3.3 Correlational studies of polymorphisms in field isolates identifies a putative ubiquitin transferase as a novel marker of reduced quinine susceptibility

Previous studies have investigated quinine and chloroquine IC_{50} values in large numbers of field isolates and lab strains (Mu et al., 2003). From the field isolates available to our labaratory, I selected 26 strains from Southeast Asia, 12 from Africa, 10 from Latin America, one from Papua New Guinea for the subsequent analyses; also included in these analyses were five lab strains (3D7, HB3, Dd2, 7G8, and GB4) (Table 3.2). I sequenced the relevant genomic regions of all of the field isolates and lab strains to investigate the polymorphisms described above. The sequences from these 50 strains were then aligned using the BioEdit program. The identified polymorphisms are listed in Appendix 1. I subsequently used PCR to check for length polymorphisms (Appendix 1). The primers used for identifying length polymorphisms are listed in table 2.3.

The *pfcrt* K76T mutation has been thought to be a key marker of resistance to chloroquine and other quinoline drugs. Ten of the analysed strains carried a wild type *pfcrt* (76K) allele, and 40 carried a mutant *pfcrt* allele (76T). The strains that carried the *pfcrt* 76T allele exhibited significantly higher chloroquine IC_{50} values than the strains carrying the WT (76K) *pfcrt* allele. With respect to the quinine IC_{50} values, however, when the cutoff for quinine resistance was set at $IC_{50} \geq 100$, three of the strains (102/1, 92-9, and GB4) were more sensitive to quinine despite the fact that they carried the mutant *pfcrt* allele. This finding suggests that the *pfcrt* substitution is not the only determinant of the reduced quinine susceptibility of *P. falciparum*. This finding is consistent with previous studies showing that quinine resistance involves multiple genes (Ferdig et al., 2004; Mu et al., 2003). I next selected 80 polymorphisms in 12 polymorphic genes within the B5M12 locus to determine their correlation with the observed quinine IC_{50} values of the 50 field isolates and lab strains.

3.3.1 Identification of candidate genes

The correlation of each polymorphism with the quinine IC_{50} values was determined by first calculating the Pearson correlation coefficient (simple regression). Then, the probabilityof the null hypothesis was calculated and converted to a logarithmic odd (LOD) score (detailed see 2.2.5). The higher the LOD score, the higher the association of a polymorphism with reduced quinine susceptibility (i.e., the IC_{50} value). The results were

simplified and presented as a histogram (Fig. 3.2). Each bar in the histogram indicates the peak correlation of each gene to the quinine IC_{50} values. A horizontal line at zero indicates no polymorphism in this gene, and a gap indicates that the gene that could not be analysed or was not selected for further analysis. Two genes with major LOD scores were significantly associated with reduced quinine susceptibility. The downstream locus contained a putative ubiquitin transferase (MAL7P1.19), while the upstream peak included the RAMA gene (Rhoptry Associated Membrane Antigen; MAL7P1.208) (Fig. 3.2).
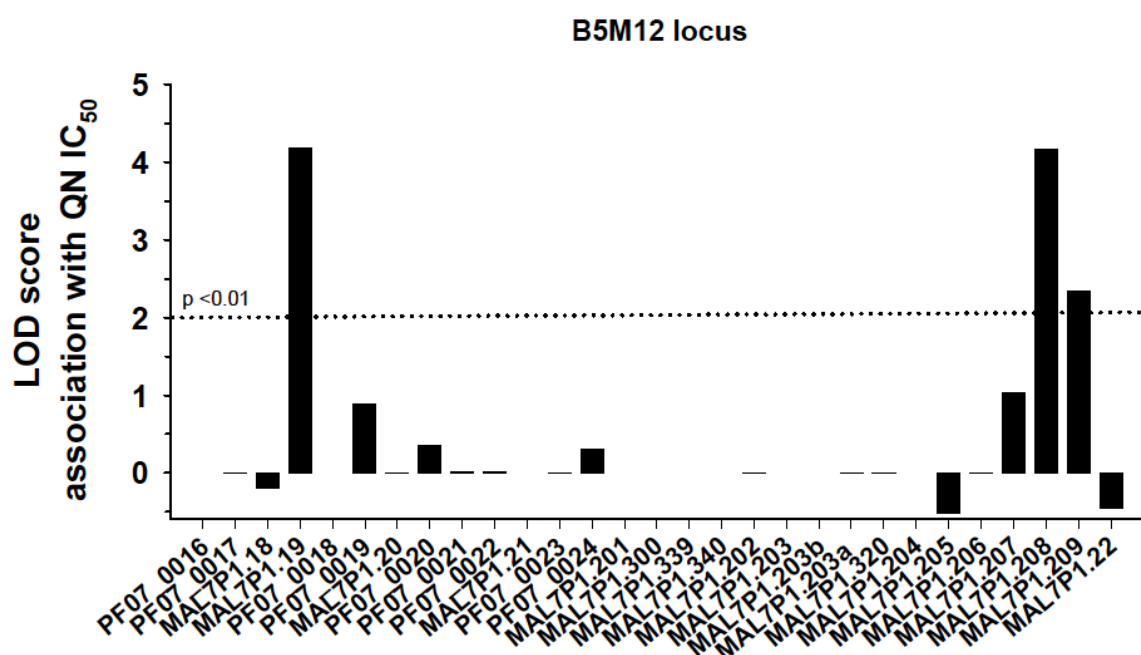

Figure 3.2 Linkage analyses of quinine IC$_{50}$ values with the annotated polymorphic genes in the B5M12 locus in 50 field isolates and laboratory strains. Horizontal lines indicate genes without polymorphisms, and gaps indicate the lack of sequencing results or genes that were not selected for further analyses. The dotted lines represent the confidence line with p<0.01. LOD, logarithmic of odd; QN, quinine. (courtesy of Dr. Sanchez)

In addition to the putative ubiquitin transferase and the RAMA gene, a putative ATPase (MAL7P1.209) was also observed to significantly associate with reduced quinine IC_{50} values. In the following studies, however, I did not observe any significant associations of the polymorphisms in this putative ATPase with IC_{50} values (data not shown). I also noticed that certain genes (MAL7P1.18, MAL7P1.205, and MAL7P1.22) had negative LOD scores, indicating that the p-value was higher than the mean p-value and therefore that the polymorphism is not associated with quinine susceptibility.

Table 3.2. Field isolates and laboratory strains, their IC$_{50}$ values to quinine and chloroquine and the selected polymorphisms within the putative ubiquitin transferase gene, RAMA and *pfcrt*.

Strain	Origin	QN IC$_{50}$ (nM)	CQ IC$_{50}$ (nM)	UT (1388)	RAMA (328)	*pfcrt* (76)
1088	Thailand	301.7	420.4	n.d.	N	T
CP250	Cambodia	282.3	317.4	F	N	T
CP313	Cambodia	262.4	161.1	F	n.d.	T
CP297	Cambodia	252.9	124.5	n.d.	N	T
Thai2	Thailand	234.5	219.2	F	N	T
713	Guinea Bissau	214.5	218.1	F	N	T
CP201	Cambodia	214.4	134.4	F	n.d.	T
CP238	Cambodia	208.3	275.8	n.d.	N	T
Thai18	Thailand	206.1	199.8	F	N	T
CP305	Cambodia	205.1	121.5	F	N	T
CP285	Cambodia	199.5	199.8	F	N	T
CP252	Cambodia	197.9	203.4	F	N	T
CP203	Cambodia	197.6	253.9	F	N	T
PNG9-1	Papua New Guinea	195.6	87.8	F	N	T
CP269	Cambodia	194.2	141.5	F	N	T
P31	Thailand	194.1	105.6	F	N	T
98-18	Thailand	190.5	85.3	n.d.	N	T
98-5	Thailand	179.5	119.7	F	N	T
ECP	Brazil	178.5	132.5	F	N	T
PBZ945	Brazil	169.9	181.1	F	N	T
CP256	Cambodia	169.6	135.8	F	N	T
Thai-19	Thailand	151.8	280.4	F	N	T
9021	Ghana	147.6	100.3	n.d.	N	T
99-18	Thailand	141.8	151.3	F	N	T
ICS	Brazil	137.9	131.4	F	N	T
98-17	Thailand	132.0	178.3	F	Y	T
7G8	Brazil	128.0	61.9	F	Y	T
DIV14	Brazil	126.2	114.9	n.d.	N	T
DIV17	Brazil	117.5	112.9	F	N	T
98-11	Thailand	115.6	85.3	F	N	T
M97	The Gambia	113.5	206.6	F	Y	T
Dd2	Indochina	111.6	99.8	F	N	T
PBZ357	Brazil	110.2	110.2	F	N	T
FCR3	Thailand	106.6	52.1	n.d.	Y	T
PAD	Brazil	104.2	115.6	F	N	T
DIV30	Brazil	97.1	103.2	F	N	T
CP271	Cambodia	83.6	87.8	F	N	T
HB3	Honduras	70.3	11.9	Y	Y	K
IF4/1	Liberia	63.5	15.3	Y	Y	K
102/1	Sudan	63.1	201.7	Y	Y	T
418	Gambia	60.2	15.8	F	Y	K
FAB6	South Africa	56.1	42.6	F	Y	K
92-9	Thailand	54.7	109.0	Y	N	T
M5	Mali	52.6	13.9	Y	Y	K
GB4	Ghana	52.4	99.5	Y	Y	T
K39	Kenya	45.8	20.7	F	Y	K
REN	Sudan	41.9	13.7	Y	Y	K
Camp	Malasya	33.4	15.1	Y	Y	K
3D7	lab. strain	27.3	6.9	Y	Y	K
M24	Kenya	14.2	8.8	Y	N	K

n.d. = no detection

3.3.2 Random omissions study

While calculating the correlation between the polymorphisms and the previously observed IC_{50} values, I wondered whether strain selection might have influenced the results of the correlation study, i.e., whether some strains had a disproportionately strong influence on the statistical results. Therefore, I challenged our correlation study 5 times by randomly omitting 5 of the 50 strains and re-performed the correlation study (Fig. 3.3). I obtained a very similar LOD score profile, with major peaks located at the ubiquitin transferase and RAMA loci; some of the minor peaks oscillated between LOD scores of -1 and 1.
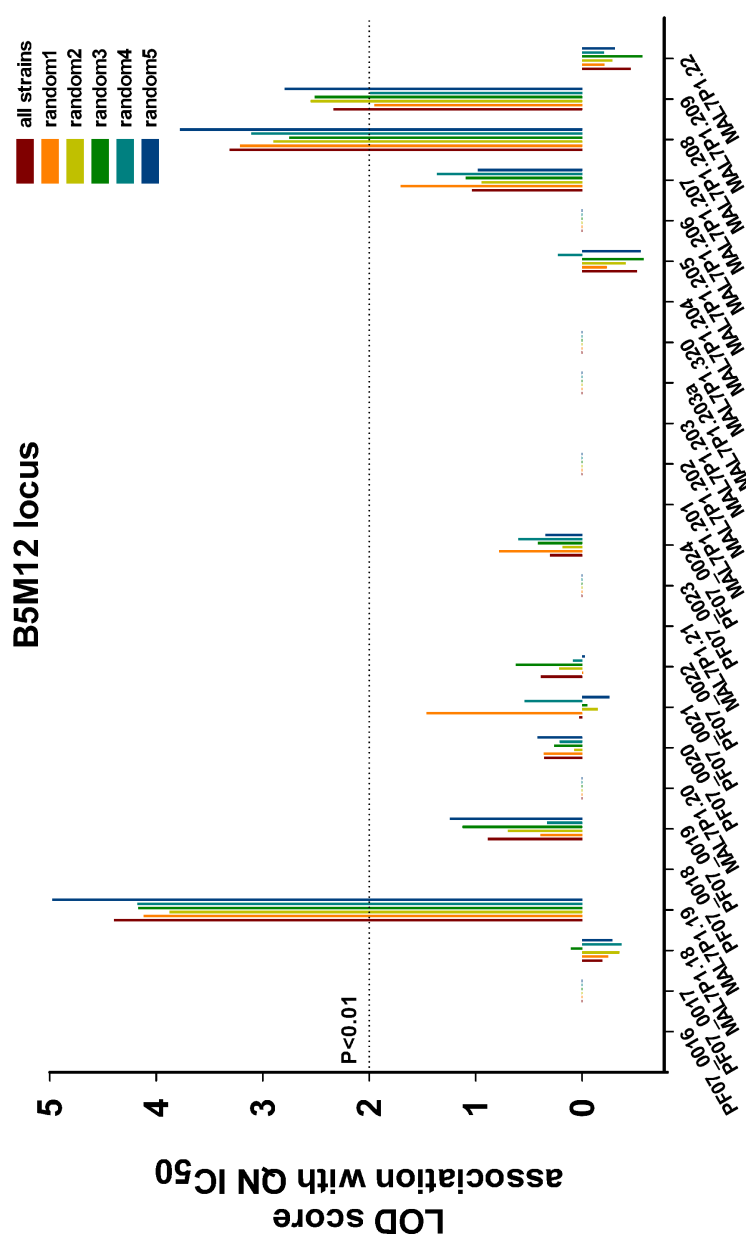

Figure 3.3 Linkage analyses of quinine IC_{50} values and polymorphisms with annotated genes in the B5M12 locus, randomly omitting 5 strains. The 5 repetitions of random omissions of field isolates and lab strains are indicated with different colors.

3.3.3 Synergism between the putative ubiquitin transferase and *pfcrt*

It has been shown that the B5M12 locus interacts synergistically with *pfcrt* to reduce quinine accumulation in HB3 x Dd2 F1 progeny (Mayer, 2009; Nurhasanah, 2009). I therefore tested whether the putative ubiquitin transferase gene determines reduced quinine susceptibility and whether *pfcrt* synergises with the putative ubiquitin transferase in conferring quinine resistance. This was performed by grouping the field isolates and lab strains according to their haplotypes for the putative ubiquitin transferase and *pfcrt*. I considered the ubiquitin transferase that encoded a Y1388F substitution (which exhibited the highest LOD score in association with the quinine IC_{50} value) and the *pfcrt* encoding a K76T substitution as mutants. A correlative box plot analysis of these groups indicated that *pfcrt* and the putative ubiquitin transferase must both be present in the mutant form to significantly increase the quinine IC_{50} value ($p < 0.001$, comparing the last box with the other three boxes; Fig. 3.4, A). As a control, grouping was also performed using the chloroquine IC_{50} value, the results of which indicated that the mutant *pfcrt* allele alone is sufficient to confer reduced chloroquine susceptibility. Mutations in the putative ubiquitin transferase were not significantly involved in the increase of the chloroquine IC_{50} value (Fig. 3.4, B). This finding indicates that there is a synergism between the *pfcrt* K76T substitution and the ubiquitin transferase Y1388F mutation in reduced quinine, but not chloroquine, susceptibility.
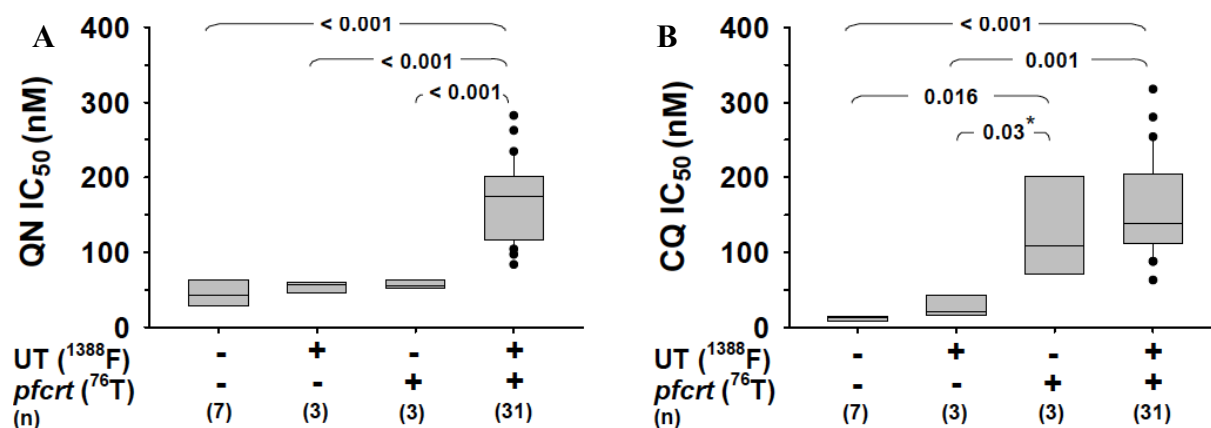

Figure 3.4 Parasites were grouped according to their haplotypes for *pfcrt* and the putative ubiquitin transferase gene and were analysed as a function of the corresponding quinine (A) and chloroquine (B) IC_{50} values. The number of strains in each group and the statistical significance between the groups are indicated. QN, quinine; CQ, chloroquine; UT, putative ubiquitin transferase. *, Fisher's LSD test. (courtesy of Dr. Sanchez)

