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Discordant Patterns of Genetic Variation at Two Chloroquine Resistance Loci in Worldwide Populations of the Malaria Parasite *Plasmodium falciparum*▽†

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Mutations in the chloroquine resistance (CQR) transporter gene of *Plasmodium falciparum* (Pf*crt*; chromosome 7) play a key role in CQR, while mutations in the multidrug resistance gene (Pf*mdr1*; chromosome 5) play a significant role in the parasite's resistance to a variety of antimalarials and also modulate CQR. To compare patterns of genetic variation at Pf*crt* and Pf*mdr1* loci, we investigated 460 blood samples from *P. falciparum*-infected patients from four Asian, three African, and three South American countries, analyzing microsatellite (MS) loci flanking Pf*crt* (five loci [~40 kb]) and Pf*mdr1* (either two loci [~5 kb] or four loci [~10 kb]). CQR Pf*mdr1* allele-associated MS haplotypes showed considerably higher genetic diversity and higher levels of subdivision than CQR Pf*crt* allele-associated MS haplotypes in both Asian and African parasite populations. However, both Pf*crt* and Pf*mdr1* MS haplotypes showed similar levels of low diversity in South American parasite populations. Median-joining network analyses showed that the Pf*crt* MS haplotypes correlated well with geography and CQR Pf*crt* alleles, whereas there was no distinct Pf*mdr1* MS haplotype that correlated with geography and/or CQR Pf*mdr1* alleles. Furthermore, multiple independent origins of CQR Pf*mdr1* alleles in Asia and Africa were inferred. These results suggest that variation at Pf*crt* and Pf*mdr1* loci in both Asian and African parasite populations is generated and/or maintained via substantially different mechanisms. Since Pf*mdr1* mutations may be associated with resistance to artemisinin combination therapies that are replacing CQ, particularly in Africa, it is important to determine if, and how, the genetic characteristics of this locus change over time.

Chloroquine-resistant (CQR) *Plasmodium falciparum* parasites were first detected in the late 1950s in Southeast Asia (Thailand-Cambodia border) and South America (Venezuela and Colombia) (64). Resistant parasites from these locations are thought to have spread steadily throughout Asia and South America, arriving in East Africa (Kenya and Tanzania) in the late 1970s and spreading across the African continent within a decade (64). Resistance to chloroquine arose on the island of New Guinea during the 1950s, when Dutch colonial officials implemented chloroquinized-salt programs on that island, long before it was detected in the mid-1970s (21, 60). Subsequent reports suggest that CQR had become widespread in Papua New Guinea (PNG) by the early 1980s (35).

CQR in *P. falciparum* is linked to point mutations in the CQR transporter gene (Pf*crt*; chromosome 7) (24, 48). The Pf*crt* K76T mutant allele confers resistance in vitro and is the most reliable molecular marker for CQR (13, 15, 24). Chloroquine-sensitive (CQS) strains from all geographic regions maintain an invariable wild-type CVMNK (amino acids 72 to 76) allele, while there are a number of predominant CQR-associated alleles: CVIET (Southeast Asia and Africa), S_{agt}VMNT (SVMNT1; Asia, South America, and Tanzania), S_{tct}VMNT (SVMNT2; South America), CVMET (Colombia), and CVMNT (South America and the Philippines) (2, 12–14, 24, 34, 58, 61, 65).

Polymorphisms, including copy number variation and point mutations, in another transporter gene of the parasite, the multidrug resistance gene (Pf*mdr1*; chromosome 5), contribute to the parasite's susceptibility to a variety of antimalarial drugs (41, 46, 47). Point mutations in this gene play a modulatory role in CQR (25, 26). Two Pf*mdr1* mutant alleles occur in CQR strains from different geographic regions: 86Y_184Y_1034S_1042N_1246D (predominant in Asia and Africa) and 86N_184F_1034C_1042D_1246Y (predominant in South America) (25, 59). Although a number of field studies have observed a significant nonrandom association between the

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CQR Pf*crt* 76T and Pf*mdr1* 86Y alleles (28), suggesting a joint contribution of these two genes to the CQR phenotype, the results of other studies have suggested that additional parasite genes are likely to be involved (11, 36).

The major aims of the present study were (i) to compare the number of origins of CQR Pf*crt* alleles with those of CQR Pf*mdr1* alleles in different locations and (ii) to determine whether the distribution of microsatellite (MS) haplotypes associated with CQR Pf*mdr1* alleles represents divergent or convergent evolution when compared with the distribution of MS haplotypes associated with CQR Pf*crt* alleles. We have two main reasons to pursue these aims. First, there seem to be marked genetic differences between Pf*crt* and Pf*mdr1* loci. In their analysis of MS loci flanking Pf*crt*, Wootton et al. (65) observed reduced allelic diversity and shared chromosomal segments (>100 kb on either side of Pf*crt*) in 48 laboratory-adapted CQR isolates obtained worldwide, suggesting CQ selection-driven "sweeps." The selective sweeps were also seen in field studies conducted in Southeast Asia (12, 39) and South America (61), where the sweep sizes may be determined by the history and strength of CQ selection pressure and the genetic structure of parasite populations (39). Based on the MS data, at least five independent origins of CQR Pf*crt* alleles worldwide have been suggested (12, 65). In contrast, the evolutionary dynamics of Pf*mdr1* are far less clear. Duraisingh et al. (19) observed significantly lower levels of variation at an intragenic MS locus associated with Pf*mdr1* N86Y alleles in Gambian parasites and suggested that a limited number of origins and a selective sweep might be responsible for this observation. On the other hand, Nair et al. (37) recently reported multiple independent origins of Pf*mdr1* copy number variation and only limited reduction in MS allelic diversity within a 170- to 250-kb region flanking Pf*mdr1*, suggesting "soft" selective sweeps, in parasites from the Thailand-Burma border. MS data from Nair's study suggest that independent origins of the Pf*mdr1* locus occur frequently, as five different haplotype groups showing 15 Pf*mdr1* amplification events were observed within single parasite populations. Thus, the reduced variation at Pf*crt* but high variation at Pf*mdr1* indicates that polymorphisms in these two regions of the *P. falciparum* genome may be generated and/or maintained via different mechanisms.

The second reason to pursue these aims is that Pf*mdr1* polymorphisms may be associated with resistance to newer artemisinin combination drugs, and it is therefore important to understand the evolutionary dynamics of the Pf*mdr1* locus under swiftly changing drug pressures. Elevated Pf*mdr1* copy number has been associated with resistance to artesunate-mefloquine (4) and artemether-lumefantrine (57) combinations in Southeast Asia. Recent clinical studies in Africa have implicated the Pf*mdr1* 86N allele as a potential marker of resistance to the artemether-lumefantrine combination (16, 30, 49). Also, a high prevalence of parasites containing a potentially novel allele, 86Y_1246Y, has been observed in East African patients after treatment with the artesunate-amodiaquine combination (40) and with amodiaquine alone (29, 30).

In this study, we analyzed 460 blood samples from *P. falciparum*-infected patients from diverse regions of Asia (PNG, Indonesia, Laos, and India), Africa (Kenya, Uganda, and Ghana), and South America (Brazil, Colombia, and Guyana) where malaria is endemic for both the Pf*crt* and the Pf*mdr1* alleles and their flanking MS haplotypes. Our results provide new insights into the evolutionary dynamics of Pf*crt* and Pf*mdr1* loci in the Asian and African parasite populations and also reveal contrasting genetic features of the Pf*mdr1* locus in the populations from Asia/Africa and South America.

MATERIALS AND METHODS

Blood samples. (i) Asia. In PNG, 143 samples were collected in the areas of Dreikikir (East Sepik Province; $n = 36$; July to September 1996), the Wosera (East Sepik Province; $n = 76$; July 1998 to January 1999), and Liksul (Madang Province; $n = 31$; May to July 2000) (34, 35). In Indonesia, 34 samples were collected from both symptomatic and asymptomatic patients in Nias Island, located off the northwestern coast of Sumatra, in August 1998 (27, 53). In Laos, 29 samples were collected from malaria patients in Luang Namtha Province from October to December 2003 (14, 50). In India, 32 samples were collected from patients with fever attending malaria clinics in the states of Goa (medium levels of CQR; $n = 2$), Assam (high levels of CQR; $n = 18$), and Uttar Pradesh (low levels of CQR; $n = 12$) in 2001 (62).

(ii) Africa. In western Kenya, 39 samples were collected in the villages of Kabobo (Uasin Gishu district, Rift Valley Province; $n = 8$; August 1996 to March 1997) (31) and Kanyawegi (Kisumu district, Nyanza Province; $n = 31$; November 2000) (32). In Uganda, 30 samples were collected from children aged 7 years or younger with uncomplicated falciparum malaria in Kampala from August 1998 to March 1999 (17, 18). In Ghana, 51 samples were collected from children aged 2 years or younger in the Navrongo area of the Kassena-Nankana District ($n = 25$ in November 1996, dry season/low transmission, and $n = 26$ in May 1997, wet season/high transmission) (8).

