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Dissecting the loci of low-level quinine resistance in malaria parasites

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Summary

Quinine (QN) remains effective against *Plasmodium falciparum*, but its decreasing efficacy is documented from different continents. Multiple genes are likely to contribute to the evolution of QN resistance. To locate genes contributing to QN response variation, we have searched a *P. falciparum* genetic cross for quantitative trait loci (QTL). Results identify additive QTL in segments of chromosomes (Chrs) 13, 7 and 5, and pairwise effects from two additional loci of Chrs 9 and 6 that interact, respectively, with the QTL of Chrs 13 and 7. The mapped segments of Chrs 7 and 5 contain *pfcrt*, the determinant of chloroquine resistance (CQR), and *pfmdr1*, a gene known to affect QN responses. Association of *pfcrt* with a QTL of QN resistance supports anecdotal evidence for an evolutionary relationship between CQR and reduced QN sensitivity. The Chr 13 segment contains several candidate genes, one of which (*pfnhe-1*) encodes a putative Na^+/H^+ exchanger. A repeat polymorphism in *pfnhe-1* shows significant association with low QN response in a collection of *P. falciparum* strains from Asia, Africa and Central and South America. Dissection of the genes and modifiers involved in QN response will require experimental strategies that can evaluate multiple genes from different chromosomes in combination.

Introduction

Quinine (QN), an alkaloid from cinchona bark, is a critical and widely used drug because of its efficacy against severe and chloroquine-resistant (CQR) malaria (World Health Organization, 2000). Yet the status of this drug is in jeopardy because of decreases in the dose responsiveness of *Plasmodium falciparum* infections in regions of south-east Asia, South America and Africa (Pukrittayakamee *et al*., 1994; Jelinek *et al*., 1995; Segurado *et al*., 1997).

Reports that decreased QN sensitivity can be associated with resistance to other structurally related drugs such as mefloquine, halofantrine and chloroquine (CQ) have led to suggestions that some heritable determinants of drug response may be shared (Simon *et al*., 1986; Warsame *et al*., 1991; Basco and Le Bras, 1992; Brasseur *et al*., 1992). Work in recent years has pointed to two such genes. One is the *pfmdr1* gene that encodes a *P. falciparum* P-glycoprotein-like protein (Pgh-1) implicated in mefloquine and halofantrine resistance, and also a possible modulator of the CQR phenotype (Cowman *et al*., 1994; Reed *et al*., 2000). The other is *pfcrt*, the actual determinant of CQR, which can carry mutations associated with stereospecific changes in quinine and quinidine responses (Fidock *et al*., 2000; Cooper *et al*., 2002). Although selection and transfection experiments have implicated these genes in QN response, their relative contribution to the trait and the way in which they interact with each other and additional genes to generate the specific drug response have not been elucidated.

QN nevertheless remains clinically effective against CQR and mefloquine-resistant *P. falciparum*. These observations and the different levels of QN susceptibility among CQR parasites indicate that the QN phenotype is complex and is probably affected by other genes in addition to *pfmdr1* and *pfcrt* (Mu *et al*., 2003). Searches for loci carrying these genes are possible through statistical methods developed to dissect complex quantitative trait loci (QTL) (Lander and Botstein, 1989; Paterson, 1995; Sen and Churchill, 2001). *P. falciparum* genetic crosses can be subjected to genome-wide scans for individual QTL-containing genes that affect the resistance phenotype.

Here, we report a QTL analysis of QN responses inherited in cloned parasite progeny from a *P. falciparum* cross. We address the genetic determinants of the QN responses of these parasites, including three additive and two pairwise QTL interactions.

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Results

Quantitative drug response traits of the HB3 × Dd2 parents and progeny

The parasites for this study were taken from the *P. falciparum* genetic cross between HB3, a CQ-sensitive (CQS) clone from Central America, and Dd2, a CQR clone of south-east Asian provenance (Wellems *et al*., 1990). Table 1 summarizes quantitative traits of the QN and CQ responses of the HB3 and Dd2 parent clones, including their QN and CQ IC_{50} and IC_{90} values, response slope parameters (Hill 3 curve-fit 'b') and IC_{90} values after verapamil (VP) chemosensitization. The values for the Dd2 clone are in the range of QN susceptibilities that our assays typically yield for culture-adapted parasites from areas where difficulties with QN treatment have been reported, including the W2 and W2-MEF ancestors of the Dd2 clone and other drug-resistant parasite lines from the Amazon and Indochina (Oduola *et al*., 1988; Pukrittayakamee *et al*., 1994; Zalis *et al*., 1998; Mu *et al*., 2003; Pickard *et al*., 2003). The response curves and Hill slope b-parameters of QN response for each parent were reproducible with high precision in more than 50 separate [^{3}H]-hypoxanthine incorporation assays. The mean b-parameters did not differ significantly between the HB3 and Dd2 clones (2.2 and 2.3, respectively, for QN).

Quantitative trait values for the QN and CQ responses of 35 independent recombinant progeny from the HB3 × Dd2 cross, based on the results of 7–22 separate assays, are provided in *Supplementary material* (Table S1). The QN b-parameters for the progeny were found to range from a low of 1.5 to a high of 3.0, well outside the parental values and indicating heritable components in the QN responses that impact the shape of the dose–response curves and, consequently, the relative information content of IC_{90} and IC_{50} values. Because median IC_{90} values are more sensitive to slope variations, these were used for subsequent QTL analyses to ensure that this curve information was included in the dose–response measure. The QN and CQ IC_{90} values of the HB3 and Dd2 parents and progeny are plotted in Fig. 1. CQ response values (*y*-axis) clearly demonstrate the effect from *pfcrt*, i.e. there is a clear gap separating the CQ IC_{90} values of CQS from CQR parasites (Fig. 1A). No such gap is evident for the QN responses, which are instead spread across a continuous range that extends to either side of the low (HB3) and high (Dd2) parent QN IC_{90} values. Considerable overlap is evident in the QN responses of CQS and CQR progeny, indicating a complex QN response trait involving multiple genes.

The QN IC_{90} values of the parasites were also examined

Table 1. Drug response traits of the HB3 and Dd2 *P. falciparum* clones.[a]

Trait	Dd2 (*n* = 65)	HB3 (*n* = 53)
QN IC_{50}	350 (334–368)	115 (110–120)
QN IC_{90}	924 (826–1038)	382 (344–424)
QN-VP IC_{90}	395 (352–444)	393 (355–433)
QN slope b (Hill)	2.3 (2.1–2.5)	2.2 (2.0–2.4)
CQ IC_{50}	433 (417–448)	25 (24–26)
CQ IC_{90}	735 (665–811)	44 (40–49)
CQ-VP IC_{90}	247 (230–266)	44 (41–49)
CQ slope b (Hill)	4.0 (3.5–4.5)	4.3 (3.5–5.0)

a. QN and CQ responses of clones derived from curve-fitted values (nM) from combined data of *n* dose–response assays. Confidence intervals (95%) are shown in parentheses. QN-VP IC_{90} and CQ-VP IC_{90} indicate responses to CQ and QN in the presence of 0.89 μM VP. The slope of QN response curve (QN slope) is the b-parameter calculated from the Hill 3 curve-fit equation.

