	6	11	78-116	0.668
TAA109	6	7	157-185	0.391
TA80	10	4	139-151	0.537
ARP2	13	7	160-184	0.445
TA1	6	12	154-190	0.570
TAA81	5	11	109-142	0.804
C1M8	1	20	150-216	0.841

Table 4.9 Number of alleles and allelic richness observed at each locus in 7 populations of *P. falciparum*

locus/sites	MAE		TAK		KAN		UBO		TRA		RAN		YAL	
n	18		48		175		18		18		38		31	
	No	AR	No	AR	No	AR	No	AR	No	AR	No	AR	No	AR
Polyα	10	(10.0)	11	(9.0)	15	(8.8)	8	(8.0)	7	(7.0)	9	(8.3)	1	(1.0)
TA60	7	(7.0)	7	(6.4)	9	(6.3)	4	(4.0)	4	(4.0)	6	(5.8)	1	(1.0)
ARA2	6	(6.0)	7	(6.4)	7	(5.5)	5	(5.0)	4	(4.0)	7	(6.2)	1	(1.0)
Pfg377	3	(3.0)	3	(2.8)	4	(3.1)	2	(2.0)	2	(2.0)	4	(3.6)	1	(1.0)
PfPK2	5	(5.0)	7	(6.5)	7	(5.5)	7	(7.0)	4	(4.0)	7	(6.3)	1	(1.0)
TAA87	7	(7.0)	8	(7.1)	8	(5.9)	7	(7.0)	6	(6.0)	6	(5.6)	1	(1.0)
TAA109	2	(2.0)	4	(3.0)	6	(3.7)	3	(3.0)	3	(3.0)	3	(2.9)	1	(1.0)
TA80	3	(3.0)	3	(2.9)	4	(3.0)	2	(2.0)	2	(2.0)	3	(3.0)	1	(1.0)
ARP2	4	(4.0)	5	(4.0)	6	(3.6)	5	(5.0)	3	(3.0)	2	(2.0)	1	(1.0)
TA1	5	(5.0)	8	(6.6)	10	(5.6)	7	(7.0)	5	(5.0)	4	(4.0)	1	(1.0)
TAA81	7	(7.0)	7	(6.6)	10	(7.5)	7	(7.0)	5	(5.0)	7	(6.5)	1	(1.0)
C1M8	7	(7.0)	12	(10.1)	18	(9.7)	10	(10.0)	8	(8.0)	7	(6.3)	1	(1.0)

No, Number of alleles; AR, allelic richness.

MAE, Maehongson; TAK, Tak; KAN, Kanchanaburi; UBO, Ubonratchathani;

TRA, Trat; RAN, Ranong; YAL, Yala.

n = number of samples analysed in each locality.

Table 4.10 Wilcoxon Signed Ranks Test of allelic richness among populations two-sided probabilities using normal approximation

(* significant p-value)

	Maehongsorn	Tak	Kanchanaburi	Ubonratchathani	Trad	Ranong	Yala
Maehongsorn	1.000						
Tak	0.433	1.000					
Kanchanaburi	0.875	0.158	1.000				
Ubonratchathani	0.875	0.272	1.000	1.000			
Trad	0.023*	0.002*	0.003*	0.008*	1.000		
Ranong	0.158	0.019*	0.099	0.480	0.158	1.000	
Yala	0.002*	0.002*	0.002*	0.002*	0.002*	0.002*	1.000

The allele frequencies for each locus in each population were shown in Figure 4.3. The prevalence of unique alleles was found in the Yala population observed with marker Polyα on chromosome 4; TAA109 on chromosome 6; ARP2 on chromosome 13 and ARA2 on chromosome 11.

Gene diversity (H_S) in each population was 0.68±0.23 in Maehongson population, 0.63±0.25 in Tak population, 0.60±0.18 in Kanchanaburi population, 0.70±0.21 in Ubonratchathani population, 0.56±0.25 in Trat population and 0.62±0.20 in Ranong population. Of course, there was no variation observed in Yala population.

No significant linkage disequilibrium among sites at *p-value* = 0.00011 in any 66 combined pairs of loci except 2 combinations in Kanchanaburi (Polyα x TAA81 and Ara2 x TAA81) and of course a total linkage disequilibrium existed in the Yala population as all samples possessed the same genotype (data not shown).

Genetic differentiation (F_{ST}) was estimated and a permutation test was applied (n=10,000) permuting alleles among samples for all loci to test whether F_{ST} significantly differ from zero. Significant differentiation (*p-value* = 0.0024) of *P. falciparum* population between sites were observed, and a very high differentiation occurred between the Yala population and all other populations (Table 4.11).

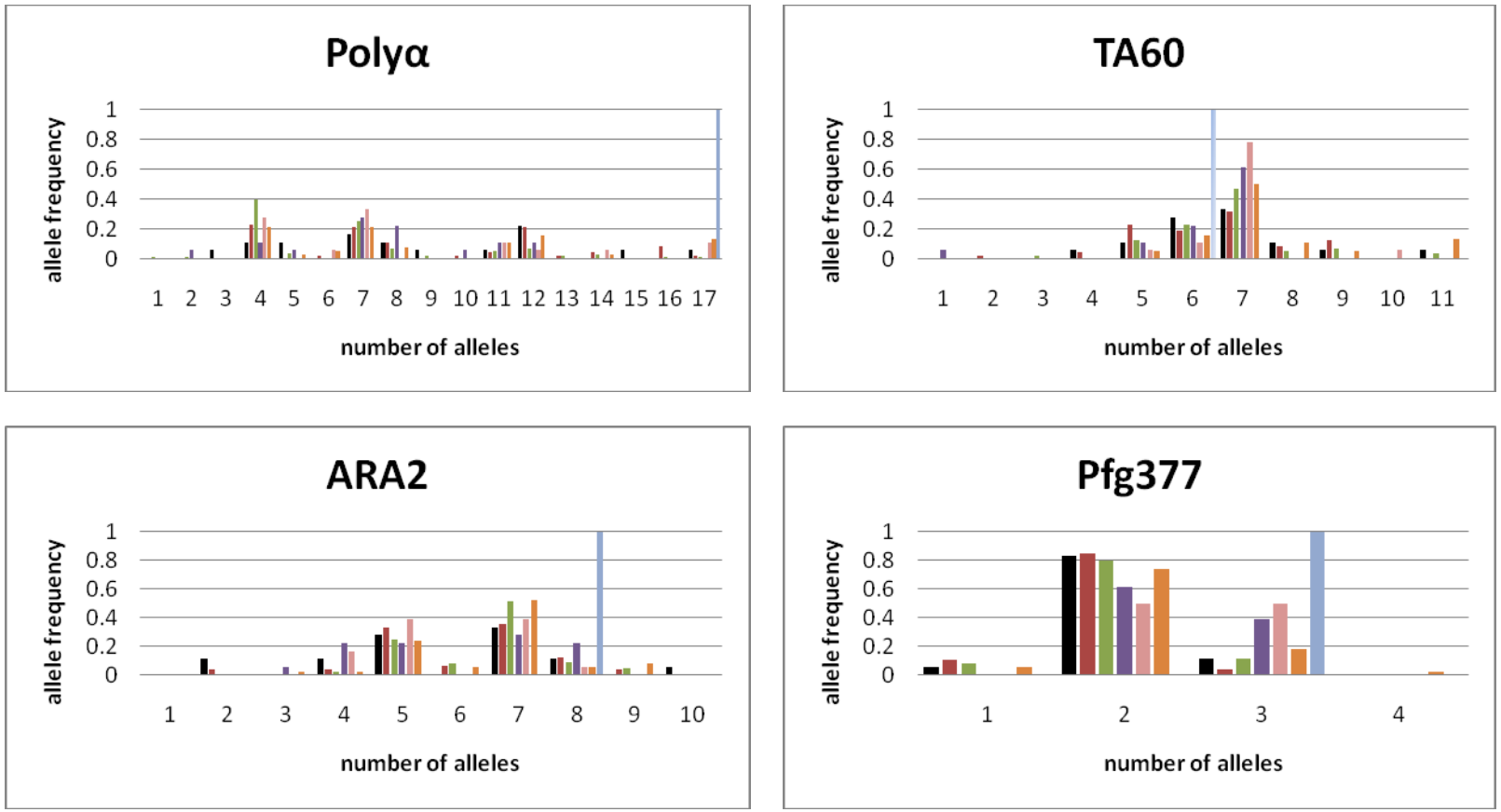


Figure 4.3 Number of alleles and allele frequencies observed for *P. falciparum* at each microsatellite locus in the 7 populations studied. Maehongson Tak Kanchanaburi Ubonratchathani Trat Ranong Yala

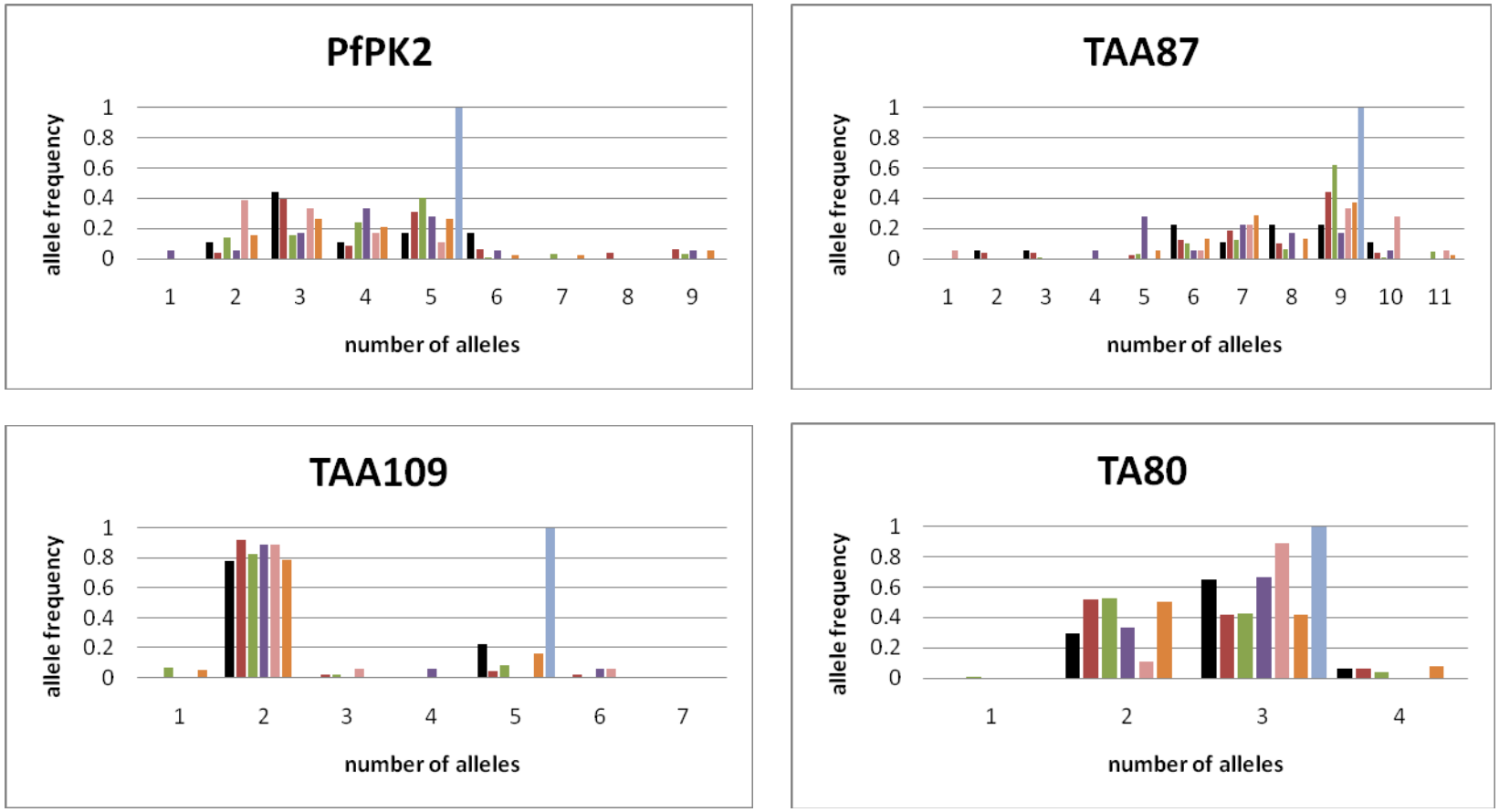


Figure 4.3 (continue) Number of alleles and allele frequencies observed for *P. falciparum* at each microsatellite locus in the 7 populations studied. Maehongson Tak Kanchanaburi Ubonratchathani Trat Ranong Yala

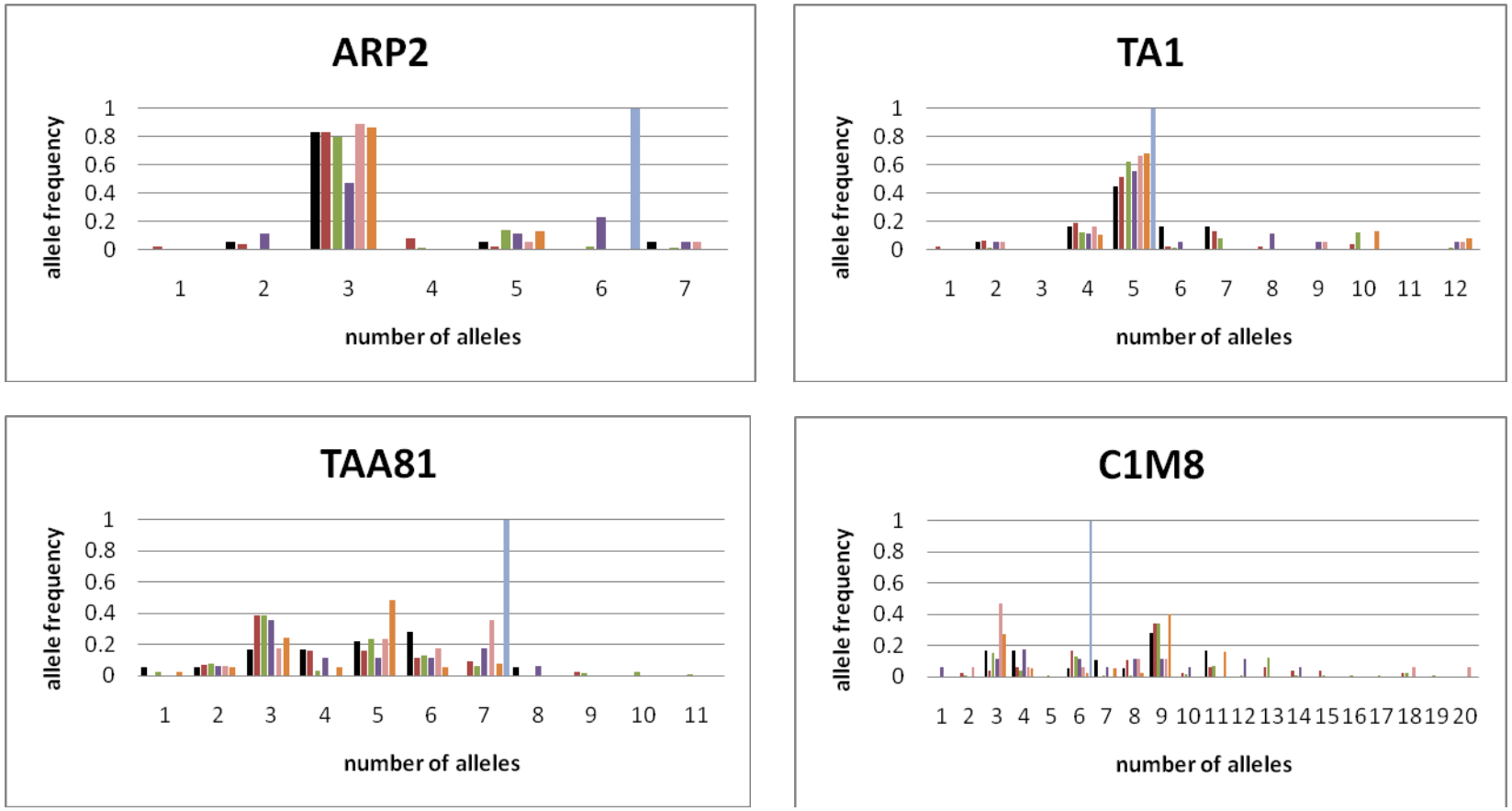


Figure 4.3 (continue) Number of alleles and allele frequencies observed for *P. falciparum* at each microsatellite locus in the 7 populations studied.

Table 4.11 Genetic differentiation (F_{ST}) between *P. falciparum* populations from different provinces of Thailand

F_{ST}	MAE (n=18)	TAK (n=48)	KAN (n=175)	UBO (n=18)	TRA (n=18)	RAN (n=38)	YAL (n=31)
MAE	-	0.0010	0.0368**	0.0152	0.0476	0.0117	0.6567**
TAK		-	0.0154**	0.0382**	0.0824**	0.0201**	0.5593**
KAN			-	0.0498**	0.0761**	0.0121	0.4739**
UBO				-	0.0297	0.0384**	0.6190**
TRA					-	0.0512**	0.6930**
RAN						-	0.5843**
YAL							-

**significant at p-value=0.0024 after Bonferroni correction for multi tests.

MAE, Maehongson; TAK, Tak; KAN, Kanchanaburi; UBO, Ubonratchathani;

TRA, Trat; RAN, Ranong; YAL, Yala

4.4.3 A comparison of *P. falciparum* in three regional areas in Thailand

We grouped 7 parasite populations along geographic locations. The Maehongson, Tak and Kanchanaburi populations were represented as the west population. The Ubonratchathani and Trat populations were represented as the east population. While the Ranong and Yala populations were represented as the south population. The distribution of allele frequency of these 3 populations was shown in Table 4.12. Most of loci revealed the contribution of different major allele in each population and some alleles could be found only in one or two populations. For example, in locus Polyα, 35% (83 individuals) of the west population had 146 bp in length allele in this locus, 31% (11 individuals) of the east population had 156 bp in length allele and 52% (36 individuals) of the south population had 189 bp in length allele (Table 4.12). Gene diversity in each region ranged from 0.290 (locus TAA109) to 0.835 (locus C1M8) in the west population, from 0.211 (locus TAA109) to 0.881 (locus C1M8) in the east population and from 0.316 (locus TA1) to 0.709 (locus C1M8) in the south population (Table 4.13). A lower diversity at locus TAA109 was observed in the west and the east populations (H_S=0.290 and 0.211, respectively) comparing with the south population (H_S=0.530). On the contrary, a lower diversity at locus TA1 was observed in the south population (H_S=0.316) comparing with the west and the east populations (H_S=0.620 and 0.611, respectively) (see in Table 4.13). A low but significant genetic differentiation was observed in all pairs of comparison (Table 4.14). This revealed the unique structure of parasite population in each region of Thailand.

Table 4.12 Allele frequency of 12 microsatellite marker distributed in three regional populations

Locus	West	East	South
Polyα			
N	241	36	69
131	0.008	-	-
136	0.008	0.028	-
142	0.004	-	-
146	0.344	0.194	0.116
150	0.033	0.028	0.014
153	0.008	0.028	0.029
156	0.237	0.306	0.116
160	0.079	0.111	0.043
163	0.021	-	-
166	0.008	0.028	-
169	0.050	0.111	0.058
172	0.108	0.083	0.087
175	0.017	-	-
178	0.029	0.028	0.014
181	0.004	-	-
183	0.025	-	-
189	0.017	0.056	0.522
TA60			
N	241	36	69
64	-	0.028	-
67	0.004	-	-
70	0.012	-	-
73	0.017	-	-
76	0.141	0.083	0.029
79	0.224	0.167	0.536
82	0.427	0.694	0.275
85	0.062	-	0.058
88	0.079	-	0.029
91	0.004	0.028	-
94	0.029	-	0.072
ARA2			
N	240	36	69
51	0.004	-	-
57	0.017	-	-
60	-	0.028	0.014

Table 4.12 (continue) Allele frequency of 12 microsatellite marker distributed in three regional populations

Locus	West	East	South
ARA2			
N	240	36	69
63	0.033	0.194	0.014
66	0.267	0.306	0.130
69	0.071	-	0.029
72	0.467	0.333	0.290
75	0.096	0.139	0.478
78	0.042	-	0.043
Pfg377			
N	240	36	69
95	0.083	-	0.029
98	0.813	0.556	0.406
101	0.100	0.444	0.551
104	0.004	-	0.014
PfPK2			
N	239	36	69
160	-	0.028	-
163	0.117	0.222	0.087
166	0.226	0.250	0.145
169	0.201	0.250	0.116
172	0.364	0.194	0.594
175	0.029	0.028	0.014
178	0.021	-	0.014
184	0.008	-	-
193	0.033	0.028	0.029
TAA87			
N	240	36	69
78	-	0.028	-
86	0.013	-	-
89	0.017	-	-
92	-	0.028	-
95	0.025	0.139	0.029
98	0.117	0.056	0.072
101	0.138	0.222	0.159
104	0.083	0.083	0.072
107	0.550	0.250	0.652

Table 4.12 (continue) Allele frequency of 12 microsatellite marker distributed in three regional populations

Locus	West	East	South
TAA87			
N	240	36	69
107	0.550	0.250	0.652
110	0.025	0.167	-
116	0.033	0.028	0.014
TAA109			
N	240	36	69
157	0.050	-	0.029
160	0.838	0.889	0.435
163	0.017	0.028	-
170	0.004	0.028	-
173	0.083	-	0.536
176	0.004	0.056	-
185	0.004	-	-
TA80			
N	215	36	69
139	0.005	-	-
145	0.507	0.222	0.275
148	0.442	0.778	0.681
151	0.047	-	0.043
ARP2			
N	236	35	69
160	0.004	-	-
169	0.017	0.057	-
172	0.809	0.686	0.478
175	0.025	-	-
178	0.110	0.086	0.072
181	0.017	0.114	0.449
184	0.017	0.057	-
TA1			
N	239	36	68
154	0.004	-	-
157	0.025	0.056	-
160	0.004	-	-
163	0.138	0.139	0.059

Table 4.12 (continue) Allele frequency of 12 microsatellite marker distributed in three regional populations

Locus	West	East	South
TA1			
N	239	36	68
166	0.586	0.611	0.824
169	0.025	0.028	-
172	0.096	-	-
175	0.008	0.056	-
178	-	0.056	-
181	0.096	-	0.074
187	0.004	-	-
190	0.013	0.056	0.044
TAA81			
N	234	34	66
109	0.021	-	0.015
112	0.073	0.059	0.030
115	0.368	0.265	0.136
118	0.068	0.059	0.030
121	0.218	0.176	0.273
124	0.141	0.147	0.030
127	0.064	0.265	0.485
130	0.004	0.029	-
133	0.017	-	-
139	0.017	-	-
142	0.009	-	-
C1M8			
N	218	34	68
150	-	0.029	-
156	0.014	0.029	-
159	0.128	0.294	0.147
162	0.055	0.118	0.029
165	0.009	-	-
168	0.133	0.088	0.471
171	0.018	0.029	0.029
174	0.032	0.118	0.015
177	0.335	0.118	0.221
180	0.018	0.029	-
183	0.078	-	0.088
189	0.009	0.059	-

Table 4.12 (continue) Allele frequency of 12 microsatellite marker distributed in three regional populations

Locus	West	East	South
C1M8			
N	218	34	68
192	0.101	-	-
195	0.014	0.029	-
198	0.014	-	-
201	0.005	-	-
204	0.005	-	-
207	0.023	0.029	-
210	0.009	-	-
216	-	0.029	-

Table 4.13 Gene diversity per locus of the west, the east and the south populations of Thailand

Locus	West	East	South
n	241	36	69
TA60	0.739	0.495	0.636
ARA2	0.697	0.759	0.677
TA87	0.658	0.852	0.546
Polyα	0.805	0.854	0.697
Pfg377	0.324	0.508	0.539
PfPK2	0.763	0.808	0.613
TAA109	0.290	0.211	0.530
TA80	0.548	0.356	0.465
ARP2	0.333	0.518	0.572
C1M8	0.835	0.881	0.709
TA1	0.620	0.611	0.316
TAA81	0.786	0.824	0.679

Table 4.14 Genetic differentiation (F_{ST}) among *P. falciparum* populations in 3 regions of Thailand

	West	East	South
West	-	0.0478*	0.1321*
East		-	0.1199*
South			-

* significant at p-value=0.017 after Bonferroni correction for multi tests.