(iii) South America. In Brazil, 29 blood samples were collected from symptomatic individuals visiting the clinic in Porto Velho, Rondônia, in July 1997 and July to September 1998. In Colombia, 40 samples were collected from symptomatic individuals visiting the clinic in El Bagre, Antioquia, in 1998. Details about the Brazilian and Colombian samples have been provided elsewhere (6). In Guyana, 33 samples were collected from an all-age population of people with clinical malaria seeking care at the Georgetown Public Hospital, Georgetown, in September 1998 (9).

Ethical approvals in these studies (6, 8, 9, 14, 17, 18, 27, 31, 32, 34, 35, 50, 53, 62) were obtained through their respective institutional review boards.

Laboratory-adapted parasite isolates. For MS analyses, genomic DNA preparations from nine *P. falciparum* laboratory isolates (HB3 [Honduras], 3D7 [unknown origin], Dd2 [Indochina], K1 [Thailand], 7G8 [Brazil], PNG1917 [PNG], PNG1905 [origin not confirmed], ECU1110 [Ecuador], and JAV [Colombia]) were included as references. Parasite strains HB3, 3D7, Dd2, K1, and 7G8 were obtained from MR4, American Type Culture Collection. Strains PNG1917 and PNG1905 were provided by Alan Cowman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. Parasite strains HB3, 3D7, Dd2, K1, 7G8, PNG1917, and PNG1905 were propagated in vitro (55). Genomic DNA preparations from strains ECU1110 and JAV were provided by Xin-zhuan Su, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases (National Institutes of Health [NIH]). DNA was extracted from cultured *P. falciparum* parasites by using a QIAamp DNA blood mini kit (Qiagen, Valencia, CA).

PCR-based analysis of Pf*crt* and Pf*mdr1* mutations. Except for the samples from Laos, Pf*crt* (codons 72 to 76 [CVMNK, CVIET, SVMNT1, SVMNT2, CVMNT, and CVMET]) and Pf*mdr1* (codons 86 [N/Y], 184 [Y/F], 1034 [S/C], 1042 [N/D], and 1246 [D/Y]) mutations were determined by post-PCR, sequence-specific oligonucleotide probe hybridization assays (34). The samples from Laos were analyzed for Pf*crt* (codons 72 to 76) and Pf*mdr1* N86Y alleles by a recently developed PCR-based enzyme-linked immunosorbent assay method (3).

Analysis of *cg2* ω repeat region and MS loci flanking Pf*crt* and Pf*mdr1*. PCR amplifications of the *cg2* ω repeat region (chromosome 7) (52), MS loci B5M77, 2E10, 9B12, and 2H4 (flanking Pf*crt*; chromosome 7), and MS loci 5-956456, 5-957861, 5-962445, and 5-966096 (flanking Pf*mdr1*; chromosome 5) were performed with seminested PCR strategies using three primers (Research Genetics, Huntsville, AL). One of the primers in each nest-2 amplification reaction was 5′ labeled with Cy5. All primer sequences, amplification conditions, and methods to evaluate single- versus multiple-allele infections at each MS locus and to construct allelic haplotypes are provided in Table S1 in the supplemental material and elsewhere (35). The MS haplotype on chromosome 7 (B5M77_2E10_Pf*crt*_9B12_*cg2*_2H4) extends ~40 kb. The MS haplotype on chromosome 5 (5-956456_5-957861_Pf*mdr1*_5-962445_5-966096) extends ~10 kb. We first an-

TABLE 1. Prevalence of Pf*crt* and Pf*mdr1* alleles in Asia, Africa, and South America

Gene and allele	Prevalence of allele in country (no. of samples)									
	PNG (143)	Indonesia (34)	Laos (29)	India (32)	Kenya (39)	Uganda (30)	Ghana (51)	Brazil (29)	Colombia (40)	Guyana (33)
Pf*crt*[a]										
CVMNK	0.385	0	0[c]	0	0.051	0	0.294	0	0	0.061
CVIET	0	0	0[c]	0.063	0.897	0.967	0.412	0	0	0.091
SVMNT1	0.615	1	0.586	0.938	0	0	0	0.069	0	0.364
SVMNT2	0	0	0	0	0	0	0.039	0.931	0	0.333
CVMET	0	0	0	0	0	0	0	0	1	0
Mixed	0	0	0.414	0	0.051	0.033	0.255	0	0	0.152
Pf*mdr1*[b]										
NYSND	0.168	0	0.923[d]	0.167	0.361	0.133	0.333	0	0	0
YYSND	0.608	0.92	0.038[d]	0.733	0.222	0.767	0.533	0	0	0.061
NFCDY	0	0	0	0	0	0	0	1	0	0.939
NFSDY	0	0	0	0	0	0	0	0	1	0
Mixed	0.224	0.08	0.038	0.1	0.417	0.1	0.133	0	0	0

[a] Codons 72 to 76.
[b] Codons 86_184_1034_1042_1246.
[c] Not observed in single infections, but found in mixed infections.
[d] Genotyping results are available only for N86Y.

alyzed loci 5-957861 and 5-962445 (extending ~5 kb), and if the diversity was moderate at these loci (locus-by-locus diversity [*h*], <0.3), then we analyzed loci 5-956456 and 5-966096.

Statistical analysis. The Arlequin 3.0 package (http://cmpg.unibe.ch/software/arlequin3/) (22) was used to compute the haplotype diversity (*H*; mean value ± standard error), locus-by-locus diversity (value ± standard deviation), mean number of pairwise differences (MPD), measure of genetic differentiation (F_{st}), and linkage disequilibrium (LD) between MS loci. We considered LD between loci to be significant only after Bonferroni correction for multiple comparisons. The Arlequin program was also used to perform analyses of molecular variance (AMOVA) to quantify Pf*crt* and Pf*mdr1* MS haplotype variation within and among parasite populations (23). In order to understand the genetic relationships among CQR Pf*crt* and Pf*mdr1* alleles, median-joining networks based on the MS haplotypes flanking Pf*crt* (four loci [B5M77_2E10_9B12_*cg2*]; ~28 kb) and Pf*mdr1* (two loci [5-957861_5-962445]; 5 kb) were constructed by using the Network 4.2 program (http://www.fluxus-engineering.com/sharenet.htm) (10).

RESULTS

Pf*crt* and Pf*mdr1* alleles and flanking MS haplotypes in *P. falciparum* laboratory isolates. We analyzed nine *P. falciparum* laboratory isolates from diverse geographic locations as references and compared their Pf*crt* and Pf*mdr1* alleles, as well as the flanking MS haplotypes (see Table S2 in the supplemental material), with those in the field samples from Asia, Africa, and South America. Among CQR isolates, different Pf*crt* alleles occurred on the same MS haplotype (SVMNT1 [isolate 7G8] and SVMNT2 [isolate PNG1905], CVMNT [isolate ECU1110], and CVMET [isolate JAV]), and the same allele occurred on different haplotypes (SVMNT1 [isolates 7G8 and PNG1917]). Interestingly, CQS isolate HB3 and CQR isolates ECU1110 and JAV shared the same Pf*mdr1* allele (NFSDD) and MS haplotype (see Table S2 in the supplemental material). For all these *P. falciparum* laboratory isolates, our results regarding Pf*crt* alleles and flanking MS loci are concordant with those of Wootton et al. (65). For isolates HB3, 3D7, Dd2, K1, 7G8, PNG1917, and PNG1905, our results regarding Pf*mdr1* alleles are concordant with those of the previous reports (25, 44); to the best of our knowledge, the data regarding Pf*mdr1* alleles in isolates ECU1110 and JAV and flanking MS loci (all isolates) are reported here for the first time.

Overview of Pf*crt* and Pf*mdr1* alleles in field samples. The Pf*crt* genotypes (codons 72 to 76) are summarized in Table 1. The CQS CVMNK allele was less prevalent than CQR alleles (Table 1). Among the CQR alleles, SVMNT1 was the predominant allele in parasites from Asia, and CVIET was the predominant allele in parasites from Africa. In parasites from South America, the predominant alleles were SVMNT2 (Brazil), CVMET (Colombia), and SVMNT1, as well as SVMNT2 (Guyana). We observed the CVIET allele in parasites from India ($n = 2$) and Guyana ($n = 3$), SVMNT1 in parasites from Brazil ($n = 2$), and SVMNT2 in parasites from Ghana ($n = 2$). The presence of the SVMNT2 allele in the parasites from Ghana was confirmed by direct DNA sequencing (34).