I repeated the grouping analyses for the RAMA gene. There was only one strain that carried a mutant RAMA allele with a wild type *pfcrt* gene (Table 3.2); therefore, a complete statistically valid grouping analysis, similar to the one performed for the putative ubiquitin transferase, could not be performed. Nevertheless, it is clear that, in contrast to the putative ubiquitin transferase, there was no significant increase in the quinine IC_{50} values (p=0.10) when comparing the strains carrying a mutant *pfcrt* allele and either a wild type or mutant RAMA gene (Fig. 3.5, A). This result suggests that there is no synergism between the RAMA Y324N mutation and the *pfcrt* K76T mutation with respect to reduced quinine susceptibility, i.e., RAMA does not contribute to reduced quinine responsiveness. Furthermore, when I performed the same grouping analyses using the chloroquine IC_{50} values as a control, the *pfcrt* K76T mutation alone was sufficient to confer chloroquine resistance (Fig. 3.5, B).
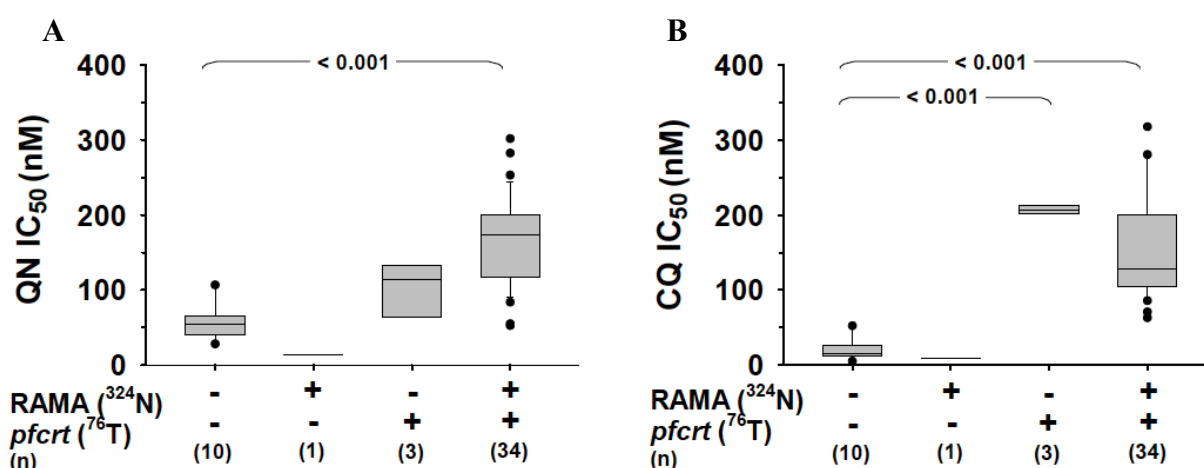

Figure 3.5 Parasites were grouped according to their haplotypes with respect to *pfcrt* and the RAMA gene and analysed as a function of the quinine (A) and chloroquine (B) IC_{50} values. The number of strains in each group and the statistical significance between the groups are indicated. QN, quinine; CQ, chloroquine; RAMA, rhoptry associated membrane antigen. (courtesy of Dr. Sanchez)

Thus, from the correlation study of the 50 field isolates, I identified two candidate genes that were associated with reduced quinine susceptibility. Both the putative ubiquitin transferase and the RAMA had comparable LOD scores. However, grouping analyses revealed that only the putative ubiquitin transferase interacted with *pfcrt* synergistically with respect to reduced quinine susceptibility. I then sought to determine whether the different associations of candidate genes with reduced quinine susceptibility resulted from linkages with *pfcrt*.

3.3.4 The ubiquitin transferase Y1388F mutation is co-selected with the *pfcrt* K76T mutation under quinine selection pressure

Previous studies have identified a conserved region near the *pfcrt* gene on chromosome 7 that is in linkage disequilibrium with the *pfcrt gene* in several chloroquine resistant field isolates and laboratory strains (Jiang et al., 2008; Dharia et al., 2009; Kidgell et al., 2006). However, this conserved region is reported to be downstream of *pfcrt* (Kidgell et al., 2006), whereas the B5M12 locus is located 45 kbp upstream of *pfcrt*. Nevertheless, I performed linkage analyses to test whether the genes within B512 locus segregate with *pfcrt* and investigate the associations of the polymorphic markers identified in the B5M12 locus and in the genes flanking this locus in the 50 strains. The putative ubiquitin transferase, RAMA, and the genes downstream of the B5M12 locus (including MAL7P1.22, *PF*07_0026, and *PF*07_0029) were significantly associated with mutated *pfcrt*. However, the 12 annotated genes located between the putative ubiquitin transferase and RAMA were not significantly associated with mutated *pfcrt* (Fig. 3.6, A).

This finding suggests that the polymorphisms in the putative ubiquitin transferase gene were co-selected with mutated *pfcrt* under quinine selection pressure and did not co-segregate with *pfcrt* due to physical linkage. Within our 50 field isolates and lab strains, 31 of 34 strains (91.2%) carried both the *pfcrt* K76T mutation and the ubiquitin transferase Y1388F mutation (Table 3.2), supporting our co-selection hypothesis.

Previous studies have identified single-feature polymorphisms (SFP) in the *P. falciparum* genome (Kidgell et al., 2006). I also performed a linkage analysis of chromosome 7 using the SFP database. Comparing the SFPs of the 8 strains that overlapped with our 50 strains, I calculated the linkage between the *pfcrt* K76T SFP and all of the other SFPs on chromosome 7. However, I did not observe any linkage between the *pfcrt* K76T mutation and either the putative ubiquitin transferase or RAMA (Fig. 3.6, B). Nonetheless, the number of strains used for these analyses (n=8) is much less than the number of strains used for the linkage analyses (n=50). Although more than 2000 SFPs were analysed, this sample was still not strong enough to disprove my hypothesis that the putative ubiquitin transferase Y1388F mutation is co-selected with the *pfcrt* K76T mutation under the pressure of quinine treatment.
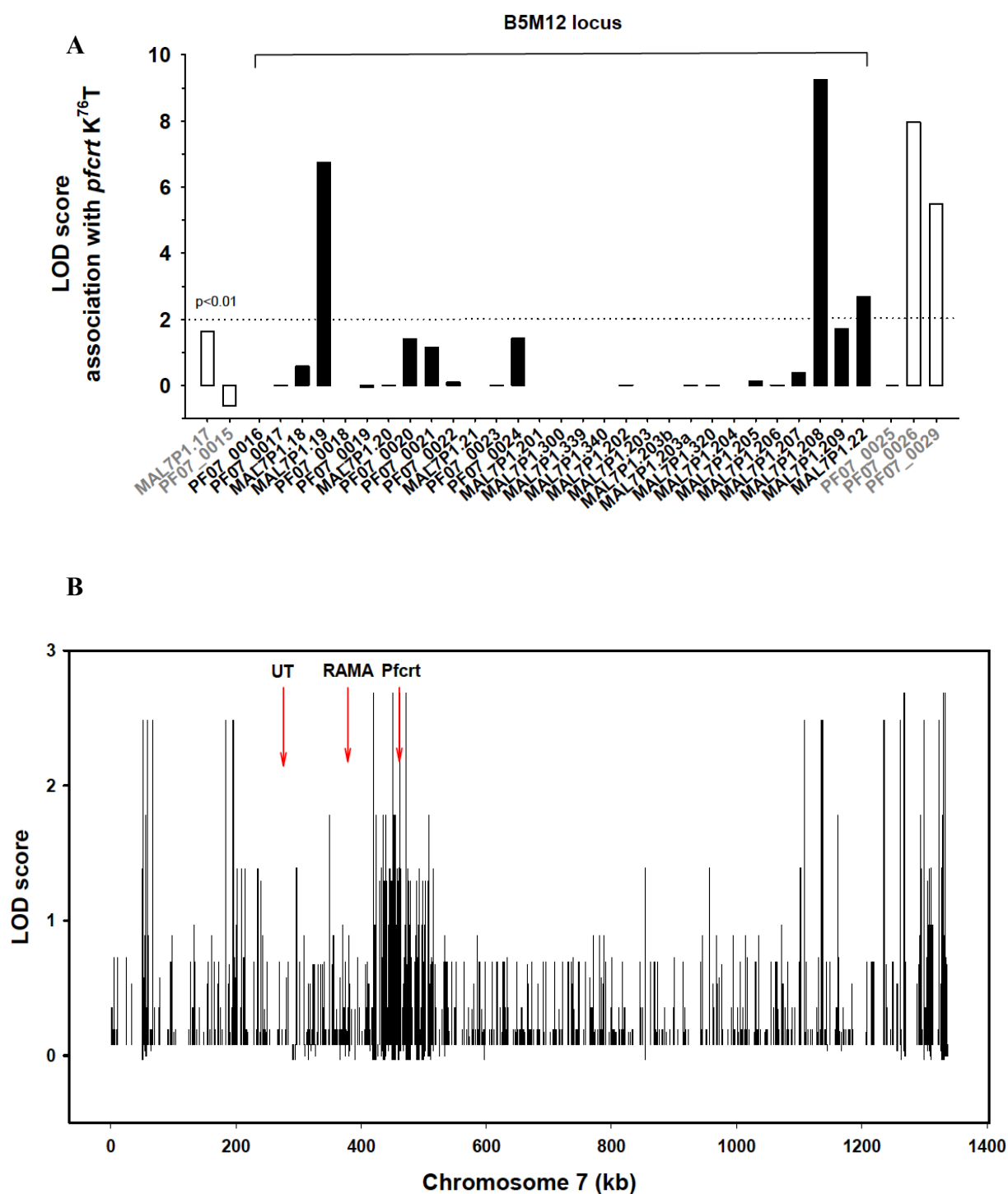
Figure 3.6 Linkage analyses of codon and length polymorphisms within the B5M12 locus (black bars), flanking regions (white bars) (A), and SFPs on chromosome 7 (B) with the mutant *pfcrt* allele. The dotted lines represent a confidence level of p <0.01. LOD, logarithmic odd score; UT, putative ubiquitin transferase; RAMA, rhoptry associated membrane antigen. (A. courtesy of Dr. Sanchez)

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3.4   Bioinformatics studies of the putative ubiquitin transferase

3.4.1 General information from the PlasmoDB website

The gene encoding the putative ubiquitin transferase is located on chromosome 7 between nucleotides 271,404 and 284,452. The gene is 13,049 nucleotides long and contains with 7 introns. The corresponding protein consists of 3893 amino acids and has a predicted molecular weight of 460,420 Da. Four transmembrane domains were predicted using the PlasmoDB website, while the TDR Target website (http://tdrtargets.org) predicted six transmembrane domains. The two additional predicted domains are located at the C-terminus of the ubiquitin transferase, which contains the E3 HECT domain. The conserved catalytic E3 HECT domain (31% similarity to the human E6AP ubiquitin ligase), which characterises the protein as a member of the ubiquitin ligase family, lies within the carboxyl terminal. In addition to a conserved HECT domain, armadillo-repeat domains throughout the entire protein were predicted using InterPro protein sequence analysis and classification. The armadillo-repeat domain consists of a multi-helical fold comprised of two curved layers of alpha helices arranged in a regular right-handed superhelix. This superhelical structure and an extensive solvent-accessible surface are well suited to bind large substrates, such as proteins and nucleic acids.

The proteomic data suggested that the putative ubiquitin transferase is expressed from the late ring stage to the schizont stage during the erythrocytic cycle, matching the stages that the quinolines target. In addition, transcriptome data has shown similar mRNA expression levels of the putative ubiquitin transferase in the Dd2 and HB3 strains, indicating that the transcription of the ubiquitin transferase is not involved in reduced quinine responsiveness. Both the N- and the C-terminal domains of the putative ubiquitin transferase are polymorphic (Fig. 3.7). In this study, I identified 19 single amino acid polymorphisms and 4 length polymorphisms within the protein (Fig. 3.7, Appendix 1). The polymorphism that demonstrated the strongest correlation with altered quinine responsiveness was a tyrosine to phenylalanine replacement at position 1388 (marked in red, Fig. 3.7).
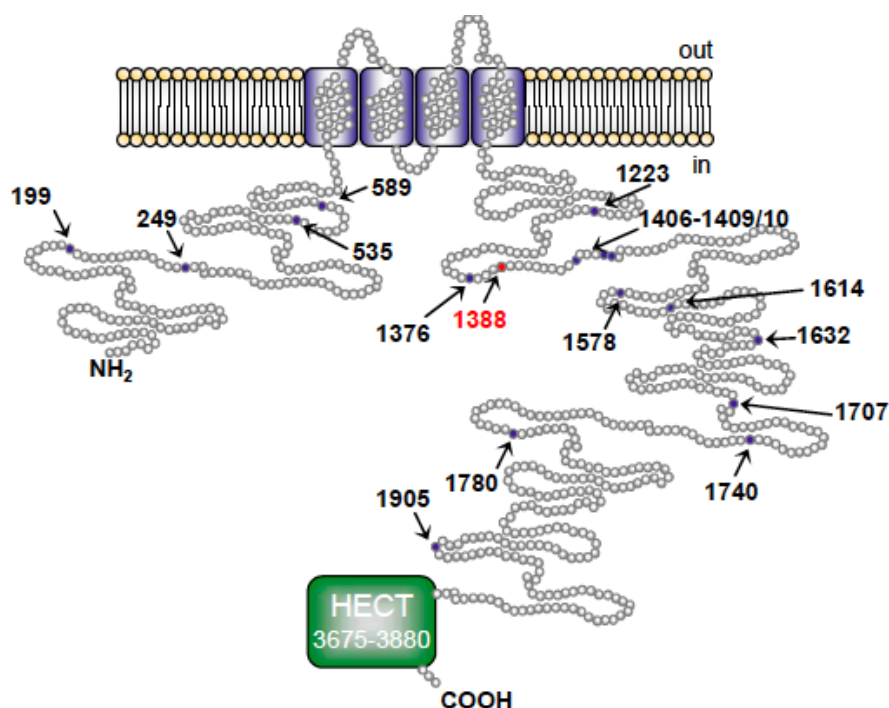
Figure 3.7 Schematic representation of the topology of the putative ubiquitin transferase. The protein consists of 3893 amino acids and has a predicted molecular mass of 460,420 kDa. The four putative transmembrane domains are indicated, as are the polymorphic sites and the catalytic domain (E3 HECT domain), which characterises the protein as a member of the ubiquitin ligase family. HECT, Homologous to the E6-AP Carboxyl Terminus. (courtesy of Dr. Sanchez)

A previous study has demonstrated that different *pfcrt* alleles found in Southeast Asia and Latin America confer different levels of resistance to chloroquine and amodiaquine (Sa et al., 2009). Grouping of the 50 field isolates and lab strains according to their geographic distribution and their quinine IC_{50} values revealed conserved haplotypes of the putative ubiquitin gene in Latin America and Southeast Asia (Fig. 3.8). The pattern of allelic frequency of the putative ubiquitin transferase was similar when position 1233 (Y to C replacement) of the putative ubiquitin transferase and positions 74 and 75 of *pfcrt* were compared. Positions 74 and 75 of pfcrt are reported as the determinant of different levels of chloroquine and amodiaquine resistance (Sa et al., 2009). However, when I grouped the quinine IC_{50} values according to the 1233 position of the putative ubiquitin transferase, I did not observed a significant difference in quinine susceptibility (data not shown). Alternatively, the quinine-sensitive strains carrying a mutant *pfcrt* gene also carry a wild type putative ubiquitin transferase, again suggesting that both mutations are required for quinine resistance (Table 3.2, Fig. 3.8).
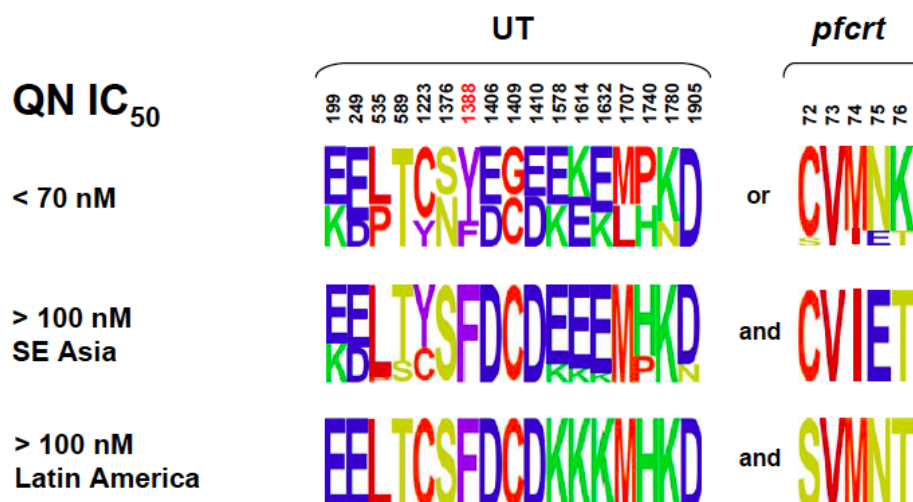
Figure 3.8 Schematic view of allelic frequency of the putative ubiquitin transferase and *pfcrt* **in field isolates and lab strains according to geographic distribution and quinine IC$_{50}$ values.** Parasites with quinine IC$_{50}$ values exceeding 100 nM were grouped according to their geographic origin. The protein residues were rendered using the enoLOGOS websites (Workman et al., 2005). The height of each letter is proportional to the frequency of amino acids found in the given position. Amino acid colour code: red, basic; purple, acidic; blue, hydrophobic; green, polar amino acids.