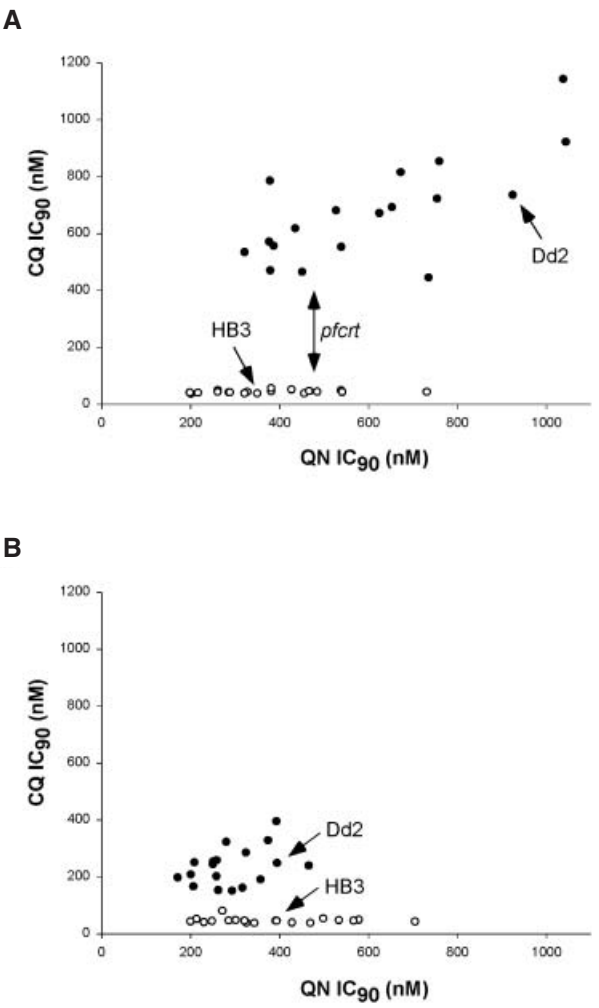


Fig. 1. QN versus CQ responses of the HB3 × Dd2 parents and progeny in the absence and presence of VP.
A. In the absence of VP, the large gap distinguishing the CQR from the CQS progeny is determined by inheritance of the *pfcrt* gene.
B. Chemosensitization by VP of QN and CQ responses occurs in CQR but not in CQS progeny. Each of the progeny data points was obtained from 7 to 22 independent dose–response assays. Results from CQR clones are shown as filled circles; results from CQS clones are shown as open circles. A concentration of 0.89 μM VP was used in the chemosensitization experiments.

for shifts in the presence of VP, a compound that potentiates the effect of CQ on CQR parasites only (Martin *et al*., 1987). The results show 30–70% VP chemosensitization of the QN response (a shift to the left in the dose–response curve) for the CQR but not the CQS parasites (Fig. 1B). This effect of VP on QN response was strictly associated with the CQR phenotype and the *pfcrt* mutant allele, irrespective of the relative QN IC_{90} values. Chemosensitization of the QN response by VP did not occur in any of the CQS progeny.

Primary and secondary scans for additive QTL associated with drug response

To search for chromosome regions involved in drug responses of the HB3 × Dd2 cross, we used the Pseudomarker QTL analysis package (Sen and Churchill, 2001) to scan the 35 progeny for phenotype associations with markers in the segregation tables of our reported 5 cM genome-wide linkage map (Su *et al*., 1999) (http://www.ncbi.nlm.nih.gov/Malaria/Mapsmarkers/PfSegData/segdata.html). The results of these scans indicated that most of the QN response variation can be attributed to two main additive QTL: 35% to a chromosome (Chr) 13 peak centred on the C13M56 marker, and another 30% to a Chr 7 peak centred on the *pfcrt* gene (Fig. 2A).

After accounting for main effects of the Chr 7 and Chr 13 loci, we searched the remaining unassigned fraction of QN response variation by secondary scans of the residual variation (Doerge and Churchill, 1996). These scans revealed a Chr 5 QTL that had not been evident in the primary scan and accounted for 30% of the remaining fraction (10.5% of total response variation). The peak height corresponding to this secondary additive QTL approaches the permutation-based significance threshold of $P = 0.05$ (Fig. 2B). The *pfmdr1* gene, previously associated with QN response in several studies (Reed *et al*., 2000; Babiker *et al*., 2001), resides within this peak near the B5M86 marker. Of interest, this Chr 5 peak is associated with increased QN IC_{90} when it is contributed by HB3, the more sensitive parent. This is in contrast to the peaks of Chr 7 and Chr 13, which are associated with increased QN IC_{90} when they are contributed by the more resistant Dd2 parent.

The contribution by the *pfcrt*-containing Chr 7 locus to QN response is evident but relatively small compared with a recently reported association in genetically heterogeneous parasite isolates from around the world (Mu *et al*., 2003). Additionally, the modest effect from this locus on QN response stands in stark contrast to its control of nearly all the CQ response variation inherited in the HB3 × Dd2 progeny. Mutations in the Dd2 *pfcrt* account for >95% of the CQ response variation and are associated in a sharp peak with LOD > 20 (Fig. 2C). No statistically significant additional peaks were identified by secondary scans of the residual variation in the CQ response after adjustment for the CQR variance associated with *pfcrt*; however, several smaller peaks that could possibly account for 20% of this variation included a peak that aligned with the major QN locus on Chr 13 (Fig. 1; data not shown). It is possible that incremental contributions from such minor loci to QN response levels could explain the slight positive correlation in CQR levels and QN response (Fig. 1A); such loci could carry incremental resistance genes or may reflect a complex adaptive role that different polymorphisms play in the CQR phenotype conferred by *pfcrt* mutations (Wellems and Plowe, 2001).

VP chemosensitization of QN response determined solely by pfcrt *mutations*

CQR may have features in common with P-glycoprotein-mediated multiple drug resistance in mammalian tumour cell lines (Krogstad *et al*., 1987; Martin *et al*., 1987). Subsequent reports suggested associations between various quinoline antimalarial responses of *P. falciparum* and mutations in the *pfmdr1*-encoded glycoprotein, Pgh-1 (reviewed by Bray and Ward, 1998); however, VP chemosensitization of CQR parasites is associated specifically with *pfcrt* mutations (Fidock *et al*., 2000; Cooper *et al*., 2002; Sidhu *et al*., 2002). To test further for genetic associations to the chemosensitization effect, we performed genome scans on the changes in QN response produced in the HB3 × Dd2 progeny by VP. Results from these scans showed that the VP chemosensitization of QN response co-segregated exactly with the Chr 7 peak containing the Dd2 *pfcrt* allele (Fig. 2D). Nearly 100% of the VP affect was assigned to this peak, demonstrating that, although *pfcrt* mutations do not alone account for QN response, they completely determine the VP chemosensitization of QN response. Secondary residual and pairwise scans did not detect any effect from the Chr 5 segment carrying *pfmdr1* or other QTL elsewhere in the genome.