The Pf*mdr1* genotypes defined by codons 86_184_1034_1042_1246 are summarized in Table 1 (lower panel). The CQS NYSND allele was present in parasites from Asia and Africa but was completely absent in parasites from South America (Table 1). The CQR YYSND allele was more frequent in parasites from both Asia (except Laos) and Africa (except Kenya). We observed a high prevalence of the CQS Pf*mdr1* 86N alleles in the Laotian (94%) and Kenyan samples (56%), which also had very high prevalences of the CQR Pf*crt* 76T alleles (91% and 92%, respectively). In the parasites from South America, we observed that the alleles NFCDY in parasites from Brazil and Guyana and NFSDY in parasites from Colombia were predominant. Two samples from Guyana were positive for the YYSND allele; these samples were also positive for the Pf*crt* CVIET allele.

MS haplotypes flanking Pf*crt* alleles. We observed predominant MS haplotypes (40 kb) associated with CQR Pf*crt* alleles in parasites from Asia (SVMNT1; 3_6_3_5_10 [42%] and 3_6_3_5_7 [28%]), Africa (CVIET; 3_7_2_4_4 [76%]), and South America (both SVMNT1 and SVMNT2; 2_3_3_6_7 [92.5%] and CVMET; 3_2_4_7_10 [96%]) (see Table S3a in the supplemental material). The haplotype associated with the Indian CVIET allele (3_7_2_11_1; $n = 2$) differed from the haplotype associated with the most-common Southeast Asian/African CVIET allele (3_7_2_4_4; seen in isolates Dd2 and K1 [see

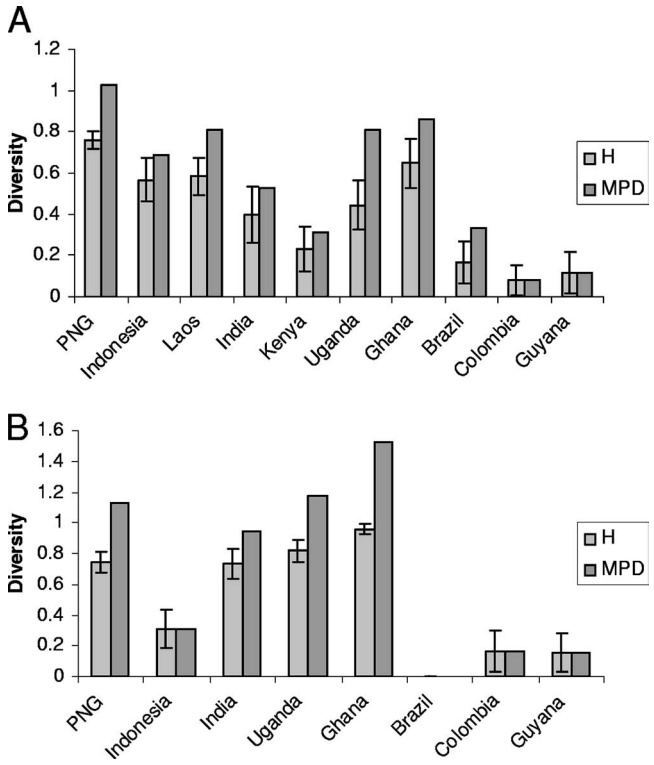

FIG. 1. (A) Haplotype diversity and MPD of CQR Pf*crt* allele-associated MS haplotypes. All Pf*crt* MS haplotypes are ~40 kb in length. The Pf*crt* (72 to 76) allele(s) and total number of associated MS haplotypes (in parentheses) for each region are Asia, SVMNT1 (23); Africa, CVIET (14); and South America, SVMNT1 and/or SVMNT2 (4) and CVMET (2). The number of unique haplotypes/total number of haplotypes for each country is PNG, 12/20; Indonesia, 1/6; India, 1/4; Laos, 1/3; Kenya, 1/4; Uganda, 7/8; Ghana, 3/6; Brazil, 1/2; Colombia, 2/2; and Guyana, 1/2 (see Table S3a in the supplemental material). (B) Haplotype diversity and MPD of CQR Pf*mdr1* allele-associated MS haplotypes. Pf*mdr1* MS haplotypes are ~5 kb in length in Asia and Africa and ~10 kb in length in South America. The Pf*mdr1* (86_184_1034_1042_1246) allele(s) and total number of associated MS haplotypes (in parentheses) for each region are Asia, YYSND (18); Africa, YYSND (19); and South America, NFCDY (3) and NFSDY (2). The number of unique haplotypes/total number of haplotypes for each country is PNG, 9/10; Indonesia, 1/2; India, 6/6; Uganda, 5/7; Ghana, 12/12; Brazil, 0/1; Colombia, 1/1; and Guyana, 0/1 (see Table S4 in the supplemental material). Laos was omitted from the analysis because all the Laotian samples carried the CQS Pf*mdr1* 86N allele.

Table S2 in the supplemental material]). We could not amplify all five MS loci in the parasites from Guyana (CVIET; $n = 3$) and Ghana (SVMNT2; $n = 2$); hence, complete haplotype determination for these samples was not possible. The MS haplotype associated with the Brazilian SVMNT1 allele (2_2_3_8_7; $n = 2$) differed from the predominant haplotypes observed in the Asian SVMNT1 samples and also from the SVMNT1 allele-carrying Brazilian laboratory isolate 7G8 (see Table S2 in the supplemental material).

The CQR Pf*crt* allele-associated MS haplotype diversity (40 kb) and MPD for each country are shown in Fig. 1A. Overall, 23 haplotypes were associated with the Pf*crt* SVMNT1 allele in parasites from Asia ($H = 0.744 \pm 0.03$, MPD = 0.993), whereas 14 haplotypes were associated with the Pf*crt* CVIET allele in parasites from Africa ($H = 0.427 \pm 0.075$, MPD = 0.645) (see Table S3a in the supplemental material). When we performed locus-by-locus analysis, we found that the locus-specific diversity at MS 2H4 was much higher in parasites from Asia ($h = 0.642 \pm 0.027$) than in parasites from Africa ($h = 0.239 \pm 0.067$). When this locus was not included in the analysis, the 4-locus (28 kb) haplotype diversities in Asian (10 haplotypes; $H = 0.314 \pm 0.054$, MPD = 0.35) and African (10 haplotypes; $H = 0.337 \pm 0.073$, MPD = 0.406) parasites were similar (see Table S3b in the supplemental material), indicating that considerably higher variation at MS 2H4 is responsible for the overall high haplotype (40 kb) diversity in Asian parasites. Compared with the haplotype diversity in parasites from Asia or Africa, much-lower haplotype (40 kb) diversity was observed in parasites from South America (Fig. 1A). Overall, there is much-lower haplotype (40 kb) diversity associated with CQR alleles (Fig. 1A) than with CQS Pf*crt* CVMNK alleles in parasites from PNG ($H = 0.995 \pm 0.01$, MPD = 3.903) and Ghana ($H = 1.000 \pm 0.034$, MPD = 4.076).

MS haplotypes flanking Pf*mdr1* alleles. Among 2-locus (5 kb) haplotypes associated with CQR Pf*mdr1* 86Y alleles, 5_6 (26%), 11_8 (18%), and 9_5 (12%) in Asian and 8_9 (22%) in African parasites were the most frequent (see Table S4 in the supplemental material). Among 4-locus (10 kb) haplotypes associated with CQR Pf*mdr1* 86N (with downstream CDY or SDY) alleles in South American parasites, 9_7_8_2 (Brazil, 100%; Guyana, 92%) and 3_14_8_4 (Colombia, 83%) were the most frequent.

The CQR Pf*mdr1* allele-associated MS haplotype diversity (5 kb) and MPD for each country are shown in Fig. 1B. Overall, the haplotype diversities were high in both Asian (18 haplotypes; $H = 0.878 \pm 0.023$, MPD = 1.559) and African (19 haplotypes; $H = 0.936 \pm 0.027$, MPD = 1.541) parasites, and only two haplotypes were common between these two geographic regions (see Table S4 in the supplemental material). Locus-by-locus analysis showed that diversities at both loci were high ($h = 0.4$ to 0.9). Within Asia, the 2-locus (5 kb) haplotype diversity was significantly higher in parasites from PNG and India than in parasites from Indonesia (Fig. 1B). However, the Indonesian samples exhibited high 4-locus (10 kb) haplotype diversity ($H = 0.765 \pm 0.075$, MPD = 1.301), whereas in South American parasites, the 4-locus (10 kb) haplotype diversity was none (Brazil) to low (Colombia and Guyana) (Fig. 1B). CQS Pf*mdr1* 86N alleles exhibited high 2-locus (5 kb) haplotype diversity in parasites from PNG ($H = 0.956 \pm 0.061$, MPD = 1.622) and Ghana ($H = 0.978 \pm 0.054$, MPD = 1.733) but only moderate ($H = 0.295 \pm 0.156$, MPD = 0.308) or no (4-locus, 10 kb) diversity in parasites from Laos.