3.4.2 Low complexity analysis

The *Plasmodium falciparum* genome is much different from other known organisms due to its high AT content and numerous repeat sequences, resulting in many low-complexity regions in the protein coding sequences. *P. falciparum* proteins are often larger than homologous proteins from other organisms. When a multiple alignment is performed, the size difference can be observed to be the result of long insertions separating well-conserved sequence blocks that are adjacent in homologous proteins (Pizzi and Frontali, 2001). The putative ubiquitin transferase also contains several long low-complexity regions with many repeated sequences containing tyrosine residues. However, whether the low-complexity region is either adjacent to or includes the Y1388F mutation, the strongest correlated polymorphism with reduced quinine responsiveness, is unknown. I performed a complexity analysis of sequence tracts (CAST) analysis using the disEMBL[TM] website (http://dis.embl.de/) and disorder analysis using the GlobPlot 2.3 website (http://globplot.embl.de/) to determine whether the Y1388F mutation is within this low-complexity region and if there are different low-complexity profiles of this ubiquitin

transferase between the Dd2 and HB3 strains due to the observed polymorphisms (Pizzi and Frontali, 2001; Promponas et al., 2000). The figures were generated using SigmaPlot 11.0 (Fig. 3.9).

The CAST and disorder analyses of the ubiquitin transferase from the HB3 and Dd2 strains revealed a similar pattern between these two strains, indicating that the polymorphisms do not influence the protein's secondary structure. The Y1388F substitution of the putative ubiquitin transferase is at the edge of the low-complexity region (Fig. 3.9), which is different from the low-complexity results obtained at the PlasmoDB website, where the low-complexity region begins at residue 1397. Nevertheless, *in silico* examination of regions other than the HECT domain did not yield any known superfamily or structural homology to proteins other than those from other *Plasmodium* species (data not shown).
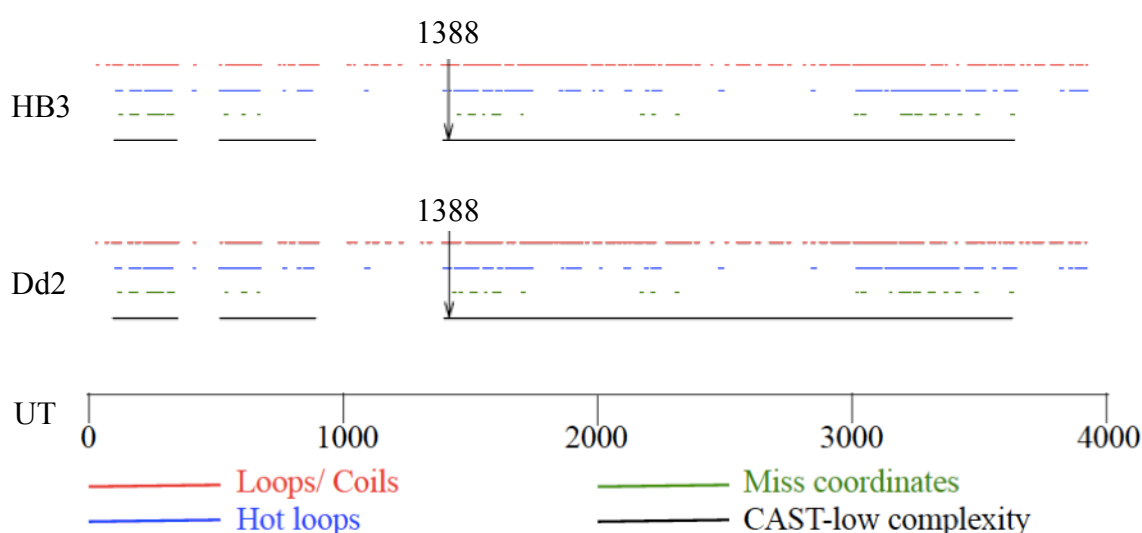

Figure 3.9 Schematics of the CAST and disorder analyses of the putative ubiquitin transferase in the HB3 and Dd2 strains. Loops/ Coils analysis indicates all non-helix or non-beta-strand structures. However, the loop is not sufficient but is required for disorder (red). Hot loops (blue) are subsets from above, indicating that the loops have a high degree of mobility, which can be considered disorder. Missing coordinates in the X-Ray structure as defined by REMARK-465, which has been used early on in disorder prediction. The position of the Y1388F substitution is indicated. The X-axis represents the linear amino acid residues of the putative ubiquitin transferase. UT, putative ubiquitin transferase; CAST, complexity analysis of sequence tracts.

3.4.3 Molecular modelling of the E3 HECT domain

The HECT domain consists of a large N-terminal lobe containing the E2-binding site and a smaller C-terminal lobe, which includes the active site cysteine residue. Structural studies have revealed that these two lobes are connected by a flexible hinge region, which is critical for juxtaposing the catalytic cysteine residues of the E2 and E3 proteins during ubiquitin transfer. A conformational change involving an alteration in the relative orientation of the two lobes is thought to facilitate the reaction (Bernassola et al., 2008). To test whether the putative ubiquitin transferase (MAL7P1.19) contains a functional E3 HECT ligase domain, I first blasted the HECT domain of the ubiquitin transferase using the PDB database, where the highest similarity was obtained for 1nd7A of the human WWP1/AIP E3 HECT ligase domain (Verdecia et al., 2003) (Fig. 3.10, A). I subsequently modelled the structure of the ubiquitin transferase E3 HECT domain (181 amino acids from the C-terminus) using Swiss-PdbViewer 4.04 and the structure of 1nd7 as a template. The predicted structure of the ubiquitin transferase E3 HECT domain was highly similar to that of 1nd7 of the human WWP1/AIP E3 HECT ligase domain (Fig. 3.10, B). The catalytic cysteine residue in the C-terminal lobe was also conserved in our modelling analysis (Fig. 3.10, C).

```
A   UT_HECT   1                                                           PVELIPNG
    1nd7A     694   KDLESIDTEF YNSLIWIRDN NIEECGLEMY FSVDMEILGK VTSHDLKLGG
                                                                 .  .*      *


    UT_HECT   9     SNIPVTNENL NLFINKTIEY SLYDGIKFQI WAFRYGFSTI APLVCTNMFD
    1nd7A     744   SNILVTEENK DEYIGLMTEW RFSRGVQEQT KAFLDGFNEV VPLQWLQYFD
                    *** **.**  .  .*     *    .  *.  *   **  **. .  **      **

                                   1                    2
    UT_HECT   59    ENEICEFLFG SNIENDEHWT KTHLSTYIKP DHGYTNDSIT FITLIEILSE
    1nd7A     794   EKELEVMLCG -----MQEVD LADWQRN-TV YRHYTRNSKQ IIWFWQFVKE
                    *.*.   * *            .          .  . ** .*  .* . ... *

                                                                3
    UT_HECT   109   FNKEERKQFV KFCTGTSALP NNGFAALKPL MKVVKKEDNN D-----LPSV
    1nd7A     838   TDNEVRMRLL QFVTGTCRLP LGGFAELMGS NGPQKFCIEK VGKDTWLPRS
                    ..* *....  * ***  **    *** *.     *    ..       **


    UT_HECT   154   MTCTNYLKIP DYKNKEKLRN RLIYAINE
    1nd7A     888   HTCFNRLDLP PYKSYEQLKE KLLFAIEETE
                    ** * * .*  **. * *.. .*..**.*
```
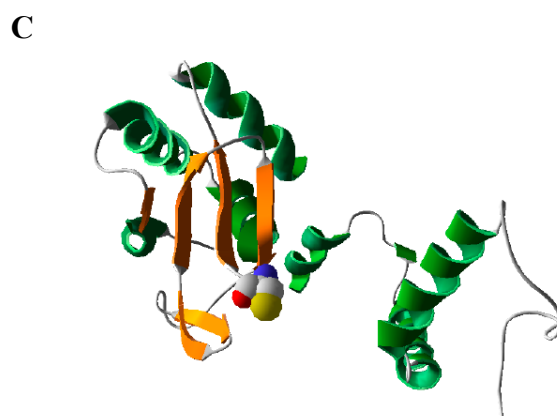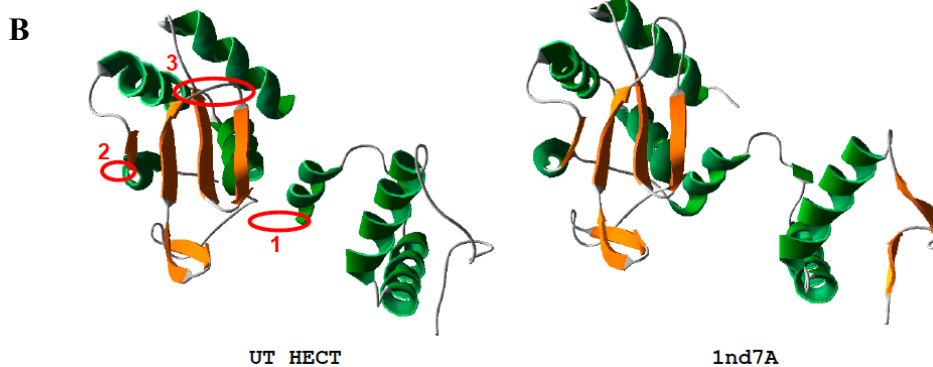

Figure 3.10 Molecular modelling of the putative ubiquitin transferase HECT domain using the human WWP1/AIP E3 HECT domain as template. (A, B) An alignment of 181 residues of the HECT domain of the putative ubiquitin transferase with 1nd7A (WWP1/AIP E3 HECT ligase domain), as well as a structural comparison between the human and parasite domains. The insertions and deletion are indicated with red numbers. **(C)** The conserved cysteine residue in the active site is represented in a CPK format.

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3.5   Biochemical analyses of the putative ubiquitin transferase

Little is known about ubiquitinylation in *P. falciparum,* and the putative ubiquitin transferase identified in this study has not been characterised. To better understand the biochemical function and cellular localisation of the putative ubiquitin transferase, I generated a specific antibody against the ubiquitin transferase. Dr. Sanchez and other colleagues in our group synthesised four peptide antibodies in rabbits and tested them in *P. falciparum* cell lysates. However, these four antibodies failed to detect the ubiquitin transferase in western blots of lysates from Dd2- or HB3-infected RBCs (data not shown). As an alternative approach, Dr. Sanchez cloned 12 fragments in the putative ubiquitin transferase into an expression plasmid. In addition, the potential antigen region of RAMA was also cloned for protein expression. From the 13 constructs, however, only two recombinant proteins could be expressed in *E. coli* (data not shown). The construct containing the E3 HECT domain was chosen for antibody generation (UT_HECT).

3.5.1 Protein expression

I first determined for the optimal conditions for UT_HECT expression in *E. coli* with respect to temperature (37ºC, 25ºC, and 18ºC), IPTG concentration (0.1 mM~1 mM), and induction duration (2-, 4-, 6-h, overnight). However, in all of the induction conditions, the recombinant protein (approximately 30 kDa) was always located in inclusion bodies, which are cytoplasmic aggregates of intermediately folded recombinant proteins (Fig. 3.11, third lane). I chose the following optimal protein induction conditions: an IPTG concentration of 0.4 mM and an overnight culture at 18ºC.
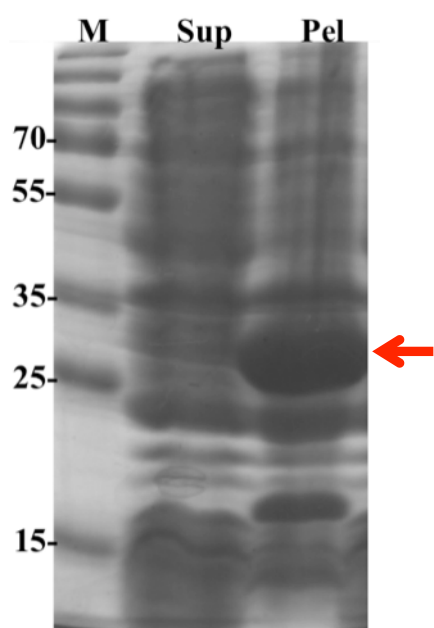
Figure 3. 11 Recombinant UT_HECT is insoluble in *E. coli*. The UT_HECT recombinant protein was induced in 0.4 mM IPTG when cultured overnight at 18ºC. The cells were lysed in a lysis buffer and sonicated, followed by centrifugation. The samples were analysed with SDS-PAGE and Coomassie blue staining. The supernatant (Sup, middle lane) contains soluble proteins, whereas the pellet (Pel, right lane) contains insoluble proteins (inclusion body). The size of UT_HECT is indicated (red arrow). M, marker.

I next took advantage of the 6x His-tag of the recombinant UT_HECT for protein purification using an Ni-NTA slurry (Invitrogen) and a denaturing protocol (see 2.2.4.5) (Fig. 3.12). Although the UT_HECT recombinant protein was successfully purified, the purity of the elution was not ideal, as I observed putative degraded proteins and/ or *E. coli* proteins that bound non-specifically to the Ni-NTA beads (lane E1~E5, Fig. 3.12, A). To increase purity, the band of our recombinant protein was removed from the acrylamide gel (without Coomassie staining) and homogenised in sterile PBS. The mixture was subsequently centrifuged at 10,000 rpm for 1 hour at 4ºC, and the resulting supernatant was subjected to SDS-PAGE electrophoresis. Following purification, I obtained a pure UT_HECT recombinant protein (lane P, Fig. 3.12, B) compared with the elution fraction obtained from column chromatography.

Figure 3.12 Column chromatography purification of UT_HECT recombinant protein (A) and acrylamide gel purification (B). The inclusion body was treated with 6 M guanidine HCl and centrifuged **(A)**. The UT_HECT band was removed from the acrylamide gel and purified **(B)**. The samples were analysed using SDS-PAGE electrophoresis and Coomassie staining. The size of UT_HECT is indicated (red arrows). M, marker; Lys, lysate; Unb, unbound; W, wash; E, elution; P, purified UT_HECT recombinant protein.

3.5.2 Antibody generation and western blot analyses

The concentration of the purified UT_HECT recombinant protein was measured using a Bradford assay, after which the protein was concentrated to approximately 1 ng/µl for injection into mice. In total, four mice were immunised three times with the recombinant protein mixed with either incomplete Freund adjuvant (1st immunisation) or complete Freund adjuvant (2nd and 3rd immunisations). The serum was collected as a polyclonal ubiquitin transferase antibody. The sera from four immunised mice were then tested against the purified UT_HECT recombinant protein (positive control, lane P), erythrocyte lysate (negative control, lane R), and *P. falciparum* (HB3) trophozoite stage lysate (lane L) using a Western blot. However, only the serum from mouse #2 was able to detect a specific band migrating at approximately 70 kDa (Fig. 3.13, A). Naïve mouse serum was used as a negative control (Fig. 3.13, B).
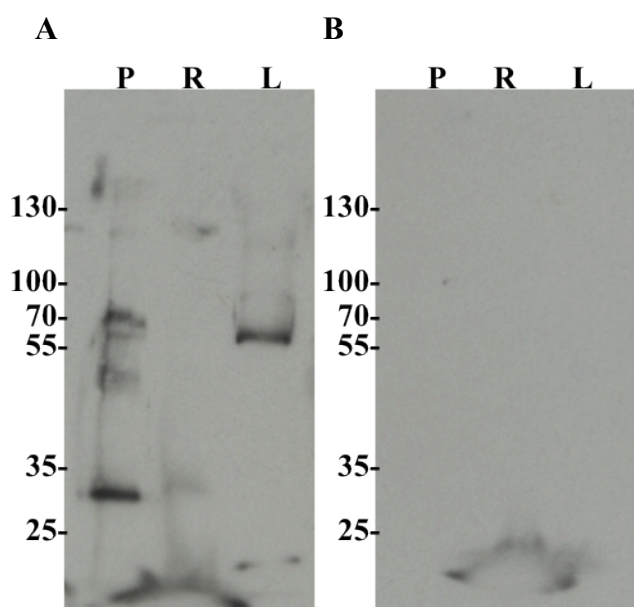

Figure 3.13 Test of putative ubiquitin transferase mouse polyclonal antibody (A) and naïve mouse serum (B) by western blot analysis. UT_HECT recombinant protein (P), erythrocyte lysate (R), and HB3 lysate (L) were analysed on a 3% to 8% gradient acrylamide gel for SDS-PAGE analyses. A western blot was then performed using either the putative ubiquitin transferase mouse polyclonal antibody **(A)** or naïve mouse serum as a negative control **(B)**.