4.4.4 Genotype distribution in Thai population

Three hundred different genotypes were obtained from the 346 single *P. falciparum* isolate infected individuals, with 14 identical haplotypes (Table 4.15). Thus, we found essentially identical genotypes from patients:

- in the same year but infected in two different areas: In Kanchanaburi, we found an identical genotype in seven individuals infected in 2007 where four of them were infected in Myanmar and three of them infected in Thailand,

- in the same site but collected in two successive years: In Ranong, we found an identical genotype in two individuals infected in 2005 and 2006, and another identical genotype in 2006 and 2007,

- in Yala, only one haplotype was found in 31 parasite samples.

These genotypes that are found in more than one individual would have an impact on the epidemiology of the disease because, (i) they can pass through several generations, and (ii) these genotypes could carry important traits such as drug-resistance genes.

Table 4.15 Spatial and temporal genotype distribution of *P. falciparum* in Thailand.

Site	Number of individuals	different haplotype	identical haplotype in 2 individuals	identical haplotype in 3 individuals	identical haplotype in 7 individuals	identical haplotype in 31 individuals
Maehongson	18	18	-	-	-	-
Tak	48	47	1 in 2007	-	-	-
Kanchanaburi	175	162	-	1 in 2004	-	-
			2 in 2005	-	-	-
			1 in 2006	-	-	-
			3 in 2007	-	1 in 2007	-
Ubonratchathani	18	17	1 in 2003	-	-	-
Trat	18	18	-	-	-	-
Ranong	38	35	1 in 2005 and 2006	-	-	-
			1 in 2006 and 2007	-	-	-
			1 in 2007	-	-	-
Yala	31		-	-	-	1 in 2007

4.4.5 A comparison of *P. falciparum* in Thailand to other *P. falciparum* blood samples from Africa and South America

We also analyzed and compared parasite population genetic in four countries which are Congo (n=15) and Cameroon (n=35) in Africa continent, Thailand (n=346) in Asia continent and French Guyana (n=137) in South America continent. The allele length data genotyped with 7 common microsatellite loci; Polyα, TA60, ARA2, Pfg377, PfPK2, TAA87 and TAA109 were analyzed. The gene diversity in Thailand population (H_S=0.65±0.17) was lower than those found in the two African countries (Congo: H_S=0.81±0.07 and Cameroon: H_S=0.78±0.18), but higher than that of French Guyana (H_S=0.54±0.08). We also used canonical correspondence analysis (CCA) to determine the relative contribution of the countries (i.e. sites) to the global genetic variability of *P. falciparum* populations. A graphic representation of the results showed overlapping distributions (i.e. centroids and ellipses of the 95% CI-Figure 4.4) of the genetic variability of *P. falciparum* observed among sites. A Monte Carlo permutation test on the first four canonical correspondence analysis axes that combines parasite genotypes and sites was statistically significant (P=0.001). This suggests some degree of geographic differentiation. Indeed, all populations in different countries displayed significant genetic differentiation except between the two African countries (i.e. Congo and Cameroon-Figure 4.4)

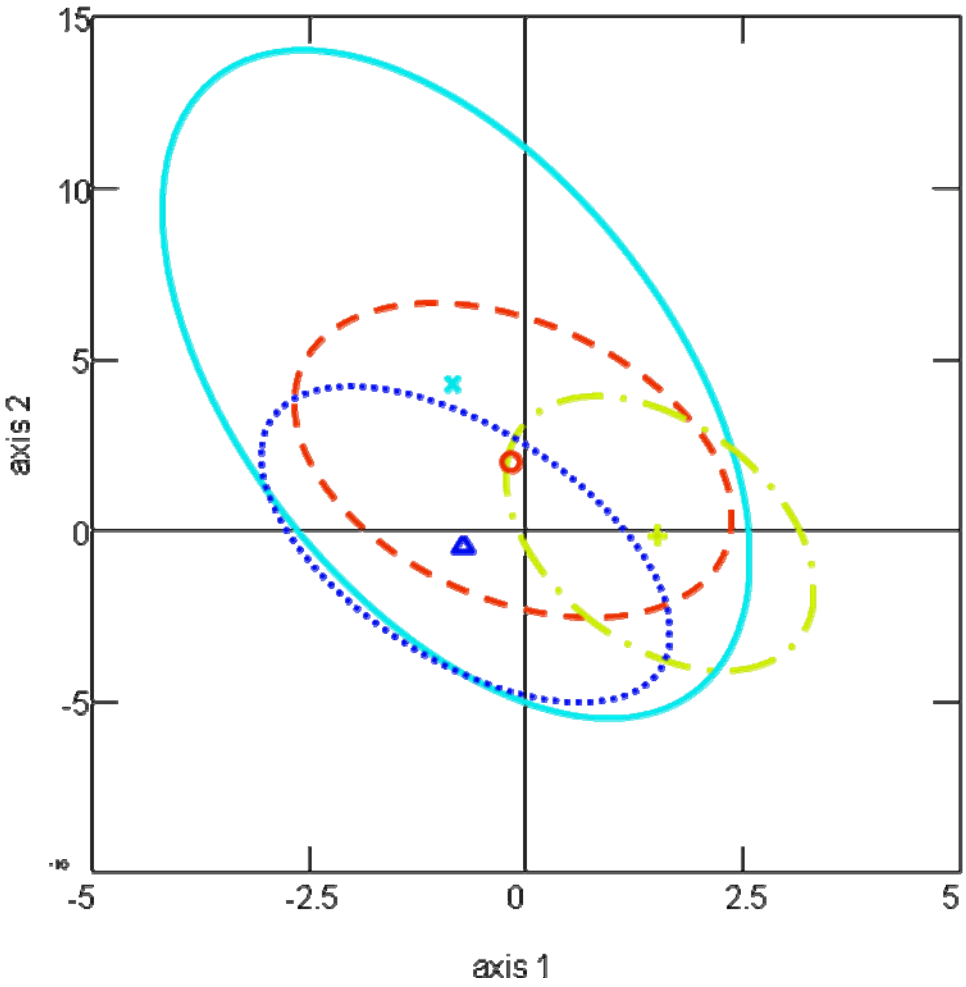


Figure 4.4 Results of the canonical correspondence analysis (CCA). Relative contribution of the variables "sites" and "genotypes" to the genetic structure of *P. falciparum* populations in four sites. *Plasmodium* populations were projected on the first two axes of the CCA. Centroids (dots) of each parasite populations are surrounded by the 95% C.I.s (ellipses). (x and solid oval stand for Congo population (n=15) ; o and dash oval stand for Cameroon population (n=35) ; + and long dash dot oval stand for French Guyana population (n=137) and Δ and round dot oval stand for Thailand population (n=286).

4.5 Genetic analysis of microsatellite loci flanking *pfmdr1* gene in Kanchanaburi samples

4.5.1 Optimization of polymerase chain reaction

In order to optimize the amplification reaction, we used approximately 300 ng of chromosomal DNA of laboratory adapted clone, T9/94RC17, and a field isolate as templates. An initial condition was the same condition as used for amplification of microsatellite loci in the previous experiment. A one-step PCR was performed in a 15 µl reaction volume contained 1.5 µl of DNA template, 80 µM of each deoxynucleoside triphosphate, 2 pmole of each primer, 4 mM $MgCl_2$ and 0.2 units of *Taq* DNA polymerase. Cycling condition was as follow, denaturation at 94^{o}C for 2 minutes, 40 cycles at 94^{o}C for 20 seconds, 45^{o}C for 20 seconds and 65^{o}C for 30 seconds and final extension step at 65^{o}C for 2 minutes. DNA product bands of locus L2, L4, L5 and L7 were observed on the agarose gel (Figure 4.5).

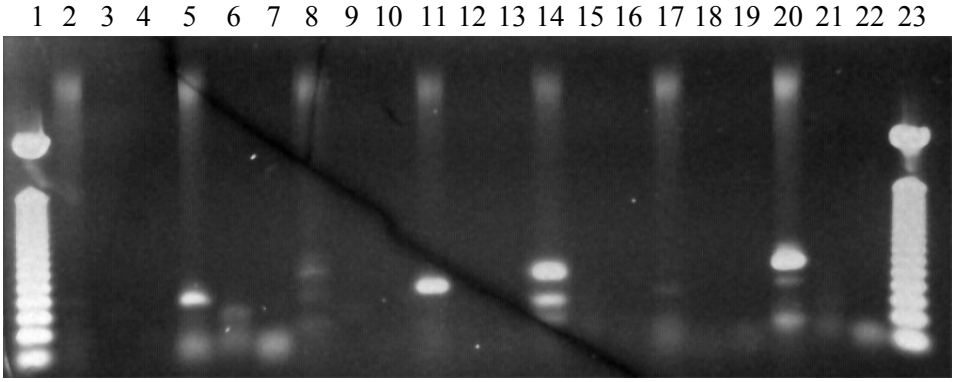


Figure 4.5 PCR products amplified from 7 microsatellite loci located inside and surround *pfmdr 1* gene. Lane 1 and 23 = 50 bp step ladder, lane 2-4 = products of locus L1, lane 5-7 = products of locus L2, lane 8-10 = products of locus L3, lane 11-13 = products of locus L4, lane 14-16 = products of locus L5, lane 17-19 = products of locus L6 and lane 20-22 = products of locus L7. Lane 2, 5, 8, 11, 14, 17 and 20 presented the PCR products from T9/94RC17. Lane 3, 6, 9, 12, 15, 18 and 21 presented the PCR products from the field isolate. Lane 4, 7, 10, 13, 16, 19 and 22 were negative control.

We varied the concentration of $MgCl_2$ in the reaction with the same cycling condition used previously. The DNA templates were undiluted or diluted at 100-fold dilution of T9/94RC17 chromosomal DNA. There was no PCR product band of locus L1 observed on the agarose gel from all varied concentration of $MgCl_2$ both amplified from undiluted and diluted DNA templates (data not shown). For locus L2, PCR product band was not observed from all varied condition except at 4 mM of $MgCl_2$ with undiluted template (data not shown). For locus L3, there were smear bands from all reaction using undiluted templates and non-specific bands from the amplification using diluted templates (Figure 4.6). For locus L4 and L7, we observed single specific band from all amplification reactions. The sharpest band was obtained from reaction contained 3 mM $MgCl_2$. For locus L5, there were non-specific bands observed from all amplification reactions (Figure 4.7). For locus L6, we observed non-specific bands from reaction using undiluted template while there was no bands observed from reaction using diluted template (Figure 4.8).

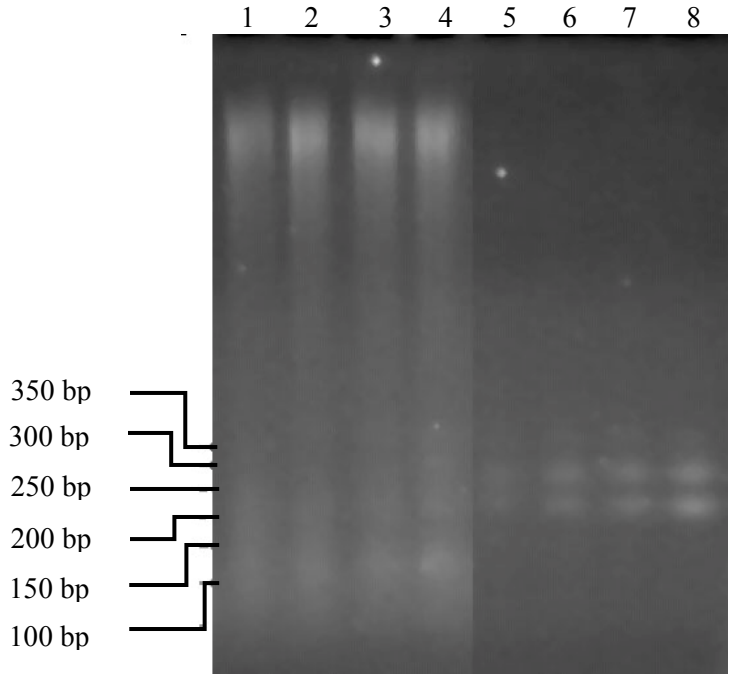


Figure 4.6 PCR products of microsatellite locus L3 with various concentration of $MgCl_2$. Lane 1 and 5 = 1.5 mM; lane 2 and 6 = 2 mM $MgCl_2$; lane 3 and 7 = 2.5 mM $MgCl_2$; lane 4 and 8 = 3 mM $MgCl_2$. Lane 1-4 presented the PCR products amplified from undiluted templates and lane 5-8 presented the PCR products amplified from diluted templates.

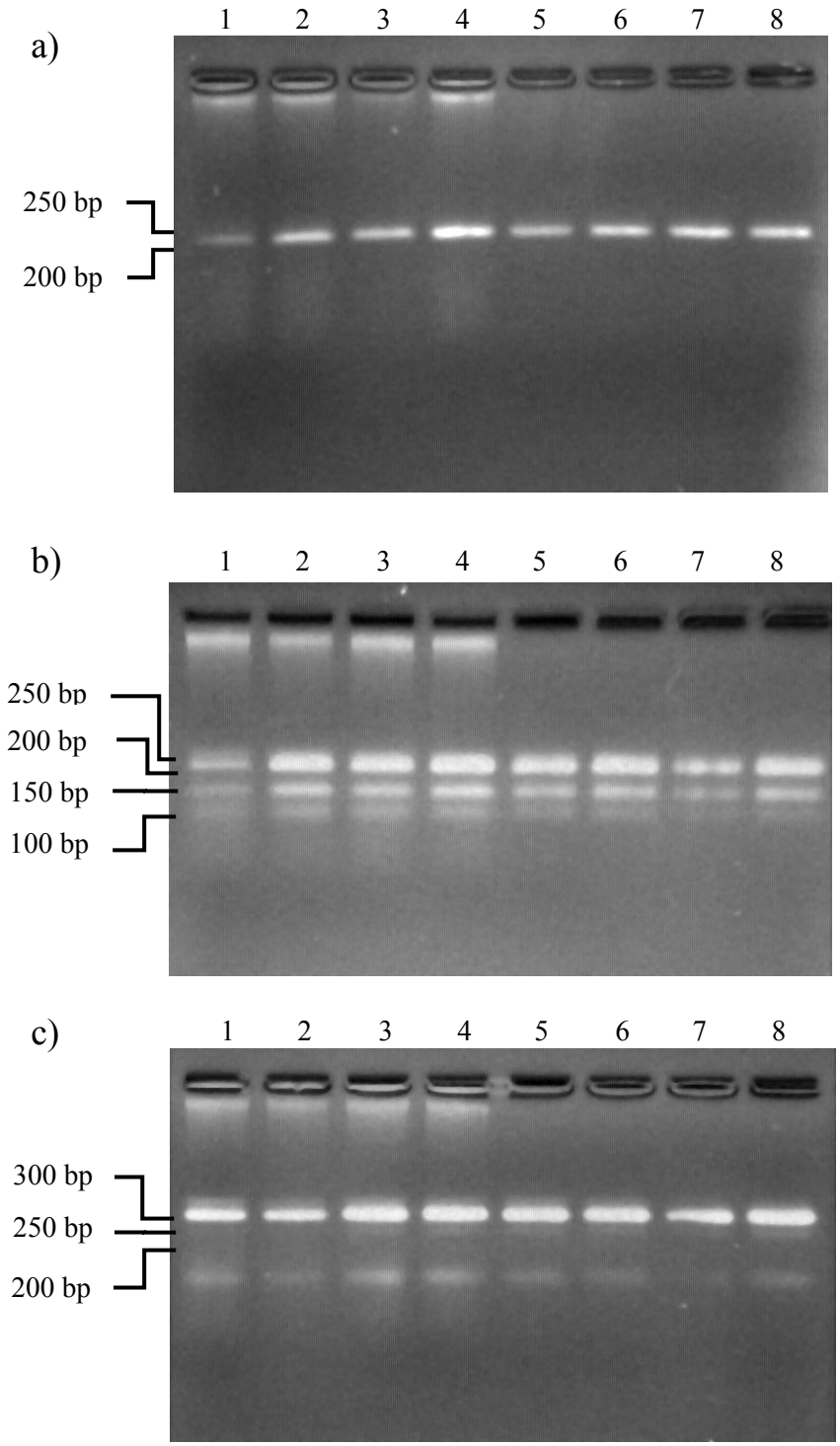


Figure 4.7 PCR products of microsatellite locus L4 (a), L5 (b) and L7 (c) with various concentration of $MgCl_2$. Lane 1 and 5 = 1.5 mM; lane 2 and 6 = 2 mM $MgCl_2$; lane 3 and 7 = 2.5 mM $MgCl_2$; lane 4 and 8 = 3 mM $MgCl_2$. Lane 1-4 presented the PCR products amplified from undiluted templates and lane 5-8 presented the PCR products amplified from diluted templates.

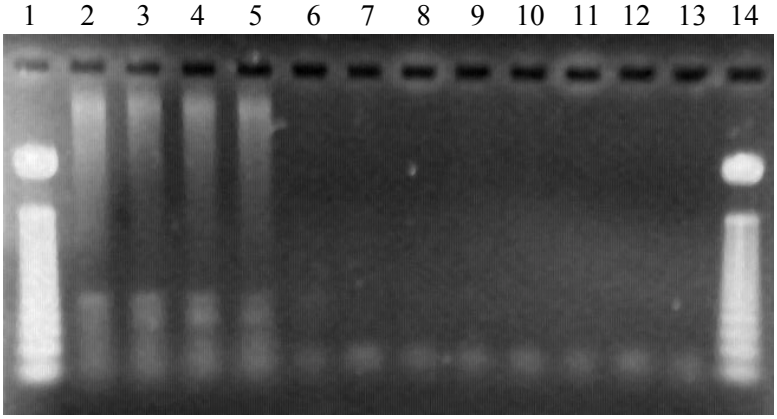
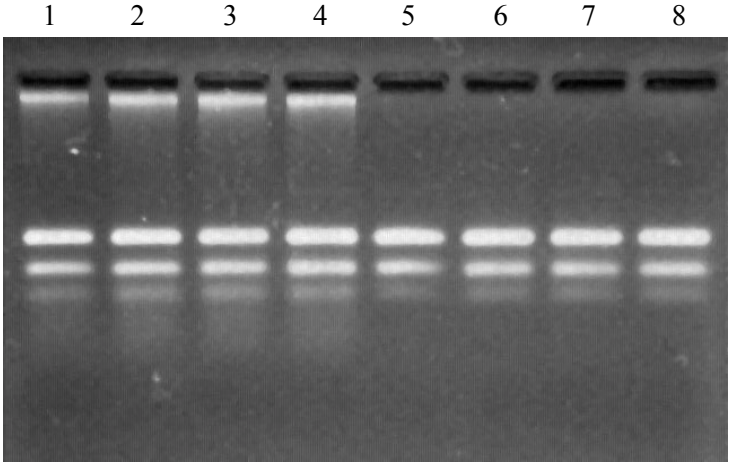


Figure 4.8 PCR products of microsatellite locus L6 with various concentration of $MgCl_2$. Lane 1 and 14 presented 50 bp step ladder; lane 2 and 7 = 1.5 mM; lane 3 and 8 = 2 mM $MgCl_2$; lane 4 and 9 = 2.5 mM $MgCl_2$; lane 5 and 10 = 3 mM $MgCl_2$ and lane 6 and 13 = negative control. Lane 2-5 presented the PCR products amplified from undiluted templates and lane 7-12 presented the PCR products amplified from diluted templates.

We reduced the annealing temperature from 45^{o}C to 42^{o}C for locus L1 but there was no PCR product obtained from all reactions (data were not shown). Thus, we stopped to optimize amplification of this locus. For locus L5 and L6, we increased the annealing temperature from 45^{o}C to 48^{o}C. The non-specific bands were obtained from the amplification reaction of locus L5. In contrast, we were able observed specific bands from amplification reaction from locus L6 when we used undiluted templates (Figure 4.9). Moreover, the intensity of products was increased when the concentration of $MgCl_2$ was increased.

We continued to increase the annealing temperature for amplification of locus L3 and L5. The annealing temperature was increased to 50^{o}C. The amount of amplified products from locus L3 decreased when the concentration of $MgCl_2$ was increased. For locus L5, the intensity of specific PCR product band increased when the concentration of $MgCl_2$ was increased. The non-specific band was still present on the agarose gel but in the lower amount compared with the products obtained from previous condition (Figure 4.10).

a)



b)

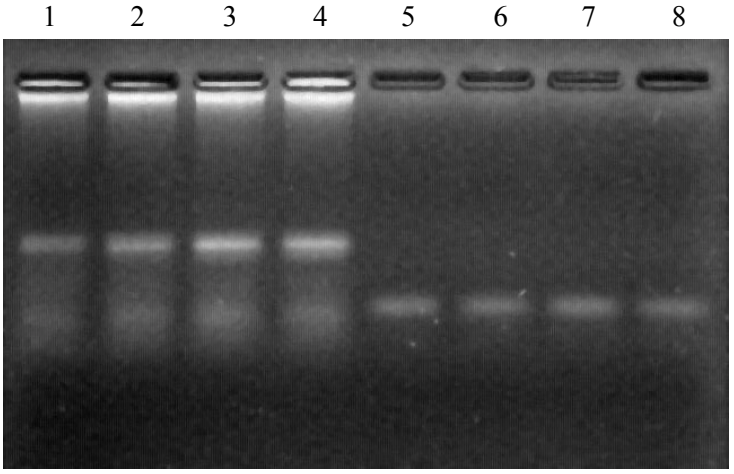


Figure 4.9 PCR products of microsatellite locus L5 (a) and L6 (b) with various concentration of $MgCl_2$. Lane 1 and 5 = 1.5 mM; lane 2 and 6 = 2 mM $MgCl_2$; lane 3 and 7 = 2.5 mM $MgCl_2$; lane 4 and 8 = 3 mM $MgCl_2$. Lane 1-4 presented the PCR products amplified from undiluted templates and lane 5-8 presented the PCR products amplified from diluted templates. The amplification was performed at 48^{o}C annealing temperature.