Candidate genes from the three additive QTL peaks associated with QN response

The *pfcrt* and *pfmdr1* genes that reside in the QTL peaks of Chrs 7 and 5 have previously been implicated in the QN responses of *P. falciparum*. For example, certain mutations in *pfcrt* generated by *in vitro* selection experiments increase sensitivity to QN but reduce sensitivity to its diastereomer, quinidine (Cooper *et al*., 2002); the results of allelic exchange studies have also shown that *pfcrt* modifications can affect QN responses (Sidhu *et al*., 2002). Additionally, amplification of and site-specific changes in *pfmdr1* can alter QN responses (Cowman

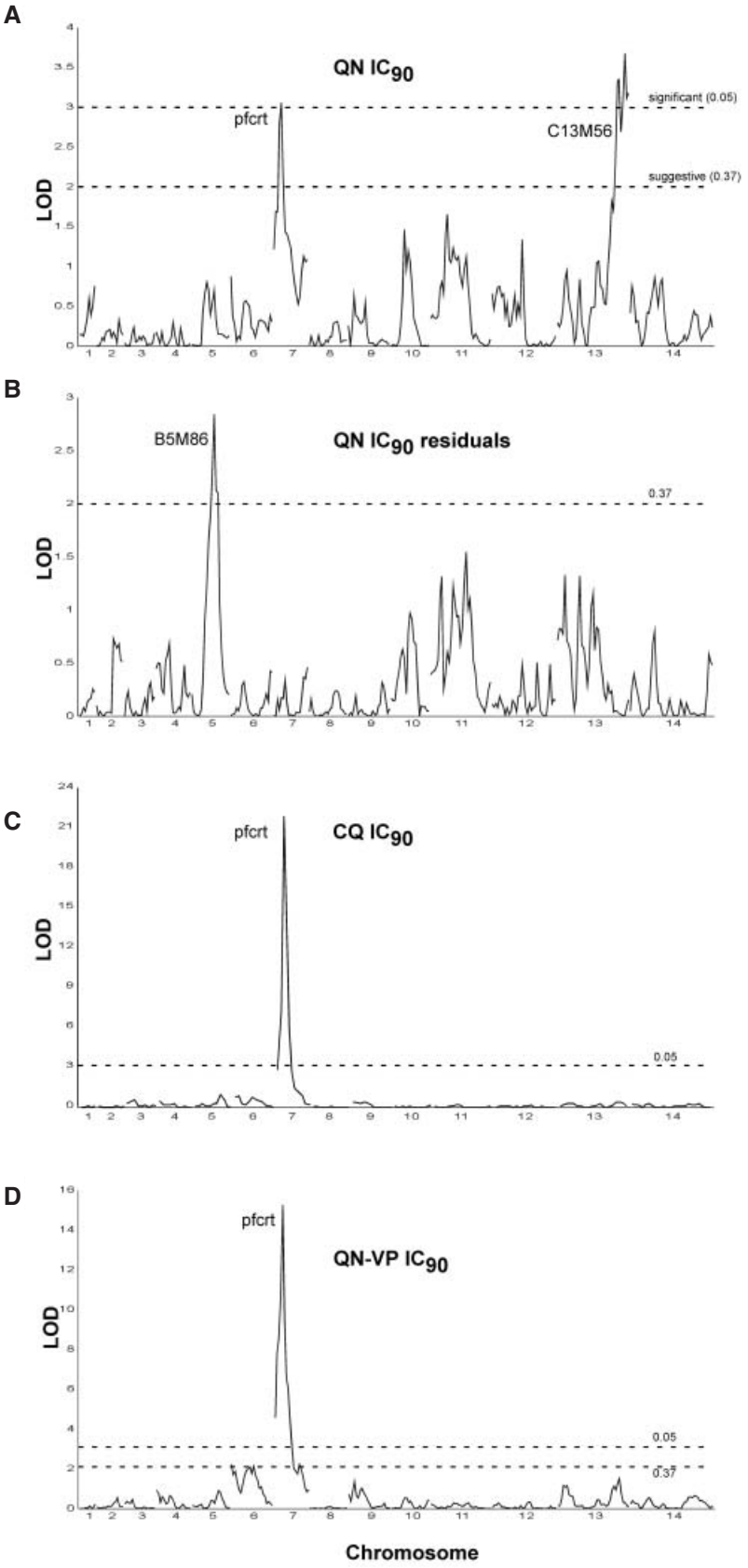


Fig. 2. Main QTL identified by one-dimensional genome-wide scans.
A. Plot showing Chr 7 (*pfcrt*) and Chr 13 (C13M56) QTL associated with the QN IC_{90}.
B. Chr 5 peak (B5M56) containing the *pfmdr1* gene identified in secondary scan after removing effects of major QTL associated with markers C13M56 and *pfcrt*.
C. Mainscan plot showing the magnitude of the effect from the *pfcrt* gene on CQ response (*Supplementary material*, Table S1) previously demonstrated to determine CQR in this cross (Fidock *et al*., 2000).
D. Complete association of the *pfcrt* peak with the VP chemosensitization phenotype, QN-VP IC_{90}.
Markers of the 14 chromosomal linkage groups occur sequentially along the horizontal axes. LOD scores are plotted as a function of genome location. Dashed lines represent threshold values from 1000 permutations.

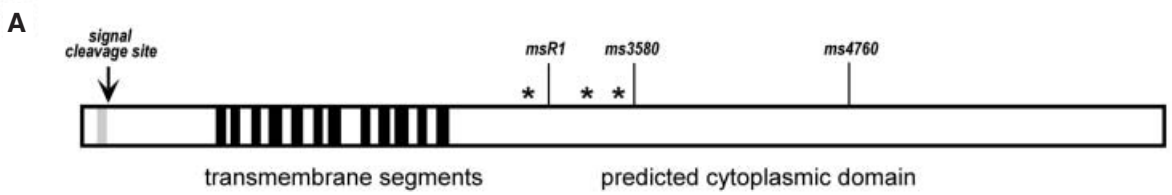
et al., 1994; Reed *et al*., 2000). The PfCRT and Pgh-1 transporter molecules encoded by these genes probably underlie the QN response variation that maps to the Chr 7 and Chr 5 QTL peaks, although our study does not exclude the possibility that QN response might also be affected by other genes within these peaks.