However, the predicted size of the putative ubiquitin transferase is approximately 460 kDa, much larger than the 70 kDa band observed in our western blot analysis. *P. falciparum* encodes many large proteins compared with the orthologues in other species. These large proteins might not migrate properly through the interface between the stacking and resolving gels. To obtain better resolution, the SDS-PAGE analysis was performed using a 3% to 8% gradient acrylamide gel (Invitrogen) and an 8% urea gel, from which I obtained similar results (Fig. 3.13).

3.5.3 Immunofluorescence Assay

The polyclonal ubiquitin transferase mouse antibody was also subjected to an immunofluorescence assay (IFA) to investigate the cellular localisation of the putative ubiquitin transferase (Fig. 3.14). Despite a strong background, the perinuclear localisation and vesicle-like staining observed using IFA suggested an ER location of the putative ubiquitin transferase. However, a co-localisation study with a suitable ER marker staining is required before reaching a definitive conclusion. In addition, *in silico* studies have revealed four to six predicted transmembrane domains in the putative ubiquitin transferase. The IFA did not reveal specific membrane staining. As a control, naïve mouse serum was also investigated using IFA, but no fluorescent signal was detected using confocal microscopy (data not shown).

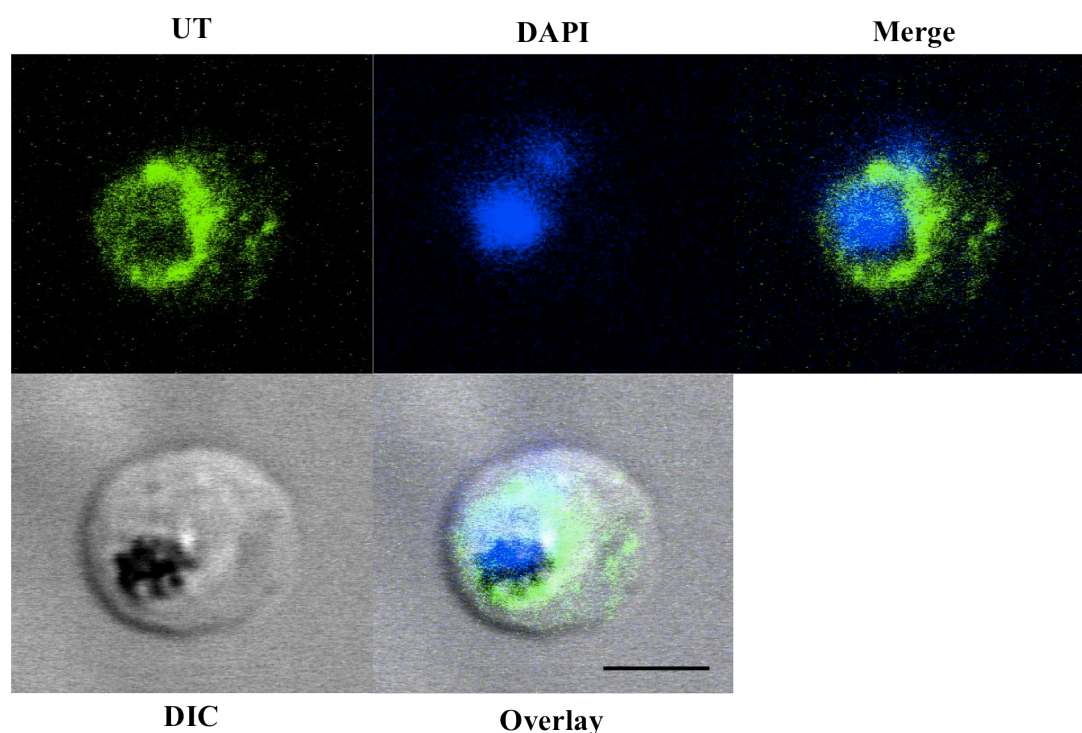**Figure 3.14 Immunofluorescence analyses of the putative ubiquitin transferase in the *P. falciparum* trophozoite stage.** Anti-putative ubiquitin transferase (UT, green) staining revealed a likely ER localisation of the putative ubiquitin transferase. The nucleus was visualised using DAPI staining (blue). The differential contrast image (DIC) image indicates the location of the parasites within the erythrocyte. Scale bar = 4 μm.

3.6 Other genetic factors involved in quinine resistance

3.6.1 *Pfmdr1* polymorphisms and correlation study

Polymorphisms in 5 positions (86, 184, 1034, 1042, and 1246) in the *pfmdr1* gene have been reported to be involved in altering susceptibility to lumefantrine, artemisinin, quinine, mefloquine, halofantrine and chloroquine (Fig. 1.6; Petersen, 2011). Among these 5 positions, the N1042D substitution appears to play a role in quinine resistance (Sidhu et al., 2005). I first used restriction fragment length polymorphism (RFLP) analysis to determine the *pfmdr1* haplotypes of the 50 field isolates and lab strains. I mapped 4 polymorphic sites but could not distinguish the polymorphism at position 184. I subsequently grouped the field isolates according to each mutation (Fig. 3.15, A~D). None of the four mutations was associated with quinine IC_{50} or chloroquine IC_{50} values (data not shown). A previous study demonstrated that different administrations of drugs in different endemic areas have driven the selection of distinct combinations of *pfcrt* and *pfmdr1* mutations (Sá et al., 2009). Thus, I grouped the quinine IC_{50} values according to the *pfmdr1* haplotypes of wild type ($86^N 1034^S 1042^N 1246^D$), SE Asian/ African ($86^Y 1034^S 1042^N 1246^D$), and Latin American (*pfmdr1*^{7G8}, $86^N 1034^C 1042^D 1246^Y$; *pfmdr1*^{HB3}, $86^N 1034^S 1042^D 1246^D$) strains (Fig. 3.15, E).

However, none of these combinations yielded any association with quinine IC_{50} values (Fig. 3.15, E). This result suggests that point mutations in *pfmdr1* are not involved in reduced quinine responsiveness. However, other studies have suggested that copy number polymorphisms are involved in antimalarial resistance (Sidhu et al., 2006; Anderson et al., 2009; Johnson et al., 2008). Unfortunately, there was insufficient genomic DNA to use real-time PCR to perform *pfmdr1* copy number analyses of the field isolates.
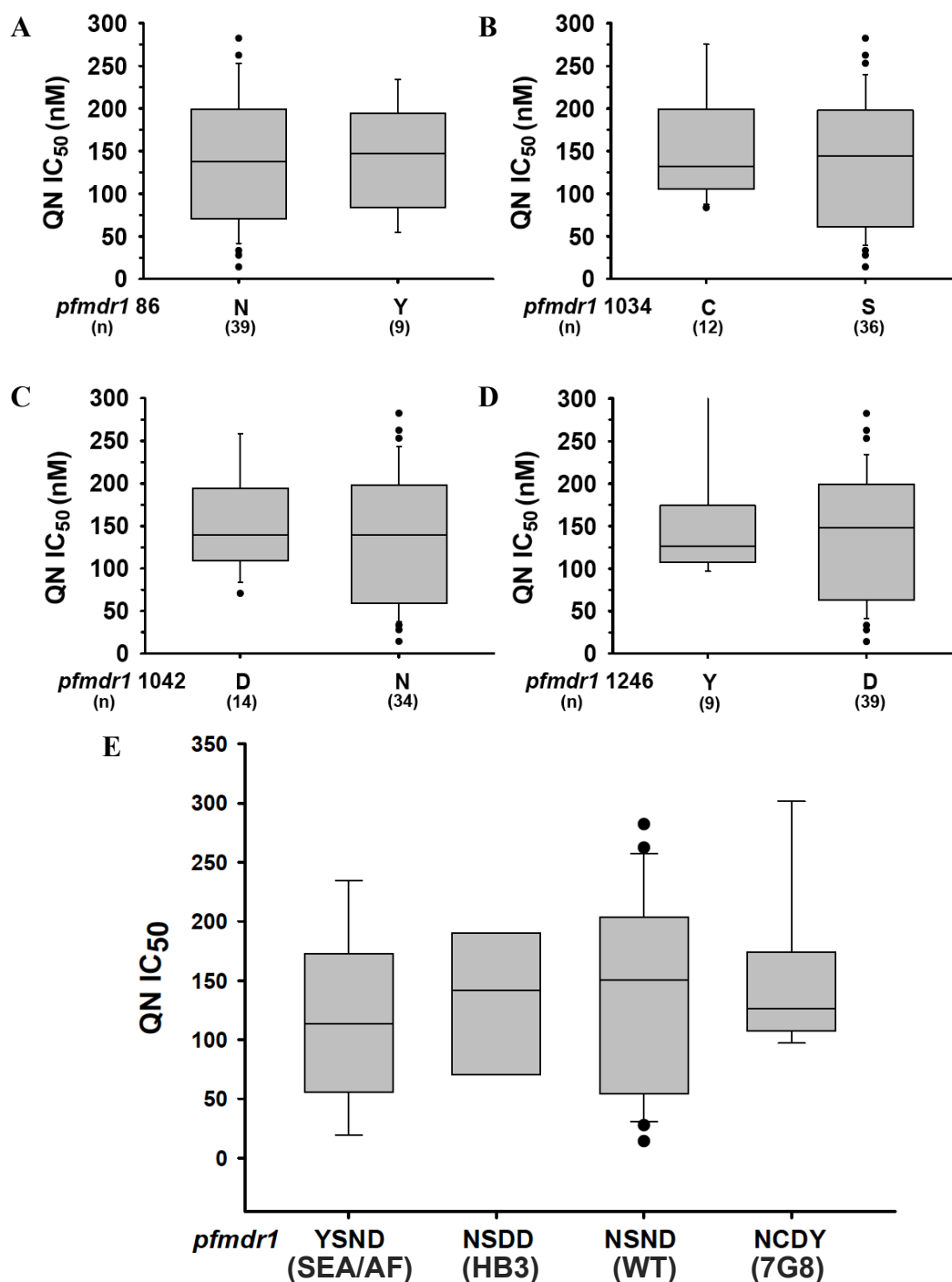
Figure 3.15 *P. falciparum* **strains were grouped according to their** *pfmdr1* **polymorphisms and analysed as a function of their quinine IC$_{50}$ values. (A~D)** Grouping according to each codon polymorphism. **(E)** Grouping according to geographic distribution: wild type (WT; $86^N1034^S1042^N1246^D$), Southeast Asian (SEA)/ African (AF; $86^Y1034^S1042^N1246^D$), and Latin American (LA; *Pf*MDR1^{7G8}, $86^N1034^C1042^D1246^Y$; *Pf*MDR1^{HB3}, $86^N1034^S1042^D1246^D$). The number of strains in each group is indicated. QN, quinine.

Table 3.4 Field isolates and laboratory strains, their IC$_{50}$ values to quinine and chloroquine and the selected polymorphisms within *pfcrt* and *pfmdr1*.

Strain	Origin	*pfcrt* (MAL7P1.27)					*pfmdr1* (PFE1150w)				QN IC$_{50}$	CQ IC$_{50}$
		72	74	75	76	326	86	1034	1042	1246		
M5	Africa	C	M	N	K	D	N	S	N	D	52.6	13.9
418	Africa	C	M	N	K	N	N	S	N	D	60.2	15.8
FAB6	Africa	C	M	N	K	N	Y	S	N	D	56.1	42.6
IF4/1	Africa	C	M	N	K	N	N	S	N	D	63.5	15.3
K39	Africa	C	M	N	K	N	N	S	N	D	45.8	20.7
M24	Africa	C	M	N	K	N	N	S	N	D	14.2	8.8
REN	Africa	C	M	N	K	N	N	S	N	D	41.9	13.7
713	Africa	S	M	N	T	D	N	C	D	D	214.5	218.1
9021	Africa	C	I	E	T	S	Y	S	N	D	147.6	100.3
102/1	Africa	C	I	E	T	S	N	S	N	D	63.1	201.7
M97	Africa	C	I	E	T	S	Y	S	N	D	113.5	206.6
DIV14	LA	S	M	N	T	n.d.	N	C	D	Y	126.2	114.9
PBZ945	LA	S	M	N	T	D	N	C	D	Y	169.9	181.1
PAD	LA	S	M	N	T	D	N	C	D	Y	104.2	115.6
ICS	LA	S	M	N	T	D	N	C	D	Y	137.9	131.4
ECP	LA	S	M	N	T	D	N	C	D	Y	178.5	132.5
DIV17	LA	S	M	N	T	D	N	C	D	Y	117.5	112.9
DIV30	LA	S	M	N	T	N	N	C	D	Y	97.1	103.2
PBZ357	LA	S	M	N	T	S	N	C	D	Y	110.2	110.2
Camp	SEA	C	M	N	K	N	N	S	N	D	33.4	15.1
92-9	SEA	S	M	N	T	D	Y	S	N	D	54.7	109
98-5	SEA	C	I	E	T	n.d.	N	S	N	D	179.5	119.7
CP250	SEA	C	I	E	T	n.d.	N	S	N	D	282.3	317.4
98-17	SEA	C	I	E	T	D	N	S	N	D	132	178.3
FCR3	SEA	C	I	E	T	N	N	S	N	D	106.6	52.1
98-11	SEA	C	I	E	T	S	N	S	N	D	115.6	85.3
98-18	SEA	C	I	E	T	S	N	S	D	D	190.5	85.3
99-18	SEA	C	I	E	T	S	N	S	D	D	141.8	151.3
CP201	SEA	C	I	E	T	S	N	S	N	D	214.4	134.4
CP203	SEA	C	I	E	T	S	N	S	N	D	197.6	253.9
CP238	SEA	C	I	E	T	S	N	S	N	D	208.3	275.8
CP252	SEA	C	I	E	T	S	N	S	N	D	197.9	203.4
CP256	SEA	C	I	E	T	S	N	S	N	D	169.6	135.8
CP269	SEA	C	I	E	T	S	N	S	N	D	194.2	141.5
CP285	SEA	C	I	E	T	S	N	S	N	D	199.5	199.8
CP297	SEA	C	I	E	T	S	N	S	N	D	252.9	124.5
CP305	SEA	C	I	E	T	S	N	S	N	D	205.1	121.5
CP313	SEA	C	I	E	T	S	N	S	N	D	262.4	161.1
P31	SEA	C	I	E	T	S	Y	S	N	D	194.1	105.6
Thai-19	SEA	C	I	E	T	S	Y	S	N	D	151.8	280.4
Thai-2	SEA	C	I	E	T	S	Y	S	N	D	234.5	219.2
CP271	SEA	C	I	E	T	S	N	C	N	D	83.6	87.8
Thai-18	SEA	C	I	E	T	S	N	C	D	D	206.1	199.8
1088	SEA	C	I	E	T	S	N	C	D	Y	301.7	420.4
PNG9-1	PNG	S	M	N	T	D	Y	S	N	D	195.6	87.8
3D7	Lab	C	M	N	K	N	N	S	N	D	27.3	6.9
HB3	Lab(LA)	C	M	N	K	N	N	S	D	D	70.3	11.9
Dd2	Lab(SEA)	C	I	E	T	S	Y	S	N	D	111.6	99.8
7G8	Lab(LA)	C	V	M	N	D	N	C	D	Y	128	61.9
GB4	Lab(AF)	C	I	E	T	S	Y	S	N	D	52.4	99.5

n.d., not detected; QN, quinine; CQ, chloroquine; LA, Latin America; SEA, Southeast Asia; PNG, Papua New Guinea

3.6.2 *Pfcrt* polymorphisms and correlational study

Currently, *pfcrt* is the prominent determinant of chloroquine resistance, which is primarily conferred by a lysine to threonine substitution at position 76. The protein consists of 10 predicted transmembrane domains, and mutations within transmembrane domains 1, 4 and 9 can alter the susceptibility to chloroquine, quinine, and quinidine. These mutations (position 72, 76, 163, and 352) and charge and hydropathy alterations affect chloroquine susceptibility and accumulation and the stereospecific responses to quinine and quinidine (Cooper et al., 2007). In addition, other studies have observed that geo-specific mutations (74,75) of *pfcrt* are involved in different levels of susceptibility to chloroquine and other quinoline drugs, such as amodiaquine (Sá et al., 2009). Nevertheless, previous studies have demonstrated that asparagine to aspartate or serine substitutions at *pfcrt* position 326 also may be involved in quinine transport in *Xenopus* oocytes that express a codon-optimised Dd2 *pfcrt* haplotype (Dave, 2011). All of these studies indicate that mutations in *pfcrt* other than K76T replacement play a role in altering antimalarial drug responsiveness. Thus, from our 50 field isolates and lab strains, I investigated positions 72 to 76 and position 326 with respect to their association with quinine susceptibility.

