

*Analyses of microsatellite polymorphisms flanking the pfcrt gene of Plasmodium falciparum showed different modes of evolution of chloroquine resistance in Thailand and Vietnam*

Moritoshi Iwagami<sup>1</sup>, Pannapa Susomboon<sup>1,2</sup>, Noppadon Tangpukdee<sup>2</sup>, Polrat Wilairatana<sup>2</sup>, Srivicha Krudsood<sup>3</sup>, Le Duc Dao<sup>4</sup>, Nguyen Van Tuan<sup>4</sup>, Bui Quang Phuc<sup>4</sup>, Nguyen Duc Giang<sup>4</sup>, Shusuke Nakazawa<sup>5</sup>, Sornchai Looareesuwan<sup>2</sup>, Shigeyuki Kano<sup>1</sup>

<sup>1</sup>Research Institute, International Medical Center of Japan, Tokyo, Japan

<sup>2</sup>Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

<sup>3</sup>Department of Tropical Hygiene, Mahidol University, Bangkok, Thailand

<sup>4</sup>Molecular Biology Department, National Institute of Malariology, Parasitology and Entomology, Hanoi, Vietnam

<sup>5</sup>Department of Protozoology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

# *Introduction*

- A mutation in *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*) gene in codon 76 (K76T) is associated with chloroquine (CQ) resistance and used to monitor the distribution and frequency of the CQ resistant malaria.
- Microsatellite DNA polymorphisms flanking the drug resistant genes can be used to study the evolution of the genes.

K76T: substitution K (lysine) to T (threonine) at 76th amino acid

# *The purpose of this study*

To identify the mode of evolution of the CQ resistant gene between the Thai and Vietnamese populations,

1. Determine sequences around the codon 72-76 of the *pfcr* gene
2. Analyze the microsatellite DNA loci flanking the *pfcr* gene

*pfcr*: *Plasmodium falciparum* chloroquine resistant transporter

# *Sampling sites*



*Western part of Thailand  
(2002-2005)*

*Southern part of Vietnam  
(2004)*

# *Materials and Methods*

## DNA sequencing of the *pfCRT* gene

### *Materials*

Blood samples from falciparum malaria patients

Thailand: 50 isolates

Vietnam: 39 isolates

### *Methods*

DNA extraction from blood samples on the filter papers/  
frozen blood



PCR of the partial *pfCRT* gene



DNA sequencing of the partial *pfCRT* gene by  
ABI 310 genetic analyzer

*pfCRT*: *Plasmodium falciparum* chloroquine resistant transporter

# *Sequences of the pfcr1 gene: codon 72-76*

## *Thailand*

Amino acid sequences 72-76	Frequency (%)	CQ susceptibility
<b>CVIET</b>	<b>100.0</b>	<b>Resistant</b>

(50 isolates)

## *Vietnam*

Amino acid sequences 72-76	Frequency (%)	CQ susceptibility
<b>CVMNK</b>	<b>69.0</b>	<b>Susceptible</b>
<b>CVIET</b>	<b>15.4</b>	<b>Resistant</b>
<b>CVIDT</b>	<b>7.7</b>	<b>Resistant</b>
<b>CVMDT</b>	<b>2.6</b>	<b>Resistant</b>
<b>CVMNK/CVIDT</b>	<b>5.3</b>	<b>-</b>

(39 isolates)

C: cysteine, V: valine, M: methionine, N: asparagine, K: lysine, T: threonine, I: isoleucine, D: aspartic acid

*Is there any difference in the mode of evolution of CQ resistant gene between the Thai and Vietnamese populations?*