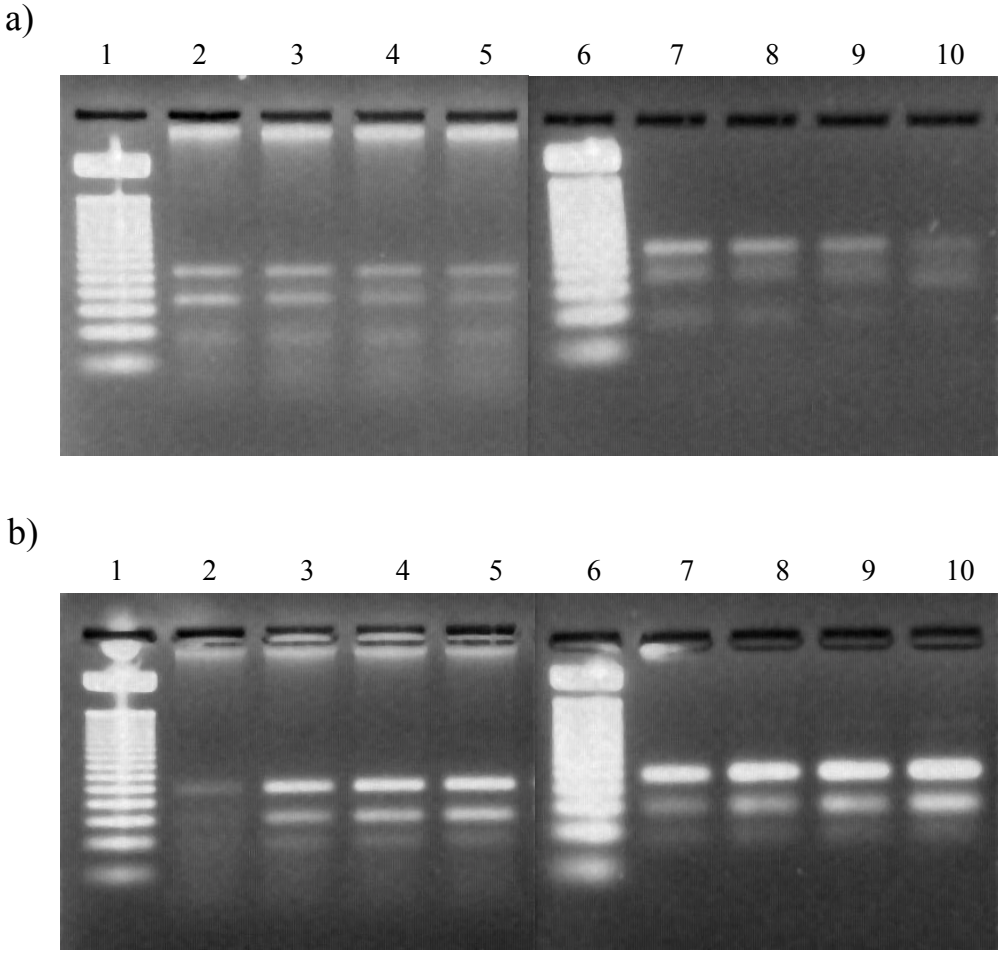


Figure 4.10 PCR products of microsatellite locus L3 (a) and L5 (b) with various concentration of $MgCl_2$. Lane 1 and 6 = 50 bp step ladder; lane 2 and 7 = 1.5 mM; lane 3 and 8 = 2 mM $MgCl_2$; lane 4 and 9 = 2.5 mM $MgCl_2$; lane 5 and 10 = 3 mM $MgCl_2$. Lane 2-5 presented the PCR products amplified from undiluted templates and lane 7-10 presented the PCR products amplified from diluted templates. The amplification was performed at 50^oC annealing temperature.

The optimize conditions for amplification each microsatellite locus were as followed; L2 was amplified with amplification reaction contained 4 mM of $MgCl_2$ at 45^{o}C annealing temperature; L4 and L7 were amplified with amplification reaction contained 3 mM of $MgCl_2$ at 45^{o}C; L3 was amplified with amplification reaction contained 1.5 mM of $MgCl_2$ at 50^{o}C; L5 was amplified with amplification reaction contained 3 mM of $MgCl_2$ at 50^{o}C; L6 was amplified with amplification reaction contained 3 mM of $MgCl_2$ at 48^{o}C.

4.5.2 Amplification microsatellite loci in field samples

We genotyped 127 mono-infected isolates of Kanchanaburi samples; 28 samples collected from 2004, 26 samples collected from 2005, 41 samples collected from 2006 and 32 samples collected from 2007. We chose all first three years samples which were successfully grown *in vitro* and tested mefloquine susceptibility for genotyping. The samples from 2007 were not tested for mefloquine susceptibility.

Of 6 microsatellite markers, we analyzed only samples which had the allele length data at 4 loci (L3, L4, L5 and L7). Twenty (71%), 18 (69%), 34 (83%) and 22 (69%) samples from 2004, 2005, 2006 and 2007, respectively, were able to determine their allele lengths in all four loci and were analyzed using FSTAT software to determine structure of *P. falciparum* populations in different year of collection.

The number of alleles ranged from 4 (locus L4) to 10 (locus L5). Genetic diversity (H_S) per locus varied from 0.416 (locus L4) to 0.690 (locus L5 and L7). The allele frequencies for each locus in each population were shown in Figure 4.11. Sixty-five percents (13 of 20) of parasite samples in the 2004 population possessed the allele 2 at microsatellite locus L3 as in the 2006 population; most of parasites (68%, 23 of 34) had allele 2 genotype at this locus. While, in the others 2 populations, there were 44% (8 of 18) and 33% (6 of 18) of the 2005 population possessed the allele 2 and allele 3, respectively, 55% (12 of 22) and 23% (5 of 22) of the 2006 population possessed the allele 2 and allele 6, respectively.

For locus L4, 50% (10 of 20) and 25% (5 of 20) of the 2004 population had the allele 2 and allele 3, respectively while majority of population in the other 3

populations had the allele 3 at this locus (70%, 13 of 18; 82%, 28 of 34; 86%, 19 of 22 in the 2005, 2006 and 2007 populations, respectively).

Locus L5, the majority of the 2004 population had the allele 1 (75%, 15 of 20) while the other populations had 2 majority of population contributed in the allele 1 and allele 9 (the 2005 and 2006 populations) and in the allele 1 and allele 5 (the 2007 population) in the proportion nearly 40% in each allele.

The last locus, L7, 65% (13 of 20) of the 2004 population had the allele 2. The 2005 population had 2 major alleles which were the allele 2 (39%, 7 of 18) and the allele 3 (33%, 6 of 18). The 2006 and 2007 populations had 3 major alleles, the allele 2 (41%, 14 of 34; 36%, 8 of 22 in the 2006 and 2007 populations, respectively), the allele 3 (26%, 9 of 34; 27%, 6 of 22 in the 2006 and 2007 populations, respectively), the allele 4 (24%, 8 of 34; 32%, 7 of 22 in the 2006 and 2007 populations, respectively) (Figure 4.11). The differences in allele distribution in all population may have an effect on the genetic differentiation of these populations.

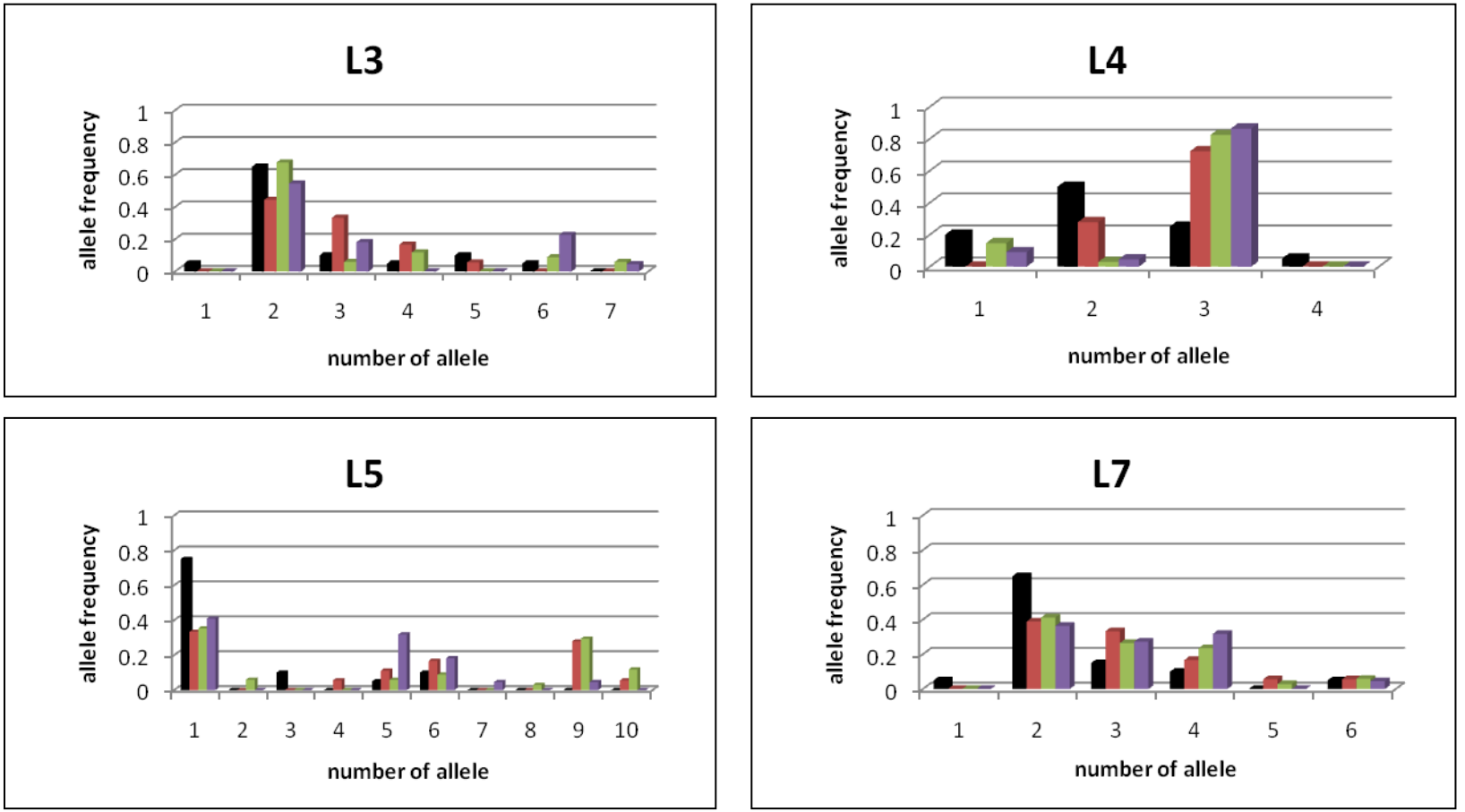


Figure 4.11 Number of alleles and allele frequencies observed for *P. falciparum* at each microsatellite locus around *pfmdr 1* gene in the different 4 year sampling. 2004 2005 2006 2007

Gene diversity (H_S) per locus in each population was shown in Table 4.16. We noticed that there was a decreasing trend in the gene diversity at the locus L4 at the different year of collection. Besides, the gene diversity at the locus L5 of the 2004 was nearly 2 times lower than those of other populations.

Table 4.16 Gene diversity per locus of 4 populations at different year of sampling

locus	2004	2005	2006	2007
n	20	18	34	22
L3	0.579	0.699	0.529	0.645
L4	0.679	0.425	0.308	0.255
L5	0.437	0.810	0.783	0.727
L7	0.568	0.745	0.722	0.723
H_S±SD	0.57±0.10	0.67±0.17	0.59±0.21	0.59±0.22

Significant linkage disequilibrium among populations was observed at *p-value* = 0.002 in 6 combined pairs of combinations in the 2006 population (L3 x L5) and the 2007 population (L3 x L5 and L3 x L7).

Genetic differentiation between years of collection was also tested; a low but significant differentiation (*p-value* = 0.008) was detected between 2004 population versus 2006 and 2007 population as shown in Table 4.17.

Table 4.17 Genetic differentiation (F_{ST}) between *P. falciparum* in Kanchanaburi populations at different year of collection observed with microsatellite loci around *pfmdr 1* gene.

F_{ST}	2004	2005	2006	2007
2004	-	0.1021	0.1412*	0.1476*
2005		-	0.0125	0.0175
2006			-	0.0140
2007				-

* significant at *p-value* = 0.008 after Bonferroni correction for multi test

For genotype distribution in four year of collection, 45 genotypes were obtained from the 94 examined individuals (see in Appendix IV). Among these different genotypes, 13 identical genotypes were observed. A number of individual with the same genotype was shown in Table 4.18. Many identical genotypes of parasites were found in the following year of parasite collection. This finding was different from those found in the examination with microsatellite loci which not under drug pressure in previous experiments.

To clarify the observation that a variety of microsatellite genotypes inside and around *pfmdr 1* gene were found in these populations, the *pfmdr 1* gene of some parasites which possessed different pattern of genotypes were sequence in the next part.

Table 4.18 The numbers of identical genotypes presented in each year

Genotype	2004 n = 20	2005 n = 18	2006 n = 34	2007 n = 22	total
1	4	-	3	1	8
2	6	2	-	-	8
3	-	-	2	-	2
4	2	-	4	3	9
5	-	2	1	3	6
6	-	-	-	3	3
7	-	1	4	1	6
8	-	1	2	-	3
9	-	-	4	-	4
10	-	1	-	2	3
11	-	1	1	-	2
12	-	1	1	-	2
13	-	-	3	3	6
	12	9	25	16	62

4.6 Sequence analysis of multidrug resistant gene of *P. falciparum* in Kanchanburi populations

4.6.1 Optimization of polymerase chain reaction

We optimized the condition of the PCR reaction by varying the concentration of $MgCl_2$ from 1.5 mM to 4 mM with the increment of 0.5. The optimal condition to amplify all fragments of *pfmdr 1* gene was as following; 300 ng of chromosomal DNA of clone T9/94RC17, 1.5 mM of $MgCl_2$, 125 μM of each deoxynucleoside triphosphate, 5 pmole of each primer and 0.4 units of *Taq* DNA polymerase in 20 μl reaction. The thermal cycling condition was as following, denaturation at 94^{o}C for 3 minutes, 30 cycles at 94^{o}C for 45 seconds, 50^{o}C for 30 seconds and 68^{o}C for 2 minutes and final extension step at 68^{o}C for 5 minutes. We found a sharp band of PCR product from all primer pairs as shown in Figure 4.12. All primer pairs were specific to *P. falciparum*, there was no PCR product obtained from human white blood cell chromosomal DNA used as negative control.

4.6.2 Amplification of *pfmdr 1* gene in field samples

For amplification of this gene in field samples, we did PCR twice with the same reaction condition except for the concentration of primers in the first round was 2.5 pmole which were reduced in a half of concentration of the second round reaction. The PCR product size was consistent with that of product from positive control (Figure 4.13). These PCR products would be further purified and sequenced.

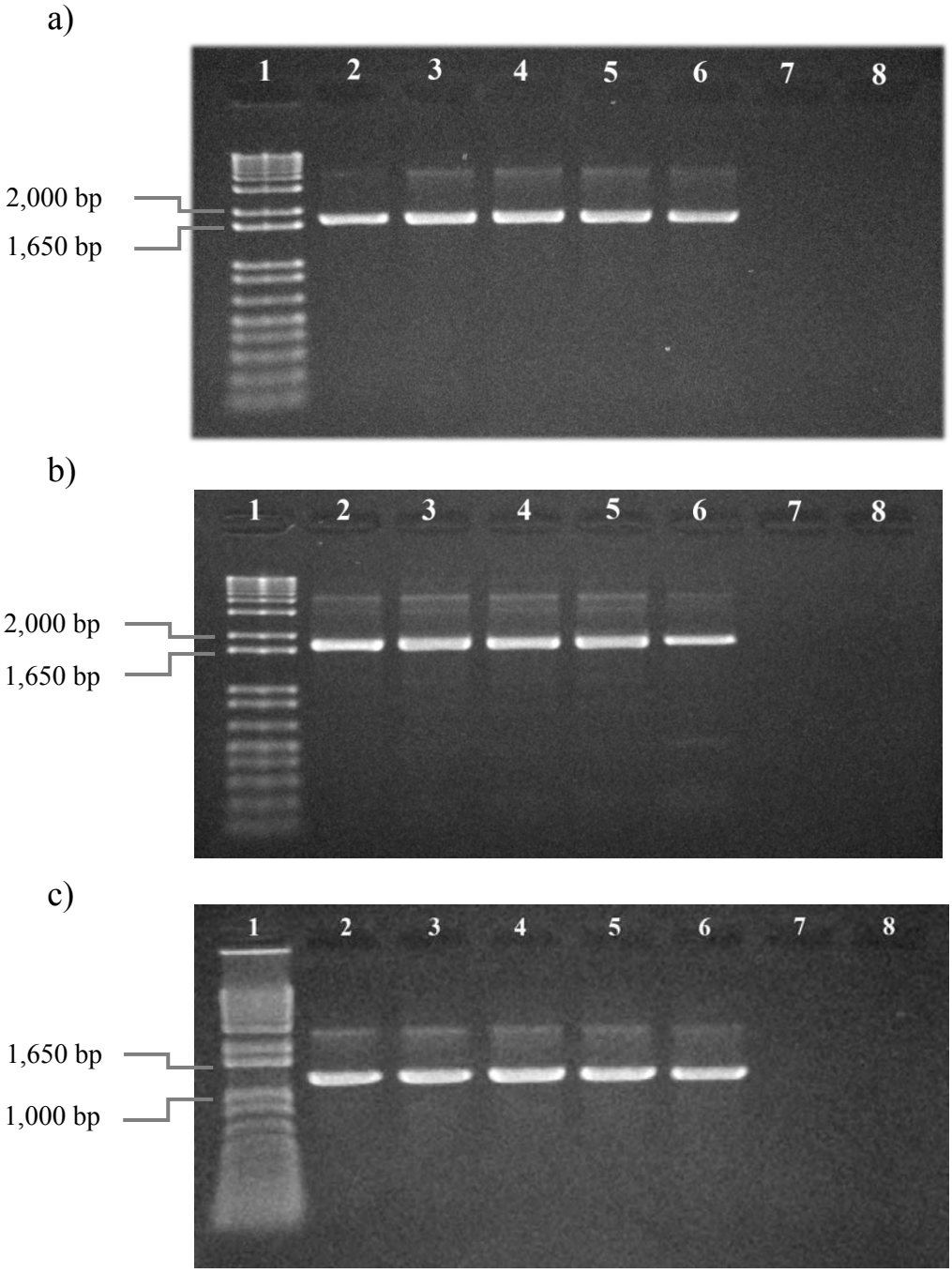


Figure 4.12 *pfmdr 1* PCR products fragment 1 (a), fragment 2 (b) and fragment 3 (c), which were amplified using DNA sample from *P. falciparum* T9/94RC17 with various concentration of $MgCl_2$. Lane 1 = 1 kb DNA ladder, lane 2 - 6 represented various concentration of $MgCl_2$ 1.5, 2, 2.5, 3 and 4 mM $MgCl_2$, lane 7 = human white blood cell chromosomal DNA and lane 8 = negative control.

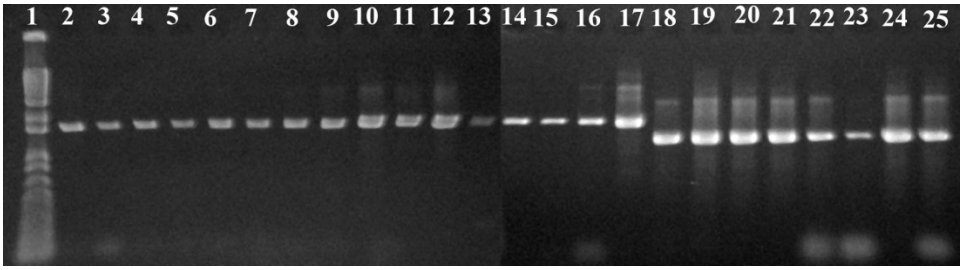


Figure 4.13 *pfmdr 1* PCR products amplified from field isolates using 2 round PCR amplification. Lane 1 = 1 kb DNA ladder, lane 2-8 = PCR products of fragment 1, lane 9-17 = PCR products of fragment 2 and lane 18-25 = PCR products of fragment 3.

4.6.3 Multidrug resistant gene sequence analysis

Sixteen field samples which had different types of genotype examined by microsatellite markers surrounding *pfmdr 1* gene and a laboratory parasite clone were amplified and sequenced. We focused on the field samples that had repetitive genotype which was found in samples collected in different years. We reasoned that this may reflect that this repetitive genotype may transfer to the next generation of parasite in this area. It is also possible that the genotype which was carried by more number of parasites would be spread more readily in this area. The selected samples had mefloquine response ranging from $7x10^{-8}$ – $5x10^{-7}$ M. The 4,200 bp in length of these gene sequences of 13 selected and the laboratory clone samples were successfully done. These sequences were edited and aligned with the sequence of reference clone 3D7 using Bioedit software as shown in Figure 4.14. Due to different number of repeats were detected in this gene of distinct samples, we edit those sequences in repetitive region in comparable number with that of 3D7. This may lead to ease for position comparison.