The polymorphisms at *pfcrt* positions 72 to 76 were examined by sequencing the nested PCR-amplified fragments (see 2.2.3.4), whereas position 326 was examined via pyrosequencing. Polymorphisms were identified in five positions: 72, 74, 75, 76, and 326. Positions 74 and 75 appear to be co-inherited given that I did not observe combinations other than the wildtype (MN) or mutant haplotype (IE) (Table 3.4). There were three haplotypes at position 326, including the wild type asparagine and the mutant forms, aspartic acid or serine. In addition to K76T replacement, mutations in *pfcrt* indicated a trend that mutations differentially occur according to their geographic distribution. This may be due to the different histories of antimalarial drug administration in these endemic areas. However, only 50 lab strains and field isolates were available, which is a relatively small sample size for drawing this conclusion.

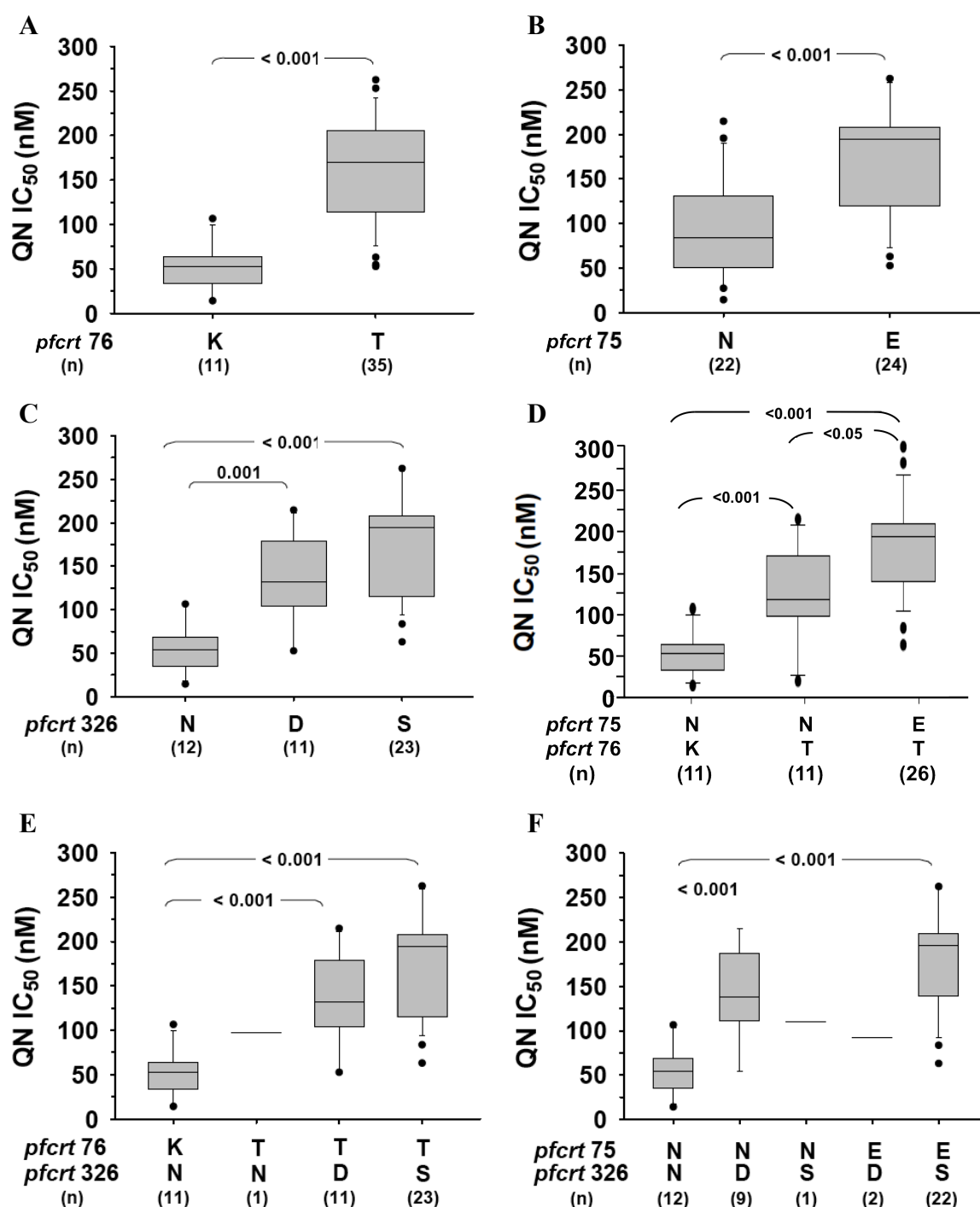**Figure 3.16 Parasites grouped according to their *pfcrt* polymorphisms and analysed as a function of their quinine IC$_{50}$ values.** Grouping according to the codon polymorphisms at position 76 **(A)**, 75 **(B)**, and 326 **(C)**. Grouping according to combinations of two of the three mutations **(D~F)**. The number of strains in each group and the statistical significance are indicated. QN, quinine.

I first grouped the field isolates and lab strains according to the quinine IC_{50} values for each position in *pfcrt* (Fig. 3.16, A~C). Interestingly, in contrast to position 72 (data not shown), I observed significant differences between the wild type and mutant haplotypes at positions 75, 76 and 326 ($p < 0.001$). However, the K76T replacement still revealed the most significant difference ($p < 10^{-7}$) compared to positions 75 and 326. There was no significant difference between the two mutant haplotypes for position 326 (Fig. 3.16, C). To investigate whether there were synergistic or additive effects between these positions, I grouped the quinine IC_{50} values of the field isolates and lab strains using two of the three positions (Fig. 3.16, D~E). Position 75 has been demonstrated to be involved in altering chloroquine and amodiaquine susceptibility (Sá et al., 2009). In the grouping analysis, in the 76T *pfcrt* background, the N75E mutation had an additive effect for quinine IC_{50} values ($p < 0.05$) (Fig. 3.16, D). However, with respect to position 326, mutations in position 76 and 326 appear to be linked given that only one of the 46 strains that inherited the K76T mutation was wild type at position 326. Thus, I was not able to investigate the possibility of a synergistic or additive effect of positions 326 and 76 with respect to quinine susceptibility (Fig. 3.16, E). When comparing groups that were wild type at positions 75 and 326 with the other groups, I again observed no synergistic or additive effects (Fig. 3.16, F).

4   Discussion

QTL analyses of quinine and quinidine accumulation in HB3 and Dd2 genetic crosses revealed a novel locus on chromosome 7, termed B5M12 (Mayer, 2009; Nurhasanah, 2009). The B5M12 locus interacts synergistically with *pfcrt* to reduce quinine and quinidine, but not chloroquine, responsiveness. The B5M12 locus is approximately 158 kbp in size and contains 29 open reading frames and four tRNAs. I performed a detailed analysis of the B5M12 locus and identified 109 polymorphisms between the HB3 and Dd2 strains, including codon and length polymorphisms (table 3.1). Two candidate genes, a putative ubiquitin transferase and RAMA, were identified in a subsequent correlation study using 50 field isolates and lab strains (Fig. 3.1). The Y1388F mutation of the putative ubiquitin transferase showed a highly significant synergism with the *pfcrt* K76T mutation in reducing quinine susceptibility (Fig 3.3, A). However, the RAMA Y328N mutation did not exhibit any synergism or additive effect with *pfcrt* mutations.

I subsequently calculated the linkage (correlation) of the polymorphisms identified in the B5M12 locus and the flanking genes with the *pfcrt* K76T mutation. Interestingly, I observed high linkages for both the Y1388F mutation in the putative ubiquitin transferase gene and RAMA (Fig. 3.5, A); the correlation between these two genes was also high (data not shown). This suggests that these two polymorphisms physically co-segregate with *pfcrt* on chromosome 7. Indeed, previous studies have identified a conserved region near *pfcrt* that exhibited linkage disequilibrium in chloroquine-resistant field isolates and lab strains (Jiang et al., 2008; Dharia et al., 2009; Kidgell et al., 2006). Although one of these studies suggested that the linkage disequilibrium region is downstream of *pfcrt* (Kidgell et al., 2006), it is still possible that RAMA is located within the region that co-segregates with *pfcrt* given that it is only 49 kb away from *pfcrt* (Table 3.1). In addition, the polymorphisms between RAMA and its downstream genes are all highly associated with mutant *pfcrt*. However, the likelihood of co-segregation of RAMA and mutant *pfcrt* and the lack of a synergistic interaction with *pfcrt* would exclude RAMA as a candidate gene for reduced quinine and quinidine responsiveness.

However, the co-segregation of the putative ubiquitin transferase with mutant *pfcrt* may be ruled out by the evidence presented in this study. First, RAMA is 97 kb away from the putative ubiquitin transferase, approximately twice the distance between RAMA and *pfcrt*, and none of the 54 polymorphisms located between these two genes were associated with mutant *pfcrt* (Table 3.1; Fig. 3.5, A). Secondly, the synergism between the putative ubiquitin transferase and the mutant *pfcrt* was observed in both Southeast Asian and Latin American strains for quinine IC_{50} values over 100 nM. Although all of these strains carry the K76T mutation, different mutant *pfcrt* alleles have arisen in different geographic locations (Table 3.4; Fig. 3.7) (Sá et al., 2009). Nevertheless, field isolates carrying *pfcrt* K76T mutations and having quinine IC_{50} values over 100 nM carry several geo-specific mutations in the putative ubiquitin transferase as well as other conserved mutations, such as the Y1388F replacement (Fig. 3.7; Table 3.4). This finding strongly supports the hypothesis that mutations in the ubiquitin transferase, similar to *pfcrt*, have emerged independently in Southeast Asia and Latin America. Third, the high level of synergism between the Y1388F mutation in the putative ubiquitin transferase and the K76T mutation in pfcrt can be detected for reduced quinine susceptibility (p <0.001) but not for chloroquine susceptibility. This result suggests that the mutations in the putative ubiquitin transferase emerged due to the pressure of quinine, rather than chloroquine, treatment. In addition, previous genome-wide studies of 189 culture-adapted field isolates have shown that the SNP in the putative ubiquitin transferase is under positive selection, possibly due to drug treatment (Mu et al., 2010).

For many years, the K76T substitution in *pfcrt* has been considered the major determinant in resistance to chloroquine and other quinoline drugs, such as quinine. However, several epidemiology studies have not observed an association between mutant *pfcrt* and quinine susceptibility (Okombo et al., 2010; 2011; Henry et al., 2009; Baliraine et al., 2011; Sanchez et al., 2011). Moreover, in this study, three strains (102/1, 92-9, and GB4) carrying a mutant *pfcrt* allele had relatively low quinine IC_{50} values (less than 100 nM) (Table 3.2). The synergism between the mutant putative ubiquitin transferase and mutant *pfcrt* may explain this phenomenon. Furthermore, previous allelic exchange experiments in which several haplotypes of mutant *pfcrt* were introduced into the Dd2 x HB3 progeny strain GCO3 demonstrated the unexpected result that mutant parasites containing the *pfcrt* K76T mutation are highly susceptible to quinine and quinidine (Sidhu et al., 2002). The same observation was observed for an allelic exchange experiment with the 3D7 strain (Valderramos et al., 2010). The synergism between mutant putative ubiquitin transferase and mutant *pfcrt* may explain

these results given that both the 3D7 and GCO3 strains carry the wild type putative ubiquitin transferase allele.

The field isolates investigated in our study were kindly provided from MR4, including 25 strains from Southeast Asia, 11 from Africa, 8 from Latin America, and one strain from Papua New Guinea. I also included 5 lab strains (3D7, Dd2, HB3, 7G8, and GB4) in this study (Table 3.2). The investigation of 50 strains was sufficient to determine allelic frequency and the correlations between polymorphisms and drug responsiveness (Fig. 3.1; Fig. 3.7). However, although I performed group studies, certain statistical analyses could not be performed due to a lack of representative strains for certain haplotype combinations of the putative ubiquitin transferase, RAMA and *pfcrt* polymorphisms (Fig. 3.4; Fig 3.14). Thus, increasing the number of field isolates may overcome this statistical limitation and validate the conclusions with respect to co-selection and co-segregation. However, the lack of strains carrying one of the mutations can be explained by the co-segregation of *pfcrt* and RAMA or the co-selection between the putative ubiquitin transferase and *pfcrt*.

In our study, the *in vitro* quinine IC_{50} values of field isolates were compared to those of a previous study (Table 3.2) (Mu et al., 2003; 2010). The range of quinine IC_{50} values was between 14.2 nM (M24, Kenya) and 301.7 nM (1088, Thailand). However, the quinine IC_{50} values I obtained were different from those reported by previous studies (Basco, 2007; Meng et al., 2010). For example, in this study, the quinine IC_{50} values of the 3D7, HB3, and 7G8 strains were 27.3 nM, 70.3 nM, and 128.0 nM, respectively, whereas in a previous study, the quinine IC_{50} values of these three strains were 108 nM, 319 nM, and 619 nM, respectively (Meng et al., 2010). According to the WHO publication, "Field application of *in vitro* assays for the sensitivity of human malaria parasites to antimalarial drugs," the definition of resistance varies from procedure to procedure. In most studies that obtained similar quinine IC_{50} values as Meng et al., the cutoff for quinine resistance was approximately 500 nM (Basco, 2007). When I normalised the cut-off quinine IC_{50} value according to the ratio of our observed quinine IC_{50} value to that of Meng's study, I obtained a cut-off IC_{50} value for quinine resistance of over 100 nM (Fig. 3.7). This also supports the use of QTL analyses of quinine accumulation and/or susceptibility with the F1 progeny of Dd2 x HB3 genetic crosses, where Dd2 is a quinine-resistant parasite line (IC_{50}= 111.6 nM) and HB3 is a quinine-sensitive line (IC_{50}= 70.3 nM).

This study is the first to show that two mutations on different genes are co-selected and synergistically interact in reducing drug responsiveness. However, there is no biochemical evidence to conclusively demonstrate the direct or indirect interaction between the putative ubiquitin transferase and *pfcrt in vivo* or *in vitro*. This limitation is because there were no suitable antibodies against either the putative ubiquitin transferase or *pfcrt*. I therefore attempted to generate an antibody against the putative ubiquitin transferase in mice. Although the antibody recognised a single band on a western blot, the size of the band (less than 100 kDa) was smaller than the predicted size of the putative ubiquitin transferase (approximately 460 kDa) (Fig. 3.12). One explanation for this result is that this antibody recognised another unknown protein rather than the putative ubiquitin transferase. Before I began the antibody generation process, I checked for antigenicity and cross-reactivity in an *in silico* study (data not shown). The results of these analyses revealed no structural similarity of UT_HECT to other *Plasmodium* proteins. The Western blot result also revealed that the antibody recognised the antigen (UT_HECT), which was a positive control. Moreover, the negative control of the erythrocyte lysate also indicated that there was no cross-reactivity of the antibody against erythrocytic proteins. The second possibility is that the putative ubiquitin transferase is alternatively spliced. *In silico* studies have predicted that there are seven introns within the putative ubiquitin transferase, one of them located in the first 500 bp of the N-terminus, while the others are located in 2 kbp at the 3' end of the gene. Thus, from the predicted locations of the introns, there is no combination of alternative splicing mRNA that could encode a protein of approximately 70 kDa. In addition, the mRNA sequencing result obtained on the GeneDB website indicated that no alternative splicing event occurs in the putative ubiquitin transferase based on the 3D7 strain sequence (data not shown). A third possible explanation is that post-translational processing of the putative ubiquitin transferase is required for maturation of the functional protein. However, I obtained no evidence to support this hypothesis.

In spite of the puzzling western blot results, I tested the putative ubiquitin transferase polyclonal antibody using IFA to investigate the subcellular localisation of the putative ubiquitin transferase (Fig. 3.13). The observed perinuclear localisation staining suggested that the putative ubiquitin transferase localises to the lumen of the endoplasmic reticulum (ER). I did not observe any membrane staining using confocal microscopy, although *in silico* studies identified 4 transmembrane domains in the N-terminal portion of the protein. Further co-

localisation studies are required to validate the localisation of the putative ubiquitin transferase.

The identification of the putative ubiquitin transferase in our study indicated the possibility of post-translational modification of *Pf*CRT, i.e., *Pf*CRT may be ubiquitinylated by the putative ubiquitin transferase. Ubiquitinylation is involved in several cellular processes, including proteasome-dependent protein degradation, cell cycle regulation, protein localisation, and protein trafficking. In this study, however, I observed a synergistic interaction between the mutant forms of both the *pfcrt* and the putative ubiquitin transferase. The possible models of interaction are discussed below. First, *Pf*CRT may be directly ubiquitinylated by the putative ubiquitin transferase. E3 ubiquitin ligases normally consist of two domains: an N-terminal substrate-binding domain, which determines the specificity of the E3 ubiquitin ligase and a C-terminal ubiquitin ligase domain, which is either a RING or HECT domain. The Y1388F replacement in the putative ubiquitin transferase may be within the substrate-binding domain, altering its specificity for mutant *Pf*CRT and transferring a ubiquitin to the mutant *Pf*CRT protein. This would promote an unknown change to *Pf*CRT dynamics and may alter quinine transport. Second, the putative ubiquitin transferase may indirectly interact with *Pf*CRT. Furthermore, if the interaction is indirect, the intermediate(s) should appear in the QTL analyses. Indeed, QTL analyses of both quinine IC_{90} values and quinine accumulation identified two non-overlapping loci on chromosome 13 (Ferdig et al., 2004; Mayer, 2009; Nurhasanah, 2009). QTL analyses of quinine IC_{90} values have also identified a sodium-hydrogen exchanger (*pfnhe*). The reduced expression level of *Pf*NHE can alter quinine susceptibility but not quinine accumulation (Nkrumah et al., 2009; Mayer, 2009). However, the actual interaction between the mutant putative ubiquitin transferase and mutant *Pf*CRT remains unknown and awaits sophisticated biochemical analyses.