The Chr 13 QTL peak did not immediately suggest a polymorphism or gene previously reported to be associated with malaria drug responses. To search for candidate genes, we used a Bayesian localization module (Sen and Churchill, 2001) to generate a posterior distribution spanning the position of this residual Chr 13 effect between the subtelomeric region of the chromosome (ta58 marker) and the proximal 164.5 cM marker, C13M14 [95% confidence interval (CI) ranging from ≈170 cM to the end of the chromosome] (http://www.ncbi.nlm.nih.gov/Malaria/Mapsmarkers/PfSegData/ch13a.html). The nucleotide sequence spanning the QTL is a 380 kb segment (Sanger Center sequence chr13-1 available at http://plasmodb.org). Approximately 100 predicted genes reside within this segment (*Supplementary material*, Table S2).

Molecules that can affect pH gradients across the membranes of cellular compartments have been proposed to have a role in responses to CQ and, by extension, to other quinoline drugs. The A and B subunits of a vacuolar ATPase (Karcz *et al*., 1993) and a sodium-hydrogen exchanger (NHE) (Sanchez *et al*., 1998; Wunsch *et al*., 1998) have been suggested as candidates for such a role. Evidence for direct involvement of the A subunit in CQR has been negative, however, and evidence for a primary role of the NHE in CQR has been refuted (Bray *et al*., 1999). We were therefore intrigued by the presence in the Chr 13 QTL of the PF13_0034 gene, encoding putative subunit H of a vacuolar ATP synthase, and gene PF13_0019, encoding a predicted *P. falciparum* NHE (PfNHE-1).

To test for an association of QN response variation to either of these genes, we resequenced PF13_0034 and PF13_0019 from the HB3 and Dd2 parents, identified coding frame polymorphisms and surveyed these polymorphisms in 71 *P. falciparum* lines from south-east Asia, Africa and Central and South America. In the putative ATP synthase H subunit gene PF13_0034, most lines from south-east Asia possessed a single codon #8 GGA (Gly) polymorphism that was largely absent from African and American lines. The presence or absence of this polymorphism did not, however, show any significant association with QN response levels in our survey.

In contrast to the putative ATP synthase H subunit gene, sequences of PfNHE-1 showed multiple and complex variations. In the 71 *P. falciparum* lines with different QN responses, these variations were evident as point polymorphisms at three separate codons (790, 894, 950) and as microsatellite variations in three different repeat sequences (msR1, ms3580, ms4760) (Fig. 3A and B).



B

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ms4760-1  KKKKISGSNNDNNDNNNDDNNND---------NHNDDKNNKNDDDNHNDNHNNDDDNHNDNHNNDDDNNNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
ms4760-2  KKKKISGSNNDNNDNNND-----------------DKNNKNDDDNHNDNHKNDDDNHNDNHNNDDDNNNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
ms4760-3  KKKKISGSNNDNNDNNND-----------------DKNNKNDDDNHNDNHNNDDDNHNDNHNNDDDNNNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
ms4760-4  KKKKISGSNNDNNDNNNDDNNNDDNNND-----NHNDDKNNKNDDD-----------DHNDNHNNDDDNNNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
ms4760-5  KKKKISGSNNDNNDNNNDDNNNDDNNNDDNNNDNHNDDKNNKNDDD-----------NHNDNHNNDDDNNNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
ms4760-6  KKKKISGSNNDNNDNNNDDNNND---------NHNDDKNNKNDDD-----------NHNDNHNNDDDNNNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
ms4760-7  KKKKISGSNNDNNDNNNDDNNNDDNNND-----NHNDDKNNKNDDD-----------NHNDNHNNDDDNNNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
ms4760-8  KKKKISGSNNDNNDNNNDDNNNDDNNND-----NHNDDKNNKNDDDNHNDNHNNDDDNHNDNHNNDDDNHNDNHNDDDNNSSHYNKEKEANFNEYLTYQNLFALQNEDNNIINFFSN
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Fig. 3. Schematic diagram of PfNHE-1 and alignment of ms4760 microsatellite variations.
A. Features of the full open reading frame include a signal peptide cleavage site (predicted by the SIGNALP program; http://genome.cbs.dtu.dk/services/) and 12 transmembrane segments in the downstream sequence (MEMSAT program; http://saier-144-37.ucsd.edu/). PfNHE-1 also possesses a long C-terminal hydrophilic tail proposed to be a cytoplasmic domain; in related bacterial and mammalian exchangers, this domain is thought to modulate transport activity (http://pfam.wustl.edu/ accession no. PF00999; Orlowski and Grinstein, 1997; Hamada *et al*., 2001). Positions of codon polymorphisms 790, 894 and 950 in the hydrophilic tail segment are marked by asterisks; positions of microsatellite variations msR1, ms 3580 and ms4760 are labelled. The drawing is scaled to the 1920-amino-acid sequence from the *P. falciparum* 3D7 gene (Pf13_0019; http://www.plasmodb.org/).
B. Alignment of ms4760 sequences from different *P. falciparum* lines. Microsatellite variation ms4760-1, represented in the South American clone 7G8 and the south-east Asian clone Dd2, is the form associated with reduced QN sensitivity in the population surveys (Table 2). DNNND repeats discussed in the text are highlighted in bold.

The three point polymorphisms and microsatellite polymorphisms msR1 and ms3580 showed no significant association with QN response (Table 2). Variable D- and N-rich repeats encoded by ms4760, however, showed an identical form (ms4760-1; Fig. 3B) in the relatively quinine-resistant Dd2 and 7G8 lines from south-east Asia and South America; this same form also appeared frequently in other parasites with low QN responsiveness

Table 2. Relative QN responses and PfNHE polymorphisms in *P. falciparum* lines.