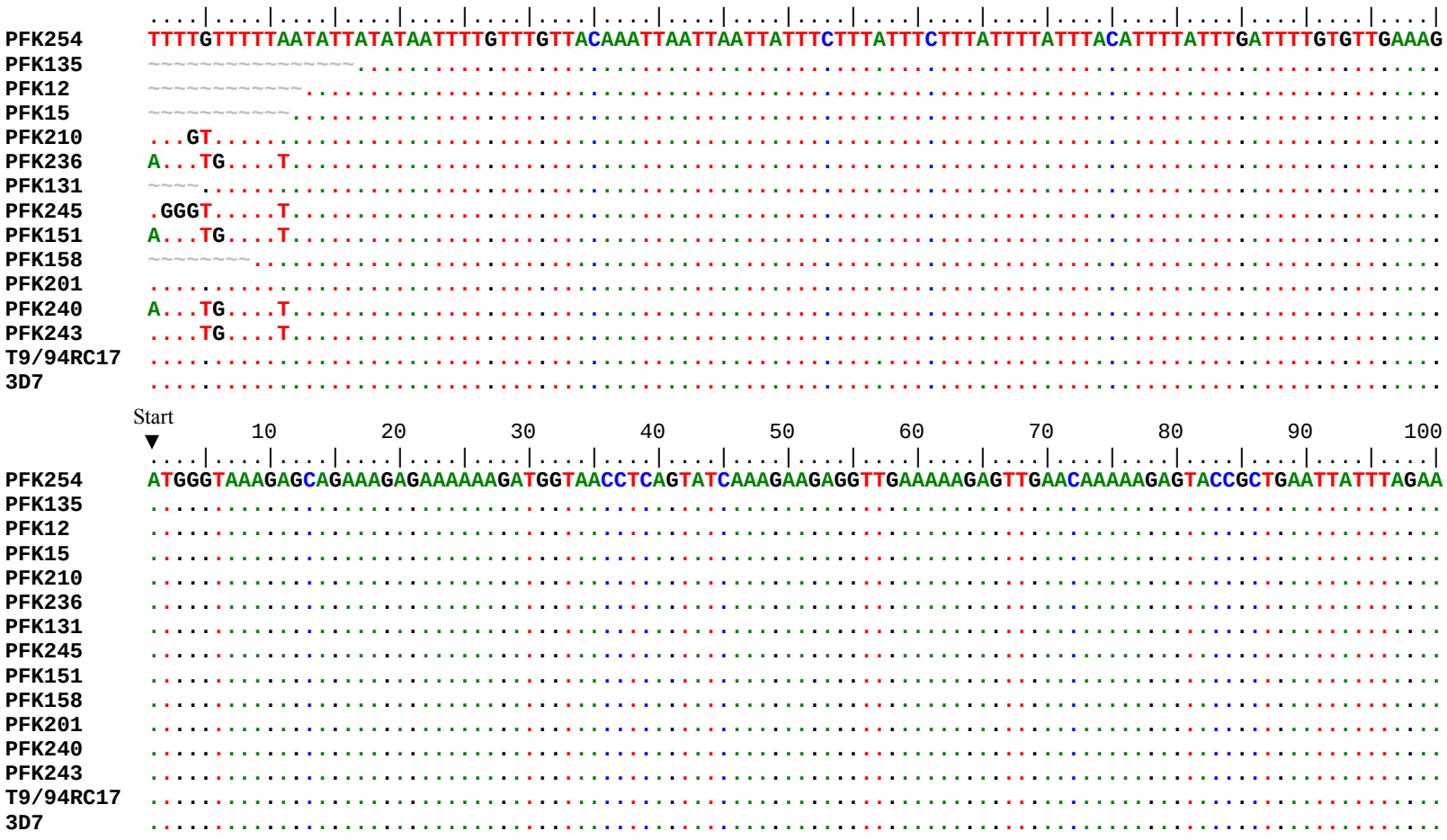


Figure 4.14 Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

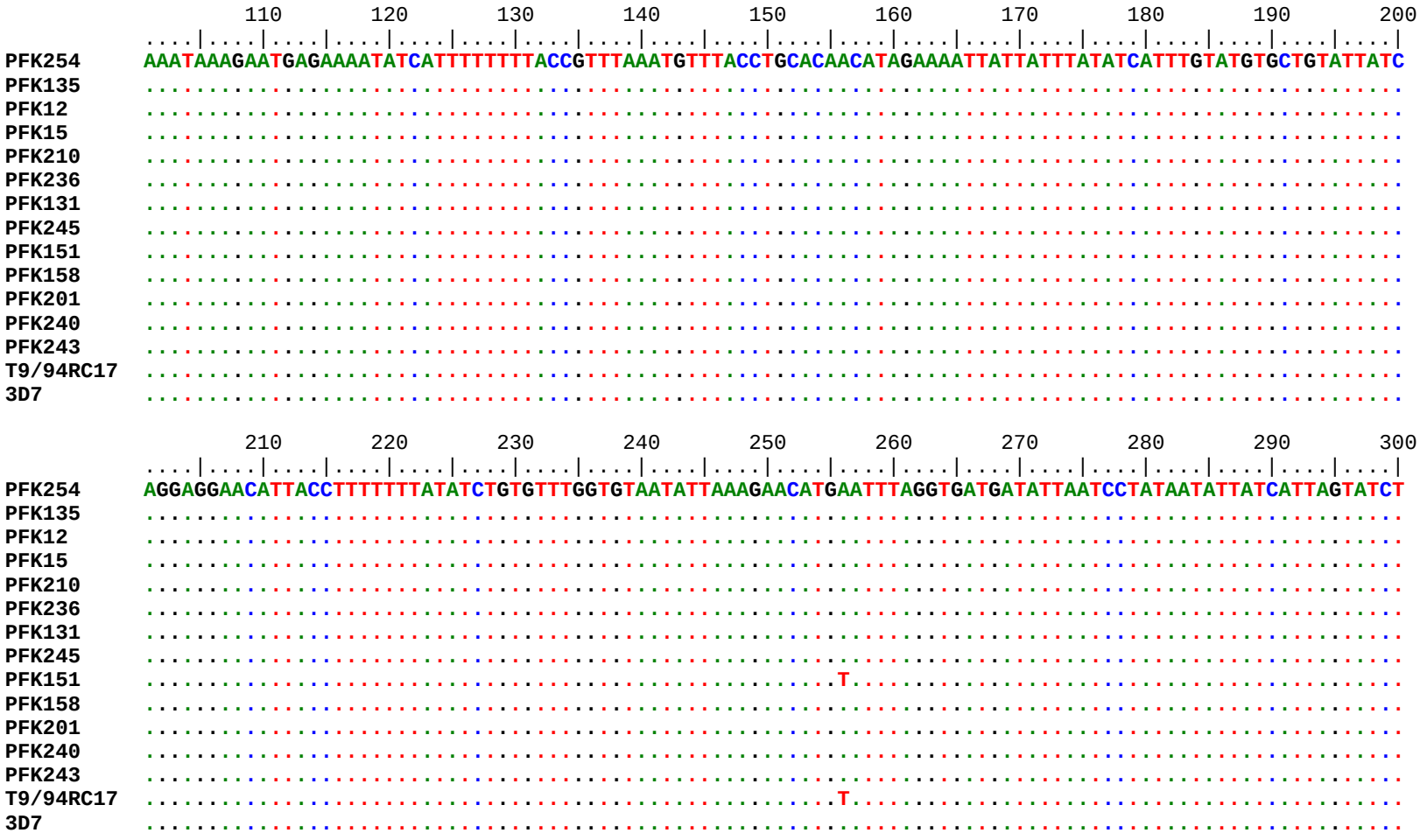


Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

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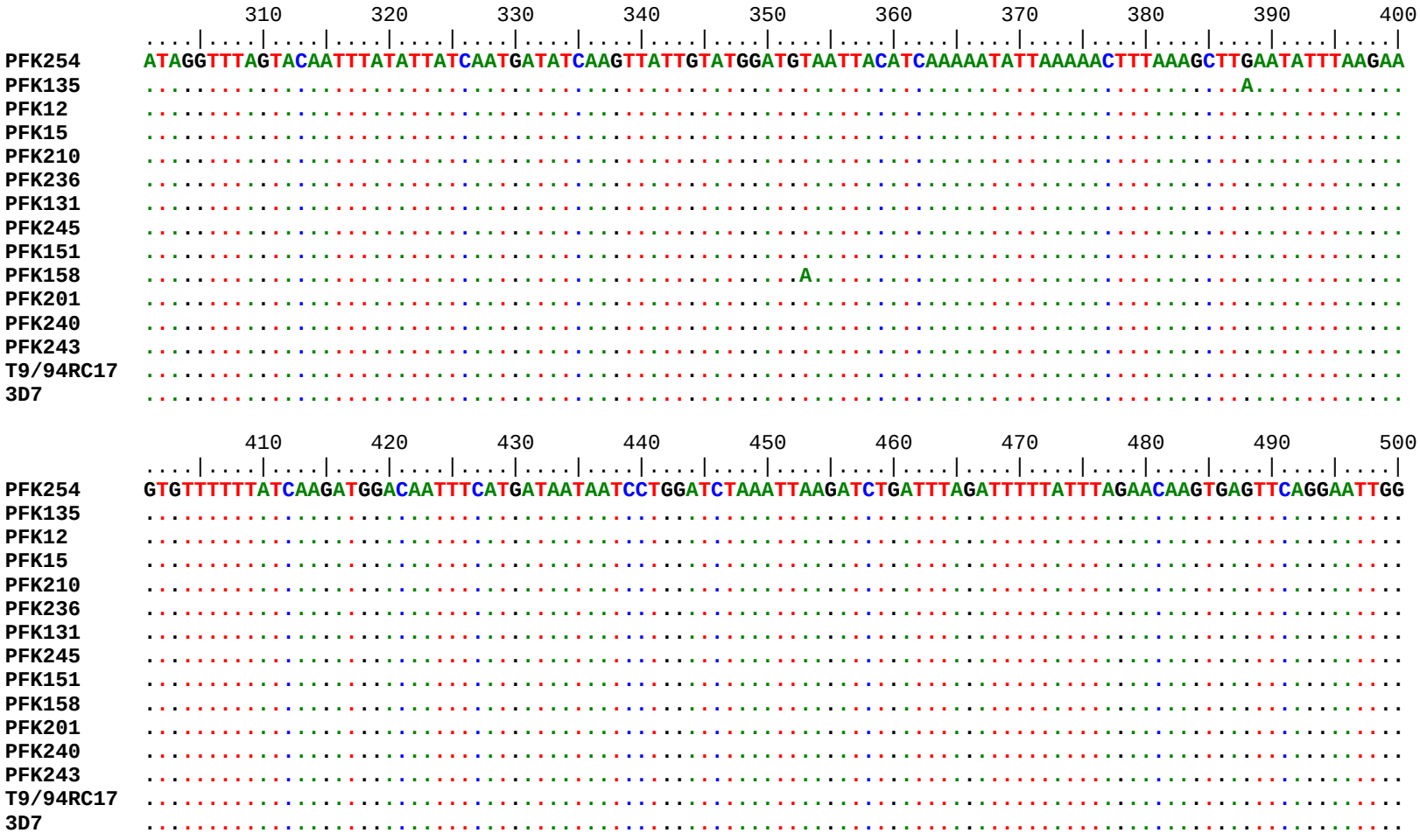