# *Microsatellite DNA is*

- *Tandem repeat sequence*

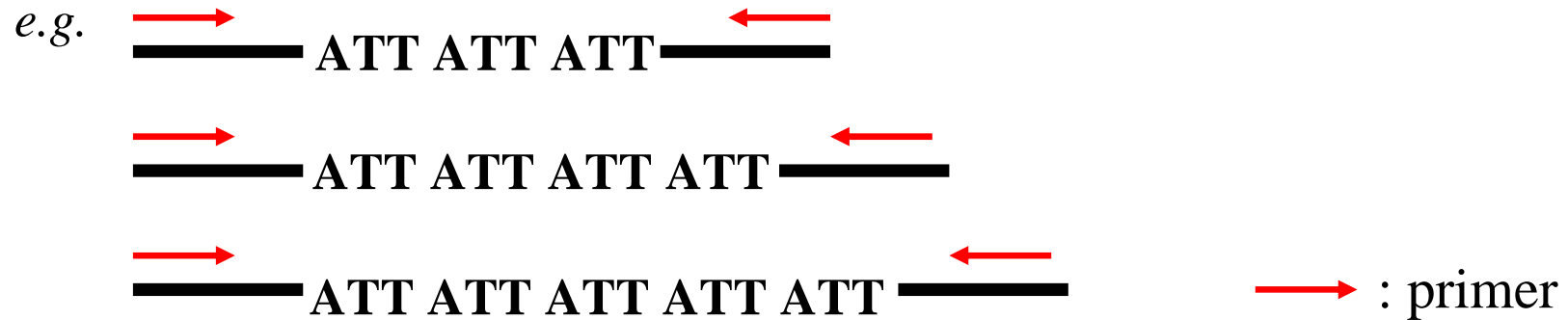
*e.g.* ATT ATT  
ATT ATT ATT  
ATT ATT ATT ATT