			Point polymorphisms			Microsatellite polymorphisms		
Isolate	Origin	QN response[a]	790	894	950	msR1	ms3580	ms4760[b]
Indo	Indochina	3.02	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-7
KMWII	Kenya	2.28	GTC	AAT	GTG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
PC15	Peru	1.75	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
FCB	SE Asia	1.61	GTC	AAA	GGG	Not present	NIH	ms4760-6
JCK	Cambodia	1.45	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
V1/S	Vietnam	1.26	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
PC09	Peru	1.15	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
C2A	Thailand	1.14	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
P31	SE Asia	1.12	GTC	AAA	GGG	Not present	NIH	ms4760-6
D5	SE Asia	1.12	GTC	AAA	GGG	Not present	NIH	ms4760-6
Dd2	Indochina	1.00	GTC	AAA	GGG	Not present	Not present	ms4760-1
TM191c	Thailand	0.98	GTC	AAA	GGG	TCDNNNMPNNNMSNNN	Not present	ms4760-7
TM284	Thailand	0.94	GTC	AAA	GGG	Not present	NIH	ms4760-1
106/1	Sudan	0.85	GTC	AAA	GGG	Not present	NIH	ms4760-6
Thai19	Thailand	0.82	GTC	AAT	GGG	Not present	NIH	ms4760-7
PC26	Peru	0.71	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
DIV17	Brazil	0.67	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
HB3	Honduras	0.63	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-5
DIV14	Brazil	0.57	GTC	AAT	GGG	Not present	NIH	ms4760-1
ICS	Brazil	0.55	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
M97	The Gambia	0.55	GTC	AAT	GTG	Not present	NIH	ms4760-3
ECP	Brazil	0.52	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
PC17	Peru	0.51	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
JAV	Colombia	0.50	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
PC49	Peru	0.46	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-6
Fab6	South Africa	0.42	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-8
7G8	Brazil	0.39	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
Thai16	Thailand	0.35	GTC	AAA	GGG	Not present	NIH	ms4760-1
K39	Kenya	0.35	GTC	AAT	GTG	Not present	NIH	ms4760-3
123/5	Sudan	0.35	GTC	AAA	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
128/4	Sudan	0.32	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
9013	Ghana	0.31	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
PAD	Brazil	0.28	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
DIV30	Brazil	0.28	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
124/8	Sudan	0.27	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-8
M5	Mali	0.27	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
3D7	Africa	0.25	GTC	AAT	GTG	Not present	NIH	ms4760-2
S35	Mali	0.23	GTC	AAT	GGG	Not present	Not present	ms4760-1
M2	R/Mali	0.23	TTC	AAT	GGG	Not present	NIH	ms4760-1
Fab9	South Africa	0.22	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-6
ECU	Ecuador81	0.21	GTC	AAT	GGG	Not present	NIH	ms4760-7
Haiti	Haiti	0.21	GTC	AAA	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
D10	Papua New Guinea	0.20	TTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-7
REN	Sudan	0.17	GTC	AAT	GTG	Not present	NIH	ms4760-3
9020	Ghana	0.17	GTC	AAT	GGG	Not present	Not present	ms4760-1
4156	The Gambia	0.17	GTC	AAT	GGG	Not present	NIH	ms4760-3
T2/c6	Thailand	0.17	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
9016	Ghana	0.16	GTC	AAT	GGG	Not present	NIH	ms4760-3
102/1	Sudan	0.16	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
M190	The Gambia	0.15	GTC	AAT	GTG	Not present	NIH	ms4760-3
LF4/1	Liberia	0.14	GTC	AAT	GGG	Not present	NIH	ms4760-1
SL/D6	Sierra Leone	0.14	GTC	AAT	GGG	Not present	NIH	ms4760-8
MR80	Vietnam	0.13	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-7
PNG3	Papua New Guinea	0.13	GTC	AAA	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-7
425	The Gambia	0.13	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
224	The Gambia	0.13	GTC	AAT	GTG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
713	Guinea Bissau	0.13	GTC	AAT	GGG	Not present	NIH	ms4760-7
418	The Gambia	0.12	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-3

Table 2. *cont.*

			Point polymorphisms			Microsatellite polymorphisms		
Isolate	Origin	QN response[a]	790	894	950	msR1	ms3580	ms4760[b]
M24	Kenya	0.11	GTC	AAT	GGG	Not present	NIH	ms4760-3
PNG13	Papua New Guinea	0.11	GTC	AAA	GGG	TCDNNNMPNNNMSNNN	Not present	ms4760-6
449	The Gambia	0.11	GTC	AAT	GTG	Not present	NIH	ms4760-3
9021	Ghana	0.11	GTC	AAT	GGG	Not present	NIH	ms4760-7
433	The Gambia	0.11	GTC	AAT	GTG	Not present	NIH	ms4760-1
MT/s-1	Thailand	0.11	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-4
Hu425	The Gambia	0.10	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-1
P13	Mali	0.09	GTC	AAT	GGG	Not present	NIH	ms4760-7
PNG 2	Papua New Guinea	0.08	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-7
PNG4	Papua New Guinea	0.08	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-7
601	The Gambia	0.08	GTC	AAT	GGG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
434	The Gambia	0.06	GTC	AAA	GAG	TCDNNNMPNNNMSNNN	NIH	ms4760-3
Camp	Malaysia	0.05	GTC	AAT	GTG	TCDNNNMPNNNMSNNN	NIH	ms4760-3

a. QN responses are expressed as median IC_{90} values relative to that of the Dd2 control value.
b. Polymorphism types are detailed in Fig. 3.

from south-east Asia, South America and Africa (Table 2). Other forms of the ms4760 region were more often found in the relatively QN-sensitive lines (Table 2). These observations, and the fact that identical ms4760-1 variations probably arose independently in the Dd2 and 7G8 drug-resistant lines from south-east Asia and South America, led us to examine the ms4760 variations in more detail.

Figure 3B shows an alignment of eight ms4760 types among the 71 worldwide isolates listed in Table 2. The insertions and deletions of degenerate repeats in this alignment occur within an ≈150 bp region of the coding frame. When the parasite isolates were grouped by the number of DNNND repeats in the ms4760 types, we found a statistical association with QN response ($F_{3,66} = 3.190$, $P = 0.0082$). Post hoc tests indicated that this relationship could be attributed to differences in QN response between isolates containing one versus two or more repeat units. When only parasites were evaluated from Asia and South America, where reduced clinical efficacy of quinine has been most clearly documented, a relationship of quinine responses and the number of DNNND repeats was still observed ($F_{1,30} = 4.485$, $P < 0.05$).

Evidence for loci that interact with the Chr 13 and Chr 7 QTL

The complexity of the QN response with respect to the additive loci of Chrs 13, 7 and 5 and its significant but incomplete association with PfNHE-1 repeat polymorphisms in some parasite lines led us to conduct additional pairwise searches for interacting QTL. These interaction effects can reveal QTL that show no effect alone, but rather have an influence dependent on a second locus. The pairwise scans, performed as described by Sen and Churchill (2001), identified significant interactions on the HB3 × Dd2 background with the main QTL of Chrs 7 and 13 (Fig. 4). These interactions were with chromosomes 6 and chromosomes 9 respectively. In each paired interaction, Chr 6 × Chr 7 and Chr 9 × Chr 13 (Table 3; Fig. 4), the maximum effect of the main locus shifting the responses higher was exerted when the interacting locus was also from the Dd2 parent. No independent main effects were detected for either of the Chr 6 and 9 loci alone (Fig. 2A and B), and little impact of the interacting locus was present if the main QTL was from the HB3 parent (Table 4, coef column; Fig. 4).