Taken collectively, these results indicate that, unlike Pf*crt* alleles (high genetic diversity for CQS and reduced genetic diversity for CQR Pf*crt* alleles), the genetic diversity is high for both CQS and CQR Pf*mdr1* alleles in parasites from Asia and Africa. However, similar to CQR Pf*crt* alleles, the genetic diversity is low for CQR Pf*mdr1* alleles in parasites from South America.

LD between Pf*crt* and Pf*mdr1* loci. In the samples showing single-allele infections, we observed various frequencies of the CQR Pf*crt* 76T and Pf*mdr1* 86Y alleles occurring together in parasites from Asia (67% to 92%) and Africa (20% to 73%) and of the CQR Pf*crt* 76T and Pf*mdr1* 86N alleles (with downstream CDY or SDY) occurring together in parasites from

TABLE 2. Frequencies of CQR Pf*crt* and Pf*mdr1* alleles occurring together[a]

Parasite population	Frequency (%)
PNG	67
Indonesia	92
India	88
Kenya	20[b]
Uganda	73
Ghana	39[c]
Brazil	100
Colombia	100
Guyana	79

[a] The Pf*crt* 76T and Pf*mdr1* 86Y alleles occurred together in parasites from PNG, Indonesia, India, Kenya, Uganda, and Ghana; the Pf*crt* 76T and Pf*mdr1* 86N (with downstream CDY or SDY) alleles occurred together in parasites from Brazil, Colombia, and Guyana. Note that data from Laos (92.3% CQS Pf*mdr1* 86N allele; see Table 1) were not included in the analysis.
[b] 41.7% mixed Pf*mdr1* alleles (see Table 1).
[c] 25.5% mixed Pf*crt* and 13.3% mixed Pf*mdr1* alleles (see Table 1).

South America (79% to 100%) (Table 2). Significant associations were observed between CQR Pf*crt* 76T and Pf*mdr1* 86Y alleles and between CQS Pf*crt* 76K and Pf*mdr1* 86N alleles in parasites from PNG (χ^2 test, 1 df = 13.99; $P < 0.001$) and Ghana (χ^2 test, 1 df = 7.69; $P < 0.01$). This analysis could not be performed for samples from the other countries because the CQS Pf*crt* 76K allele was absent or was low in prevalence (Table 1).

We also tested the significance of LD for a complete data set of Pf*crt* and Pf*mdr1* MS loci which included samples with missing data for one or more loci and multiple-allele infections; the latter were coded as missing data. After correction for multiple comparisons, significant LD between Pf*crt* and Pf*mdr1* MS loci was observed only in parasites from India (9B12-962445, *cg2*-957861, and 2H4-962445 [total number of Pf*crt*–Pf*mdr1* pairwise comparisons was 10]) and Guyana (B5M77-957861, 2E10-966096, 2H4-956456, and 2H4-957861 [total number of Pf*crt*–Pf*mdr1* pairwise comparisons was 12]). These observations, although limited in their interpretation, suggest that the associations between Pf*crt* and Pf*mdr1* genetic polymorphisms may not correspond to the pattern and extent of LD between Pf*crt*–Pf*mdr1* MS loci.

Hierarchical analysis of genetic variation at CQR Pf*crt* and Pf*mdr1* loci. The worldwide comparison of genetic variation between CQR Pf*crt* and Pf*mdr1* loci using AMOVA shows substantial differences (Table 3). While the Pf*crt* locus has only 25% of the variation within populations, the Pf*mdr1* locus shows 50% of the variation within populations. Further, Pf*crt* has much more between-group variation than between-population variation, whereas Pf*mdr1* has much more between-population variation than between-group variation. In fact, the between-group variation in Pf*mdr1* was not significantly different from zero. The AMOVA for each separate geographic region shows that most (Africa and South America) or almost all (Asia) of the variation in the Pf*crt* locus was contained within populations, with a minimal subdivision among populations (Africa, 4.72%, and South America, 2.68%) (Table 3). For the Pf*mdr1* locus, although the variation within populations was considerably higher than the variation among populations, higher subdivision among populations was noticed for Asia (37.26%) and Africa (11.47%) than for the Pf*crt* locus (Table 3). Note that the results for parasites from South America depend on whether or not Colombia is included and that parasites from Brazil and Guyana are quite similar for both the Pf*crt* and Pf*mdr1* loci.

Genetic relationships among Pf*crt* and Pf*mdr1* MS haplotypes in parasites worldwide. We explored the genetic relationships among CQR Pf*crt* and Pf*mdr1* MS haplotypes in parasites sampled worldwide by constructing networks under the assumption of a stepwise mutation model for the MS loci. While many MS loci in *P. falciparum* parasites seem to be evolving in a manner consistent with a stepwise mutation model (7), the mode of MS evolution is uncertain (7, 38). Since the 2H4 locus exhibited much-higher diversity than other loci for most of the populations and may not conform to the stepwise mutation model, as it contains three types of repeat units

TABLE 3. Distribution of Pf*crt* and Pf*mdr1* MS haplotype diversity among and within geographic populations by AMOVA

Locus	Source of variation	Haplotype diversity[e]							
		Worldwide		Asian		African		South American	
		% Variation	*P* value	% Variation	*P* value	% Variation	*P* value	% Variation	*P* value
Pf*crt*	Among groups[a]	56.97	<0.001						
	Among populations[b]	17.63	<0.001	0.41	0.307	4.72	0.045	2.68,[f] 90.94[g]	0.496,[f] <0.001[g]
	Within populations	25.39	<0.001	99.59		95.28		97.32,[f] 9.06[g]	
Pf*mdr1*	Among groups[c]	8.07	0.123						
	Among populations[d]	39.51	<0.001	37.26	<0.001	11.47	<0.001	2.14,[f] 90.39[g]	<0.001[f,g]
	Within populations	52.42	<0.001	62.74		88.53		97.86,[f] 9.61[g]	

[a] The groups are Asia (SVMNT1), Africa (CVIET), and South America (Brazil and Guyana [SVMNT1 and/or SVMNT2] and Colombia [CVMET]). All MS haplotypes are ~28 kb. The locus 2H4 was not included in the haplotypes.
[b] The populations are parasites from PNG, Indonesia, Laos, and India (Asia); Kenya, Uganda, and Ghana (Africa); and Brazil, Colombia and Guyana (South America).
[c] The groups are Asia and Africa (YYSND) and South America (Brazil and Guyana [NFCDY] and Colombia [NFSDY]). The length of the MS haplotypes among groups is ~5 kb, irrespective of the population. However, in each geographic region, the length of the MS haplotypes in Asia (PNG, Indonesia, and India) and Africa (Uganda and Ghana) is ~5 kb, while in South America it is ~10 kb.
[d] The populations are parasites from PNG, Indonesia, and India (Asia); Uganda and Ghana (Africa); and Brazil, Colombia, and Guyana (South America). Data for Laos (CQS Pf*mdr1* 86N allele) were not included in the AMOVA.
[e] *P*, probability of % variation equal to or greater than that observed by chance alone.
[f] Comparison between Brazil and Guyana.
[g] Comparison between Brazil and Guyana (combined) and Colombia.

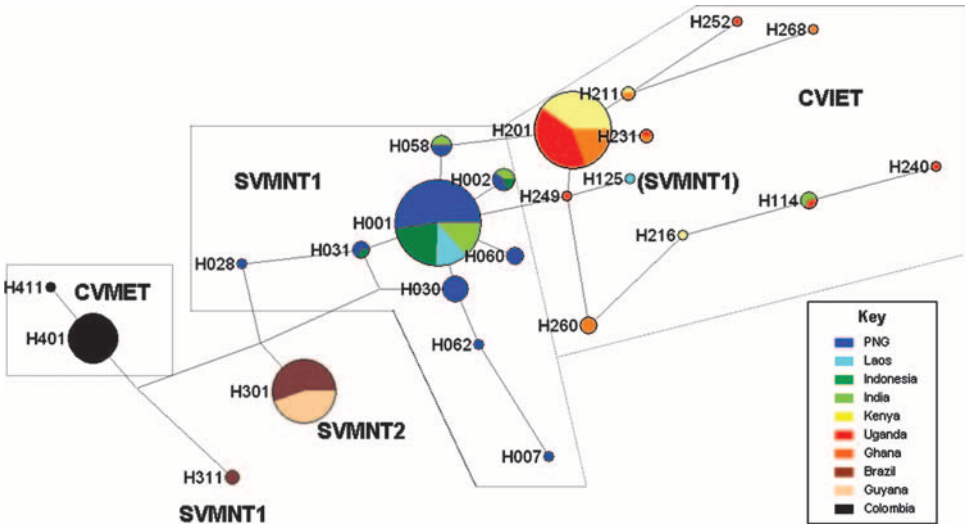

FIG. 2. Median-joining network of CQR Pf*crt* allele-associated MS haplotypes. All Pf*crt* MS haplotypes are ~28 kb in length. H001 to H200, Asia; H201 to H300, Africa; and H301 to H400, South America (see Table S3b in the supplemental material); the associated Pf*crt* allele and geographic distribution are indicated for each haplotype.