- *Highly polymorphic*



# Strategy

Analysis of the microsatellite loci flanking the *pfcr* gene



*CQ* susceptible



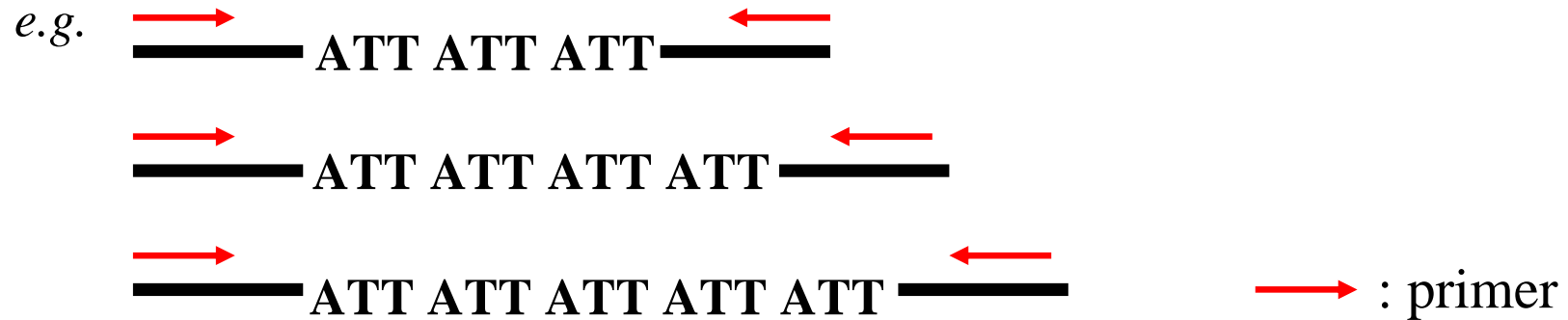
*CQ* resistant



■ : Microsatellite DNA loci, ■ : *pfcr* gene, CQ: chloroquine

# Strategy

Analysis of the microsatellite loci flanking the *pfcr* gene



*CQ* susceptible

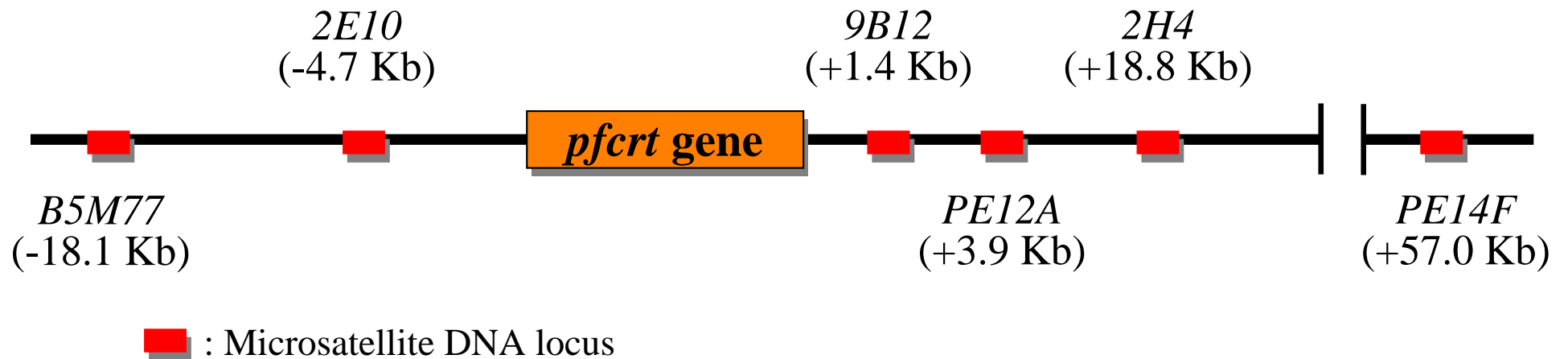


*CQ* resistant



■ : Microsatellite DNA loci, ■ : *pfcr* gene, CQ: chloroquine

# *pfCRT* gene & flanking microsatellite loci



1 cM : 15 - 30 Kb (Trend Parasitol 18; 266-272)

# *Analyses of the polymorphisms of the microsatellite loci*

**Expected Heterozygosity ( $H$ )** : Measurement of genetic diversity of the each locus  $H = [n/(n - 1)][1 - \sum p_i^2]$  ( $0 \leq H \leq 1$ )

where  $n$  : the number of isolates examined and  $p_i$ : the frequency of the  $i$ th allele

$H$  is low



less genetic diversity



Strong selective pressure

$H$  is high



high genetic diversity



Weak selective pressure

# *Results: the polymorphisms of the microsatellite loci of Thai isolates*

Sample ID	Year	B5M77	2E10	PfCRT 72-76	9B12	PE12A	2H4	PE14F
AQ1164	2004	135	130	CVIET	166	308	193	mix
AQ1165	2004	mix	158	CVIET	166	308	193	mix
AQ1167	2004	137	130	CVIET	166	308	193	mix
AQ1169	2004	137	130	CVIET	166	308	193	132
AQ1171	2004	mix	158	CVIET	166	308	189	mix
AQ1172	2004	139	158	CVIET	166	308	193	mix
AQ1174	2004	137	158	CVIET	166	308	193	132
AQ1175	2004	137	158	CVIET	166	308	193	129
AQ1176	2004	137	158	CVIET	166	308	mix	126
AQ1180	2004	137	158	CVIET	166	308	mix	126
AQ1037	2003	137	158	CVIET	166	308	193	132
AQ1040	2003	137	158	CVIET	166	308	193	132
AQ1048	2003	137	158	CVIET	166	308	175	132
AQ1049	2003	137	158	CVIET	166	308	193	126
AQ1053	2003	137	158	CVIET	166	308	175	126
AQ1054	2003	137	158	CVIET	166	309	175	126
AQ1071	2003	137	158	CVIET	166	308	204	132
AQ1072	2003	137	158	CVIET	166	308	193	132
AQ1082	2003	137	158	CVIET	166	308	193	132
AQ1097	2003	137	158	CVIET	166	308	175	132
AQ1098	2003	137	158	CVIET	166	308	ND	135
AQ1141	2003	137	158	CVIET	166	308	193	132
AQ1142	2003	137	158	CVIET	168	308	193	138
AQ1157	2003	137	158	CVIET	166	308	205	126
AQ1163	2003	137	158	CVIET	166	308	205	132