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

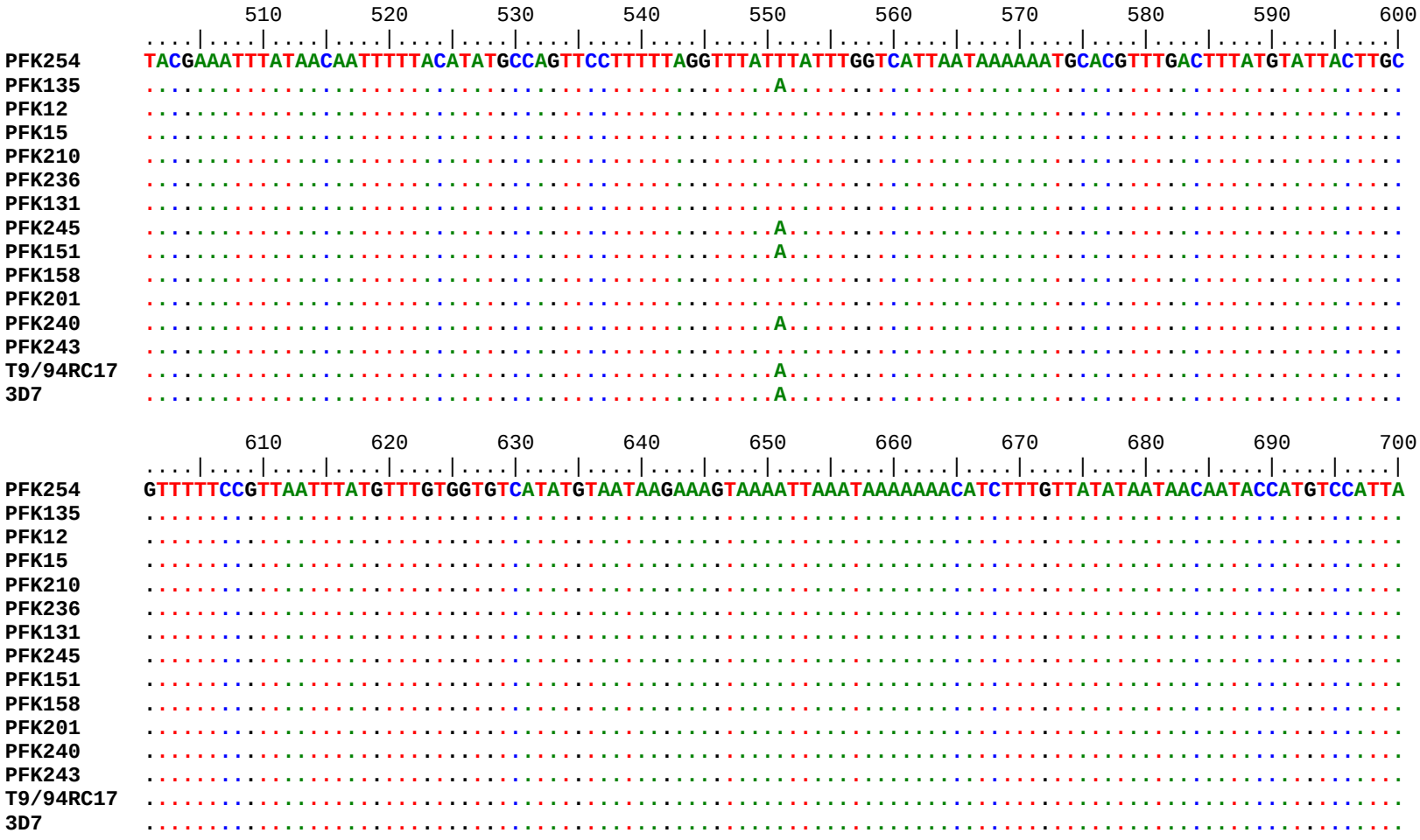

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

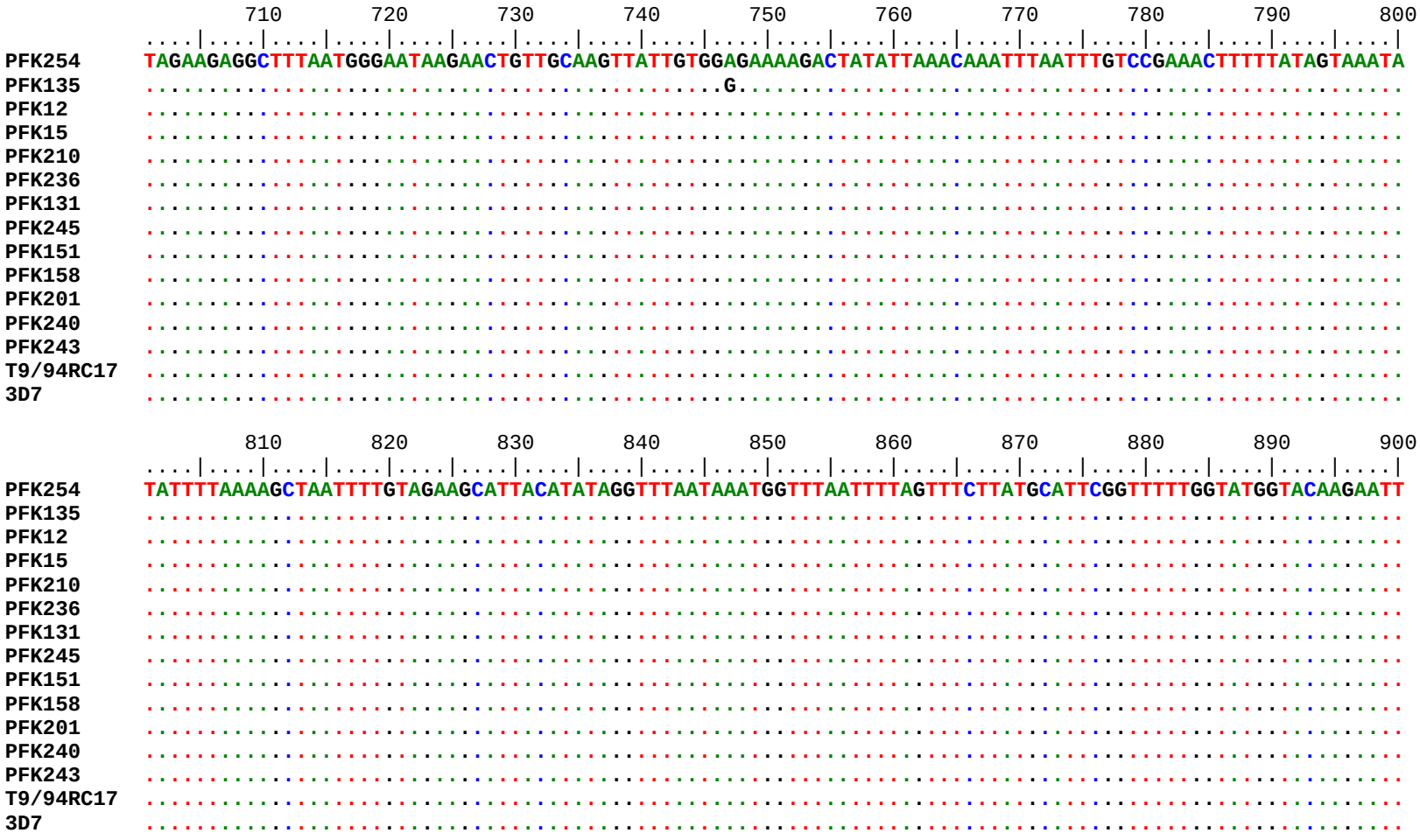

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

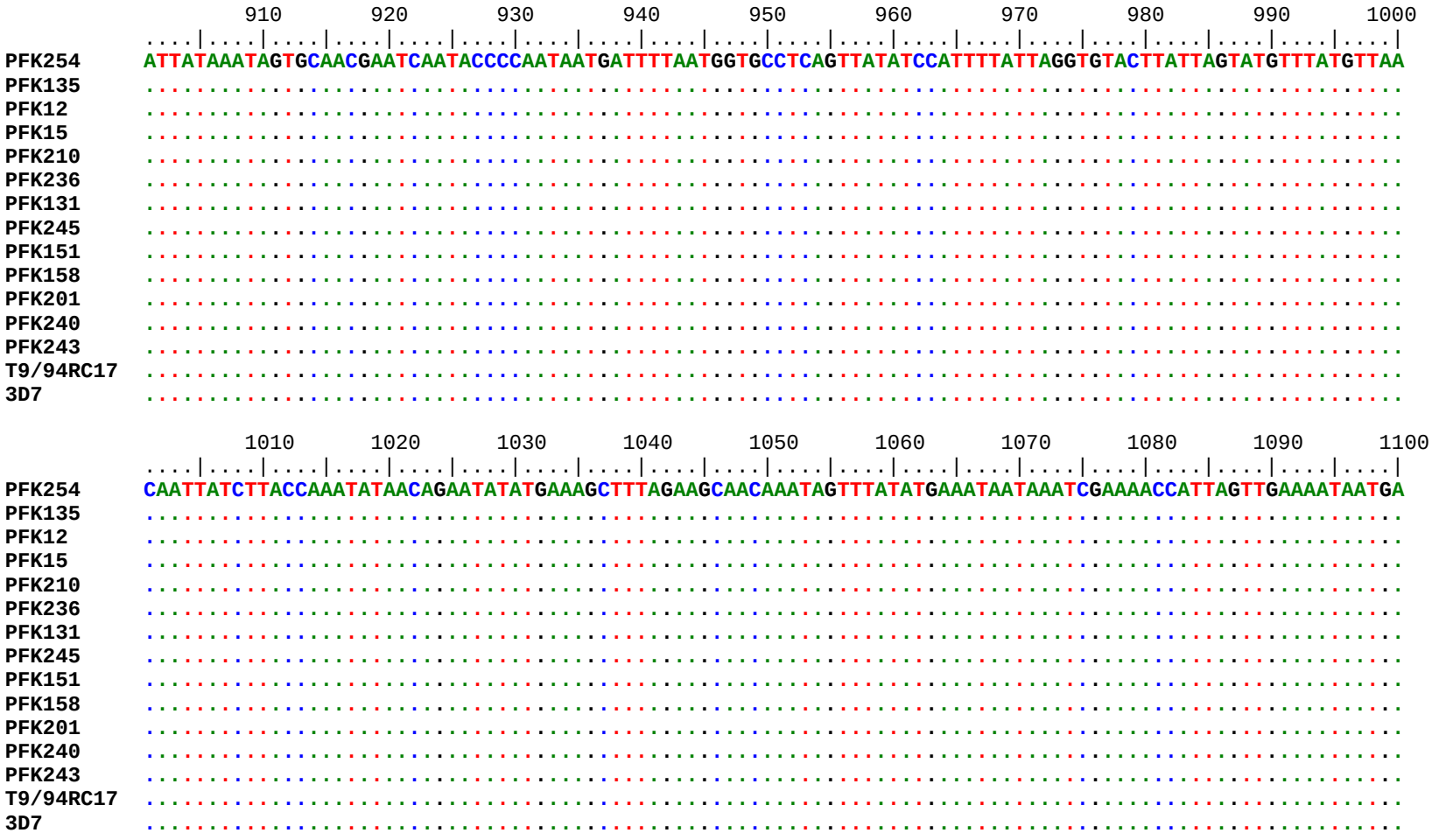

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

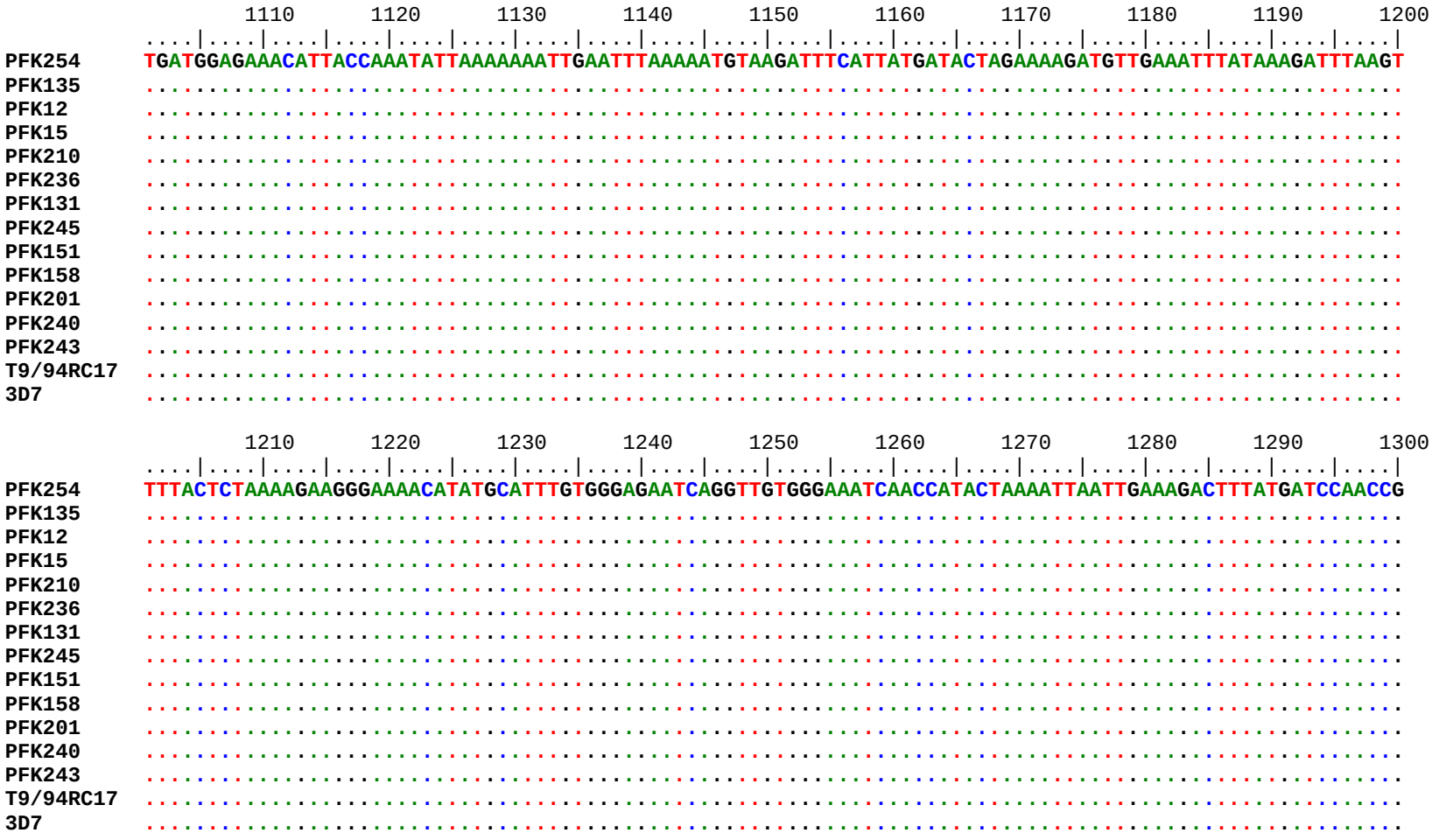

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

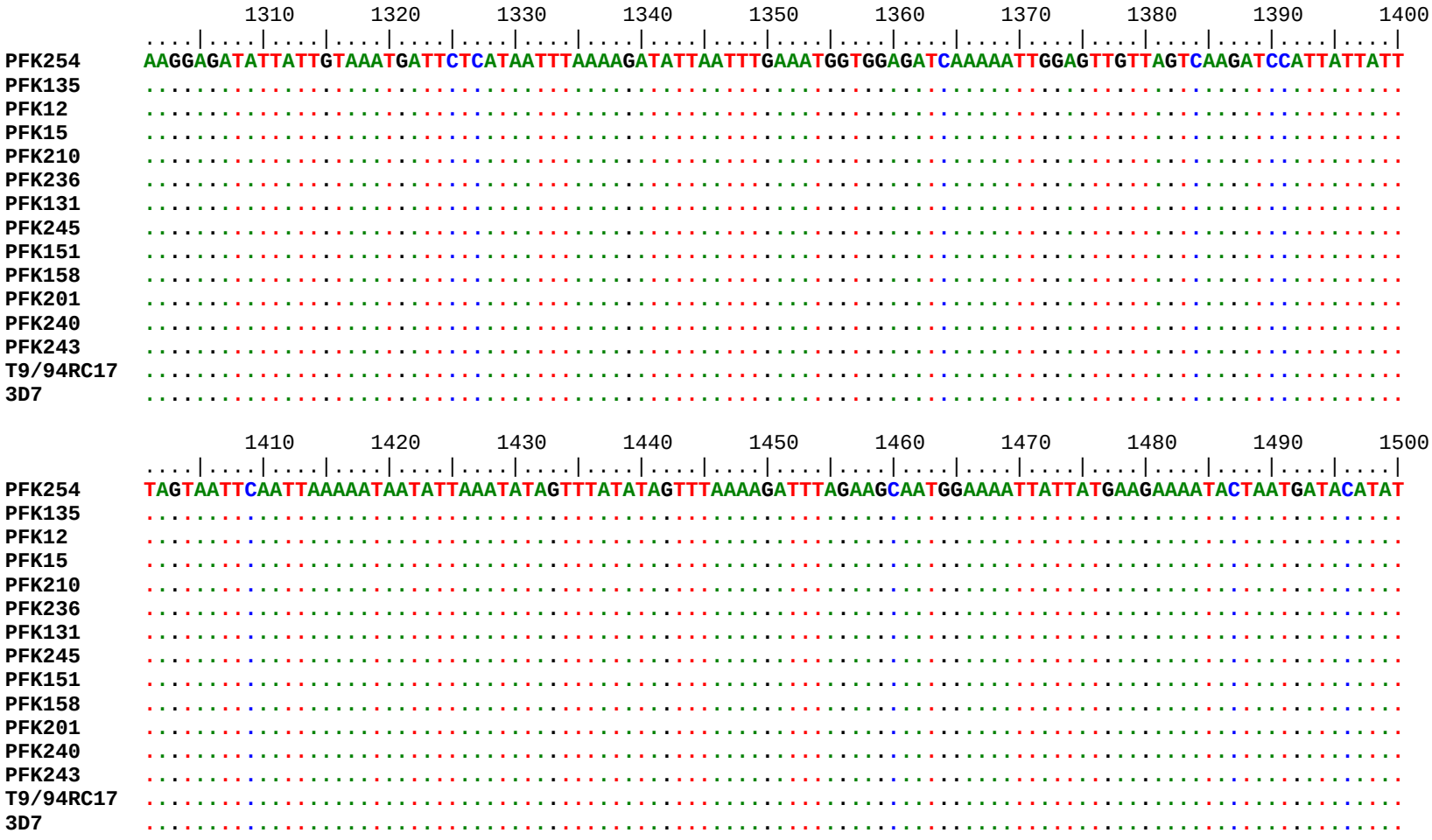

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

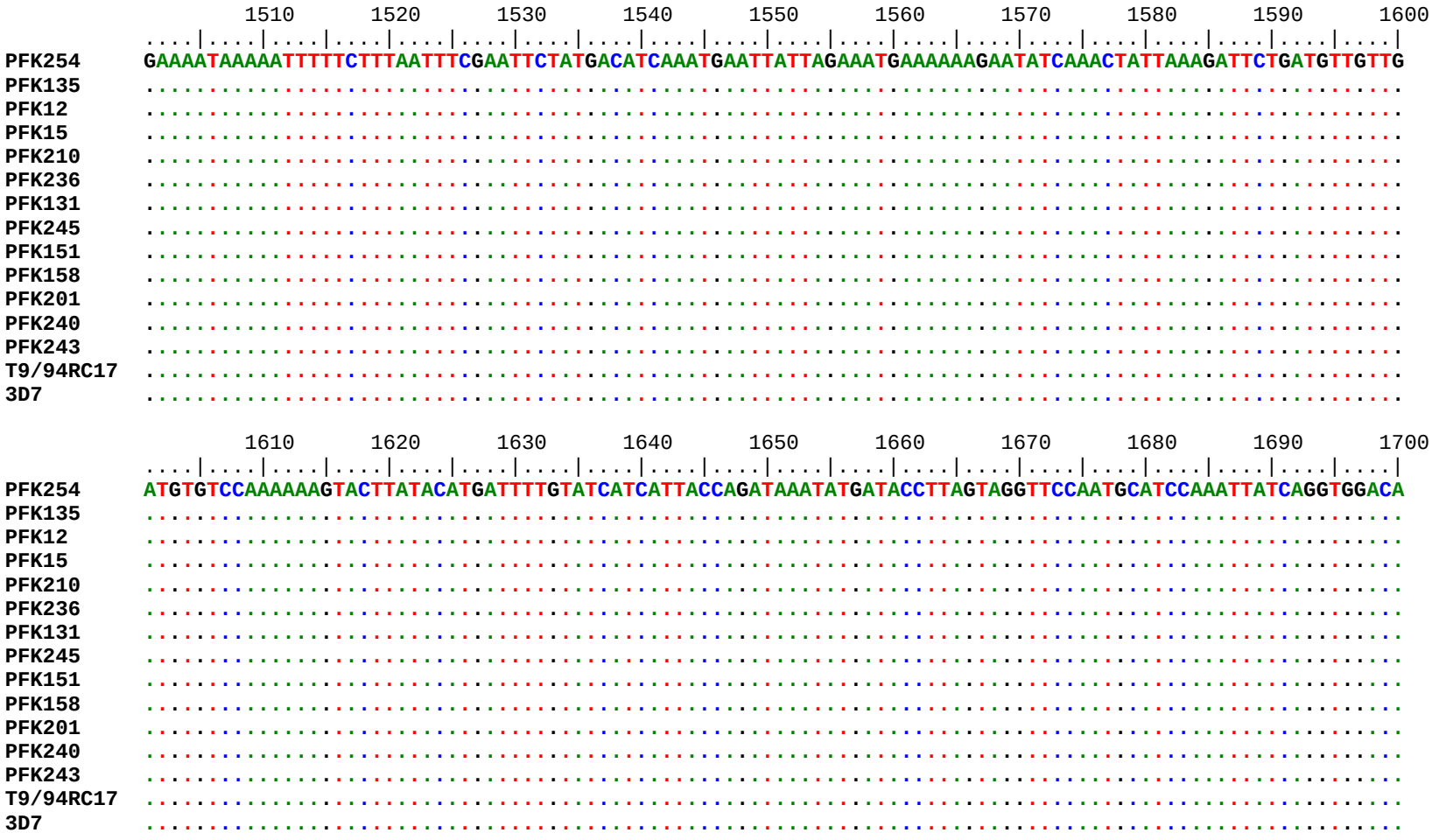

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

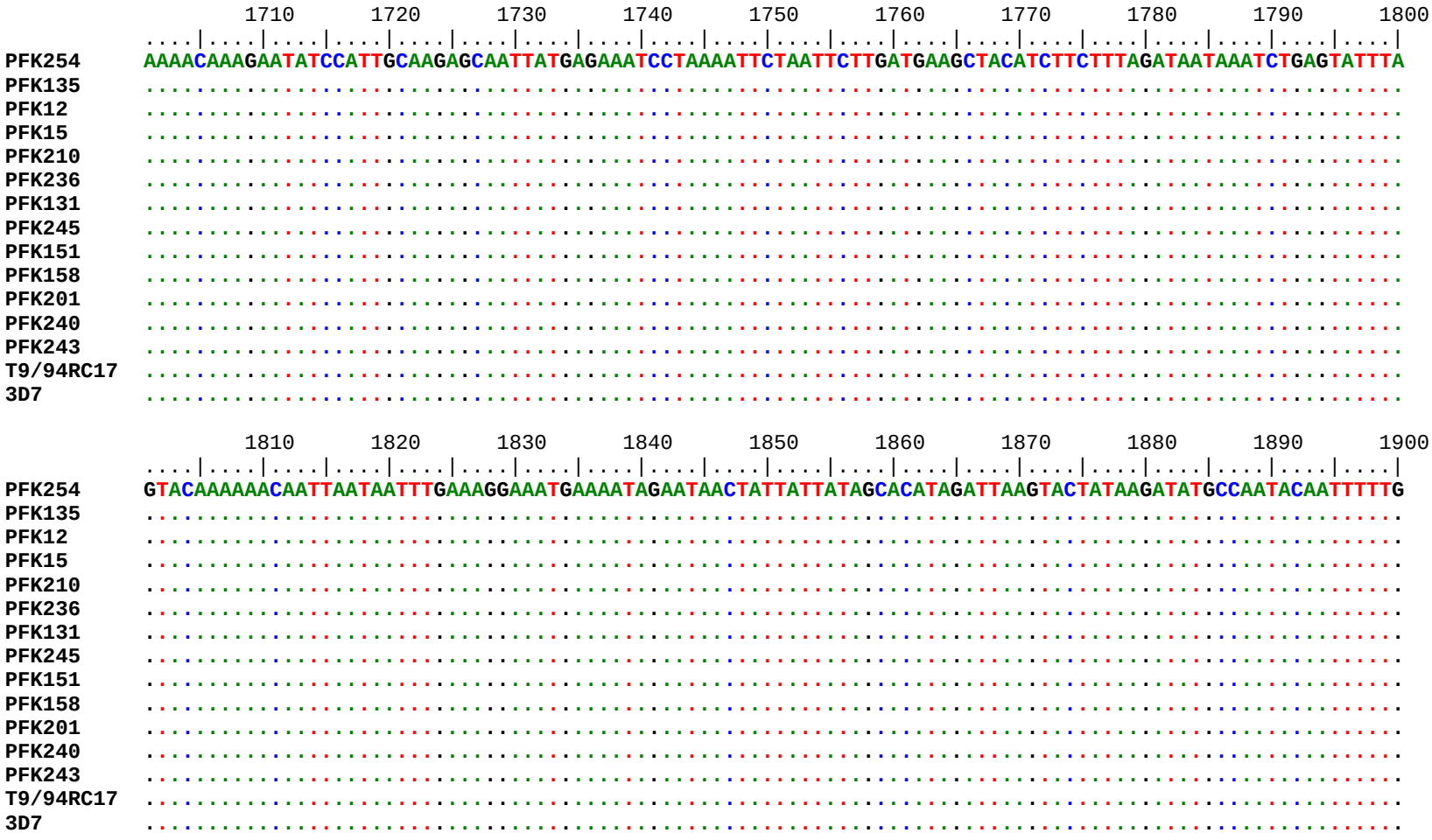

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

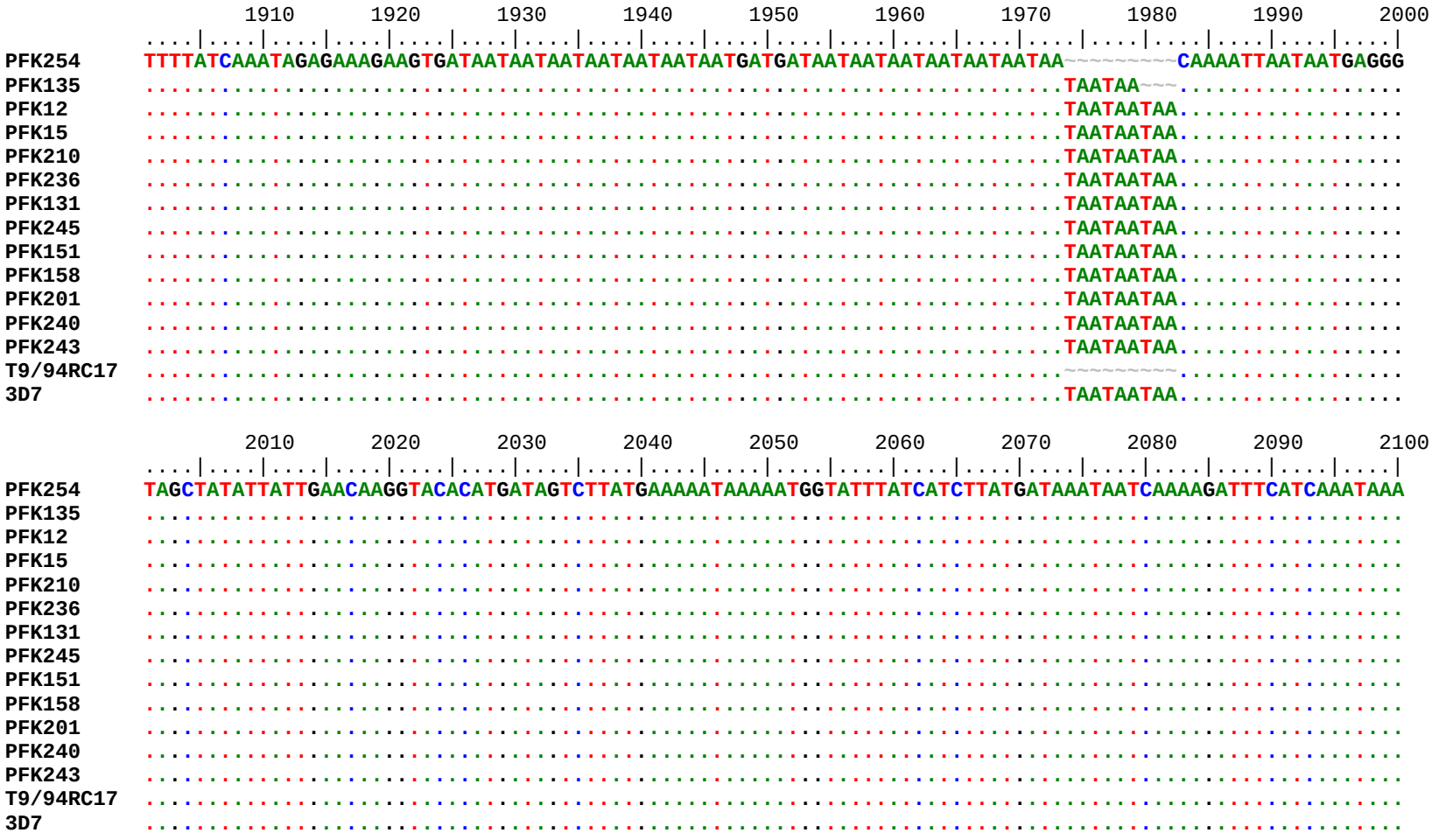

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

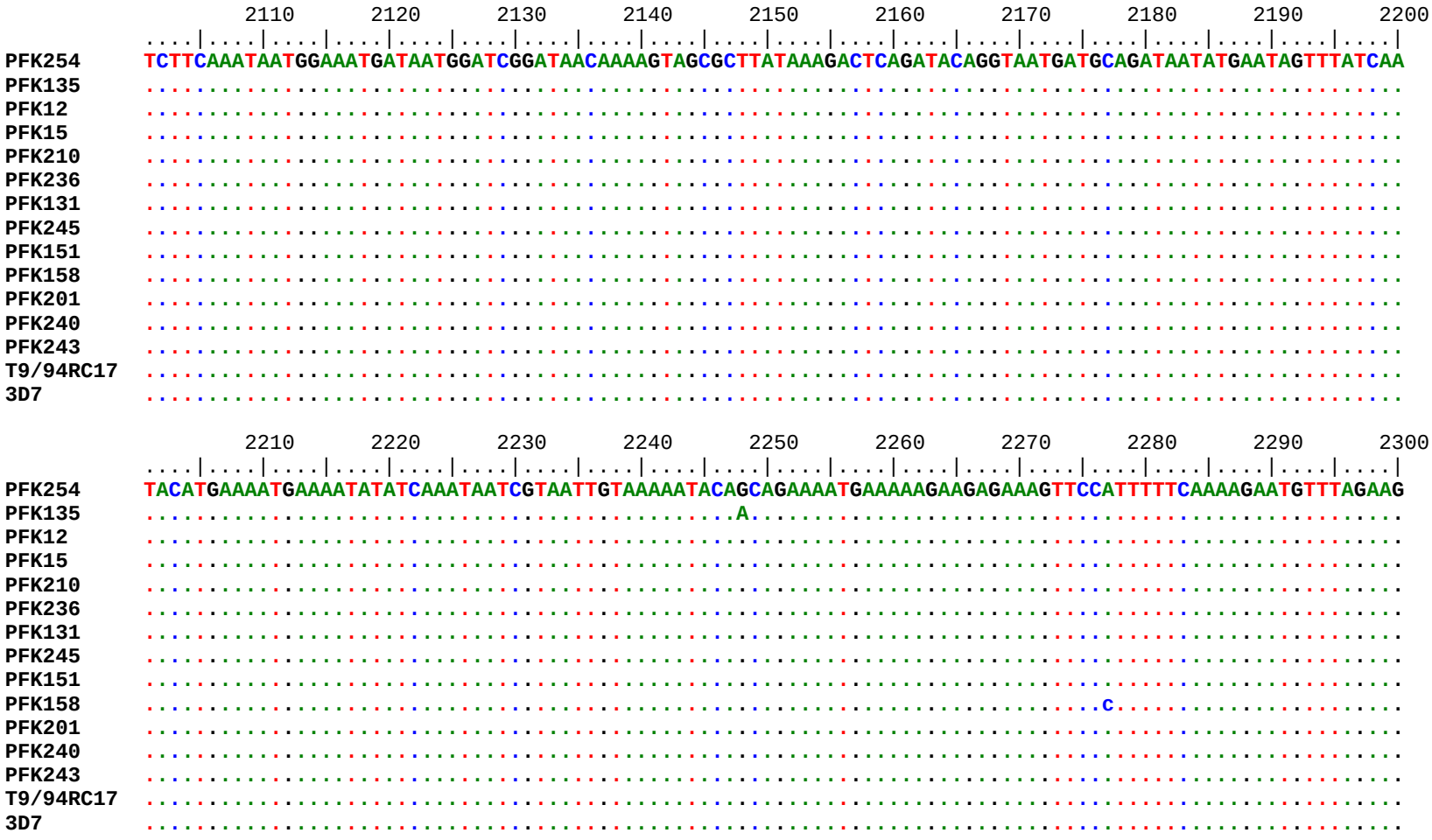

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

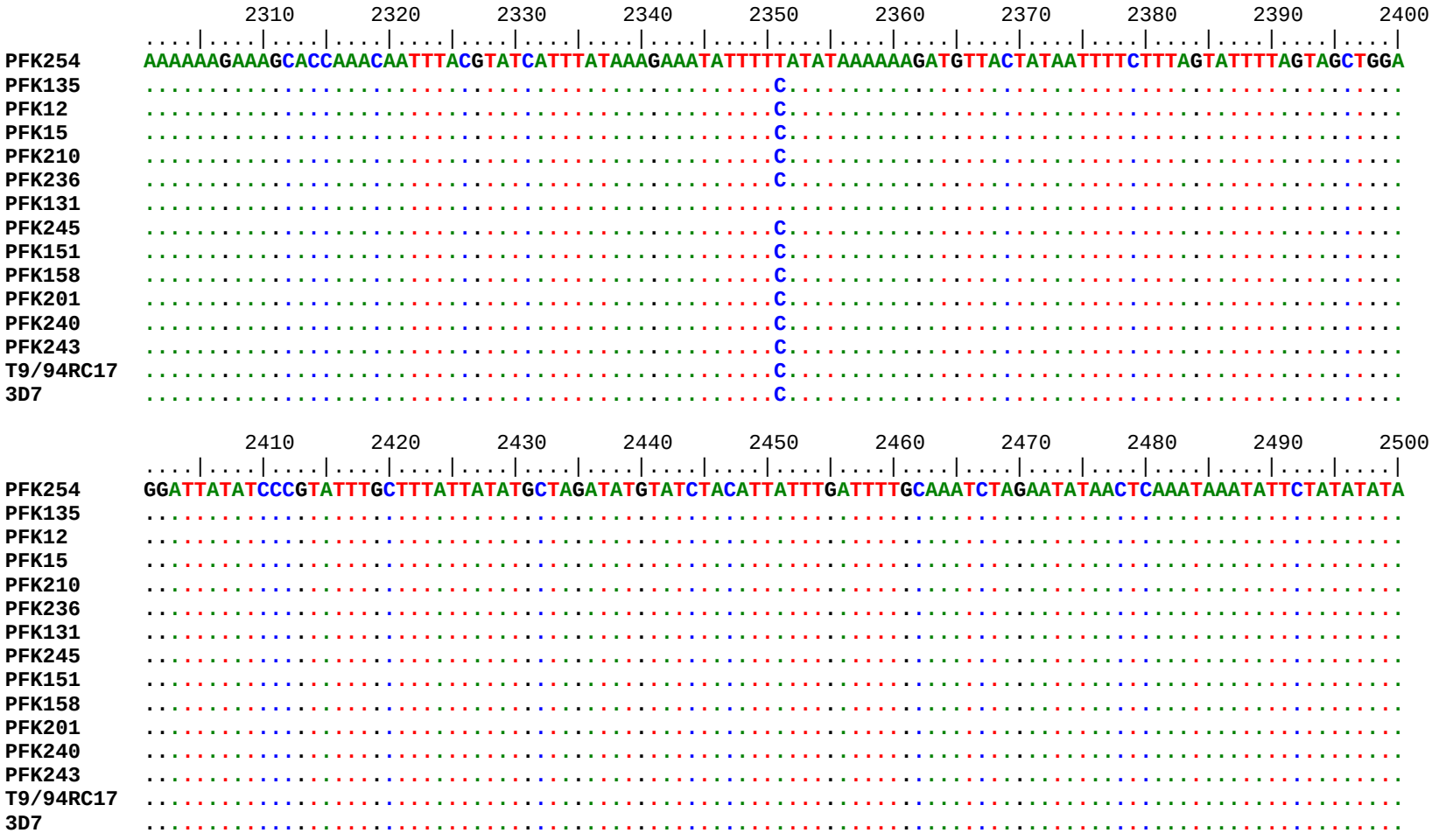

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

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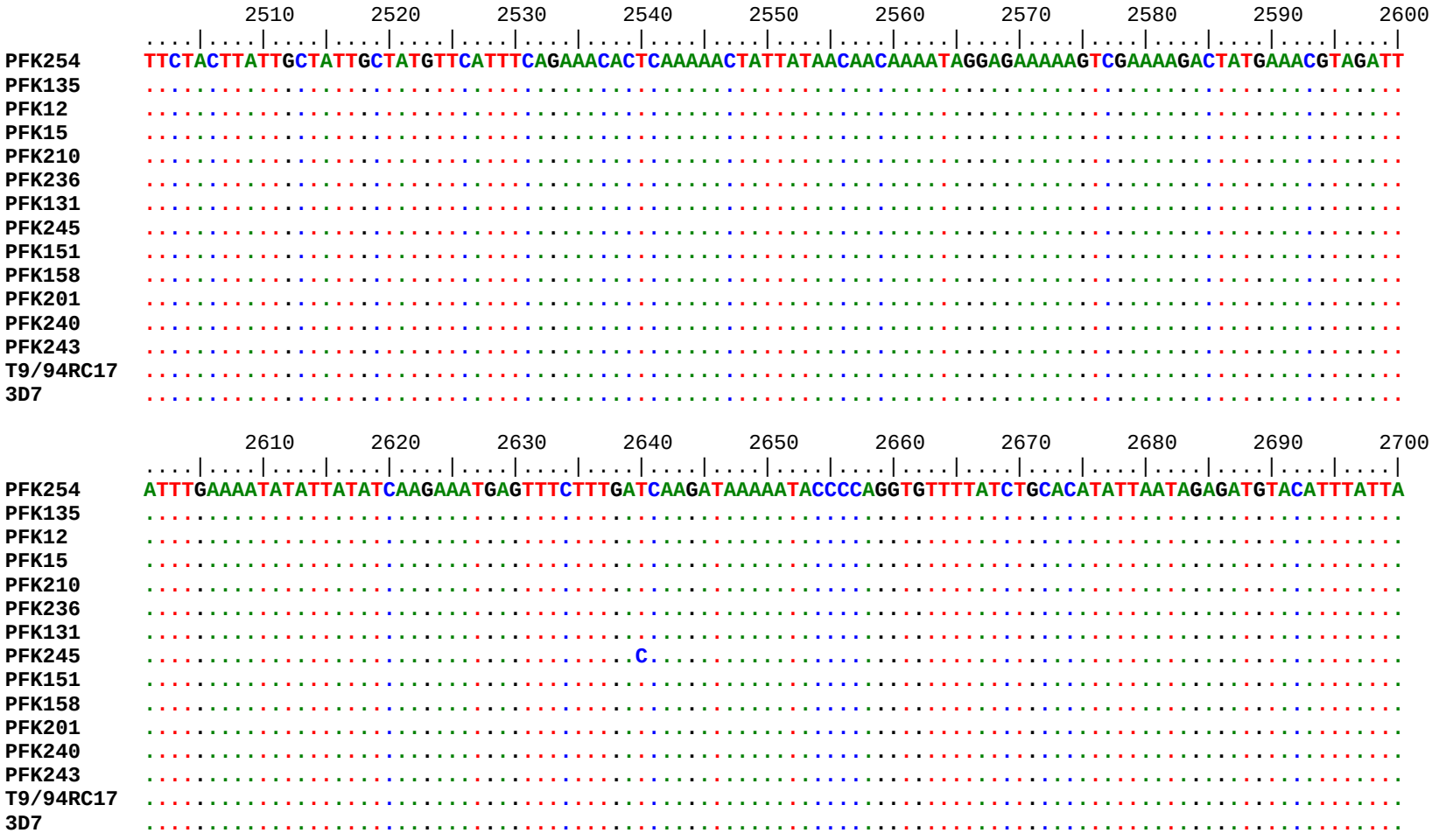