These observations suggest that gene interactions could reflect either co-operative drug response genes or compensatory mutations selected in the Dd2 parent clone. Although we do not know how these pairs function, an observation is possibly relevant: prolonged cloning periods and slow *in vitro* expansion rates were observed for the six independent recombinants with the HB3-type Chr 6 QTL and the Dd2-type Chr 7 QTL (*pfcrt*), which raises the possibility that parasite fitness is affected by this QTL combination. An additional preliminary complex growth phenotype based on radiolabelled hypoxanthine (Hx) incorporation as a growth indicator shows a QTL on the same arm of Chr 13 as the QN response locus (M. T. Ferdig, unpublished. observation). Because our dose–response assays rely on Hx incorporation, we took steps to rule out the possibility that an assay-specific artifact could be represented in the drug response QTL scan: we conducted parallel drug assays from a subset of nine progeny representing the range of QN responses using the lactate dehydrogenase indicator of parasite viability (Makler *et al*., 1993) and the standard Hx tests (data not shown). We observed no difference in the association between drug response and marker C13M56 at the Chr 13 peak using these two methods. Additionally, QTL mapping of another quinoline-based antimalarial compound, mefloquine, using the Hx assay

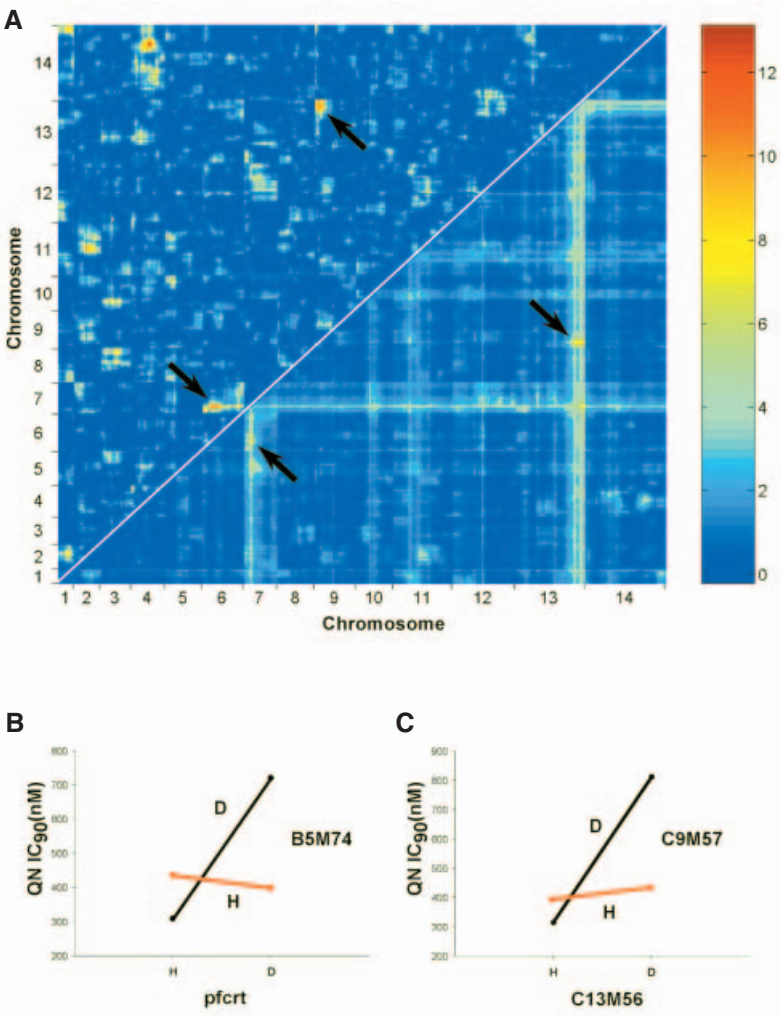

Fig. 4. Pairwise interactions located by diagonally symmetric signals in a two-dimensional genome-wide scan.
A. Output from a simultaneous search at 5 cM intervals for QTL pairs affecting QN IC_{90} in the HB3 × Dd2 cross. Markers in the 14 linkage groups occur in order along the axes. The LOD plot for a two-QTL model with interactions is displayed below the diagonal; the vertical spectrum at the right shows the corresponding LOD score colour code. For the pairwise plot below the diagonal, the $P = 0.05$ significance threshold based on 1000 permutations is LOD = 6.9. Significant interactions between Chrs 9 and 13 (LOD = 8.7) and between Chrs 6 and 7 (LOD = 7.1) are detected as bright yellow signals (arrows). Above and to the left of the diagonal is a plot of the difference in the variance between an interactive two-QTL model and a non-interactive, additive model. For this plot, a nominal cut-off of $P = 0.01$ was used, and the LOD score values were expanded by a factor of three to enhance colour detection. Chr 6 × Chr 7 and Chr 9 × Chr 13 interactions are indicated by the red signals diagonally symmetrical with those below the diagonal (LOD score ≈4; arrows).
B and C. Graphic representations of the interactive effects between the B5M74 and *pfcrt* markers (Chr 6 × Chr 7) and between the C9M57 and C13M56 markers (Chr 9 × Chr 13). The inheritance of the Dd2 parent alleles at each locus for each interaction results in substantially higher QN IC_{90} values than inheritance of other possible allele combinations. Corresponding means, standard deviations and pairwise comparisons are provided in Table 2.

shows no peak in this region of Chr 13 (M. T. Ferdig, unpublished observation).

Discussion

QN and CQ have related core quinoline structures and are thought to exert similar toxic effects through interactions with haem in malaria parasite food vacuoles; however, the historical use of these major antimalarial drugs and the consequent selection pressure on natural parasite populations has been distinct. QN has been used in the form of cinchona bark extracts or purified alkaloid prepa-

Table 3. QN IC_{90} QTL positions and effects.

QTL	Chr	Effect	Interacting QTL	Best locus (cM)[a]	Peak range (cM)	Closest marker	Allele associated with higher QN IC_{90}	QN IC_{90} at closest marker (mean, SD)[b]	*P*[c]
QN IC90-1	13	Main		180	155–185	C13M56	D	628 (222)	0.0001
QN IC90-2	7	Main		20	10–30	*pfcrt*	D	613 (228)	0.0005
QN IC90-3	5	Main		60	30–75	B5M86	H	548 (231)	0.09[d]
QN IC90-4	6	Interaction	QN IC_{90}-2	25	5–60	B5M74	DxD	718 (203)	0.002
QN IC90-5	9	Interaction	QN IC_{90}-1	15	0–50	C9M57	DxD	829 (148)	<0.0001

a. Based on a 5 cM grid.
b. Mean QN IC_{90} values and standard deviations (nM) of progeny containing the indicated marker (or marker combination). Mean QN IC_{90} for entire population: 491 ± 219 nM.
c. *P*-values are based on Student's *t*-tests comparing group means of progeny containing the D or H marker of the indicated QTL. Interaction *P*-values compare the H and D marker group means for each interacting locus (B5M74 or C9M57) when the Dd2 allele is present at the main effect locus (*pfcrt* or C13M56 respectively).
d. Calculations with a model incorporating QTL 1 and 2 yielded a *P*-value of 0.0005 when the H and D B5M86 marker groups were compared.