*CQ  
resistant*

ND: no data, mix: multiple genotype infection

# Results: the polymorphisms of the microsatellite loci of Vietnamese isolates

Sample ID	Year	B5M77	2E10	PfCRT 72-76	9B12	PE12A	2H4	PE14F
Lo C 211	2004	ND	159	CVIDT	166	308	ND	165
Lo C 191	2004	mix	164	CVIDT	166	309	ND	166
Lo C 241	2004	137	164	CVIDT	165	253	ND	165
Lo C 269	2004	137	164	CVMMDT	ND	308	ND	ND
Lo C 209	2004	135	167	CVIET	166	mix	ND	ND
Lo C 206	2004	ND	168	CVIET	ND	309	ND	112
Ben Tre 124	2004	ND	mix	CVIET	164	308	ND	165
Ben Tre 125	2004	ND	158	CVIET	160	ND	ND	160
Ben Tre 106	2004	ND	164	CVIET	160	mix	ND	160
Phu Binh 41	2004	ND	mix	CVIET	97	ND	ND	97
Lo C 195	2004	135	171	CVMNK	166	328	ND	166
Lo C 204	2004	ND	177	CVMNK	160	308	ND	160
Lo C 210	2004	ND	174	CVMNK	ND	308	ND	164
Lo C 214	2004	135	160	?VMNK	160	ND	ND	ND
Lo C 228	2004	mix	170	CVMNK	ND	308	ND	112
Lo C 230	2004	ND	169	CVMNK	ND	mix	ND	163
Lo C 231	2004	139	187	CVMNK	171	308	ND	171
Lo C 235	2004	ND	168	CVMNK	ND	mix	ND	ND
Lo C 236	2004	135	164	CVMNK	ND	290	ND	167
Lo C 219	2004	ND	165	CVMNK	ND	289	ND	ND
Lo C 244	2004	ND	155	CVMNK	166	308	ND	166
Lo C 248	2004	ND	176	CVMNK	160	mix	ND	160
Lo C 249	2004	ND	141	CVMNK	ND	mix	ND	ND
Lo C 251	2004	135	160	CVMNK	160	308	ND	160
Lo C 252	2004	ND	169	CVMNK	ND	305	ND	ND
Lo C 253	2004	135	170	CVMNK	160	308	ND	160
Lo C 255	2004	mix	156	CVMNK	164	ND	ND	164
Lo C 247	2004	mix	174	CVMNK	160	ND	ND	160
Lo C 261	2004	ND	176	CVMNK	160	308	ND	160
Lo C 283	2004	ND	mix	CVMNK	ND	308	ND	165
Lo C 279	2004	ND	154	CVMNK	ND	308	ND	160
Ben Tre 142	2004	ND	mix	CVMNK	161	mix	ND	161
Ben Tre 122	2004	133	177	CVMNK	162	308	ND	163
Ben Tre 129	2004	ND	133	CVMNK	ND	308	ND	160
Clinic 521	2004	135	171	CVMNK	169	308	ND	169
Clinic 523	2004	ND	154	CVMNK	166	308	ND	166
Dong Tam 711	2004	ND	167	CVMNK	167	308	ND	168
Lo C 271	2004	ND	166	CVMNK/CVIDT mix	ND	308	ND	ND
Dong Tam 708	2004	ND	179	CVMNK/CVIDT mix	160	308	ND	160