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

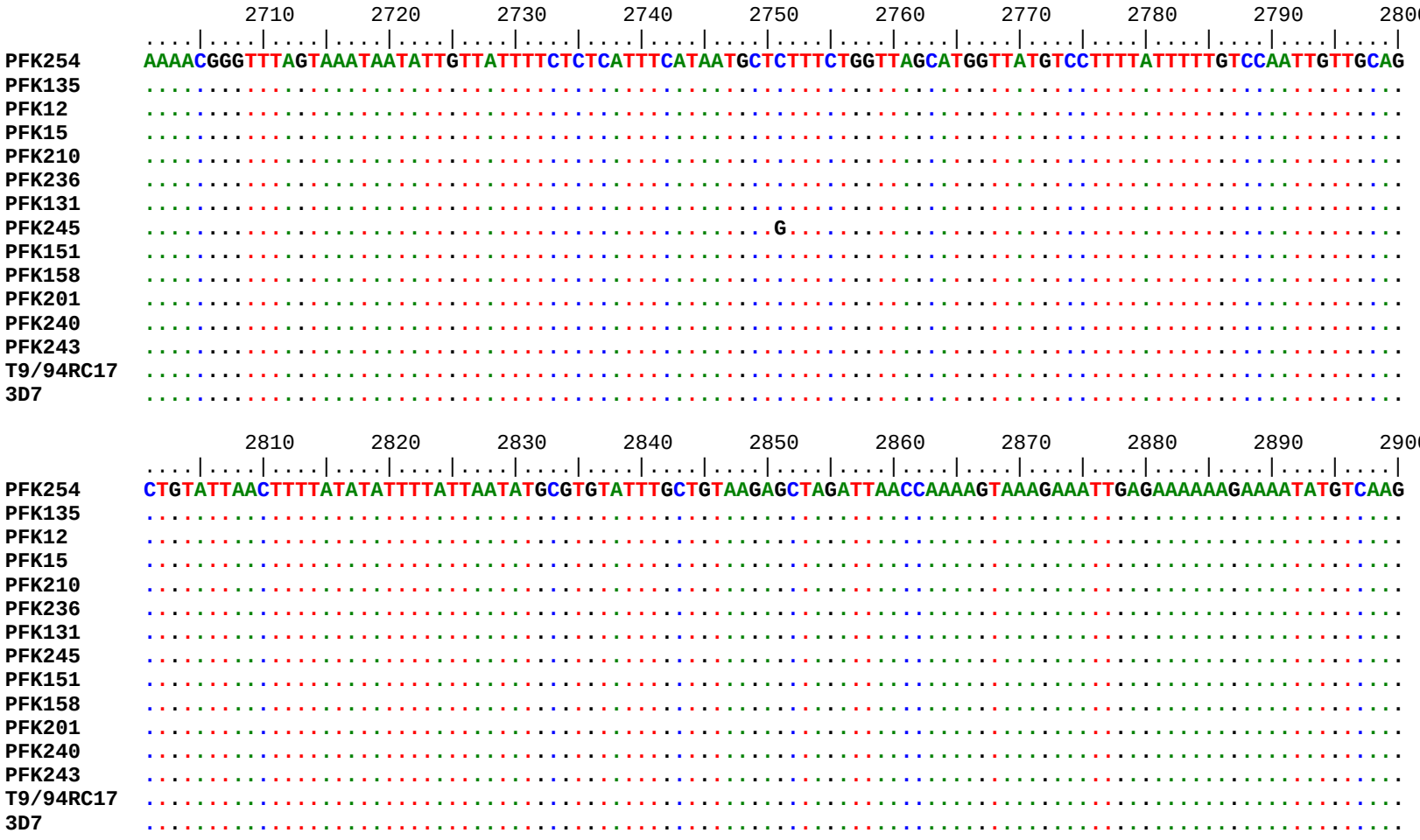

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

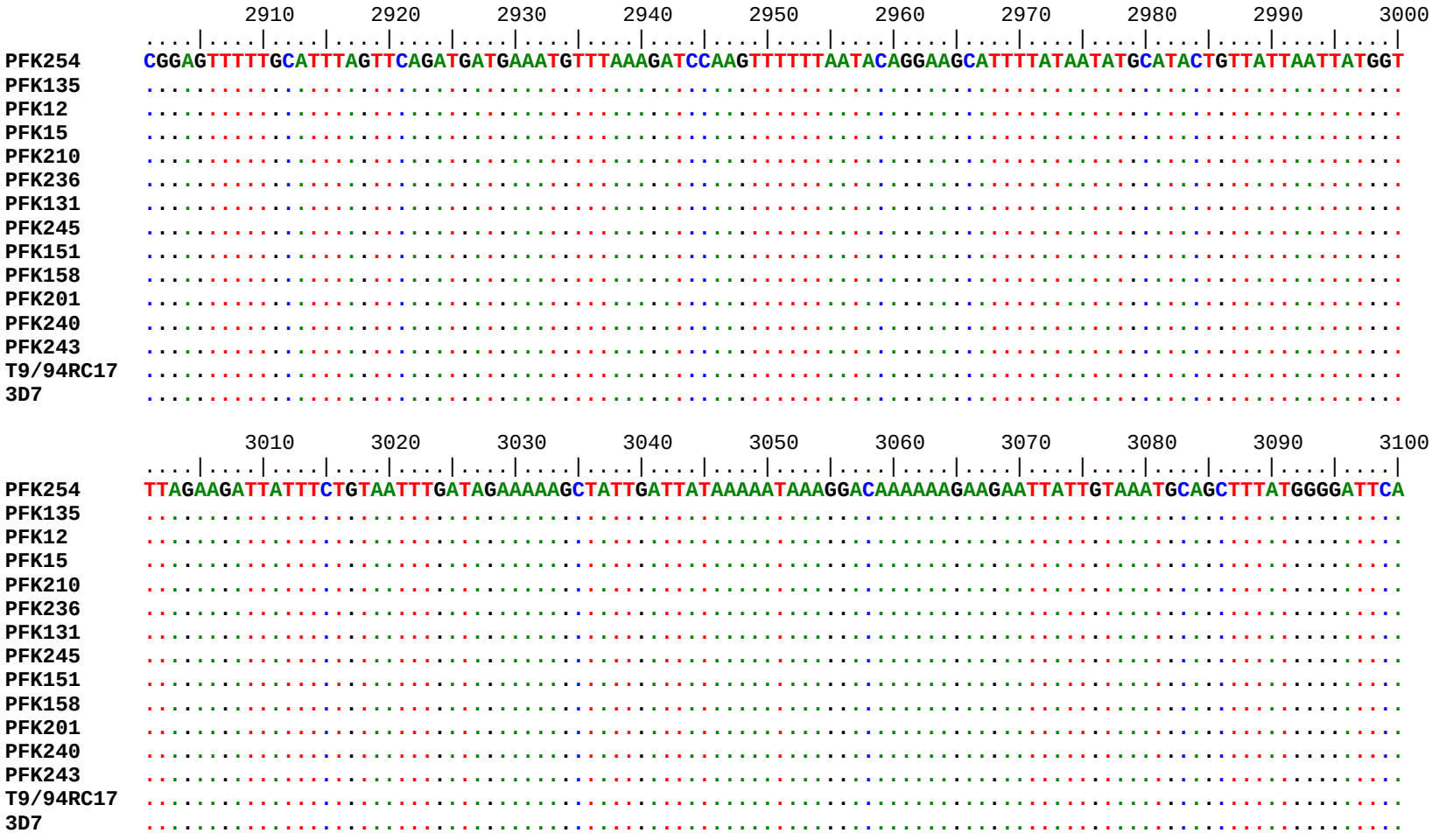

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

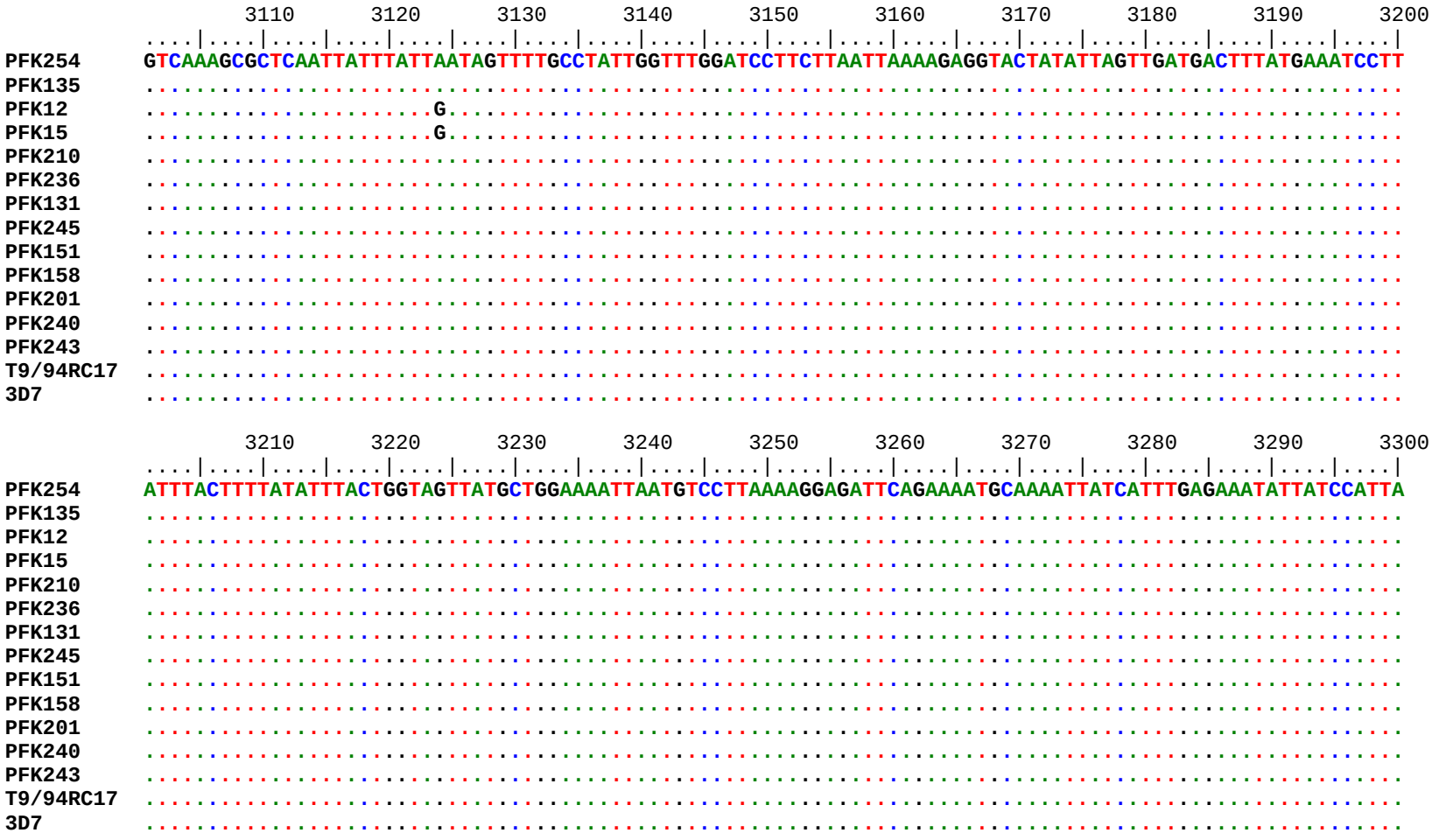

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

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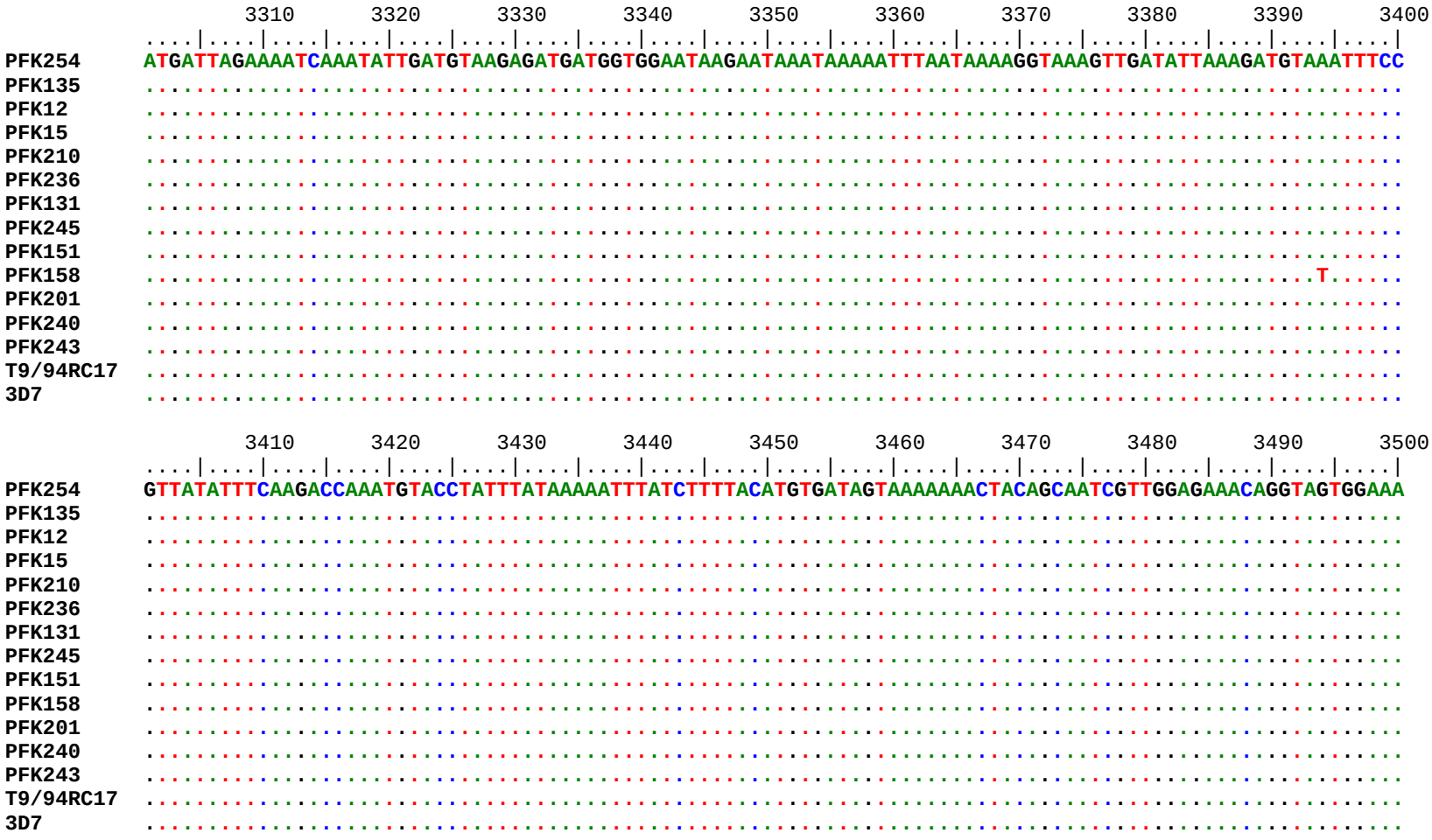