(T, TA, and TTTA) (52), this locus was omitted from the Pf*crt* network analysis.

Figure 2 displays the evolutionary relationships among all 24 Pf*crt* haplotypes (four loci, 28 kb) (see Table S3b in the supplemental material). The median-joining network roughly separates into three groups, which correspond with geography. One group consists entirely of South American haplotypes (H301 [SVMNT1 and/or SVMNT2], H311 [SVMNT1], and H401 and H411 [both CVMET]). The second group contains all but two Asian haplotypes, and the third group contains all African haplotypes plus one Asian (H125 [SVMNT1]) and one shared Asian/African (H114 [CVIET]) haplotype. In the Asian group, all SVMNT1-associated haplotypes can be derived from the most-prevalent haplotype (H001; frequency, 83%) and are connected to each other mostly by one-step mutations, with the exception that H028-H031, H031-H030, and H062-H007 exhibit two-step mutations. In the African group, all CVIET-associated haplotypes can be derived from the most-prevalent haplotype (H201; frequency, 81%) and are connected to each other by one-step, two-step, or three-step mutations. H114, observed in one Ugandan and two Indian samples, seems to have arisen from the most-prevalent Southeast Asian/African haplotype (H201; seen in isolates Dd2 and K1 [see Table S2 in the supplemental material]) by either a multistep mutation pathway or a recombination event; distinguishing between these two explanations would require further analysis.

Even though information on Pf*crt* (codons 72 to 76) alleles was not used in constructing the network, there is a considerable association between Pf*crt* alleles and the groups in the network, with two exceptions, both involving the SVMNT1 allele: H311 (SVMNT1) is in the network with H301 (SVMNT1 and/or SVMNT2) and H401 (CVMET), and H125 (SVMNT1) is connected to H249 (CVIET). H311 is equidistant (three steps) from both H301 and H401. Although the origins of SVMNT2, the most-widespread Pf*crt* allele in parasites from the Amazon region, and SVMNT1, with limited distribution, are uncertain (61), it is more likely that H311 has emerged from H301. H125 may reflect homoplasy, as it differs by one step from H249 and by only three steps from H001 (SVMNT1), which is in the range of variation of other SVMNT1-associated haplotypes.

Figure 3A displays the evolutionary relationships among all 37 Pf*mdr1* haplotypes (two loci, 5 kb) (see Table S4 in the supplemental material). These haplotypes include those associated with the CQS Pf*mdr1* 86N allele from Laos. In a stark contrast with the Pf*crt* network, the Pf*mdr1* network shows many reticulations, even though there are only two loci, indicating that there have been many parallel changes at these loci. Moreover, there is no correlation with either geography or Pf*mdr1* alleles; the one Brazilian/Guyanan haplotype (H101, NFCDY) is quite distant from the one Colombian haplotype (H201, NFSDY), which is identical to a haplotype observed in some Ghanaian samples (H033, YYSND; $n = 3$). All haplotypes from India (YYSND) and Laos (86N) are together with all of the haplotypes except one from PNG (YYSND), but haplotypes from Uganda and Ghana also are found in this part of the network. We also constructed a network for the Pf*mdr1* 4-locus (10 kb) haplotype data available for Indonesia (YYSND), Laos (86N), Brazil (NFCDY), Colombia (NFSDY), and Guyana (NFCDY). In this network, Brazilian/Guyanan haplotypes grouped with Laotian haplotypes and Colombian haplotyes grouped with Indonesian haplotypes (Fig. 3B).

The principal outcome of the network analysis is that Pf*crt* MS haplotypes correlate well with geography and CQR Pf*crt* alleles, whereas there is no distinct Pf*mdr1* MS haplotype that correlates with geography and/or CQR Pf*mdr1* alleles. Also, these analyses suggest that CQR Pf*mdr1* 86Y alleles in parasites from Asia and Africa had multiple independent origins and have further diversified extensively, much more so than the CQR Pf*crt* alleles.

DISCUSSION

In the present study, utilizing samples from diverse regions where malaria is endemic, two major observations were made

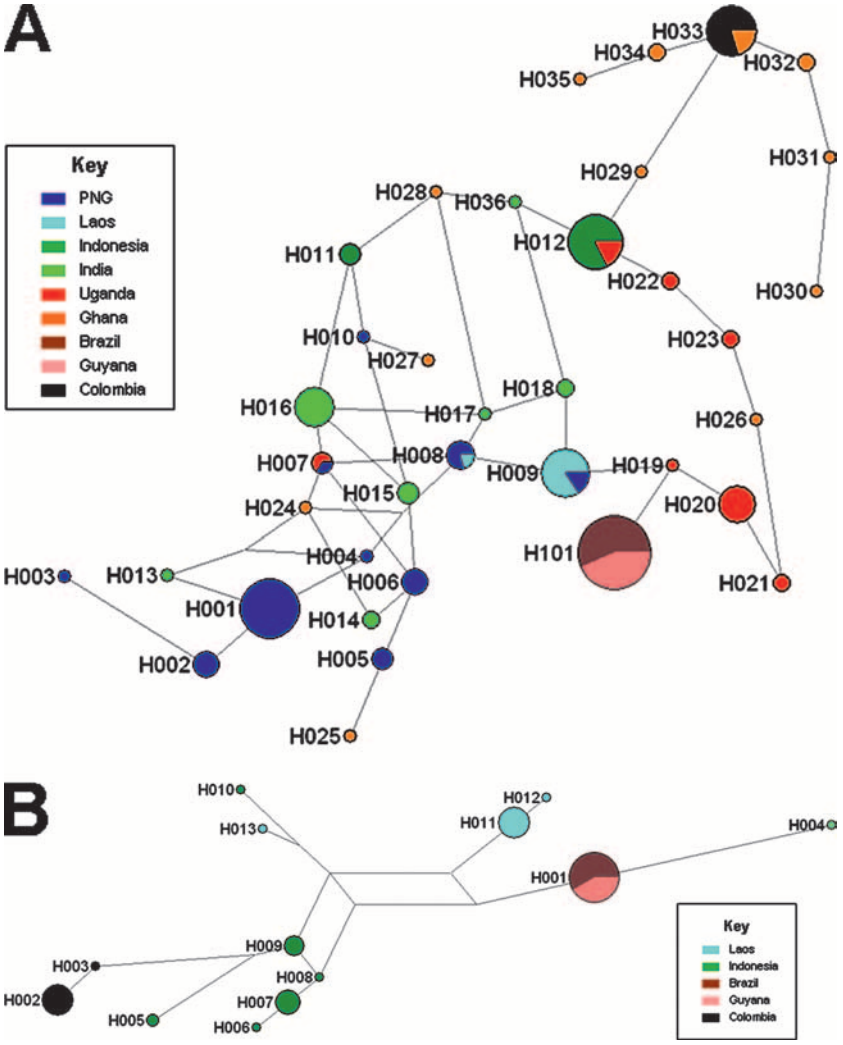

FIG. 3. (A) Median-joining network of Pf*mdr1* allele-associated MS haplotypes (~5 kb in length). See Table S4 in the supplemental material for haplotype numbering information. Note that all samples, except those from Laos, carry CQR Pf*mdr1* alleles. (B) Median-joining network of Pf*mdr1* allele-associated MS haplotypes (~10 kb in length). H001 is 9_7_8_2 (Brazil, $n = 17$, and Guyana, $n = 12$), H002 is 3_14_8_4 (Colombia, $n = 11$), H003 is 4_14_8_4 (Colombia, $n = 1$), H004 is 15_7_8_2 (Guyana, $n = 1$), H005 is 4_11_5_3 (Indonesia, $n = 2$), H006 is 4_11_8_1 (Indonesia, $n = 1$), H007 is 5_11_8_1 (Indonesia, $n = 7$), H008 is 5_11_8_2 (Indonesia, $n = 1$), H009 is 5_11_8_3 (Indonesia, $n = 5$), H010 is 7_11_5_3 (Indonesia, $n = 1$), H011 is 7_8_7_4 (Laos, $n = 11$), H012 is 7_8_6_4 (Laos, $n = 1$), and H013 is 7_11_7_4 (Laos, $n = 1$). While samples from Brazil, Guyana, Colombia, and Indonesia carry CQR Pf*mdr1* alleles, Laotian samples carry the CQS Pf*mdr1* 86N allele.

that are of relevance to public health: (i) in Asian and African *P. falciparum* parasite populations, CQR Pf*crt* and Pf*mdr1* loci exhibited strikingly different patterns of variation, with CQR Pf*crt* loci exhibiting reduced variation and CQR Pf*mdr1* loci exhibiting high variation, and (ii) in South American *P. falciparum* parasite populations, both the CQR Pf*crt* and Pf*mdr1* loci exhibited considerably lower variation.