Table 4. Calculations of fit for QTL model of QN response.[a]

Source	d.f.[b]	SS[c]	Adj. SS[d]	%[e]	F[f]	*P*[g]	Coef[h]	SE[i]
B5M86	1	44 912.6	21 227.4	3.7	7.8	0.009	−103.5	37.3
B5M74	1	36 670.5	2 131.4	0.4	0.78	0.384	32.3	36.7
pfcrt	1	214 435.8	50 650.6	8.9	18.6	0.0001	155.2	36.0
C9M57	1	25 610.2	30 975.6	5.4	11.4	0.002	118.3	35.1
C13M56	1	90 937.0	93 678.2	16.4	38.4	0.000002	198.4	33.9
B5M74 × *pfcrt*	1	63 521.9	12 313.8	2.2	4.5	0.042	193.4	90.9
C9M57 × C13M56	1	15 206.0	15 206.0	2.7	5.6	0.025	207.9	89.3
Residual	29	78 968.1						
Total	36	570 262.0						

a. Regression variables were coded as described previously (Kim *et al*., 2001).
b. One degree of freedom (d.f.) was allowed in the test of each term under type III calculations that assume that all other contributing QTL are correct.
c. SS is the associated sum of squares.
d. Adj. SS is the adjusted sums of squares (type III sum of squares adjusting for all other terms in the model).
e. % is the total variation present in the adjusted sums of squares.
f. F-statistic is based on the adjusted sums of squares.
g. *P*-values derive from the F distribution.
h. Coef is the estimated effect size of the QTL. An estimated main effect of −103.5 implies that, after adjusting for all other terms in the model, the effect of inheriting the Dd2- versus the HB3-type B5M86 marker is to decrease the QN IC_{90} by 103.5 nM. A pairwise interaction coefficient of 193.4 between two markers implies that the effect of a Dd2-type first marker when the second marker has the Dd2 type is to increase the QN IC_{90} by 193.4 nM, compared with the effect of a Dd2-type first marker when the second marker is HB3 type.
i. SE is the standard error of this estimate.

rations for four centuries. Malaria parasite strains with reduced QN sensitivity have been reported from different malarious regions only since the beginning of the twentieth century (Nocht and Werner, 1910; James *et al*., 1932; Clyde *et al*., 1970). Today, despite the reduced drug sensitivity of some strains in South America and south-east Asia, all strains remain treatable with dosing regimens that include an increased QN concentration and a second drug for radical cure. This pattern is in stark contrast to that of the global sweep of resistance to CQ: within a few decades of chloroquine's introduction at the end of the World War II, fully CQ-resistant *P. falciparum* strains spread from at least five foci into nearly all malarious regions (Wootton *et al*., 2002; Chen *et al*., 2003). The slow progression towards QN resistance reflects more complex underlying genetic changes in response to the selective drug pressure than was required for CQR.

Our results assign >95% of the CQR phenotype in the HB3 × Dd2 cross to a sharp peak on Chr 7 (LOD score >15; Fig. 2C). This peak contains *pfcrt*, the determinant of CQR, as demonstrated by linkage analysis and gene complementation (Fidock *et al*., 2000), drug selection studies (Cooper *et al*., 2002), population studies (Wootton *et al*., 2002), direct allelic modification (Sidhu *et al*., 2002) and clinical outcome trials (Djimdé *et al*., 2001). QN response in the same cross, in contrast, is controlled by multiple genes and shows no overwhelming influence by a single locus. Our model incorporates three additive effects, one of which is the Chr 7 peak containing *pfcrt*, and two pairwise interactions from a total of five QTL (Table 4). Such a multicomponent phenotype in the HB3 × Dd2 cross is consistent with the pattern observed in the field: QN continues to be effective against CQR and mefloquine-resistant *P. falciparum* in spite of likely subsets of shared genes controlling these responses. That is, the *pfcrt* and *pfmdr1* genes associated with CQ and mefloquine resistance in a variety of laboratory and field studies can affect dose–responses and still not lead to high-level QN-resistant phenotypes, depending on the local selection history, multigene context and genetic background in which these mutations function.

While the gene mutations that can influence QN response in the Chr 7 and Chr 5 loci include *pfcrt* and *pfmdr1*, the Chr 13 peak is new and an effector gene remains to be identified. Approximately 100 genes are present within the 95% CI QTL peak, one of which encodes the probable sodium-hydrogen exchanger PfNHE-1. A sodium-hydrogen exchanger was previously postulated to mediate CQR, based on the kinetics of drug uptake and its inhibition by amiloride derivatives (Sanchez *et al*., 1998). Although its relationship to QN response was not examined, drug resistance in CQR parasites was attributed to elevated cytoplasmic pH values from a constitutively activated NHE (Wunsch *et al*., 1998); however, subsequent experiments using sodium-free buffer that presumably shut down the exchanger found no dependence of CQ uptake on exogenous sodium. Moreover, amiloride derivatives were shown to alter CQ uptake by blocking CQ–haem binding rather than by inhibiting NHE activity (Bray *et al*., 1999). This evidence that NHE activity does not underpin CQR is consistent with our finding that PfCRT variants are central to CQR and that PfCRT is not an NHE homologue (Wellems *et al*., 1998). With regard to QN response in the HB3 × Dd2 cross, however, the pres-

ence of *pfnhe-1* in the Chr 13 QTL peak, along with our observed statistical correlation in global parasite populations, could revive the possibility of a role for this gene and its interacting QTL in Chr 9. Genetic transformation techniques will be required to dissect the Chr 13 QTL and to test for a role for PfNHE-1 in QN response variation. Our studies indicate that complete dissection of these QTL effects will require experimental designs addressing the need to account for multiple genes from different chromosomes working in combination.

As QN response is a complex trait that includes multiple modifiers and paired interactions with other loci, a single genetic cross may capture only a subset of the genetic components that exist in the general population. Depending on genetic background effects associated with distinct geographies and drug selection histories, QTL from different regions may vary dramatically in their contributions to the phenotype of QN response. This is consistent with reports of candidate genes underlying certain quantitative traits that have been difficult to dissect in other systems (Steinmetz *et al*., 2002). The results of our population survey of parasite strains from south-east Asia, Africa and South America support a model in which multiple genes can combine in different ways to produce similar phenotypes of reduced QN response, as has been suggested recently in a study using outbred parasite isolates to examine drug response associations with transporter gene mutations (Mu *et al*., 2003). Such a model incorporates a requirement for mutations in multiple loci and may preclude jumps to clinical levels of QN resistance such as have occurred against other antimalarial drugs as a result of mutations in single genes. Further characterizations of QN response determinants may provide ways to monitor for incipient resistance in multiple loci and support action against QN-resistant strains should they arise.

This first use of QTL mapping in *P. falciparum* demonstrates how QTL mapping can provide a critical layer of information to an integrated genomics approach for dissecting the molecular components of complex phenotypes. In addition to identifying loci, QTL mapping methods can highlight the broader genetic architecture by specifying the number of genes and the relative contributions of the specific parental alleles as well as how the genes may be interacting. This strategy in turn provides a context for understanding mutations in populations and for designing appropriate functional gene manipulation experiments.