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

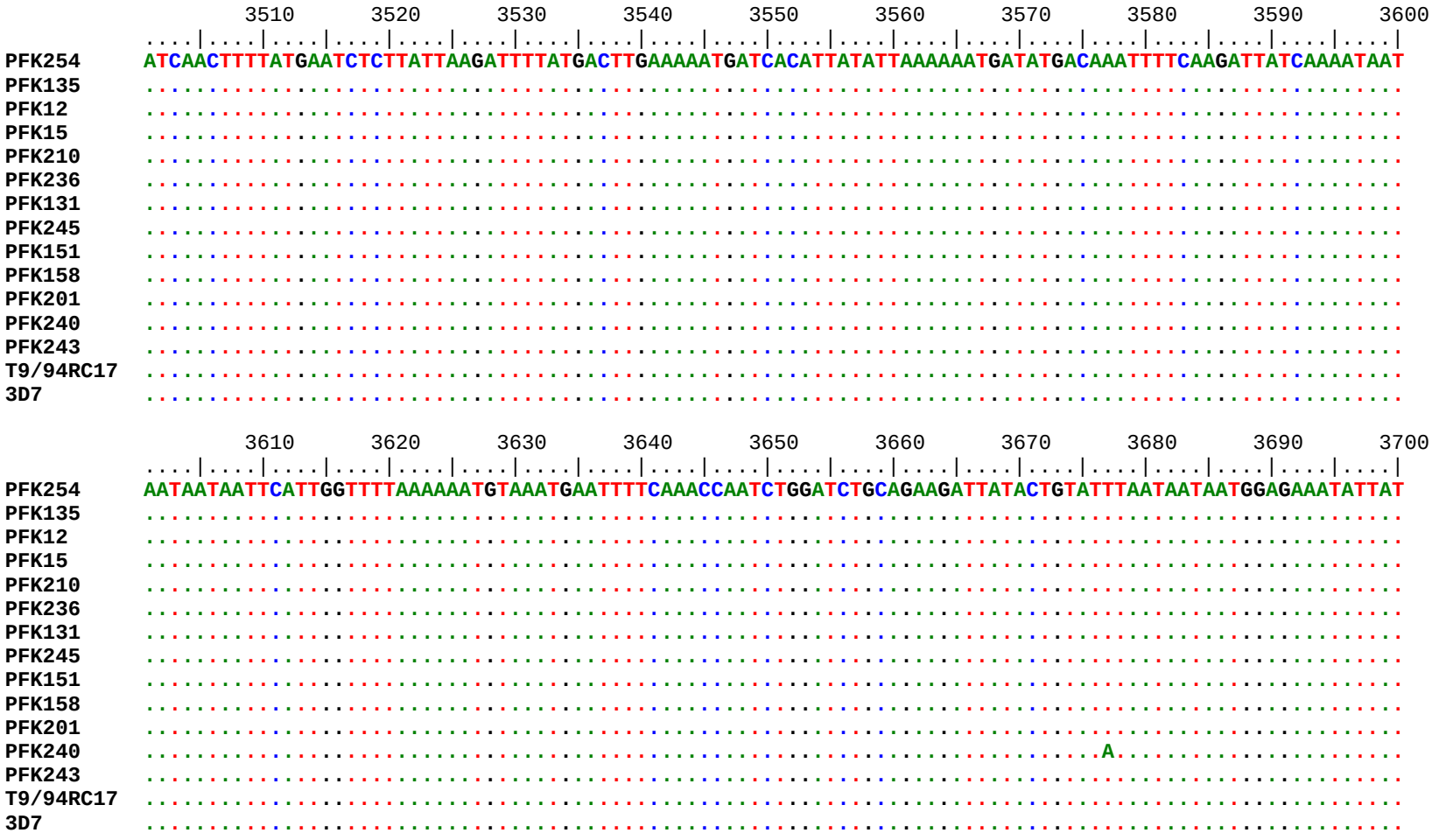

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

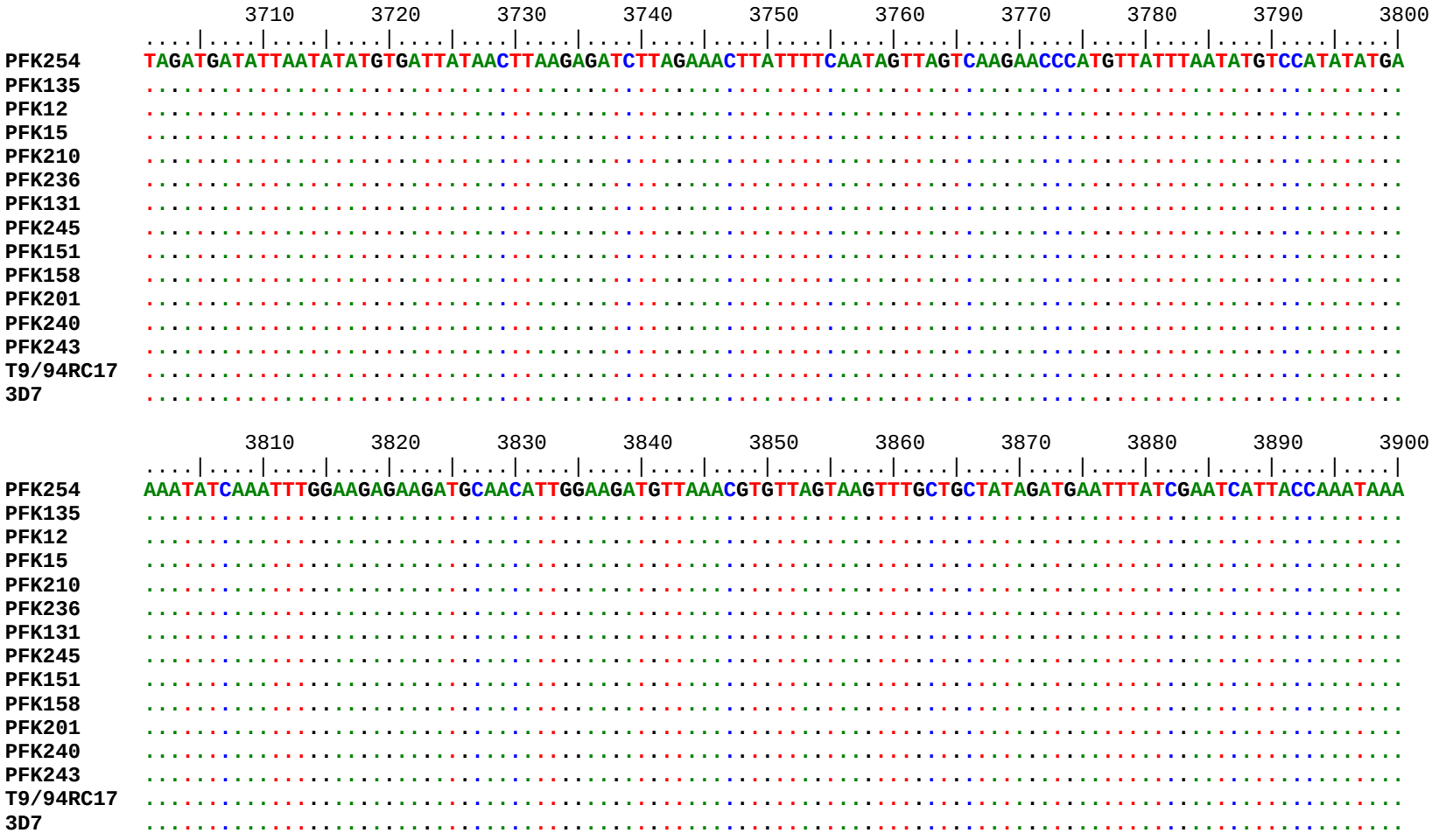

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

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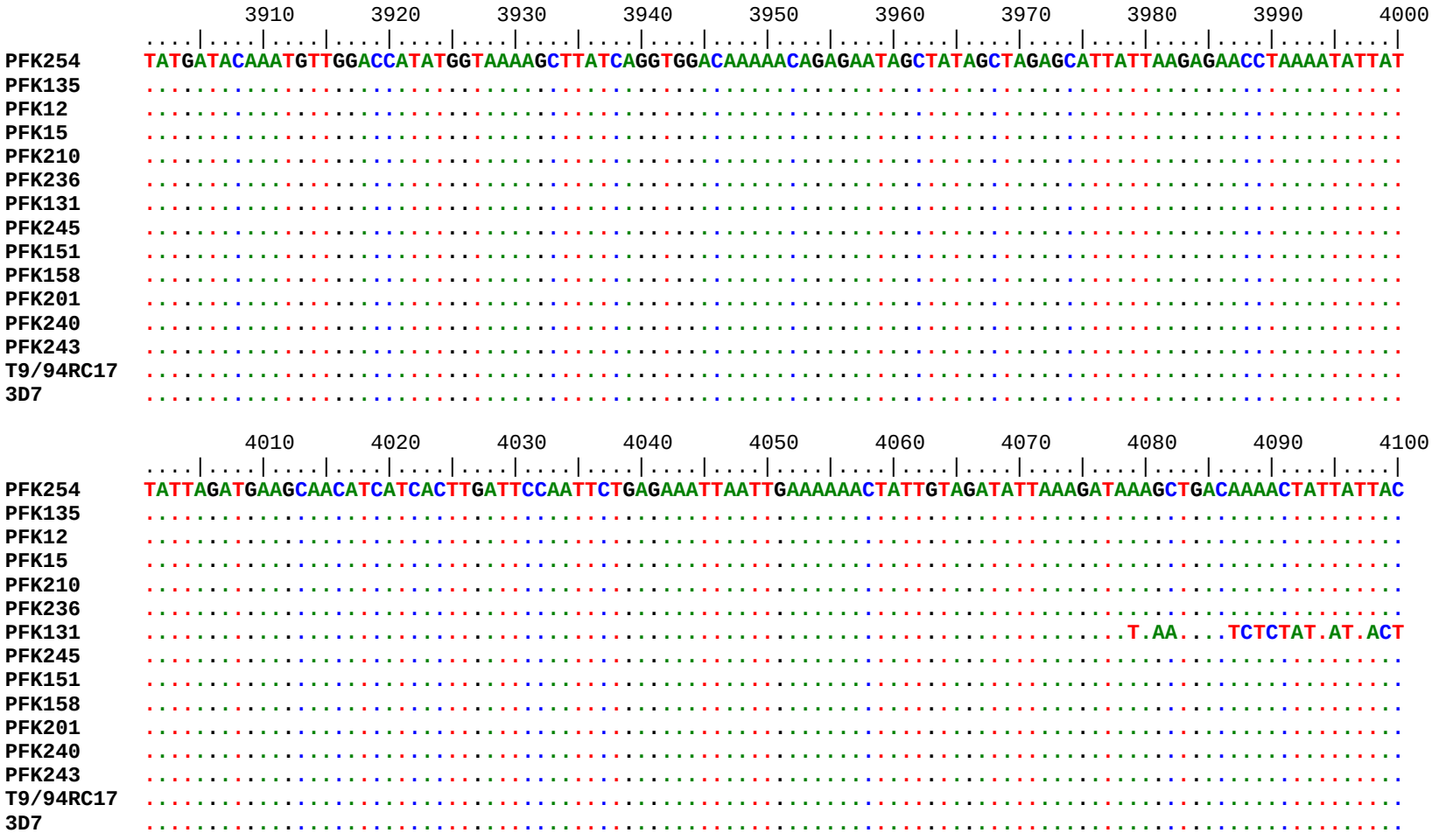

Figure 4.14 (continue) Sequences alignment of *pfmdr 1* gene obtained from 13 field samples and a laboratory clone with completed sequence of 3D7.

We started to compare the sequences at 83 bases (Figure 4.14) before the starting codon of the gene and ended at base 4075. We could not amplify the entire gene but this length covered the amino acid position 1246, a last amino acid codon at 3' region of the gene that has been reported involving in mefloquine response in several studies. From the alignment, we found 13 point mutations (see in Table 4.19). Among these mutations, nine were nonsynonymous substitution which allowed amino acid alteration as shown in Table 4.20, three of them (N86Y, Y184F and N1042D) had been reported in several studies. There were 8 genotypes characterized by the point mutations occurred in this gene. The genotype of each isolate observed with microsatellite markers (both located within and flanking *pfmdr1* gene) was inconsistent with that of characterized by nucleotide sequence in this gene. For example, the four parasite isolates, PFK12, PFK15, PFK210 and PFK236, possessed the identical microsatellite genotype; they had very high homogeneity of base composition of this gene, with exception at position 3124, two of them possessed a base Guanine while the others had a base Adenine at this position. Moreover, all these samples responded to mefloquine at different of drug level (Table 4.19). Another pair of samples (PFK131 and PFK245) with the same microsatellite genotype, they own four different point mutations in their sequences. Thus, we assumed that no relationship between microsatellite genotype and point mutation found in the sequence of this gene. This no association with these two kinds of marker may be a result of differ in mutation rate of these two markers. Besides, the information of genotype observed with 4 loci of microsatellite was not informative enough to distinguish or characterize an individual parasite under character consider. Therefore, the microsatellite markers flanking this *pfmdr1* gene were not used to track a dynamics of *P. falciparum* mefloquine resistance in population even if there was significant genetic differentiation between populations in different times of collection observed with these markers. Another proposed mechanism which had strongly associated with mefloquine response was gene amplification. We would examine this association in future to document which mechanism contributed to drug resistant characteristic in this area studied.

Consideration of nonsynonymous mutation, two codons (S1034C and D1246Y) of five known nonsynonymous polymorphisms found as wild-type genotype in all samples studied. Of 13, we found the mutant-type at amino acid codon 86, 184 and 1042; one Y86N (PFK151), one F184N (PFK254) and two D1042N. We also found six novel nonsynonymous substitutions comparing with the amino acid sequence of 3D7 reference clone. The combined mutant codon E118V and Y1132N; K130E and T750A were found in PFK158 and PFK135, respectively. The other two substitutions, L784S and Y1126F, were found in two (PFK131 and PFK254) and one (PFK240) samples, respectively (Table 4.20). Because of a small number of gene sequences examined and a little in differences drug concentration in each level of drug response could not allowed us to use these data to determine a correlation between drug response and nonsynonymous substitution in this gene.

Table 4.19 Nucleotide substitution observed in *pfmdr 1* gene

Sample	Mefloquine response (M)	Identical MS genotype	Variable sites (nucleotide position)												
			256	358	388	551	747	2248	2277	2351	2640	2751	3124	3394	3677
PFK254†	-	1	A	T	G	T*	A	G	A	T*	T	C	A	A	T
PFK135	2 x 10^{-7}	11	A	T	A*	A	G*	A*	A	C	T	C	A	A	T
PFK12‡	7 x 10^{-8}	4	A	T	G	T*	A	G	A	C	T	C	G*	A	T
PFK15‡	5 x 10^{-7}	4	A	T	G	T*	A	G	A	C	T	C	G*	A	T
PFK210¶	2 x 10^{-7}	4	A	T	G	T*	A	G	A	C	T	C	A	A	T
PFK236¶	-	4	A	T	G	T*	A	G	A	C	T	C	A	A	T
PFK131†	2 x 10^{-7}	9	A	T	G	T*	A	G	A	T*	T	C	A	A	T
PFK245	-	9	A	T	G	A	A	G	A	C	C*	G*	A	A	T
PFK151§	7 x 10^{-8}	-	T*	T	G	A	A	G	A	C	T	C	A	A	T
PFK158	2 x 10^{-7}	7	A	A*	G	T*	A	G	C*	C	T	C	A	T*	T
PFK201¶	2 x 10^{-7}	5	A	T	G	T*	A	G	A	C	T	C	A	A	T
PFK240	-	12	A	T	G	A	A	G	A	C	T	C	A	A	A*
PFK243¶	-	6	A	T	G	T*	A	G	A	C	T	C	A	A	T
RC17§	2 x 10^{-7}	-	T*	T	G	A	A	G	A	C	T	C	A	A	T

***** nucleotide substitution comparing with the 3D7 sequence

†, ‡, ¶ and § indicate each repetitive genotype observed; MS, microsatellite

Table 4.20 Nonsynonymous substitution observed in *pfmdr 1* protein

Sample	Mefloquine response (M)	Identical MS genotype	Variable sites (amino acid codon)										
			86[a]	118	130	184[a]	750	784	1034[a]	1042[a]	1132	1226	1246[a]
PFK254[†]	-	1	N	V	E	F*	A	L*	S	N	N	F	D
PFK135	2×10^{-7}	11	N	V	K*	Y	T*	S	S	N	N	F	D
PFK12[‡]	7×10^{-8}	4	N	V	E	F*	A	S	S	D*	N	F	D
PFK15[‡]	5×10^{-7}	4	N	V	E	F*	A	S	S	D*	N	F	D
PFK210[¶]	2×10^{-7}	4	N	V	E	F*	A	S	S	N	N	F	D
PFK236[¶]	-	4	N	V	E	F*	A	S	S	N	N	F	D
PFK131[†]	2×10^{-7}	9	N	V	E	F*	A	L*	S	N	N	F	D
PFK245	-	9	N	V	E	Y	A	S	S	N	N	F	D
PFK151[§]	7×10^{-8}	-	Y*	V	E	Y	A	S	S	N	N	F	D
PFK158	2×10^{-7}	7	N	E*	E	F*	A	S	S	N	Y*	F	D
PFK201[¶]	2×10^{-7}	5	N	V	E	F*	A	S	S	N	N	F	D
PFK240	-	12	N	V	E	Y	A	S	S	N	N	Y*	D
PFK243[¶]	-	6	N	V	E	F*	A	S	S	N	N	F	D
RC17[§]	2×10^{-7}	-	Y*	V	E	Y	A	S	S	N	N	F	D

***** nonsynonymous substitution comparing with the 3D7 sequence; [a] nonsynonymous substitution sites reported by others

†, ‡, ¶ and § indicate each repetitive genotype observed; MS, microsatellite

CHAPTER V

DISCUSSION

5.1 The *Plasmodium falciparum* population in Kanchanaburi province

5.1.1 Temporal structure of Kanchanaburi population

Among the four studied sub-districts in study area of Kanchanaburi, the inhabitants in Bongtee sub-district had the highest malaria incidence and a high risk of malaria infection. This sub-district is located in a valley covered with forests and small streams. On the west bordering Myanmar is a high mountain and on the east is a smaller mountain, which separates this sub-district from the district center. The *P. falciparum* population in this area at different periods of time revealed a similar level of genetic diversity (H_s, 0.61-0.62) between 2002 and 2005. The diversity tended to slightly decline since 2006. Furthermore, low but significant genetic differentiation between parasite populations collected in 2004 and 2005 versus 2007 was noted. This maybe because the Thai government allowed Thai people to legally pass through the Thai-Myanmar border in Bongtee sub-district for forest industry in Myanmar since 2006. Thus, new parasite strains carried by Thai workers from Myanmar were introduced and may affect parasite population in this area. If these parasites had high mosquito infectivity or carried gene that made more advantage (fit to the environment under area of study) than local parasites, they would spread more rapidly. In fact, we found the same genotype in seven samples collected in 2007 in Kanchanaburi, from patients infected in Thailand and Myanmar. This genotype was not found in samples collected from any other period before. These imported cases may play an important role on the *P. falciparum* gene pool and affect the epidemiology of the *P. falciparum* parasites in this area. The dynamics of *P. falciparum* related to the consequence of migration will be of great concern in planning malaria control in each endemic area.

5.1.2 The magnitude of mefloquine resistance in Kanchanaburi

After the occurrence of mefloquine treatment failure in endemic areas on the border of Thailand with Myanmar, the combination of mefloquine and artesunate was used as a first-line drug regimen for uncomplicated malaria in these areas including Kanchanaburi since 1995 (Na-Bangchang and Congpuong, 2007). Nowadays, treatment failure of these drug combinations was rarely found in this study area. The *in vitro* susceptibility test for mefloquine showed that most of Kanchanaburi populations had a response to mefloquine at a high level ($1x10^{-7}<MIC\leq5x10^{-7}$). Though, this drug sensitivity of Kanchanaburi population is comparable with that of the mefloquine sensitive clone 3D7 reported by Babiker and colleagues (1991) and they implied that these levels of drug response appeared to be sensitive to mefloquine. The decline in proportion of the parasite with highest response of mefloquine was observed in each year even if the means of MICs in the last two year were not distinct. The findings indicated that artesunate combined with mefloquine can overcome the multidrug resistance occurred in this area and will continue to be the first-line drug regimen for uncomplicated malaria treatment.

Even though, the value of mefloquine susceptibility obtained from our study cannot compare directly with others because of the difference in the method of detection such as the incubation time (72 h versus 48 h) and reported value (MIC versus the half maximal inhibitory concentration (IC_{50})), its trend can be compared.

5.1.3 The temporal Kanchanaburi population structure in consideration of mefloquine resistance dynamics

With the attempt to explore the dynamics of mefloquine resistance by utilization of microsatellite loci located within and flanking the *pfmdr1* gene, proposed to be responsible for mefloquine resistance. We successfully used only few markers (4 loci) and found less genetic diversity of microsatellite settled within this gene comparing with those flanking gene as observed in several selective sweep around drug target gene studies (Wootton et al., 2002; Nair et al., 2003; Nash et al., 2005; Nair et al., 2007). However, difference in the frequency of each allele distribution in each time period was able to be noticed especially that of microsatellite

locus within the *pfmdr1* gene. The pattern of allelic distribution observed at microsatellite within gene at the first year of collection (2004) significantly distinct from those of the last two years (2006 and 2007). This had resulted in significant genetic differentiation between populations investigated at different time periods. This may imply that the migration had occurred in this area not only had an effect to changing in structure of parasite population, but also reflect to the dynamics of drug resistance. Furthermore, the effect of migration is more sensitive for drug resistance dynamic as shown in this study, the alteration in population structure under consideration of microsatellite linked to drug response gene was observed since 2006 while those observed with neutral loci markers was clearly investigated in 2007.

Linkage disequilibrium observed in Kanchanaburi population may indicate that there was no recombination in the *pfmdr1* gene (between marker located 4.6 kb and 8.4 kb apart, in 2006 and 2007, respectively). This is consistent with the weak linkage disequilibrium (2 of 66 combined pairs) found in 2007 detected by using neutral 12 microsatellite loci. The findings may imply that the inbreeding preferably occurred in Kanchanaburi population. If the sensitive *pfmdr1* allele was selected and spread in this population (the declining in mefloquine response and means MICs was shown), the malaria treatment and control program in this area will be continually effective.

5.2 The genetic structure observed in each Thai locality

In Thailand, the malaria incidence rate shows a high spatial heterogeneity across the country, with high incidence regions found nearby the borders with Myanmar and Cambodia (Zhou et al., 2005). This study reports the genetic structure of *P. falciparum* analysis with 12 polymorphic nuclear microsatellite loci in isolates from multiple populations in seven provinces of Thailand.

The *P. falciparum* populations in the areas bordering with Myanmar revealed a similar level of genetic diversity ($0.60 < H_s < 0.68$), whilst the populations bordering with Laos and Cambodia had slightly higher or lower levels of genetic diversity at 0.70 and 0.56 in Ubonratchathani and Trat, respectively. Although the multiple isolate infection rates in those areas bordering with Myanmar (mean, 34%) were higher than

that of Ubonratchanthani (22%) and comparable to that in Trat (36%), this could be explained by the notion that (i) the parasite mating in those areas is preferentially inbreeding, or (ii) the relatively small amount of sampled individuals in Trat and Ubonratchathani leading to a stochastic masking of the true population structure. The Yala population, however, showed an extreme difference in the gene diversity compared the other samples since it showed only a unique haplotype in all sampled individuals, but again may be a transient clonal expansion of an immigrant infection.

In contrast to the Yala population, a low but significant population structure was observed amongst the provinces that border with Myanmar, Laos and Cambodia. In the case of the Myanmar bounding provinces, the significant genetic structure detected between populations (Kanchanaburi, Tak, Maehongson and Ranong) was likely to be due to the fact that the pattern of human movement from neighboring countries into each of these four provinces was different. In the Tak and Ranong provinces, the numerous movements in to Thailand from Myanmar from immigrant laborers, and from patients resident in Myanmar who cross the border to seek free malaria treatment, account for the high proportion (approximately 70%) of sampled individuals being likely to have been infected in Myanmar. Indeed, we found the same genotype in seven samples collected in 2007 in Kanchanaburi, from patients infected in Thailand and Myanmar. These, imported cases may play an important role on the *P. falciparum* gene pool in each endemic area and affect the epidemiology of the *P. falciparum* caused malaria in these areas. Besides this migration rate of humans in each foci, there is additionally the factor of the geographic barrier between Tak and Kanchanaburi provinces which may be another cause of genetic differentiation by partial isolation by distance and breaking down of panmixia. Thus, the genetic differentiation found between populations within each province of Thailand may reflect both a variation of the transmission rate in each location and the migration rate of people from one location to another. Certainly seasonal migration has been suspected as a leading cause of malaria transmission in these areas (Wongsrichanalai et al., 2001). However, not only does the transmission rate and immigration of inhabitants play a role, but the differential dispersal of the Anopheles vectors in local areas may also have an effect on the parasite structure. In Thailand, *An. aconitus sensu lato*, *An. baimaii*, *An. dirus*, *An. maculatus*, *An. minimus* and *An.*

pseudowillmori have been incriminated as important vectors of *P. falciparum* across humans, and they are differentially distributed throughout the country (O'Loughlin, Somboon and Walton, 2007; Rattanarithikul et al., 2006; Walton et al., 2000), and may each have different vectoring capabilities for different isolates of *P. falciparum*. Thus, although differences in the population structure of *P. falciparum* between two vectors (*An. gambiae* and *An. fenustrus*) have been preliminarily investigated (Annan et al., 2007), no such information currently exists for the different vector species in each region of Thailand.

Importantly, the same multilocus genotypes of *P. falciparum* infected blood samples collected one year apart (2005 - 2006 and 2006 - 2007) was observed only in Ranong, where one case was found in a patient infected from Myanmar and the following year was found from a patient infected in Thailand. If we assume a generation time of two months, this parasite genotype has been transmitted through six generations without detectable change across the 12 loci (eight / 14 chromosomes) due to recombination. This may imply that if this *P. falciparum* strain has an advantage (fitness) in that particular environment, it will propagate and expand in this population.

5.3 The genotype distribution of *P. falciparum* in Thailand and its consequence

In this study we detected both a spatial and a temporal distribution of *P. falciparum* haplotypes collected in the same year and over two years. A unique spatial haplotype, distinct from the other populations, was found in all patients in the Yala population, which were obtained from individual residents in four different sub-districts in Bannang Sata and other three nearby districts during May - July 2007. Three possible explanations are available to explain this trend. Firstly, that a *P. falciparum* isolate with this genotype had a high infectivity in the mosquito's midgut or carried some genetic characteristics, such as drug resistance, which gave it a significant benefit over other haplotypes. Secondly, that there is no inhabitant movement into this area due to the political violence, and so as a result no new haplotype parasite was introduced into this area (no gene flow). Thirdly there maybe a rare new gene influx by immigration but which was subsequently lost due to selection or stochastic failure to progress to a stable infection threshold. Finally, is the

possibility that the decrease in sanitary cover in this area allowed this particular genotype to spread into the population rapidly. Thus, a longitudinal study in the population dynamics in this area is required. Nevertheless, this is the first study to reveal the potential; clonal expansion of a single haplotype in a malaria endemic area.

5.4 Comparison of Thai *P. falciparum* genetic structure with other countries

At the level of continents, the genetic structure of *P. falciparum* populations in different continents revealed that African countries with high transmission intensities had the highest population genetic diversity. The genetic diversity decreased with low transmission intensities, as in Thailand (Asia) and French Guyana (South America). The average genetic diversity of *P. falciparum* populations in Thailand ($H_s = 0.65 \pm 0.17$) was, however, close to that of the high transmission intensity level of Papua New Guinea (Anderson et al., 2000), and slightly higher than that reported for populations in Malaysia (Anthony et al., 2005) and Brazil (Machado et al., 2004), also based on assaying genetic diversity at these seven microsatellite loci.

P. falciparum's (deduced) mating patterns in Thailand showed a deviation from random mating, in congruence with the French Guyana population, whilst, in contrast, the two African populations showed likely panmixia. The proportion of multiple isolate infections, and the number of clones in an individual host, which are related to the transmission intensity of malaria parasites in any endemic area, are important parameters that affect *P. falciparum* mating, because fertilization may occur between gametes of the same or different genotypes in mosquitoes. Therefore, an inverse correlation between the proportion of multiple clone infections and the degree of linkage disequilibrium is expected. Even though the genetic diversity of Thai populations was observed at an intermediate level, and multiple clone infections occurred frequently, a significant linkage disequilibrium was found which could be caused by, (i) a higher chance of inbreeding (selfing) in this parasite population, or (ii) structure formation within populations into sub-populations of each foci in Thailand (i.e. the Wahlund effect). The latter reason would be more likely to explain our finding because we pooled samples from all the foci from across the country (the seven different provinces) in the analysis.

5.5 Do the microsatellite loci be utilized as markers for tracking mefloquine resistance dynamics?

According to microsatellite around the *pfmdr1* gene revealed the changes of genetic structure of Kanchanaburi population over time which compatible with that of neutral microsatellite observation. We try to determine an association between microsatellite genotype, point mutation in the *pfmdr1* gene and mefloquine susceptibility level of parasite and no association was found. This is consistent with previous study by Nair and colleagues (2007). They found the same pattern of point mutations in more than one clade of microsatellite pattern. Besides, they determined a copy number of *pfmdr1* gene and its relationship to mefloquine resistance; they found distinctive amplicons on identical microsatellite backgrounds. This evidence may be due to (i) the unequivalent mutation rates of microsatellite sequence and point mutation. The high level of length variation of microsatellite repeats is proposed be caused by the replication slippage or slip-strand mispairing. The mean rate of microsatellite mutation is 1.59×10^{-4} which higher than single-nucleotide substitution by a factor of 10^5-10^6 (Hartl et al., 2002). (ii) a small number of sampling sequences in which each microsatellite pattern provided insufficient data for the association analysis. Thus, more samples will be sequenced and analyzed to provide the precise association result.