Global distribution of CQR Pf*crt* and Pf*mdr1* alleles. Our results, together with the results of several previous studies, suggest that although various CQR Pf*crt* and Pf*mdr1* alleles predominate in *P. falciparum* populations in different geographic regions and countries, none of these alleles is restricted to any one particular location (2, 13, 14, 25, 34, 58, 61). Instead, a variety of alleles can be found in many populations worldwide. In this study, among the CQR Pf*crt* alleles, SVMNT1 in Asian parasites and CVIET in African parasites were highly prevalent, with some CVIET and some SVMNT2, respectively. In South American parasites, the distribution of CQR alleles varied. In parasites from Brazil, SVMNT2 predominates, with some SVMNT1. The Colombian parasite population was fixed for the CVMET allele. The Guyanan parasite population was quite heterogeneous, with high frequencies of both the SVMNT1 and SVMNT2 alleles and lower but still appreciable frequencies of the CVIET allele. Regarding the distribution of CQR Pf*mdr1* alleles, only YYSND in both Asian and African parasites, NFCDY in Brazilian parasites, and NFSDY in Colombian parasites were observed. In Guyanan parasites, NFCDY predominates, with some YYSND. Given modern means of transportation and greater frequency of travel throughout the world, such a distribution of parasite alleles is not surprising. On the other hand, certain alleles, such as SVMNT1 in parasite populations in Asia and South America, may have more

than one origin (Fig. 2) (65). Furthermore, as observed with the Laotian and Kenyan samples in the present study, the prevalence of sensitive and resistant alleles of both Pf*crt* and Pf*mdr1* in the parasite population in any given location could be heterogeneous due to several local factors: the history of drug selection pressure (16, 30, 49), parasite population genetic structure (33), and, possibly, host-intrinsic factors (42, 54).

Genetic variation at CQR Pf*crt* and Pf*mdr1* loci in parasites from Asia and Africa. (i) Dissemination of Pf*crt* SVMNT1 allele in parasites from Asia. Despite the fact that CVIET is the predominant CQR Pf*crt* allele in parasites from Southeast Asia (13, 65), SVMNT1 was the predominant allele in our Asian samples. This Pf*crt* SVMNT1 allele was associated with an MS haplotype, 3_6_3_5_10, that was observed to be predominant in all four countries (PNG [21%], Indonesia [64%], Laos [57%], and India [78%]) (see Table S3a in the supplemental material). Therefore, parasites with this or similar haplotypes are likely to be the source of the SVMNT1 founder allele in Asian parasites. The network analysis (Fig. 2) also supports the identification of the 4-locus haplotype 3_6_3_5 as the founder. In a previous study in which we analyzed samples collected in PNG between 1982 and 1984 (during the early years of the spread of CQR in PNG), we observed that 97% of the samples carried the SVMNT1 allele and 83% of those samples carried the 3_6_3_6_10 haplotype (35). The present predominant 3_6_3_5_10 haplotype can be derived from the earlier predominant 3_6_3_6_10 haplotype by just a one-step mutation. Previously, the SVMNT1 allele-carrying parasites from PNG were found to closely share the Pf*crt*-flanking MS haplotypes with those from the Philippines and the Solomon Islands (12). Taking these data together, it appears that the SVMNT1 allele-carrying parasites, with similar genetic backgrounds flanking Pf*crt*, are widespread in the Asia/Pacific region.

It is interesting to note that between the two CQR Pf*crt* alleles, CVIET and SVMNT1, that are highly prevalent in Asian parasites, it is CVIET that moved into Africa and is now widespread in that continent (13, 65; this study). Recently, the SVMNT1 allele has been reported in parasites from Iran (58) and Tanzania (2). This may represent westward movement of the Asian SVMNT1 allele and may have significance from a malaria treatment standpoint. The SVMNT1 allele may be associated with resistance to amodiaquine (48, 63), and therefore, the efficacy of artesunate-amodiaquine treatment, which is becoming increasingly popular in Africa, might be compromised (2).

(ii) Genetic variation of Pf*crt* versus Pf*mdr1* in Asia and Africa. In contrast with CQR Pf*crt* alleles, the haplotype diversities for CQR Pf*mdr1* alleles were very high in both Asian and African parasites. Moreover, in the AMOVA analysis, the differentiation among both Asian (about 37%) and African (about 12%) populations for the CQR Pf*mdr1* allele-associated haplotypes was significantly high (Table 3). These observations suggest that the mechanisms responsible for generating and/or maintaining genetic variation at Pf*crt* and Pf*mdr1* loci are very different. First, differences in the genomic characteristics of Pf*crt* and Pf*mdr1* loci may generate different patterns of genetic variation at these loci; Pf*crt* is a single-copy locus (24), whereas Pf*mdr1* can occur as a single- or multiple-copy locus (56). However, we did not evaluate the Pf*mdr1* copy number in the present study. It is possible that the local recombination rate differs between Pf*crt* (chromosome 7) and Pf*mdr1* (chromosome 5). The genome average recombination rate in *P. falciparum* is 17 kb/centimorgan (51). However, recombination is unlikely to be even across the genome, and the impact of selective events will depend on the local recombination rate rather than the genome average recombination rate (5). In addition, it is known that the MS mutation rate in *P. falciparum* varies considerably for different types of loci (1.59×10^{-4} [95% confidence interval, 6.98×10^{-5} to 3.47×10^{-4}]) (6, 7). However, whether the mutation rate varies between neutral loci and loci under selection, varies from one chromosome to another, or varies in different allelic lineages, which may contribute to the extent of hitchhiking around Pf*crt* and Pf*mdr1* loci, is not clear.

Second, differences in drug selection history, as well as selection strength, may alter patterns of genetic variation. It is possible that Pf*mdr1* 86Y alleles were already present at an appreciable frequency prior to the spread of resistant Pf*crt* 76T alleles. A recent study conducted in Madagascar observed a high prevalence (67.5%) of the Pf*mdr1* 86Y allele, despite the absence of the Pf*crt* 76T allele, in a population supposedly exposed to CQ for the last 60 years (43). The results of in vitro tests revealed that all isolates except one were sensitive to CQ, regardless of the status of Pf*mdr1* codon 86 (43).

It is also possible that the strength of CQ selection is different for these genes; Pf*crt* may be under strong selection pressure, whereas Pf*mdr1* may be under weak selection pressure. Limited studies that have analyzed the prevalence of both the Pf*crt* 76T and Pf*mdr1* 86Y alleles along with the spread of CQR in an area over a period of time have found that Pf*crt* 76T alleles spread more rapidly and reached high frequencies faster than Pf*mdr1* 86Y alleles (1), suggesting that CQ selection acts more strongly on Pf*crt* than on Pf*mdr1*. This is likely, considering the physiologic relevance of PfCRT in the case of CQ transport and CQR. Both PfCRT and PfMDR1 are integral membrane proteins, localized to the parasite's digestive vacuole membrane (59). While PfCRT is considered to be the primary transporter for CQ, PfMDR1 is considered to be the primary transporter for mefloquine and a variety of other antimalarials (20, 44). Although the precise mechanism of CQR is still not clear, the efflux of CQ out of the parasite's digestive vacuole via an energy-coupled transporter is one of the promising models of CQR (59). Using allelic-exchange mutant parasites, Sanchez et al. (45) showed that stimulated CQ accumulation was associated with Pf*crt* alleles CVIET and SVMNT, but not with Pf*mdr1* allele 184F_1034C_1042D_1246Y. They suggested that PfCRT is, but PfMDR1 is not, directly or indirectly involved in carrier-mediated CQ efflux from resistant parasites.

Finally, there may indeed be multiple origins of Pf*mdr1* polymorphisms (37, 56), in contrast with only a few origins of CQR Pf*crt* alleles (65). If resistance alleles have evolved repeatedly on different genetic backgrounds, then the difference in diversity associated with resistant versus sensitive alleles will be diminished and the association of individual haplotypes with resistance mutations will be less clear (5). That is exactly what we observed regarding Pf*mdr1* alleles in parasites from PNG (for 86Y, $H = 0.741 \pm 0.066$, and for 86N, $H = 0.956 \pm 0.06$;

F_{st} = 0.126) and Ghana (for 86Y, H = 0.958 ± 0.036, and for 86N, H = 0.978 ± 0.054; F_{st} = 0.085), further supporting multiple origins of CQR Pf*mdr1* alleles. Considering that CQ selection strength may be weaker for Pf*mdr1*, multiple origins represent signs of "soft" selection of CQR Pf*mdr1* alleles.