Little is known regarding the ubiquitin system in *Plasmodium falciparum*. A recent ubiquitome study of *P. falciparum* predicted the ubiquitinylation profile in *P. falciparum*. The percentage of ubiquitin-conjugated proteins in *P. falciparum* (65%) is much higher than in yeast (52%) or plants (39%). This may be due to the high lysine composition (12%) in the *P. falciparum* proteome, which is approximately twice that of yeast (6%) and plants (5%). The functional classification of experimentally verified ubiquitin conjugates indicates that ubiquitinylation in *P. falciparum* is involved in many cellular process, including several parasite-specific processes, such as invasion and haemoglobin metabolism (Ponts et al., 2011). However, despite a high degree of ubiquitinylation in the *P. falciparum* proteome, there are

only 39 annotated genes involved in ubiquitinylation: 6 are ubiquitin/polyubiquitin/ubiquitin-like proteins, 7 are putative E1 ubiquitin-activating enzymes, 16 are putative E2 ubiquitin-conjugating enzymes, and 10 are putative ubiquitin ligases/transferases (including 4 with E3 RING ligase domains, 4 with an E3 HECT ligase domain, and 2 possessing other domains) (Table 4.1). In addition, although previous *in silico* studies have identified 114 putative E3 enzymes in the *P. falciparum* proteome, the majority of them have unknown function or their predicted ubiquitin ligase domains have low E-values (Ponts et al., 2008). These results indicate that *P. falciparum* E3 enzymes may have a broad specificity to various substrates rather than targeting a single protein or protein subgroup. Indeed, the putative ubiquitin transferase contains a long armadillo repeat domain, which is suitable for binding multiple substrates. Previously, a high throughput yeast two-hybrid screen identified nine proteins that interact with the putative ubiquitin transferase, including the 20S proteasome β-subunit, a translation elongation factor, two invasion-related proteins (MSP-1, and RON2), two Maurer's cleft proteins (ETRAMP, exported protein 2), two proteins of unknown function, and a chloroquine resistance marker protein (LaCount et al., 2005). Another study identified a homologous E3 enzyme in yeast, named UFD4, which consists of an armadillo-repeat domain at the N-terminus and a HECT domain at the C-terminus. UFD4 is involved in the ubiquitin fusion degradation pathway, resulting in the polyubiquitinylation of ubiquitin-conjugated proteins that are not processed by the N-end rule pathway (Ponts et al., 2008; Ju et al., 2007; Hwang et al., 2010; Gatti et al., 2011). I did not identify any homologues of the putative ubiquitin transferase in other non-apicomplexan species in our *in silico* study. However, the native function of the putative ubiquitin transferase remains to be elucidated.

Quinine resistance is a complex multi-factorial trait. Previous QTL analyses of the F1 progeny of the HB3 x Dd2 genetic cross have identified three genetic markers involved in reduced quinine susceptibility, including *pfcrt*, *pfnhe*, and *pfmdr1* (secondary scan) (Ferdig et al., 2004). These three genetic markers all encode transporters. Both *Pf*MDR1 and *Pf*CRT localise to the membrane of the digestive vacuole, in which quinine appears to target the haem detoxification pathway. Therefore, the transportation and accumulation of quinine appears to be associated with quinine resistance. In our group, Drs. Mayer and Nurhasanah measured the accumulation of quinine and its stereoisomer, quinidine, in the F1 progeny of HB3 x Dd2 genetic crosses for QTL analyses. Interestingly, these analyses indicated that the locus containing *pfnhe* is not significantly associated with quinine or quinidine accumulation (Nkrumah et al., 2009; Mayer, 2009). Consistent with this finding, accumulation assays of

pfnhe mutants with altered expression suggest that there is no significant difference between the mutants and wildtype in terms of quinine and quinidine accumulation (Mayer, 2009). In addition, a recent QTL study of quinine and quinidine susceptibility and accumulation in the F1 progeny of 7G8 x GB4 genetic crosses demonstrated that *pfcrt* is involved in quinine accumulation, whereas *pfmdr1* is the major determinant of reduced quinine susceptibility (Sanchez et al., 2011). To investigate the role of *pfmdr1* and *pfcrt* in reduced quinine responsiveness, I identified polymorphisms in 50 field isolates and lab strains (Table 3.4) and their association with quinine susceptibility (Fig. 3.14, 3.15; Mayer, 2009).

Table 4.1 Annotated *P. falciparum* proteins involved in ubiquitinylation. Different types of E3 ubiquitin ligases/ transferase are highlighted. TMD, predicted transmembrane domain.

	Gene ID	Product description	Length	TMD
Ubiquitin	*PF*08_0067	ubiquitin	373	4
	*PF*L0585w	polyubiquitin	381	0
	*PF*L1830w	ubiquitin-like protein, putative	73	0
	*PF*13_0084	ubiquitin-like protein, putative	177	0
	*PF*11_0329	ubiquitin-like protein, putative	882	0
	*PF*I1085w	ubiquitin-like protein, putative	1542	2
E1	*PF*13_0344	ubiquitin-activating enzyme	584	2
	*PF*13_0182	ubiquitin-activating enzyme	1838	3
	*PF*13_0264	ubiquitin-activating enzyme e1, putative	706	0
	*PF*L1245w	ubiquitin-activating enzyme e1, putative	1140	0
	MAL8P1.75	ubiquitin-activating enzyme, putative	389	0
	*PF*L1790w	ubiquitin-activating enzyme, putative	686	0
	*PF*11_0457	ubiquitin activating enzyme (E1) subunit Aos1, putative	338	0
E2	MAL13P1.227	ubiquitin conjugating enzyme	278	1
	*PF*E1350c	ubiquitin conjugating enzyme 13, putative	152	0
	*PF*C0255c	ubiquitin conjugating enzyme E2, putative	142	0
	*PF*L0190w	ubiquitin conjugating enzyme E2, putative	147	0
	*PF*F0305c	ubiquitin conjugating enzyme E2, putative	155	0
	*PF*L2175w	ubiquitin conjugating enzyme E2, putative	157	0
	*PF*L2100w	ubiquitin conjugating enzyme E2, putative	465	0
	*PF*I1030c-a	ubiquitin conjugating enzyme, putative	148	0
	*PF*I0740c	ubiquitin conjugating enzyme, putative	159	0
	*PF*C0855w	ubiquitin conjugating enzyme, putative	160	0
	*PF*I1030c-b	ubiquitin conjugating enzyme, putative	163	0
	*PF*08_0085	ubiquitin conjugating enzyme, putative	163	0
	*PF*10_0330	ubiquitin conjugating enzyme, putative	191	0
	*PF*13_0301	ubiquitin conjugating enzyme, putative	202	0
	*PF*14_0128-b	ubiquitin conjugating enzyme, putative	299	0
	*PF*14_0128-a	non-canonical ubiquitin conjugating enzyme, putative	91	0
E3	*PF*14_0215	ubiquitin ligase, putative **(RING)**	510	5
	*PF*C0845c	ubiquitin-protein ligase, putative **(RING)**	107	0
	*PF*10_0117	ubiquitin-protein ligase e3, putative **(RING)**	781	0
	*PF*C0550w	ubiquitin-protein ligase, putative	807	0
	*PF*14_0462	ubiquitin-protein ligase, putative	851	0
	*PF*07_0026	ubiquitin-protein ligase e3, putative **(RING)**	961	0
	*PF*11_0201	ubiquitin-protein ligase, putative **(HECT)**	2147	2
	MAL7P1.19	ubiquitin transferase, putative **(HECT)**	3893	4
	MAL8P1.23	ubiquitin-protein ligase 1, putative **(HECT)**	8591	2
	*PF*F1365c	**HECT**-domain (ubiquitin-transferase), putative	10287	2

*Pf*MDR1 may directly transport drugs either out of or into the food vacuole; alternatively, *Pf*MDR1 may indirectly influence drug partitioning by altering the transport of other substrates (Duraisingh and Cowman, 2005). Studies of lab strains and field isolates have indicated that the N86Y, S1034C, N1042D and D1246Y mutations may be associated with decreased quinine susceptibility (Reed et al., 2000; Sidhu et al., 2005). However, in the correlation study of *pfmdr1* polymorphisms in the 50 field isolates and lab strains we examined, no significant associations of codon polymorphisms with quinine IC$_{50}$ values were observed (Fig. 3.14). In addition, the association of *pfmdr1* codon and copy number polymorphisms with quinine resistance has been questioned in several epidemiological studies in malaria endemic areas (Pickard et al., 2003; Anderson et al., 2005; Price et al., 2004; Basco and Ringwald, 2002; Zalis et al., 1998). The ambiguous role of *pfmdr1* polymorphisms may be due to the different experimental designs used by the studies. Allelic exchange of *pfmdr1* in lab strains may obscure the contribution of other genetic factors. Moreover, the number and diverse geographic distribution of the field isolates may also lead to various results of correlation studies due to differences in both allelic frequency and the resistance phenotype (Anderson et al., 2005). In addition, neither the single codon polymorphisms nor the geo-specific *pfmdr1* haplotypes were observed to contribute to reduced chloroquine susceptibility (data not shown). Although the role of *pfmdr1* in quinine transport remains unclear, an *in vitro* assay of P-gp (*Pf*MDR1 homolog) in mice confirmed that quinine is a substrate for P-gp (Pussard et al., 2007).

Chloroquine resistance has been widely studied for the past decade, and the chloroquine resistant phenotype is strongly associated with the K76T mutation in the gene encoding a chloroquine resistance transporter (*Pf*CRT). *Pfcrt* polymorphisms have also been associated with reduced accumulation of quinine and its stereoisomer quinidine in *P. falciparum* parasites (Sanchez et al., 2008a) (Cooper et al., 2002; 2007). The heterologous expression of *Pf*CRT in *Xenopus laevis* oocytes has demonstrated the uptake of chloroquine by a codon-optimised Dd2 *pfcrt* allele and that quinine and quinidine can inhibit chloroquine uptake (Martin et al., 2009; Lehane and Kirk, 2010). A subsequent study conclusively demonstrated that *Pf*CRT transports quinine and quinidine. The direction of quinine and quinidine transport was observed from the digestive vacuole to the cytosol, consistent with the resistant phenotype, with less quinine accumulation in the resistant strains (Dave, 2011). Interestingly, quinine transport was only observed in specific *pfcrt* alleles (Dd2 and GB4). The phenomenon of allelic-specific transport of quinine suggests that *pfcrt* polymorphisms

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other than the K76T mutation may underlie the different levels of quinine accumulation and susceptibility.

PfCRT is composed of ten predicted transmembrane domains, and the topology remains unknown. However, it has been reported that mutations in transmembrane domains 1, 4 and 9 of PfCRT can alter susceptibility to chloroquine, quinine and quinidine (Cooper et al., 2007). In addition, geo-specific $pfcrt$ alleles in Southeast Asia and Latin America result in different levels of chloroquine resistance (Sa et al., 2009). Furthermore, quinine uptake assays in $Xenopus laevis$ oocytes expressing different PfCRT alleles have demonstrated that position 326 may be involved in quinine transport (Dave, 2011). In our study, we investigated the association of polymorphisms between positions 72 and 76 and at position 326 with reduced quinine susceptibility (Table 3.4). With respect to position 72 (C to S replacement), no significant correlation with quinine responsiveness was observed (data not shown). However, mutations at the other four positions (M74I, N75E, N326D, and N326S) were significantly and independently correlated with reduced quinine susceptibility (p <0.001) (Fig. 3.16, B, C). Interestingly, the mutations resulting in quinine resistance (IC_{50} values greater than 100 nM) appear to have developed independently in different endemic areas (Table 3.4). The quinine resistant field isolates from Southeast Asia carry the $pfcrt$ allele with I^{74}, E^{75}, T^{76}, and S^{326}. In contrast, quinine-resistant strains in Latin America carry the $pfcrt$ allele with M^{74}, N^{75}, T^{76}, and D^{326}. When I performed grouping analysis using combinations of two of three polymorphisms, I observed an additive effect of the N75E and K76T mutations in increasing quinine IC_{50} values (i.e., more resistant to quinine) (Fig. 3.16, D). This phenomenon was also observed in QTL analyses of quinine resistance in 7G8 x GB4 genetic crosses (Sanchez et al., 2011). However, this additive effect was not detected when I performed grouping analyses of position 326 with positions 75 or 76. The explanations for this phenomenon are either that there were insufficient samples to detect the additive effect or the mutations at position 326 were highly linked with the K76T mutation according to geographic distribution, unlike the N75E mutation, for which Latin American strains carry the wild type haplotype.

Ubiquitinylation in drug resistance has been studied in cancer research. There are several previous studies reporting that mutations in a ubiquitin ligase or a deubiquitinylation enzyme affect drug susceptibility by regulating the stability of a resistance-mediating drug transporter (Zhang, 2004; Zhang et al., 2009b; Liu et al., 2007). An example from malaria is a $Plasmodium chabaudi$ deubiquitinylation enzyme that is associated with increased resistance to artemisinin and chloroquine (Hunt et al., 2007). A recent genome-wide study in yeast

identified several *P. falciparum* homologues involved in enhanced or reduced quinine susceptibility, where two homologous genes in *P. falciparum* were identified as being involved in ubiquitinylation (Santos and Sá-Correia, 2011). The first of these genes is *PF*10_0330, a putative ubiquitin-conjugating enzyme. The yeast homologue encodes the protein Ubc8, which has been shown to possess decreased rapamycin resistance and is required for rapamycin-induced degradation of Hxt7p (Snowdon et al., 2008). The second gene is *PF*07_0026, a putative E3 ubiquitin ligase with a RING domain. Its yeast homologue, Aft1, encodes a transcription factor that is involved in iron homeostasis and utilisation (Yamaguchi-Iwai et al., 1996). Interestingly, *PF*07_0026 is a downstream flanking gene of the B5M12 locus and is highly associated with the *pfcrt* K76T mutation (Table 3.1; Fig 3.5, A). In addition, other ubiquitin ligases with RING domains were also shown to be involved in antimalarial drug resistance (Ribacke, unpublished).

To summarise, by dissecting the B5M12 locus, I identified a novel genetic marker encoding a putative ubiquitin transferase. The polymorphisms identified in the putative ubiquitin transferase, especially the Y1388F mutation, are highly associated with reduced quinine responsiveness. In addition, this mutation was co-selected with the *pfcrt* K76 mutation under the pressure of quinine treatment. It is possible that these polymorphisms may be developed into a novel molecular marker for improving molecular surveillance of quinine resistance in endemic areas. The strict synergism between the mutant putative ubiquitin transferase and the mutant *pfcrt* remains unclear and awaits further analysis. In addition, our results demonstrate the geo-specific *pfcrt* N75E mutation has an additive effect with the K76T mutation in conferring reduced quinine susceptibility. Overall, these results may help define the mechanism of quinine resistance and the epidemiological research of quinine resistance, thereby preserving the efficacy of this valuable antimalarial drug.