However, in our 13 sampling parasites, we found six novel nonsynonymous substitutions in the *pfmdr1* gene sequence. The novel nonsynonymous substitution was also found in the *pfmdr1* gene of mefloquine resistance induced in laboratory (Nishiyama et al., 2004). This mutation resulted in decrease mefloquine sensitivity as well as artemisinin and halofantrine sensitivity but this mutation was not found in our field samples. The examination of the role of three mutations at 3' region of the *pfmdr1* gene in controlling parasite sensitivity and resistance to antimalarial using plasmid transformation was conducted by Reed and colleagues in 2000. The Cys1034, Asp1042 and Tyr1246 increased sensitivity to mefloquine. Conversely, the *pfmdr1* allele encoded for Ser1034, Asn1042 and Asp1246 conferred resistance against mefloquine. All our samples possessed resistant allele except two of them had Asp1042. These two isolates differently responded to mefloquine, one had the lowest level (7×10^{-8} M) while another had the highest level (5×10^{-7} M). Even the controversy

in the role of point mutation on mefloquine resistance mechanism based on field study was observed in many reports but there were several findings in common. The predominant *pfmdr1* wild-type allele was detected in mefloquine resistance of Thai population especially on the Thailand-Myanmar border as observed in our study (Nair et al., 2007; Congpuong et al., 2005; Price et al., 1999). At present, many studies proposed a strong association between increased multidrug resistant gene copy number and mefloquine resistance (Nair et al., 2007; Nelson et al., 2005; Price et al., 2004). However, there were the mefloquine resistant parasites with one copy of this gene and wild-type allele left for investigation. Most studies worked on the association between substitution in the *pfmdr1* gene and mefloquine response were focused only on five major substitutions. If we analyzed (i) the whole gene sequence; (ii) the sequence of upstream and downstream of this gene; (iii) gene(s) that co-amplified with the *pfmdr1* gene (Nair et al., 2007) , an association may be detected. Even though, this study cannot reveal any relationship between molecular marker and mefloquine response, molecular data, such as new substitutions, may be have a possibility to be responsible for mefloquine resistance. A development of determinant marker for mefloquine resistance is needed. This will ease the detection of drug resistance emergence and its extent. This information can guide policy maker to promptly direct malaria treatment and control in the proper way.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

1. In local malaria endemic area, Kanchanaburi province, changes in *P. falciparum* population structure occurred frequently at the different periods of time. These changes may be accelerated by the migration which is an important factor in population structure. After an introduction of imported parasites, the genetic differentiation was observed in following year. Fortunately, these new parasites did not carry more mefloquine resistant genotypes, thus, the means of mefloquine response slightly decline after the appearance of immigrants. Besides, the parasite population tended to undergo inbreeding as we found weak linkage disequilibrium in the last two year of study. According to these findings, we can assume that the malaria treatment and control programs in this endemic area may be adequate.

2. *P. falciparum* populations are highly structured in Thailand suggesting local and differential routes of genetic evolution for these parasites. Although Thailand is considered as a low transmission area, a high level of genetic diversity and weak linkage disequilibrium were found in most sites, except the transient infections in the Yala province in one year where only a single genotype was found. Moreover, (i) identical genotypes were encountered in the same site but from patients coming from different localities (i.e. Myanmar and Thailand), and (ii) two identical genotypes were observed from two successive years at the same site (i.e. Ranong). These findings are particularly relevant in the context of malaria control. They can help understand specific dynamics of malaria and parasite populations in areas displaying different histories of drug applications.

3. The attempt to utilize microsatellite markers for the determination of the dynamics of mefloquine resistance in Kanchanaburi population was unsuccessful. Although, these markers can reveal the distinct genetic characteristics among parasite populations at different time, they are not informative enough to distinguish the uniqueness of each mefloquine characteristic comparing with other molecular determinants. This can be partly explained by nonequivalent mutation rates of these molecular markers.

6.2 Recommendations

Malaria has a complex relation between three factors of the chain of transmission: hosts, parasites and vectors. Thus, a study only based on parasite population was insufficient to point out which epidemiological process should be targeted for disease control in each endemic area. Extensive samplings should be continued both from the human host and from the *Anopheles* mosquito vector. It will be best if the sampling is done in the same transmission areas at the same transmission cycle. Only good design to obtain a holistic transmission can we further our understanding of link between recombination in the vector and population structure in humans. However, the current findings do shed light on an important tool that may be used if such a design for holistic understanding is attempted.

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APPENDICES

APPENDIX I

Allele frequency of 12 microsatellite markers distributed in Kanchanaburi population

Locus	2002	2004	2005	2006	2007
Polyα					
N	14	39	34	54	34
131	-	-	0.029	0.019	-
136	-	-	-	0.037	-
146	0.214	0.641	0.382	0.278	0.412
150	-	-	0.059	0.019	0.088
153	-	-	0.029	-	-
156	0.643	0.179	0.235	0.259	0.176
160	-	0.051	-	0.111	0.118
163	-	-	0.029	0.056	-
166	-	0.026	-	-	-
169	0.071	0.051	0.029	0.093	-
172	0.071	0.026	0.088	0.037	0.147
175	-	-	-	0.056	-
178	-	-	0.118	0.019	-
183	-	-	-	-	0.059
189	-	0.026	-	0.019	-
TA60					
N	14	39	34	54	34
70	-	-	-	0.056	-
73	-	0.026	-	-	-
76	-	0.051	0.059	0.148	0.265
79	0.286	0.256	0.235	0.222	0.176
82	0.357	0.487	0.500	0.500	0.412
85	0.214	0.077	-	-	0.088
88	-	0.051	0.147	0.056	0.059
91	0.071	-	-	-	-
94	0.071	0.051	0.059	0.019	-

Locus	2002	2004	2005	2006	2007
ARA2					
N	14	38	34	54	34
51	-	0.026	-	-	-
63	0.143	-	0.029	0.019	-
66	0.357	0.342	0.147	0.241	0.206
69	0.143	0.026	0.235	0.037	0.029
72	0.357	0.368	0.500	0.556	0.676
75	-	0.079	0.059	0.130	0.088
78	-	0.158	0.029	0.019	-
Pfg377					
N	14	39	34	54	34
95	-	0.128	0.147	0.056	0.029
98	0.714	0.744	0.765	0.833	0.882
101	0.286	0.103	0.088	0.111	0.088
104	-	0.026	-	-	-
PfPK2					
N	14	37	34	54	34
163	0.071	0.297	0.176	0.111	-
166	0.143	0.162	0.118	0.148	0.206
169	0.357	0.135	0.235	0.259	0.294
172	0.286	0.351	0.382	0.426	0.471
175	-	-	-	0.019	-
178	0.143	0.054	0.029	-	-
193	-	-	0.059	0.037	0.029
TAA87					
N	14	38	34	54	34
89	-	-	-	0.019	-
95	-	0.026	-	0.074	-
98	0.143	0.105	0.147	0.111	0.029
101	0.071	0.105	0.206	0.111	0.118
104	0.143	0.026	0.147	0.037	0.029
107	0.643	0.658	0.471	0.593	0.735
110	-	-	-	0.019	0.029
116	-	0.079	0.029	0.037	0.059

Locus	2002	2004	2005	2006	2007
TAA109					
N	14	39	33	54	34
157	-	0.103	0.212	0.019	-
160	0.857	0.821	0.636	0.870	0.912
163	0.071	-	-	0.019	0.029
170	-	-	0.030	-	-
173	0.071	0.077	0.091	0.093	0.059
185	-	-	0.030	-	-
TA80					
N	13	33	28	43	33
139	-	0.030	-	-	-
145	0.538	0.424	0.464	0.558	0.636
148	0.462	0.455	0.464	0.419	0.364
151	-	0.091	0.071	0.023	-
ARP2					
N	14	37	33	52	34
169	0.071	-	-	-	-
172	0.643	0.649	0.939	0.788	0.912
175	-	-	-	0.038	-
178	0.143	0.297	0.061	0.154	0.029
181	0.071	0.054	-	-	0.029
184	0.071	-	-	0.019	0.029
TA1					
N	14	39	33	54	34
157	0.071	0.026	-	-	-
160	-	0.026	-	-	-
163	0.214	0.103	0.121	0.148	0.059
166	0.714	0.436	0.606	0.648	0.765
169	-	0.026	0.030	-	-
172	-	0.128	0.152	0.056	0.029
175	-	0.026	-	-	-
181	-	0.205	0.061	0.130	0.118
187	-	-	0.030	-	-
190	-	0.026	-	0.019	0.029

Locus	2002	2004	2005	2006	2007
TAA81					
N	14	37	34	54	33
109	0.071	-	-	0.056	-
112	0.071	0.054	0.088	0.074	0.091
115	0.357	0.486	0.412	0.259	0.455
118	-	-	-	0.074	0.061
121	0.214	0.324	0.206	0.315	0.030
124	0.071	0.054	0.147	0.148	0.212
127	0.071	0.054	0.147	0.037	0.030
133	0.071	0.027	-	-	0.030
139	0.071	-	-	0.037	0.030
142	-	-	-	-	0.061
C1M8					
N	13	36	32	39	33
156	-	-	-	-	0.061
159	0.231	0.278	0.063	0.103	0.121
162	0.154	-	-	0.077	0.030
165	-	0.028	0.031	-	-
168	0.077	0.083	0.063	0.179	0.212
171	-	0.028	-	0.026	-
174	-	-	-	-	0.030
177	0.385	0.444	0.406	0.231	0.273
180	-	0.056	0.031	-	-
183	0.154	0.028	0.188	0.026	0.030
189	-	0.028	0.031	-	-
192	-	-	0.188	0.179	0.182
195	-	0.028	-	-	-
198	-	-	-	0.026	-
201	-	-	-	0.026	-
204	-	-	-	0.026	-
207	-	-	-	0.077	0.030
210	-	-	-	0.026	0.030

APPENDIX II

Allele frequency of 12 microsatellite markers distributed in seven province populations

Locus	MAE	TAK	KAN	UBO	TRA	RAN	YAL
Polyα							
N	18	48	175	18	18	38	31
132	-	-	0.011	-	-	-	-
135	-	-	0.011	0.056	-	-	-
141	0.056	-	-	-	-	-	-
147	0.111	0.229	0.400	0.111	0.278	0.211	-
150	0.111	-	0.034	0.056	-	0.026	-
153	-	0.021	0.006	-	0.056	0.053	-
156	0.167	0.208	0.251	0.278	0.333	0.211	-
160	0.111	0.104	0.069	0.222	-	0.079	-
163	0.056	-	0.023	-	-	-	-
166	-	0.021	0.006	0.056	-	-	-
169	0.056	0.042	0.051	0.111	0.111	0.105	-
172	0.222	0.208	0.069	0.111	0.056	0.158	-
175	-	0.021	0.017	-	-	-	-
178	-	0.042	0.029	-	0.056	0.026	-
181	0.056	-	-	-	-	-	-
183	-	0.083	0.011	-	-	-	-
189	0.056	0.021	0.011	-	0.111	0.132	1.000
TA60							
N	18	48	175	18	18	38	31
64	-	-	-	0.056	-	-	-
67	-	0.021	-	-	-	-	-
70	-	-	0.017	-	-	-	-
73	0.056	0.042	0.006	-	-	-	-
76	0.111	0.229	0.120	0.111	0.056	0.053	-
79	0.278	0.188	0.229	0.222	0.111	0.158	1.000
82	0.333	0.313	0.469	0.611	0.778	0.500	-
85	0.111	0.083	0.051	-	-	0.105	-
88	0.056	0.125	0.069	-	-	0.053	-
91	-	-	0.006	-	0.056	-	-
94	0.056	-	0.034	-	-	0.132	-

Locus	MAE	TAK	KAN	UBO	TRA	RAN	YAL
ARA2							
N	18	48	174	18	18	38	31
51	-	-	0.006	-	-	-	-
57	0.111	0.042	-	-	-	-	-
60	-	-	-	0.056	-	0.026	-
63	0.111	0.042	0.023	0.222	0.167	0.026	-
66	0.278	0.333	0.247	0.222	0.389	0.237	-
69	-	0.063	0.080	-	-	0.053	-
72	0.333	0.354	0.511	0.278	0.389	0.526	-
75	0.111	0.125	0.086	0.222	0.056	0.053	1.000
78	-	0.042	0.046	-	-	0.079	-
81	0.056	-	-	-	-	-	-
Pfg377							
N	18	47	175	18	18	38	31
95	0.056	0.106	0.080	-	-	0.053	-
98	0.833	0.851	0.800	0.611	0.500	0.737	-
101	0.111	0.043	0.114	0.389	0.500	0.184	1.000
104	-	-	0.006	-	-	0.026	-
PfPK2							
N	18	48	173	18	18	38	31
160	-	-	-	0.056	-	-	-
163	0.111	0.042	0.139	0.056	0.389	0.158	-
166	0.444	0.396	0.156	0.167	0.333	0.263	-
169	0.111	0.083	0.243	0.333	0.167	0.211	-
172	0.167	0.313	0.399	0.278	0.111	0.263	1.000
175	0.167	0.063	0.006	0.056	-	0.026	-
178	-	-	0.029	-	-	0.026	-
184	-	0.042	-	-	-	-	-
193	-	0.063	0.029	0.056	-	0.053	-
TAA87							
N	18	48	174	18	18	38	31
78	-	-	-	-	0.056	-	-
86	0.056	0.042	-	-	-	-	-
89	0.056	0.042	0.006	-	-	-	-
92	-	-	-	0.056	-	-	-
95	-	0.021	0.029	0.278	-	0.053	-
98	0.222	0.125	0.103	0.056	0.056	0.132	-
101	0.111	0.188	0.126	0.222	0.222	0.289	-
104	0.222	0.104	0.063	0.167	-	0.132	-

Locus	MAE	TAK	KAN	UBO	TRA	RAN	YAL
TAA87							
N	18	48	174	18	18	38	31
107	0.222	0.438	0.615	0.167	0.333	0.368	1.000
110	0.111	0.042	0.011	0.056	0.278	-	-
116	-	-	0.046	-	0.056	0.026	-
TAA109							
N	18	48	174	18	18	38	31
157	-	-	0.069	-	-	0.053	-
160	0.778	0.917	0.822	0.889	0.889	0.789	-
163	-	0.021	0.017	-	0.056	-	-
170	-	-	0.006	0.056	-	-	-
173	0.222	0.042	0.080	-	-	0.158	1.000
176	-	0.021	-	0.056	0.056	-	-
185	-	-	0.006	-	-	-	-
TA80							
N	17	48	150	18	18	38	31
139	-	-	0.007	-	-	-	-
145	0.294	0.521	0.527	0.333	0.111	0.500	-
148	0.647	0.417	0.427	0.667	0.889	0.421	1.000
151	0.059	0.063	0.040	-	-	0.079	-
ARP2							
N	18	48	170	17	18	38	31
160	-	0.021	-	-	-	-	-
169	0.056	0.042	0.006	0.118	-	-	-
172	0.833	0.833	0.800	0.471	0.889	0.868	-
175	-	0.083	0.012	-	-	-	-
178	0.056	0.021	0.141	0.118	0.056	0.132	-
181	-	-	0.024	0.235	-	-	1.000
184	0.056	-	0.018	0.059	0.056	-	-
TA1							
N	18	47	174	18	18	38	30
154	-	0.021	-	-	-	-	-
157	0.056	0.064	0.011	0.056	0.056	-	-
160	-	-	0.006	-	-	-	-
163	0.167	0.191	0.121	0.111	0.167	0.105	-
166	0.444	0.511	0.621	0.556	0.667	0.684	1.000
169	0.167	0.021	0.011	0.056	-	-	-
172	0.167	0.128	0.080	-	-	-	-
175	-	0.021	0.006	0.111	-	-	-

Locus	MAE	TAK	KAN	UBO	TRA	RAN	YAL
TA1							
N	18	47	174	18	18	38	30
178	-	-	-	0.056	0.056	-	-
181	-	0.043	0.121	-	-	0.132	-
187	-	-	0.006	-	-	-	-
190	-	-	0.017	0.056	0.056	0.079	-
TAA81							
N	18	44	172	17	17	37	29
109	0.056	-	0.023	-	-	0.027	-
112	0.056	0.068	0.076	0.059	0.059	0.054	-
115	0.167	0.386	0.384	0.353	0.176	0.243	-
118	0.167	0.159	0.035	0.118	-	0.054	-
121	0.222	0.159	0.233	0.118	0.235	0.486	-
124	0.278	0.114	0.134	0.118	0.176	0.054	-
127	-	0.091	0.064	0.176	0.353	0.081	1.000
130	0.056	-	-	0.059	-	-	-
133	-	0.023	0.017	-	-	-	-
139	-	-	0.023	-	-	-	-
142	-	-	0.012	-	-	-	-
C1M8							
N	18	47	153	17	17	37	31
150	-	-	-	0.059	-	-	-
156	-	0.021	0.013	-	0.059	-	-
159	0.167	0.043	0.150	0.118	0.471	0.270	-
162	0.167	0.064	0.039	0.176	0.059	0.054	-
165	-	-	0.013	-	-	-	-
168	0.056	0.170	0.131	0.118	0.059	0.027	1.000
171	0.111	-	0.013	0.059	-	0.054	-
174	0.056	0.106	0.007	0.118	0.118	0.027	-
177	0.278	0.340	0.340	0.118	0.118	0.405	-
180	-	0.021	0.020	0.059	-	-	-
183	0.167	0.064	0.072	-	-	0.162	-
189	-	-	0.013	0.118	-	-	-
192	-	0.064	0.124	-	-	-	-
195	-	0.043	0.007	0.059	-	-	-
198	-	0.043	0.007	-	-	-	-
201	-	-	0.007	-	-	-	-
204	-	-	0.007	-	-	-	-
207	-	0.021	0.026	-	0.059	-	-
210	-	-	0.013	-	-	-	-
216	-	-	-	-	0.059	-	-

APPENDIX III

Allele frequency of four microsatellite markers located within and flanking *pfmdr1* gene distributed in Kanchanaburi population

Locus	2004	2005	2006	2007
L3				
N	20	18	34	22
237	0.050	-	-	-
243	0.650	0.444	0.676	0.545
247	0.100	0.333	0.059	0.182
249	0.050	0.167	0.118	-
251	0.100	0.056	-	-
253	0.050	-	0.088	0.227
255	-	-	0.059	0.045
L4				
N	20	18	34	22
200	0.200	-	0.147	0.091
206	0.500	0.278	0.029	0.045
212	0.250	0.722	0.824	0.864
224	0.050	-	-	-
L5				
N	20	18	34	22
222	0.750	0.333	0.353	0.409
226	-	-	0.059	-
228	0.100	-	-	-
230	-	0.056	-	-
232	0.050	0.111	0.059	0.318
234	0.100	0.167	0.088	0.182
236	-	-	-	0.045
246	-	-	0.029	-
248	-	0.278	0.294	0.045
250	-	0.056	0.118	-
L7				
N	20	18	34	22
272	0.050	-	-	-
274	0.650	0.389	0.412	0.364
276	0.150	0.333	0.265	0.273
278	0.100	0.167	0.235	0.318
280	-	0.056	0.029	-
282	0.050	0.056	0.059	0.045

APPENDIX IV

Allele length of four microsatellite markers located within and flanking *pfmdr1* gene distributed in Kanchanaburi population

Samples	Year of collection	Identical haplotype	Allele length at each locus			
			L3	L4	L5	L7
PFK45	2004		237	206	222	274
PFK20	2004		243	200	222	274
PFK36	2004		243	200	222	274
PFK42	2004		243	200	222	274
PFK58	2004	1	243	200	222	274
PFK143	2006		243	200	222	274
PFK181	2006		243	200	222	274
PFK200	2006		243	200	222	274
PFK254	2007		243	200	222	274
PFK253	2007		243	200	222	282
PFK180	2006		243	200	248	276
PFK9	2004		243	206	222	274
PFK18	2004		243	206	222	274
PFK23	2004		243	206	222	274
PFK28	2004	2	243	206	222	274
PFK62	2004		243	206	222	274
PFK63	2004		243	206	222	274
PFK70	2005		243	206	222	274
PFK71	2005		243	206	222	274
PFK191	2006	3	243	212	222	276
PFK206	2006		243	212	222	276
PFK12	2004		243	212	222	278
PFK15	2004		243	212	222	278
PFK166	2006		243	212	222	278
PFK174	2006		243	212	222	278
PFK187	2006	4	243	212	222	278
PFK210	2006		243	212	222	278
PFK218	2007		243	212	222	278
PFK222	2007		243	212	222	278
PFK236	2007		243	212	222	278
PFK212	2006		243	212	226	274
PFK104	2005		243	212	230	274
PFK8	2004		243	212	232	274
PFK79	2005		243	212	232	276
PFK107	2005		243	212	232	276
PFK201	2006	5	243	212	232	276
PFK248	2007		243	212	232	276
PFK249	2007		243	212	232	276
PFK250	2007		243	212	232	276

Samples	Year of collection	Identical haplotype	Allele length at each locus			
			L3	L4	L5	L7
PFK234	2007		243	212	232	278
PFK243	2007	6	243	212	232	278
PFK252	2007		243	212	232	278
PFK190	2006		243	212	248	274
PFK119	2005		243	212	248	276
PFK118	2005		243	212	248	278
PFK134	2006		243	212	248	278
PFK158	2006	7	243	212	248	278
PFK161	2006		243	212	248	278
PFK163	2006		243	212	248	278
PFK225	2007		243	212	248	278
PFK117	2005		243	212	248	282
PFK148	2006	8	243	212	248	282
PFK184	2006		243	212	248	282
PFK142	2006		243	212	250	274
PFK150	2006	9	243	212	250	274
PFK176	2006		243	212	250	274
PFK178	2006		243	212	250	274
PFK235	2007		247	206	222	274
PFK26	2004		247	206	222	276
PFK114	2005		247	212	222	274
PFK131	2005		247	212	222	274
PFK245	2007	10	247	212	222	274
PFK256	2007		247	212	222	274
PFK124	2005		247	212	222	278
PFK151	2006		247	212	226	280
PFK14	2004		247	212	228	276
PFK219	2007		247	212	232	274
PFK132	2005	11	247	212	248	276
PFK138	2006		247	212	248	276
PFK77	2005		247	212	248	280
PFK127	2006		247	212	250	276
PFK144	2006		249	200	222	274
PFK75	2005	12	249	206	222	274
PFK135	2006		249	206	222	274
PFK88	2005		249	206	234	274
PFK183	2006		249	212	222	274
PFK47	2004		249	212	222	276
PFK211	2006		249	212	232	276

Samples	Year of collection	Identical haplotype	Allele length at each locus			
			L3	L4	L5	L7
PFK95	2005		249	212	234	276
PFK54	2004		251	206	234	274
PFK80	2005		251	206	234	278
PFK55	2004		251	224	228	272
PFK17	2004		253	206	234	282
PFK223	2007		253	212	234	274
PFK133	2006		253	212	234	276
PFK168	2006		253	212	234	276
PFK198	2006	13	253	212	234	276
PFK232	2007		253	212	234	276
PFK240	2007		253	212	234	276
PFK259	2007		253	212	234	276
PFK258	2007		253	212	236	274
PFK231	2007		255	212	222	274
PFK189	2006		255	212	246	274
PFK137	2007		255	212	248	274

BIOGRAPHY

Miss Tepanata Pumpaibool was born on August, 26, 1973 in Bangkok, Thailand. After graduating from Horwang School, Tepanata enrolled in the Department of Microbiology, Faculty of Science, Chulalongkorn University and received her Bachelor's degree in May 1994. She enrolled in the same department, majoring in Industrial Microbiology in June 1994 and conducted a research on fungal fermentation in the itaconic acid production. She finished her Master degree in May 1998. She continued her Ph.D. study in Biomedical Sciences Program, Graduate School Chulalongkorn University in June 2003. She was awarded the scholarship from the Golden Jubilee Ph.D. Program, Thailand Research Fund for her study at Chulalongkorn University.