Thus, it is possible that reduced variation at Pf*crt* is the result of strong CQ selection together with low local recombination rates and/or MS mutation rates, whereas high variation at Pf*mdr1* is the result of weak CQ selection together with high local recombination rates and/or MS mutation rates. Regardless of the reasons, the results of the present study show that there are significant differences between the patterns of genetic variation at the Pf*crt* and Pf*mdr1* loci in Asian and African parasite populations.

Genetic variation at CQR Pf*crt* and Pf*mdr1* loci in South America. Compared with the Asian or African parasite populations, the South American parasite populations were relatively homogeneous for CQR Pf*crt*, as well as Pf*mdr1*, loci.

Although the first reports of CQR came almost simultaneously from South America and Southeast Asia (64), the probable factors and/or mechanisms governing the dynamics of CQ-driven selective sweeps in South America may have been substantially different from those in Southeast Asia; a multifocal origin of CQR in South America due to high and widespread drug pressure in the form of chloroquinized salt may have led to the homogenization of CQR-associated genotypes in a short period of time (64; T. E. Wellems, personal communication). Furthermore, the background level of genetic variation in the *P. falciparum* population is quite low in South America (mean heterozygosities of 12 putatively neutral MS loci, H = 0.3 to 0.4) compared with the levels of variation in Southeast Asia/Pacific (H = 0.51 to 0.65) and Africa (H = 0.76 to 0.8) (6), due to small, structured parasite populations with fewer multiple-clone infections, high levels of inbreeding, and considerably lower recombination rates (5). Although we did not estimate the mean number of parasite clones per sample, MS genotyping revealed that only <1% and 2.9% of all samples from South America carried multiple alleles at the Pf*crt* and Pf*mdr1* loci, respectively (cf. Asia [5% and 5.5%, respectively] and Africa [15% and 18.5%, respectively]). By using one putatively neutral locus, PfPK2 (chromosome 12), we observed similar levels of multiple-allele infections in the samples from South America (3%), Asia (7.6%), and Africa (25.8%). Thus, it is not surprising to observe that both the CQR Pf*crt* and Pf*mdr1* alleles exhibited low diversities in South American parasites.

Conclusions and implication for malaria treatment. A number of studies, focused on Pf*crt*, have provided convincing evidence regarding how this gene evolved under CQ selection pressure (39, 61, 65). So far, two studies, focused on the evolutionary dynamics of Pf*mdr1* from two different angles, have reported different yet complementary outcomes (19, 37). While the results of the study by Duraisingh et al. (19), using an intragenic MS locus, indicate a limited number of origins and a strong selective sweep of Pf*mdr1* N86Y alleles in Gambian parasites, the results of the study by Nair et al. (37), using flanking MS loci, indicate 5 to 15 independent origins and "soft" selective sweeps of Pf*mdr1* amplification events in parasites from the Thailand-Burma border. Since we used the same sets of samples to analyze genetic variation at both the Pf*crt* and Pf*mdr1* loci, our results for samples from worldwide locations provide a unique insight into the evolutionary dynamics of these genes. We found that there are marked differences between the evolutionary dynamics of Pf*crt* and Pf*mdr1* in both Asia and Africa and in the evolutionary dynamics of both genes, particularly Pf*mdr1*, between Asia/Africa and South America. We observed strong selective sweeps and a limited number of origins of CQR Pf*crt* alleles, a hallmark of selection at the Pf*crt* locus, whereas high levels of variation and multiple origins of the most-prevalent CQR Pf*mdr1* allele (86Y) were observed in Asia and Africa. In South America, we observed reduced variation and only a few origins of CQR Pf*crt*, as well as Pf*mdr1*, alleles. It is most likely that genetic variation at Pf*crt* and Pf*mdr1* loci in both Asian and African parasite populations is generated and/or maintained via substantially different mechanisms.

CQ is being replaced by newer artemisinin-based combination drugs, such as artesunate-mefloquine, artemether-lumefantrine, and artesunate-amodiaquine, in several countries where malaria is endemic (http://www.rbm.who.int/). Pf*mdr1* is a major modulator of resistance to these drugs. Understanding the mechanisms that regulate the genetic variation at Pf*mdr1*, separately or in conjunction with Pf*crt*, is important, as this would usefully complement our current efforts to understand the evolution of the malaria parasite genome under changing drug pressure. With increased understanding, we might be able to augment our success in treating this global killer despite our limited arsenal of drugs.

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SUPPLEMENTAL MATERIAL

TABLE S2. *Pfcrt* and *pfmdr1* alleles and flanking MS loci in *P. falciparum* laboratory isolates[†]

Isolate	CQ phenotype	*pfcrt* allele[a]	MS loci flanking *pfcrt*					*pfmdr1* allele[b]	MS loci flanking *pfmdr1*			
			B5M77 (-18 kb)[c]	2E10 (-5 kb)[c]	9B12 (+2 kb)[c]	*cg2* (+7 kb)[c]	2H4 (+19 kb)[c]		5-956456 (-1.8 kb)[d]	5-957861 (-400 bp)[d]	5-962445 (+700 bp)[d]	5-966096 (+4.3 kb)[d]
HB3	S	CVMNK	4	5	1	1	14	NFSDD	3	14	8	4
3D7	S	CVMNK	1	8	3	3	9	NYSND	9	11	8	2
Dd2	R	CVIET	3	7	2	4	4	YYSND	4	11	2	4
K1	R	CVIET	3	7	2	4	4	YYSND	4	11	2	4
7G8	R	SVMNT1	2	3	3	6	7	NFCDY	9	7	8	2
PNG1917	R	SVMNT1	3	6	3	5	10	NFSND	11	5	1	1
PNG1905	R	SVMNT2	2	3	3	6	7	NFCDY	9	7	8	2
ECU1110	R	CVMNT	3	2	4	7	10	NFSDD	3	14	8	4
JAV	R	CVMET	3	2	4	7	10	NFSDD	3	14	8	4

[†] MS allele numbers correspond to relative positions of Cy5-labeled PCR amplicons on a 6% denaturing polyacrylamide gel.

[a] Codons 72–76, [b] Codons 86_184_1034_1042_1246

[c] Distance from *pfcrt* , [d] Distance from *pfmdr1*

S = sensitive, R = resistant

SVMNT1 = S(agt)VMNT, SVMNT2 = S(tct)VMNT

SUPPLEMENTAL MATERIAL

TABLE S3a. Distribution and prevalence of *pfcrt* MS haplotypes (~40 kb) in various parasite populations[†]

Haplotype I.D.	*pfcrt* (72–76) allele	Location (number of samples)	*pfcrt*-flanking microsatellite alleles				
			B5M77 (-18 kb)[e]	2E10 (-5 kb)[e]	9B12 (+2 kb)[e]	*cg2* (+7 kb)[e]	2H4 (+19 kb)[e]
H001	SVMNT1	PNG (31), Indonesia (5)	3	6	3	5	7
H002	SVMNT1	PNG (1), Indonesia (1)	4	6	3	5	7
H005	SVMNT1	PNG (1)	3	6	3	5	13
H006	SVMNT1	PNG (3), Laos (5)	3	6	3	5	11
H007	SVMNT1	PNG (1)	3	6	3	9	7
H011[a]	SVMNT1	PNG (15), Indonesia (16), India (14), Laos (8)	3	6	3	5	10
H015	SVMNT1	PNG (2), Indonesia (1)	3	6	3	5	4
H028	SVMNT1	PNG (1)	3	3	3	5	7
H030	SVMNT1	PNG (1)	3	6	3	6	11
H031	SVMNT1	PNG (1, 1[**]), Indonesia (1)	3	5	3	5	7
H043	SVMNT1	PNG (1)	3	6	3	5	12
H048	SVMNT1	PNG (1), India (2)	4	6	3	5	10
H053	SVMNT1	PNG (2, 1[**])	3	6	3	6	10
H058	SVMNT1	PNG (1), India (1)	3	6	3	4	4
H059	SVMNT1	PNG (2)	3	6	3	5	5
H060	SVMNT1	PNG (2)	3	6	4	5	7
H062	SVMNT1	PNG (1)	3	6	3	7	7
H064	SVMNT1	PNG (1)	3	6	3	6	6
H066	SVMNT1	PNG (1)	3	6	4	5	10
H069	SVMNT1	PNG (1)	3	6	3	6	7
H083	SVMNT1	Indonesia (1)	3	6	3	5	6
H106	SVMNT1	India (1), PNG (1[**])	3	6	3	4	10
H114	CVIET	India (2)	3	7	2	11	1