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6   Appendix

Appendix 1 polymorphism table of B5M12 locus

gene	position	strain												
		Dd2	HB3	713	REN	FAB6	M5	M97	Camp	IF4/1	102/1	FCR3	K39	418
MAL7P1.17	1389	I	N	N	N	N	N	N	I	N	N	N	D	N
	1390	D	N	N	N	N	N	N	D	N	N	N	H	N
	1391	K	D	D	D	D	D	D	K	D	D	D	del	D
	1393	K	I	I	I	I	I	I	K	I	I	I	N	I
	1395-1398	del.	NNLY	NNLY	NNLY	NNLY	NNLY	NNLY	del.	NNLY	NNLY	NNLY	HKND	NNLY
	1403	N	D	D	D	D	D	D	N	D	D	D	E	D
	1417-1421	del	del	del	del	LYDNY	del	del	del	del	del	del	LYDNL	del
	1444-1452	del	del	del	NYYDNYYD	del	del	del	del	del	del	del	NYYDNYYD	del
PF07_0015	714-719	ILKSEN	-ILKSEN	ILKSEN	ILKSEN	del	del	del	del	del	ILKSEN	ILKSEN	ILKSEN	ILKSEN
PF07_0016	1016-1017	NN	del											
	1474	I	K											
MAL7P1.18	309-311	KYN	del	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN
	784-807	1	0	1	0	0	0	0	1	1	1	1	0	1
	808	H	D	D	D	D	D	D	D	D	D	D	D	D
	815	V	G	V	V	V	V	V	V	V	V	V	G	V
	823	V	G	V	V	V	V	V	V	V	V	V	V	V
	1351-1352	NN	del	del	del	del	del	del	del	del	del	del	del	del
MAL7P1.19	174-175	del	N	del	NI	del	del	NI	del	NI	NI	NI	del	del
	199	K	E	K	E	K	K	E	K	E	E	K	K	K
	249	D	del	D	D	D	D	del	D	del	D	D	D	D
	309	Q	E	E	E	E	E	E	E	E	E	E	E	E
	535	L	P	L	L	L	P	L	L	P	L	P	L	P
	589	S	T	T	T	T	T	T	T	T	T	T	T	T
	1233	C	Y	C	C	C	C	Y	C	C	C	Y	C	C
	1376	S	S	S	N	S	N	N	N	N	S		S	S
	1388	F	Y	F	Y	F	Y	F	Y	Y	Y		F	F
	1406	D	E	D	E	D	D	D	E	del	E		D	D
	1409	C	G	C	C	C	C	C	G	C	G		C	C
	1410	D	E	D	D	D	D	D	E	D	E		D	D
	1578	E	del	K	K	K	E	E	K	K	K	E	E	K
	1614	K	K	del	K	K	K	K	del	K	E	E	K	K
	1632	K	E	E	E	K	K	K	E	K	K	K	K	K
	1692	I	V	I	V	I	I	I	V	V	I	I	I	I
	1707	L	M	L	M	L	L	M	M	M	L	L	L	L
	1740	P	P	H	P	H	H	P	del	P	P	P	P	H
	1780	N	K	N	K	N	N	K	N	N	N	N	N	K
	1905	N	D	D	D	D	D	D	D	D	D	D	D	D
	1960-1967	NDNNNDNM	del	NDNNNDNM	del	del	del	NDNNNDNM	del	del	del	del	del	del
	3118-3122	YEDDY	YEDDY	del	del	del	del	del	del	del	del	del	del	del
	3189-3192	NKNK	NKNK	NKNK	del	del	NKNK	del	NKNK	NKNK	NKNK	NKNK	NKNK	del
PF07_0018	751-755	del	NNNNN											
	1039	S	R											
PF07_0019	397	S	P											
	435-439	1	0	0	0	0	0	1	1	0	0	1	0	0
	455-459	1		0	0	0	0	1	1	0	0	1	0	0
PF07_0020	712-751	1		0	0	1	0	0	0	1	1	1	1	1
PF07_0021	29	I	M	I	I	I	I	I	I	I	I	I	I	I
	51-53	del	INE	del	del	del	del	del	del	del	del	del	del	del
	232	E	K	K	K	K	K	K	K	K	K	K	K	K
	376	L	V	V	V	V	V	V	V	V	V	V	V	V
	468	T	I	T	I	T	I	I	I	I	T	T	I	T
	500	G	D	G	D	G	D	D	D	D	G	G	D	D
	503	K	N	K	N	K	N	N	N	N	K	K	N	N
	519	K	N	K	N	K	N	N	N	K	K	K	K	K
	542	del	F	Y	Y	F	Y	F	F	F	Y	Y	Y	del
	595	del	R	S	R	R	R	R	R	S	R	R	R	del
	657	R	R	S	R	R	R	R	S	S	R	S	R	S
	568-629	1	0	0	0	0	0	0	0	0	0	0	0	1
PF07_0022	264	S	L											
	417	E	D											
	546	S	A											
	550	Y	L											
	551	Q	Y											
	552	Y	I											
	553	I	M											
	554	Y	P											
	555	P	L											
	604	S	N											
	1168-1170	del	del	NNI	del	del	del	del	del	del	NNI	del	del	del
	1208	L	F											
MAL7P1.21	101	T	A											

gene	position	Dd2	HB3	713	REN	FAB6	M5	M97	Camp	IF4/1	102/1	FCR3	K39	418
PF07_0024	443	I	M	M	M	I	M	M	M	M	M	M	M	M
	598-600	NNN	del	del	del	del	del	del	del	del	del	del	del	del
	1006	NNN	del	del	del	del	del	del	del	del	del	del	del	del
	1213-1215	del	del	del	del	del	III	III	III	III	III	III	del	del
	1738	E	DEDDE	E	Y	E	E	Y	E	E	Y	E	E	E
	1742-1746	DEDDE	DEDDE	DEDDE	del	DEDDE	DEDDE	del	DEDDE	DEDDE	del	DEDDE	DEDDE	DEDDE
	2592	Y	H	H	H	Y	H	Y	H	H	H	H	H	H
	2744	G	D	G	D	G	D	D	D	D	G	G	D	D
MAL7P1.201	126	N	del											
MAL7P1.203	638	D	E											
	1334	R	S											
MAL7P1.204	300	del	N											
	819-820	NN	del											
	1054-1055	del	NN											
	1228-1229	NN	del											
	1344	D	N											
	1345-1348	del	NNDN											
	1353	D	N											
	1360	del	N											
	1371	K	N											
MAL7P1.205	190-193	INKI	INKI	INKI	INKI	INKI	INKI	INKI				INKI	INKI	INKI
MAL7P1.207	928	I	F	F	F	I	F	F	F	F	F	F	F	F
	942	M	I	M	I	M	M	M	M	M	M	M	M	M
	981	S	C	C	C	C	C	C	C	C	C	C	C	C
	982	D	H	H	H	H	H	H	H	H	H	H	H	H
	984	G	D	D	D	D	D	D	D	D	D	D	D	D
	1038-1049	1	0	1	0	0	0	0	0	0	1	0	0	1
	2009-2015	1		1	0	0	0	0	0	0	1	0	0	1
MAL7P1.208	196	A	T	T	A	A	A	A	A	A	A	A	A	A
	272	E	K	E	E	E	E	E	E	E	E	E	E	E
	286	M	I	M	I	M	M	M	M	M	M	M	M	M
	315	Q	V	E	V	V	E	E	V	-	V	V	V	V
	318	Y	D	D	D	D	D	D	D	D	D	D	D	D
	321	F	M	M	M	M	M	M	M	M	M	M	M	M
	328	N	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	330	E	Q	E	Q	Q	E	E	Q	E	Q	Q	Q	Q
	331	F	M	F	M	M	F	F	M	F	M	M	M	M
	333	N	Y	Y	Y	Y	N	N	Y	N	Y	Y	Y	N
	394	E	del	Q	del	E	del	del	E	del	Q	E	Q	E
	322-326	del	del	KYEEF	del	del	del	del	del	del	del	del	del	del
	381-432	1	0	1	0	1	0	0	1	0	1	1	1	1
	623	M	I	M	I	M	M	M	M	M	M	M	M	M
MAL7P1.209	348	S	S	S	S	S	S	S	S	S	S	S	S	S
	409	L	L	L	del	L	L	L	del	del	del	L	del	L
	516	T	N	T	T	T	T	T	T	T	T	T	T	T
MAL7P1.22	286-287	del	NK	del	NK	NK	NK	NK	NK	NK	del	NK	del	del
	1420	I	V	I	I	I	I	I	I	I	I	I	I	I
PF07_0026	547	S	S	S	S	S	S	S	N	S	S	S	S	S
	553	G	G	G	G	G	G	G	S	G	G	G	G	G
	555	N	S	S	S	S	N	S	N	del	S	S	S	S
	561	S	G	G	G	G	S	G	S	G	G	G	G	G
	562	N	S	N	S	N	N	S	N	S	N	S	S	S
	565	T	N	T	N	T	T	N	T	N	T	N	N	N
	571	N	S	N	S	N	N	S	N	S	N	S	S	S
	548-572	1	0	0	0	0	0	0	1	0	0	0	0	0
PF07_0029	Microsatellite	1	0	1	0	0	0	0	0	0	1	0	0	0
MAL7P1.27 (*pfcrt*)	72	C	C	S	C	C	C	C	C	C	C	C	C	C
	74	I	M	M	M	M	M	I	M	M	I	I	M	M
	75	E	N	N	N	N	N	E	N	N	E	E	N	N
	76	T	K	T	K	K	K	T	K	K	T	T	K	K
	326	S	N	D	N	N	D	S	N	N	S	N	N	N
PFE1150w (*pfmdr1*)	86	Y	N	N	N	Y	N	Y	N	N	N	N	N	N
	1034	S	S	C	S	S	S	S	S	S	S	S	S	S
	1042	N	D	D	N	N	N	N	N	N	N	N	N	N
	1246	D	D	D	D	D	D	D	D	D	D	D	D	D

gene	position	CP269	CP238	CP250	CP203	CP256	CP285	CP313	CP252	CP201	CP305	CP297	CP271	9021	DIV14
MAL7P1.17	1389	N	I	I	I	N	N	N	N	N	N	I	N	I	I
	1390	N	D	D	D	N	N	N		N	N	D	N	D	D
	1391	D	K	del	K	D	D	D	D	D	D	K	D	K	del
	1393	I	K	N	K	I	I	I	N	I	I	K	I	K	N
	1395-1398	NNLY	del.	del.	del.	NNLY	NNLY	NNLY		NNLY	NNLY	del.	NNLY	del.	KKID
	1403	D	N	D	N	D	D	D		D	D	N	D	N	K
	1417-1421	del	del	del	del	del	del	del		del	del	del	del	del	LYDNL
	1444-1452	del	del	del	del	del	del	del	del	del	del	del	del	del	NYYDNYYD
PF07_0015	714-719	ILKSEN	ILKSEN	ILKSEN	ILKSEN	-ILKSEN	-ILKSEN	-ILKSEN	ILKSEN	ILKSEN	ILKSEN	del	ILKSEN	del	del
PF07_0016	1016-1017														
	1474														
MAL7P1.18	309-311	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	del	IYN
	784-807	1	1	1	1	0	0	0	1	0	0	1	0	1	1
	808	D	D	D	H	D	D	D	D	H	D	D	D	D	D
	815	V	V	V	V	V	V	V	V	V	V	V	V	V	V
	823	V	V	V	V	V	V	V	V	V	V	V	V	V	V
	1351-1352	del	del	del	del	del	del	del	del	del	del	del	del	del	del
MAL7P1.19	174-175	del	del	del	del		N	N	del	N	N	del	N	del	del
	199	K	K	X	K		E	E	K	E	E	K	E	K	K
	249	D	D	D	D	D	del	del	D	del	del	D	del	D	D
	309	E	E		E	E	E	E	E	E	E	E	E	E	E
	535	L	L		L	L	L	L	L	L	L		L	L	L
	589	T	T		T	T	T	T	T	T	T	del	T	S	T
	1233	C	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	C	C
	1376	S		S	S	S	S	S	S	S	S		S		S
	1388	F		F	F	F	F	F	F	F	F		F		F
	1406	D		D	D	D	D	D	D	D	D		D		D
	1409	C		C	C	C	C	C	C	C	C		C		C
	1410	D		D	D	D	D	D	D	D	D		D		D
	1578	E	K	K	K	K	K	K	K	K	K		K	E	K
	1614	K	E	E	E	E	E	E	E	E	E	del	E	K	del
	1632	K	K	del	K	K	K	K	K	K	K	del	K	K	E
	1692	I	I	I	I	I	I	I	I	I	I	del	I	I	I
	1707	L	L	del	L	L	L	L	L	L	L	L	L	L	L
	1740	H	H	del	H	H	H	H	H	H	H	H	H	P	H
	1780	N	N	N	N	N	N	N	N	N	N	del	N	N	N
	1905	D	D	D	D	D	D	D	D	D	D	D	D	N	D
	1960-1967	del	del		del	del	del	del	del	del	del		del	NDNNNDNM	NDNNNDNM
	3118-3122	YEDDY	del	del	del	del	del	del	del	del	del	del	del	YEDDY	del
	3189-3192	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK
PF07_0018	751-755														
	1039														
PF07_0019	397														
	435-439	1	1	1	1	0	0	0	1	0	0	1	0	1	0
	455-459	1	1	1	1	0	0	0	1	0	0	1	0	1	0
PF07_0020	712-751	1	1												
PF07_0021	29	I	I	I	I	I	I	I	I	I	I	I	I	I	M
	51-53	del	del	del	del	del	del	del	del	del	del	del	del	del	INE
	232	K	K	K	K	K	K	K	K	K	K	K	K	K	K
	376	V	V	V		V	V	V	V	V		V	V	V	V
	468	T	T		T		I	I	T	I	I	T	I	T	T
	500	G	D		D		D	D	D	D	D	G	D	G	G
	503	K	N		N		N	N	N	N	N	K	N	K	K
	519	K	N		N		N	N	N	N	N	K	N	K	K
	542	del	F		F		F	F	F	F	F	del	F	del	Y
	595	del	R		R		R	R	R	R	R	del	R	del	S
	657	S	S		S		S	S	S	S	S	R	S	R	S
	568-629	1	0		0		0	0	0	0	0	1	0	1	0
PF07_0022	264														
	417														
	546														
	550														
	551														
	552														
	553														
	554														
	555														
	604														
	1168-1170	del	NNI	NNI	NNI		del	del	NNI	del	del	del	del	del	del
	1208														
MAL7P1.21	101														

gene	position	strain													
		CP269	CP238	CP250	CP203	CP256	CP285	CP313	CP252	CP201	CP305	CP297	CP271	9021	DIV14
PF07_0024	443	M	M	M	M	I	M	M	M	M	M	M	M	M	M
	598-600	NNN	NNN	NNN	NNN	del	del	del	del	del	del	del	del	del	del
	1006	NNN	NNN	NNN	NNN	del	del	del	del	del	del	del	del	del	del
	1213-1215	del	del	del	del	del	del	del	del	del	del	del	del	del	del
	1738	E	E	E	E	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	1742-1746	DEDDE	DEDDE	DEDDE	DEDDE	del	del	del	del	del	del	del	del	del	del
	2592	H	H	H	H	H	H	H	H	H	H	H	H	Y	H
	2744	G	D		D		D	D	D	D	D	G	D	G	G
MAL7P1.201	126														
MAL7P1.203	638														
	1334														
MAL7P1.204	300														
	819-820														
	1054-1055														
	1228-1229														
	1344														
	1345-1348														
	1353														
	1360														
	1371														
MAL7P1.205	190-193	INKI	del			INKI	INKI	INKI	del	INKI	INKI	INKI	INKI	INKI	INKI
MAL7P1.207	928	F	F	F	F	I	F	F	F	F	F	F	F	F	F
	942	M	M	M	M	M	M	M	M	M	M	M	M	M	M
	981	C	C	C	C	C	C	C	C	C	C	C	C	C	C
	982	H	H	H	H	H	H	H	H	H	H	H	H	H	H
	984	D	D	D	D	D	D	D	D	D	D	D	D	D	D
	1038-1049	1	1	1	1	1	1	1	1	1	1	1	1	1	0
	2009-2015	1	1	1	1	1	1	1	1	1	1	1	1	1	0
MAL7P1.208	196	A	A	A	A	A	A		A		A	A	A	A	T
	272	E	E	E	E	E	E		E		E	E	E	E	E
	286	M	M	M	M	M	M		M		M	M	M	M	M
	315	Q	Q	Q	Q	Q	Q		Q		Q	Q	Q	Q	E
	318	Y	Y	Y	Y	Y	Y		Y		Y	Y	Y	Y	D
	321	F	F	F	F	F	F		F		F	F	F	F	M
	328	N	N	N	N	N	N		N		N	N	N	N	N
	330	E	E	E	E	E	E		E		E	E	E	E	E
	331	F	F	F	F	F	F		F		F	F	F	F	F
	333	N	Y	Y	Y	Y	Y		Y		Y	Y	Y	Y	Y
	394	E	E	Q	E	E	del		E		E	E	E	E	Q
	322-326	del	del	del	del	del	del		del		del	del	del	del	KYEEF
	381-432	1	1	1	1	1	1		1		1	1	1	1	1
	623	M	M	M	M	M	M		M		M	M	M	M	M
MAL7P1.209	348	del	del	S	S	S	del	S	S	S	S	del	S	S	S
	409	L	L	L	L	L	L	L	L	L	L	L	L	L	L
	516	T	T	T	T	T	T	T	T	T	T	T	T	T	T
MAL7P1.22	286-287	del	del	del	del	NK	NK	NK	NK		NK	NK	NK	NK	del
	1420	I	I	I	I	I	I	I	I		I	I	I	I	V
PF07_0026	547	S	S	S	S	N	N	N	S	N	N	S	N	S	S
	553	G	G	G	G		S	S	G	S	S	G	S	G	G
	555	N	N	N	N	N	N	N	N	N	N	N	N	N	S
	561	S	S	S	S	S	S	S	S	S	S	S	S	S	G
	562	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	565	T	T	T	T	T	T	T	T	T	T	T	T	T	T
	571	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	548-572	1	1	1	1	1	1	1	1	1	1	1	1	1	0
PF07_0029	intron length polymorphism	1	1	1	1	1	1	1	1	1	1	1	1	1	0
MAL7P1.27 (pfcrt)	72	C	C	C	C	C	C	C	C	C	C	C	C	C	S
	74	I	I	I	I	I	I	I	I	I	I	I	I	I	M
	75	E	E	E	E	E	E	E	E	E	E	E	E	E	N
	76	T	T	T	T	T	T	T	T	T	T	T	T	T	T
	326	S	S		S	S	S	S	S	S	S	S	S	S	
PFE1150w (pfmdr1)	86	N	N	N	N	N	N	N	N	N	N	N	N	Y	N
	1034	S	S	S	S	S	S	S	S	S	S	S	C	S	C
	1042	N	N	N	N	N	N	N	N	N	N	N	N	N	D
	1246	D	D	D	D	D	D	D	D	D	D	D	D	D	Y