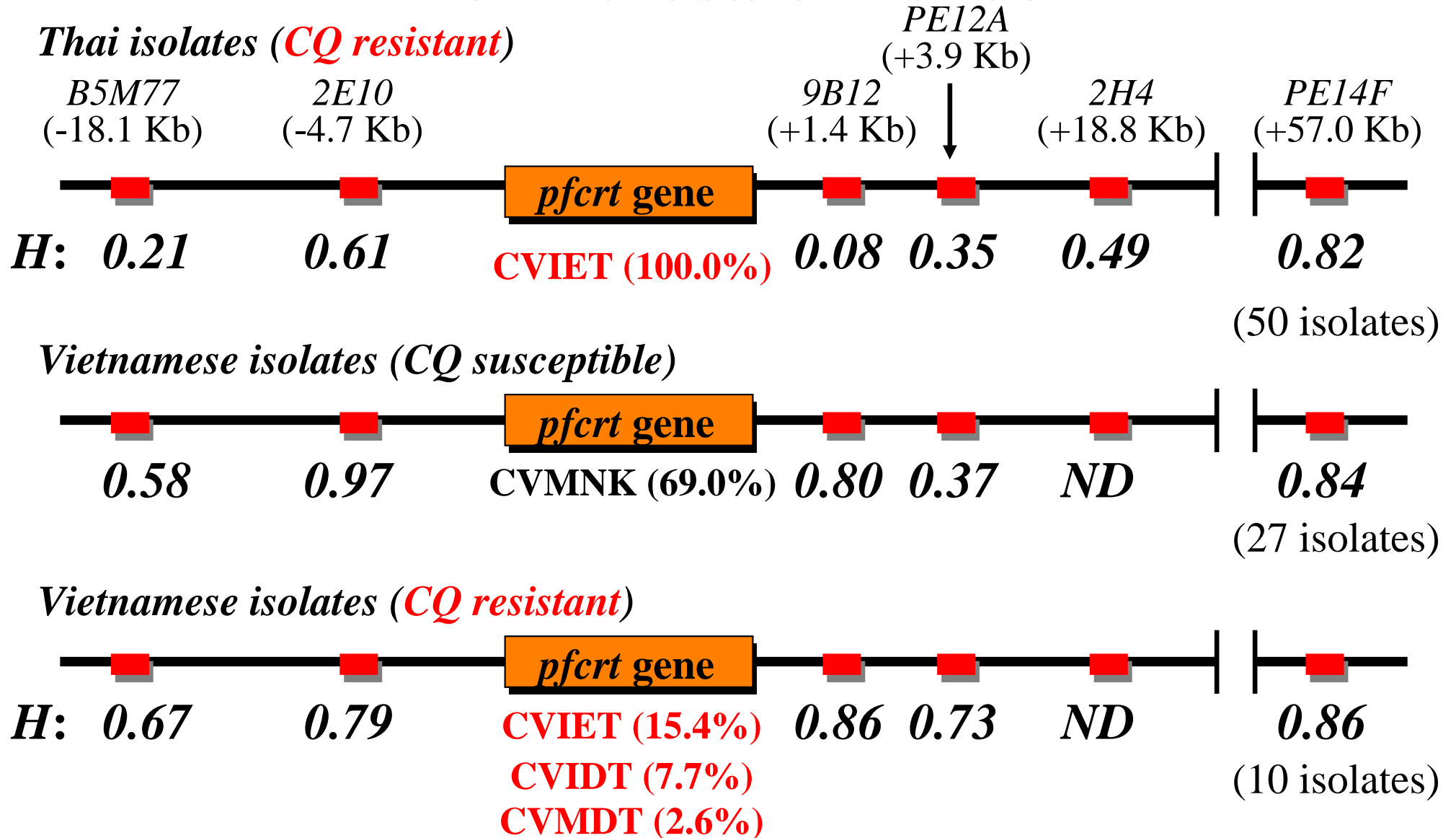
ND: no data, mix: multiple genotype infection

*CQ*  
*resistant*  
*(10)*

*CQ*  
*susceptible*  
*(27)*

*Mixed*  
*infection*  
*(2)*

# Results: genetic diversity of the microsatellite loci



■ : Microsatellite DNA locus, *H*: expected heterozygosity (genetic diversity)

## *Summary and Discussion*

1. The CQ resistant genotype (CVIET) was fixed in the Thai population, but not in the Vietnamese population.
2. The tendency of  $H$  of the microsatellite loci flanking the *pfcr* gene of the Thai population were lower than those of the Vietnamese population.
3. The CQ pressure in Thailand has been higher than that in Vietnam.
4. The mode of evolution of the *pfcr* gene in Thailand and Vietnam seems to be different from each other.



## *Future Plan*

1. We suspect that the origin of CQ resistant Vietnamese isolates may not be from the Thai isolates.

The sizes of some microsatellite loci were different each other

- Isolates from Thai-Cambodia border or isolates from Cambodia

# *Acknowledgments*

We thank all the study participants both Thailand and Vietnam for their kind cooperation.

This study was supported by :

A Grant-in-Aid for Scientific Research (B) (16406012 and 19406013) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.