Experimental procedures

Dose–response assays

Clones from the genetic cross between the Dd2 clone from Indochina and the HB3 clone from Honduras (Wellems *et al*., 1990) were cultivated *in vitro* by standard methods (Haynes *et al*., 1976; Trager and Jensen, 1976). Red blood cells (RBCs) were purified from leucocytes and platelets by Sepacell R-500II sets (Baxter, Fenwal Division) and suspended in complete medium [CM; RPMI 1640 with L-glutamine (Life Technologies), 370 μM hypoxanthine (Hx) and 25 mM Hepes; 0.5% Albumax I (Life Technologies), 10 μg ml^{-1} gentamicin and 0.225% $NaHCO_3$) at 4% haematocrit. Cultures were maintained in six-well plates at 37°C under an atmosphere of 5% CO_2/5% O_2/90% N_2 in environmental chambers (Billups-Rothenberg). Parasites recovered from cryopreservates were cultivated for at least three cycles to ascertain thriving growth with a minimum doubling of parasitaemia in each 48 h period before assays. The parasite stages in these cultures were generally synchronous as a result of ring stage-specific survival from the freeze–thaw process. Samples for drug assays were diluted from cultures consisting of >75% ring stages.

Drug assays are sensitive to variations in haematocrit, starting parasitaemia, medium constituents (especially different sources of serum, when used), pH and parasite life cycle stage (Bickii *et al*., 1998; Duraisingh *et al*., 1999). We minimized these sources of non-heritable variation by the following precautions: Albumax from the same lot was used as a serum substitute; RBCs from one of two donors and CM were prepared in bulk and used within 1 and 2 weeks respectively; media stocks were refreshed with gas mixture daily to limit pH variation; after smear preparation, medium was changed and cultures were returned to gassed incubator chambers at 37°C for 2 h before initiating tests; Dd2 and HB3 parental lines were included in each test as internal controls; up to nine parasite clones at carefully established parasitaemias were tested simultaneously and housed in the same environmental chamber; assays of each line were replicated on different occasions with cultures originating from at least two separate frozen stocks thawed several weeks apart. Drug stocks (1 mg ml^{-1}) of QN hydrochloride, CQ diphosphate and VP hydrochloride were prepared in tissue culture-grade dimethyl sulphoxide (DMSO) or 70% ethanol (Sigma) and preserved at −70°C until use. Dilutions from these stocks were established in modified low-hypoxanthine CM (low-Hx CM; RPMI 1640 with L-glutamine, 2.94 μM Hx and 50 mM Hepes; 0.5% Albumax I, 10 μg ml^{-1} gentamicin and 0.225% $NaHCO_3$). Drug assays based on [^{3}H]-hypoxanthine incorporation (Desjardins *et al*., 1979) were conducted in flat-bottomed 96-well plates (Corning Life Sciences). Control wells in each assay included: parasitized RBCs in CM without QN, CQ or VP; unparasitized RBCs in CM; and parasitized RBCs in CM containing 0.89 μM VP (no QN or CQ). Cells from the wells were lysed after 64–68 h by freezing and thawing, transferred onto glass fibre filtermats, placed in sample bags containing scintillation fluid and counted using a 1450 Micro beta plate reader (Perkin-Elmer Life Sciences). Counts per minute (c.p.m.) from all test wells were adjusted by subtracting the counts from RBC-only control wells. Percentage inhibition was calculated by the formula:

$$100 \times [(\text{c.p.m. of no drug control}) - (\text{c.p.m. of treated sample})] / (\text{c.p.m. of no drug control}).$$

IC_{50}, IC_{90}, slope values and confidence intervals (CI) were computed from the sigmoid dose–response curves derived from fitting a three-parameter Hill function (Hill 3 equation

from SigmaPlot; SPSS) to the data from repeated assays. VP chemosensitization ratios were computed from the IC_{50} and IC_{90} values in the presence and absence of 0.89 μM VP. Mean values used for genome scanning were calculated from 7 to 22 independent assays of each progeny clone, 65 assays of the Dd2 parent and 53 assays of the HB3 parent.

Genome-wide scans

QTL analysis was carried out by computational approaches described previously (Sen and Churchill, 2001). The erythrocyte stages of the parasites used for genotyping and phenotyping are haploid; consequently, only two genetic classes are present for each locus, and the computational approach is equivalent to an analysis of recombinant inbred lines. Programs (http://www.jax.org/research//churchill) were run under the MATLAB package (MathWorks), and interval mapping was carried out at the average resolution (5 cM) of the *P. falciparum* genetic linkage map (Su *et al*., 1999) (http://www.ncbi.nlm.nih.gov/Malaria/Index.html). This resolution ensured that each mapping interval closely corresponded to a mapped microsatellite marker. Significance thresholds for all genome scans were determined by permutation analyses (Churchill and Doerge, 1994; Lander and Kruglyak, 1995). Because our set of progeny showed regions of skewed parental allele inheritance (Su *et al*., 1999), this permutation analysis helped to ensure that drug response loci were not falsely assigned to chance marker arrangements in the pedigree. Main QTL and corresponding mean trait values from the primary scans were used to obtain estimates of residual empirical thresholds (Doerge and Churchill, 1996) to search by secondary scans for additional QTL. This secondary scan procedure was equivalent to installing the a priori QTL as covariates, thereby attributing a large portion of the overall variation to these QTL and allowing for detection of additional loci controlling the remaining variation.

Pairwise scans

To search for QTL affecting the QN phenotype only in the context of specific variants at second loci, we performed pairwise scans at 5 cM spacing, testing all possible pairs of QTL locations. The likelihood calculated for each pair (two main loci and an interaction term) was compared with that of the null model (no pairwise effects). Significance was assessed by permutation analysis of every pairwise combination of marker alleles. Where an interaction was identified, a sequential series of statistical tests was performed to distinguish a true epistatic interaction from additive effects (independent gene actions) and hitchhiking (false positives resulting from related genotypes). These subsequent tests used a stringent nominal (comparison-wise) cut-off ($P = 0.01$).

Statistics

S-PLUS 2000 (Insightful) statistical analysis software was used to compute correlation coefficients and linear regression analyses of phenotype relationships. An ANOVA for the final model was conducted by incorporating into a multiple regression all the main and interacting QTL identified by the main, secondary and pairwise scans. F-statistics were calculated from adjusted (type III) sums of squares to make final determinations on the contribution of a QTL (or interaction) in combination with all other QTL. For this analysis, inheritance of the Dd2 parent allele was coded as +0.5 and inheritance of the HB3 parent allele was coded as −0.5 in the regression analysis. Accordingly, the main effect estimate for any locus was taken to be the difference between the effect of the Dd2 genotype and the HB3 genotype. Each interaction parameter was computed from the different effects on a main QTL of its paired Dd2- or HB3-type interacting QTL.

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Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4035/mmi4035sm.htm

Table S1. Progeny phenotypes and markers of QTL involved in drug responses.

Table S2. Genes and markers in the chromosome 13 segment spanning the QN response QTL peak.

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