gene	position	strain										
		PBZ945	98-18	ECP	Thai-19	Thai-2	PBZ357	DIV30	PAD	PNG9-1	98-17	98-5
MAL7P1.17	1389	I	I	N	I	I	N	N	N	I	I	N
	1390	D	D	N	D	D	N	N	N	D	D	N
	1391	del	K	D	K	K	D	D	D	K	K	D
	1393	N	K	I	N	N	I	I	I	K	I	I
	1395-1398	KKID	del.	NNLY	DNIY	DNIY	NNLY	NNLY	NNLY	del.	EKND	NNLY
	1403	K	K	N	D	D	D	D	D	N	K	D
	1417-1421	LYDNL	del	del	LYDNY	FFXNX	del	del	del	del	LYDNL	del
	1444-1452	NYYDNYYD	del	del	del	del	del	del	del	del	NYYDNYYD	del
PF07_0015	714-719	del	ILKSEN	ILKSEN	ILKSEN	ILKSEN	-ILKSEN	-ILKSEN	ILKSEN	ILKSEN	ILKSEN	ILKSEN
PF07_0016	1016-1017											
	1474											
MAL7P1.18	309-311	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN
	784-807	1	1	1	1	1		1	1	1	1	
	808	D	D	D	D	D	D	D	D	D	D	D
	815	V	G	V	V	V	G	V	V	V	V	V
	823	V	V	G	V	V	V	V	V	V	V	V
	1351-1352	del	del	del	del	del	del	del	del	del	del	del
MAL7P1.19	174-175	del	N	del	del	del	del	del	del	del		
	199	K	E	K	K	X	K	K	K	K		
	249	D	del	D	D	D	D	D	D	D	D	D
	309	E	E	E	E	E	E		E	E	E	E
	535	L	L	L	L	L	L		L	L	L	
	589	T	T	T	S	T	T		T	S	T	
	1233	C	del	C	C	C	C	C	C	C	Y	C
	1376	S		S	S	S	S	S	S	S	S	S
	1388	F		F	F	F	F	F	F	F	F	F
	1406	D		D	D	D	D	D	D	D	D	D
	1409	C		C	C	C	C	C	C	C	C	C
	1410	D		D	D	D	D	D	D	D	D	D
	1578	K	K	K	E		K	K	K	K	K	E
	1614	del	del	del	K		del	del	del	E	E	K
	1632	E	E	E	K		E	E	E	K	K	K
	1692	I	I	I	I		I	I	I	I	I	I
	1707	L	L	L	L		L	L	L	L	L	L
	1740	H	del	H	P		H	H	H	P	H	H
	1780	N	N	N	N		N	N	N	N	N	N
	1905	D	D	D	N		D	D	D	N	D	D
	1960-1967	del	del	NDNNNDNM	NDNNNDNM		NDNNNDNM	NDNNNDNM	NDNNNDNM	NDNNNDNM	del	del
	3118-3122	del	YEDDY	del	YEDDY	YEDDY	del	del	del	YEDDY	YEDDY	del
	3189-3192	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK	NKNK
PF07_0018	751-755											
	1039											
PF07_0019	397											
	435-439	0	1	0	1	1	0	0	0	1	0	
	455-459	0	1	0	1	1	0	0	0	1	0	
PF07_0020	712-751											
PF07_0021	29	I	I	I	I	I	I	I	I	I	M	M
	51-53	del	del	del	del	del	del	del	del	del	INE	INE
	232	K	K	K	E	K	K	K	K	K	K	K
	376	L	V	V	L	V	V	V	V	V	V	V
	468	T	T	T	T	T	T	T	T	T	T	T
	500	G	G	G	G	G	G	G	G	G	D	G
	503	K	K	K	K	K	K	K	K	K	N	K
	519	K	K	K	K	K	K	K	K	K	N	K
	542	Y	del	del	del	Y	Y	Y	del	del	F	del
	595	S	del	del	del	S	S	S	del	del	R	del
	657	S	R	S	S	S	S	S	S	R	S	S
	568-629	0	1	1	1	0	0	0	1	1	0	1
PF07_0022	264											
	417											
	546											
	550											
	551											
	552											
	553											
	554											
	555											
	604											
	1168-1170	del	del	del	del	del			NNI	del		
	1208											
MAL7P1.21	101											

gene	position						strain					
		PBZ945	98-18	ECP	Thai-19	Thai-2	PBZ357	DIV30	PAD	PNG9-1	98-17	98-5
PF07_0024	443	M	M	M	M	M	M		M	M	M	
	598-600	del	del	del	del	del	del		del	del	del	
	1006	del	del	del	del	del	del		del	del	del	
	1213-1215	del	III	del	III	III	del		del	III	III	
	1738	Y	E	Y	Y	Y	E		del	del	DEDDE	
	1742-1746	del	DEDDE	del	del	del	DEDDE		del	del	DEDDE	
	2592	H	H	H	Y	Y	H		H	H	Y	
	2744	G	G	G	G	G	G		G	G	D	
MAL7P1.201	126											
MAL7P1.203	638											
	1334											
MAL7P1.204	300											
	819-820											
	1054-1055											
	1228-1229											
	1344											
	1345-1348											
	1353											
	1360											
	1371											
MAL7P1.205	190-193	INKI	INKI	INKI	INKI	INKI			del	del		del
MAL7P1.207	928	F	F	F	F	F	F	F	F	F	F	F
	942	M	M	M	M	M	M	M	M	M	M	M
	981	C	C	C	C	C	C	C	C	C	C	C
	982	H	H	H	H	H	H	H	H	H	H	H
	984	D	D	D	D	D	D	D	D	D	D	D
	1038-1049	0	1	0	1	0	0	1	1	0	0	
	2009-2015	0	1	0	1	0	0	1	1	0	0	
MAL7P1.208	196	A	A	A	A	A	A	A	A	A	A	A
	272	E	E	E	E	E	E	E	E	E	E	E
	286	M	M	M	M	M	M	M	M	M	M	M
	315	E	Q	E	Q	Q	E	E	E	Q	V	Q
	318	D	Y	D	Y	Y	D	D	D	Y	D	Y
	321	M	F	M	F	F	M	M	M	F	M	F
	328	N	N	N	N	N	N	N	N	N	Y	N
	330	E	E	E	E	E	E	E	E	E	Q	E
	331	F	F	F	F	F	F	F	F	F	M	F
	333	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
	394	Q	E	Q	E	Q	Q	Q	Q	Q	Q	Q
	322-326	KYEEF	del	KYEEF	del	del	KYEEF	KYEEF	KYEEF	del	del	del
	381-432	1	1	1	1	1	1	1	1	1	1	1
	623	M	M	M	M	M	M	M	M	M	M	M
MAL7P1.209	348	del	S	S	S	S	S	S	S	S	S	del
	409	L	L	del	del	L	L	L	L	L	L	L
	516	T	T	T	T	T	T	T	T	T	T	T
MAL7P1.22	286-287	del	NK	del	NK	NK	del	del	del	NK	NK	
	1420	I	I	I	I	I	I	I	I	I	V	
PF07_0026	547	S	S	S	S	S	S	S	S	S	S	N
	553	G	G	G	G	G	G	G	G	G	G	S
	555	S	S	S	N	N	S	S	S	S	N	N
	561	G	G	G	S	del	G	G	G	S	S	S
	562	N	N	N	N	del	N	N	N	N	N	N
	565	T	T	T	T	T	T	T	T	T	T	T
	571	N	N	N	N	N	N	N	N	N	N	N
	548-572	0	0	0	1	0	0	0	0	0	1	1
PF07_0029	intron length polymorphism	0	1	0	1	1			1	0		1
MAL7P1.27 (*pfcrt*)	72	S	C	S	C	C	S	S	S	S	C	C
	74	M	I	M	I	I	M	M	M	M	I	I
	75	N	E	N	E	E	N	N	N	N	E	E
	76	T	T	T	T	T	T	T	T	T	T	T
	326	D	S	D	S	S	S	N	D	D	D	
PFE1150w (*pfmdr1*)	86	N	N	N	Y	Y	N	N	N	Y	N	N
	1034	C	S	C	S	S	C	C	C	S	S	S
	1042	D	D	D	N	N	D	D	D	N	N	N
	1246	Y	D	Y	D	D	Y	Y	Y	D	D	D

gene	position	strain									
		99-18	Thai-18	P31	ICS	M24	1088	98-11	92-9	DIV17	3D7
MAL7P1.17	1389	I	N	I	N	I	N	I	N	N	N
	1390	D	N	D	N	D	N	D	D		N
	1391	K	D	K	D	del	D	K	K	del	D
	1393	N	I	N	I	N	I	K	I	I	I
	1395-1398	DNIY	NNLY	DNIY	NNLY	KKID	NNLY	del.	KKID		NNLY
	1403	D	D	D	D	K	D	N	D		D
	1417-1421	LYDNY	del	FXDNY	del	LYDNL	del	del	LYDNL		del
	1444-1452	NYYDNYYD	del	NYYDNYYD	del	NYYDNYYD	del	del	NYYDNYYD	del	del
PF07_0015	714-719	ILKSEN	del	ILKSEN	ILKSEN	ILKSEN	del	ILKSEN	ILKSEN	del	ILKSEN
PF07_0016	1016-1017										
	1474										
MAL7P1.18	309-311	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN	IYN
	784-807	1	1	0	1	0	1	1	1	1	1
	808	D	D	D	D	D	D	D	D	D	D
	815	V	V	G	V	V	V	V	V	V	V
	823	V	V	V	V	V	V	V	V	V	V
	1351-1352	del	del	del	del	del	del	del	del	del	del
MAL7P1.19	174-175	del	del	N	del	N	del			del	N
	199	K	K	E	K	E	K			K	K
	249	D	D	del	D	del	D	del	D	del	D
	309	E	E	E	Q	E	E	E	E	E	Q
	535	L	L	L	L	L	L	L	L	L	P
	589	S	T	S	T	T	T	S	T	T	T
	1233	C	Y	C	C	Y	del	C	C	C	Y
	1376	S	S	S	S	S		S	N	S	N
	1388	F	F	F	F	Y		F	Y	F	Y
	1406	D	D	D	D			D	E	D	E
	1409	C	C	C	C	G		C	G	C	G
	1410	D	D	D	D	E		D	E	D	E
	1578	K	K	K		K	E	E	K	K	K
	1614	del	E	K		K	K	K	E	del	del
	1632	E	K	K	E	E	K	K	K	E	K
	1692	I	I	I	I	V	I	I	I	I	I
	1707	L	L	L	L	M	L	L	L	L	L
	1740	del	H	H	H	P	P	P	H	H	P
	1780	N	N	N	N	N	N	N	N	N	N
	1905	D	D	D	D	D	N	N	D	D	D
	1960-1967	del	del	del	del	del	NDNNNDNM	NDNNNDNM	del	NDNNNDNM	del
	3118-3122	YEDDY	del	del	del	del	YEDDY	YEDDY	del	del	del
	3189-3192	NKNK	NKNK	NKNK	NKNK	del	NKNK	NKNK	NKNK	NKNK	NKNK
PF07_0018	751-755										
	1039										
PF07_0019	397										
	435-439	1	1	1	0	0	1	1	0	0	1
	455-459										
PF07_0020	712-751										
PF07_0021	29	I	I	I	I	I	I	I	I	I	I
	51-53	del	del	del	del	del	del	del	del	del	del
	232	K	K	E	K	K	K	K	K	K	E
	376	V	V	V	V	V	V	V	V	V	V
	468	T	T	T	T	T	T	T	T	T	T
	500	G	G	G	G	G	G	G	G	G	G
	503	K	K	K	K	K	K	K	K	K	K
	519	K	K	K	K	N	K	K	K	K	K
	542	del	del	Y	Y	F	del	del	del	Y	Y
	595	del	del	S	S	R	del	del	del	S	R
	657	R	S	S	S	S	R	del	S	S	S
	568-629	1	1	0	0	0	1	1	1	0	0
PF07_0022	264										
	417										
	546										
	550										
	551										
	552										
	553										
	554										
	555										
	604										
	1168-1170	del	del		del	del	NNI	NNI	NNI	del	NNI
	1208										
MAL7P1.21	101										

gene	position	strain									
		99-18	Thai-18	P31	ICS	M24	1088	98-11	92-9	DIV17	3D7
PF07_0024	443	M	M	M	M	M	I	M	M	M	M
	598-600	del	del	del	del	del	del	del	del	del	del
	1006	del	del	del	del	del	del	del	del	del	del
	1213-1215	NNN	NNN	NNN	del	del	del	NNN	NNN	del	del
	1738	del	DEDDE	DEDDE	del	del	DEDDE	DEDDE	DEDDE	DEDDE	DEDDE
	1742-1746	del	DEDDE	DEDDE	del	del	DEDDE	DEDDE	DEDDE	DEDDE	DEDDE
	2592	H	H	Y	H	H	H	H	Y	H	H
	2744	G	G	G	G	G	G	G	G	G	G
MAL7P1.201	126										
MAL7P1.203	638										
	1334										
MAL7P1.204	300										
	819-820										
	1054-1055										
	1228-1229										
	1344										
	1345-1348										
	1353										
	1360										
	1371										
MAL7P1.205	190-193	del	del			del	del	del	del	del	INKI
MAL7P1.207	928	F	F	F	F	F	I	F	F	F	F
	942	M	M	M	M	M	M	M	M	M	M
	981	C	C	C	C	C	C	C	C	C	C
	982	H	H	H	H	H	H	H	H	H	H
	984	D	D	D	D	D	D	D	D	D	D
	1038-1049	0	0	0	1	1	0	0	0	1	0
	2009-2015										
MAL7P1.208	196	A	A	A	A	A	A	A	A	A	T
	272	E	E	E	E	E	E	E	E	E	K
	286	M	M	M	M	M	M	M	M	M	M
	315	Q	Q	Q	E	E	Q	Q	Q	E	V
	318	Y	Y	Y	D	D	Y	Y	Y	D	D
	321	F	F	F	M	M	F	F	F	M	M
	328	N	N	N	N	N	N	N	N	N	Y
	330	E	E	E	E	E	E	E	E	E	Q
	331	F	F	F	F	F	F	F	F	F	M
	333	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
	394	E	E	E	Q	Q	E	E	E	Q	E
	322-326	del	del	del	KYEEF	KYEEF	del	del	del	KYEEF	del
	381-432	1	1	1	1	1	1	1	1	1	1
	623	M	M	M	M	M	M	M	M	M	M
MAL7P1.209	348	S	S	S	S	S	S	S	del	S	S
	409	L	L	L	del	del	L	L	L	del	del
	516	T	T	T	T	T	T	T	T	T	T
MAL7P1.22	286-287	NK	NK	NK	del	del	del	del	del	del	del
	1420	I	I	I	I	I	I	I	I	I	I
PF07_0026	547	S	S	S	S	S	S	S	S	S	S
	553	G	G	G	G	G	G	G	G	G	G
	555	N	N	N	S	S	N	N	N	S	S
	561	S	S	S	G	G	S	S	S	G	G
	562	N	T	N	N	del	N	N	N	N	S
	565	T	T	T	T	N	T	T	T	T	N
	571	N	N	N	N	S	N	N	N	N	S
	548-572	1	1	1	0	0	1	1	1	0	0
PF07_0029	intron length polymorphism	1	1		0	0	1		1	0	
MAL7P1.27 (pfcrt)	72	C	C	C	S	C	C	C	S	S	C
	74	I	I	I	M	M	I	I	M	M	M
	75	E	E	E	N	N	E	E	N	N	N
	76	T	T	T	T	K	T	T	T	T	K
	326	S	S	S	D	N	S	S	D	D	N
PFE1150w (pfmdr1)	86	N	N	Y	N	N	N	N	Y	N	N
	1034	S	C	S	C	S	C	S	S	C	S
	1042	D	D	N	D	N	D	N	N	D	N
	1246	D	D	D	Y	D	Y	D	D	Y	D

del, deletion; ins, insertion; 1, haplotype the same as Dd2; 0, haplotype the same as HB3