H125	SVMNT1	Laos (1)	3	8	2	5	11
H201[b]	CVIET	Kenya (22), Uganda (21), Ghana (10)	3	7	2	4	4
H211	CVIET	Kenya (1), Ghana (1)	4	7	2	4	4
H216	CVIET	Kenya (1)	3	7	2	9	7
H217	CVIET	Kenya (1), Ghana (1)	3	7	2	4	7
H230	CVIET	Uganda (1)	3	7	2	4	2
H231	CVIET	Uganda (1)	3	7	2	3	17
H240	CVIET	Uganda (1)	1	7	2	11	17
H245	CVIET	Uganda (1)	3	7	2	11	4
H249	CVIET	Uganda (1)	3	7	2	5	7
H252	CVIET	Uganda (1)	4	9	2	4	4
H253	CVIET	Uganda (1)	3	7	2	4	16
H260	CVIET	Ghana (3)	3	7	2	7	4
H268	CVIET	Ghana (1)	7	7	2	4	4
H270	CVIET	Ghana (1)	3	7	2	3	9
H301[c]	SVMNT1/2[*]	Brazil (21), Guyana (16)	2	3	3	6	7
H311	SVMNT1	Brazil (2),	2	2	3	8	7
H330	SVMNT1-2	Guyana (1)	2	3	3	6	14
H401[d]	CVMET	Colombia (24)	3	2	4	7	10
H411	CVMET	Colombia (1)	3	1	4	7	10

[†] Samples with single infections only

[a] Present in PNG1917 (PNG) isolate (Table S2)

[b] Present in Dd2 (Indochina) and K1 (Thailand) isolates (Table S2)

[c] Present in 7G8 (Brazil [SVMNT1]) and PNG1905 (origin not confirmed [SVMNT2]) isolates (Table S2)

[d] Present in ECU1110 (Ecuador [CVMNT]) and JAV (Colombia [CVMET]) isolates (Table S2)

[e] Distance from *pfcrt*

[*] Brazil = SVMNT2; Guyana = SVMNT1 or SVMNT2

[**] Samples carry CQS *pfcrt*- CVMNK allele.

SUPPLEMENTAL MATERIAL

TABLE S3b. Distribution and prevalence of *pfcrt* MS haplotypes (~28 kb) in various parasite populations[†]

Haplotype I.D.	*pfcrt* (72–76) allele	Location (number of samples)	*pfcrt*-flanking microsatellite alleles			
			B5M77 (-18 kb)[e]	2E10 (-5 kb)[e]	9B12 (+2 kb)[e]	*cg2* (+7 kb)[e]
H001[a]	SVMNT1	PNG (55), Indonesia (23), India (14), Laos (13)	3	6	3	5
H002	SVMNT1	PNG (2), Indonesia (1), India (2)	4	6	3	5
H007	SVMNT1	PNG (1)	3	6	3	9
H028	SVMNT1	PNG (1)	3	3	3	5
H030	SVMNT1	PNG (5, 1[**])	3	6	3	6
H031	SVMNT1	PNG (1, 1[**]), Indonesia (1)	3	5	3	5
H058	SVMNT1	PNG (1, 1[**]), India (2)	3	6	3	4
H060	SVMNT1	PNG (3)	3	6	4	5
H062	SVMNT1	PNG (1)	3	6	3	7
H114	CVIET	India (2), Uganda (1)	3	7	2	11
H125	SVMNT1	Laos (1)	3	8	2	5
H201[b]	CVIET	Kenya (23), Uganda (23), Ghana (11)	3	7	2	4
H211	CVIET	Kenya (1), Ghana (1)	4	7	2	4
H216	CVIET	Kenya (1)	3	7	2	9
H231	CVIET	Uganda (1), Ghana (1)	3	7	2	3
H240	CVIET	Uganda (1)	1	7	2	11
H249	CVIET	Uganda (1)	3	7	2	5
H252	CVIET	Uganda (1)	4	9	2	4
H260	CVIET	Ghana (3)	3	7	2	7
H268	CVIET	Ghana (1)	7	7	2	4
H301[c]	SVMNT1/2[*]	Brazil (21), Guyana (17)	2	3	3	6
H311	SVMNT1	Brazil (2)	2	2	3	8
H401[d]	CVMET	Colombia (24)	3	2	4	7
H411	CVMET	Colombia (1)	3	1	4	7

[†] Samples with single infections only

[a] Present in PNG1917 (PNG) isolate (Table S2)

[b] Present in Dd2 (Indochina) and K1 (Thailand) isolates (Table S2)

[c] Present in 7G8 (Brazil [SVMNT1]) and PNG1905 (origin not confirmed [SVMNT2]) isolates (Table S2)

[d] Present in ECU1110 (Ecuador [CVMNT]) and JAV (Colombia [CVMET]) isolates (Table S2)

[e] Distance from *pfcrt*

[*] Brazil = SVMNT2; Guyana = SVMNT1 or SVMNT2; one sample from Guyana contained SVMNT1-SVMNT2 mixed allele.

[**] Samples carry CQS *pfcrt*-CVMNK allele.

SUPPLEMENTAL MATERIAL

TABLE S4. Distribution and prevalence of *pfmdr1* MS haplotypes (~5 kb) in various parasite populations [†]

Haplotype I.D.	*pfmdr1* allele[a]	Location (number of samples)	*pfmdr1* -flanking microsatellite alleles	
			5-957861 (-400 bp)[c]	5-962445 (+700 bp)[c]
H001	YYSND	PNG (20)	5	6
H002	YYSND	PNG (4)	5	7
H003	YYSND	PNG (1)	5	9
H004	YYSND	PNG (1)	6	6
H005	YYSND	PNG (3)	8	3
H006	YYSND	PNG (4)	8	4
H007	YYSND	PNG (1), Uganda (2)	8	5
H008	YYSND	PNG (4), Laos (1[*])	8	6
H009	YYSND	PNG (2), Laos (11[*])	8	7
H010	YYSND	PNG (1)	11	4
H011	YYSND	Indonesia (3)	11	5
H012	YYSND	Indonesia (14), Uganda (3)	11	8
H013	YYSND	India (1)	5	5
H014	YYSND	India (2)	7	4
H015	YYSND	India (3)	9	4
H016	YYSND	India (9)	9	5
H017	YYSND	India (1)	9	6
H018	YYSND	India (2)	9	7
H019	YYSND	Uganda (1)	8	8
H020	YYSND	Uganda (8)	8	9
H021	YYSND	Uganda (2)	8	10
H022	YYSND	Uganda (2)	11	9
H023	YYSND	Uganda (2)	11	10
H024	YYSND	Ghana (1)	7	5
H025	YYSND	Ghana (1)	8	2
H026	YYSND	Ghana (1)	10	10
H027	YYSND	Ghana (1)	11	3
H028	YYSND	Ghana (1)	11	6
H029	YYSND	Ghana (1)	12	8
H030	YYSND	Ghana (1)	14	5
H031	YYSND	Ghana (1)	14	6
H032	YYSND	Ghana (2)	14	7
H033[b]	YYSND	Ghana (3)	14	8
H034	YYSND	Ghana (2)	14	9
H035	YYSND	Ghana (1)	14	10
H036	NYSND	Laos (1)	11	7
H101	NFCDY	Brazil (17), Guyana (13)	7	8
H201[b]	NFSDY	Colombia (12)	14	8

[†] Samples with single infections only

[a] Codons 86_184_1034_1042_1246

[b] Same ~5 kb haplotypes, different *pfmdr1* alleles

[c] Distance from *pfmdr1*

[*] Samples carry CQS *pfmdr1* -86N allele

Note. Due to exhaustion of the Kenyan genomic DNA preparations, *pfmdr1* MS analysis could not be performed.

SUPPLEMENTAL MATERIAL

TABLE S1. Primer sequences, amplification conditions, and the method for genotyping various MS loci

Locus (chromosome)	Primer sequences [a,b,c]	Amplification conditions
cg2 ω repeat region (7)	Nest-1: Up 5'-TTCTTGAATACTCCTCCCCACACACCTCAC-3' Dn 5'-TCATAAGAATAATAAAATGGATACTGCACAACAAT-3' Nest-2: Dn Cy5-5'-GATGAGGAGGATGCCTGGTTTTACTGTCTT-3'	94°C 2 min; 94°C 30 sec, 60°C 30 sec, 72°C 45 sec (25x) 94°C 2 min; 94°C 30 sec, 60°C 30 sec, 72°C 30 sec (25x)
B5M77 (7)	Nest-1: Up 5'-GAAATAATTTCATATACACAC-3' Dn 5'-TATTTCATGTCTCAGTAAAG-3' Nest-2: Dn Cy5-5'-TAAAGTCTTTCAATACATATG-3'	94°C 2 min; 94°C 30 sec, 54°C 30 sec, 60°C 30 sec (25x) Same as for nest-1
2E10 (7)	Nest-1: Up 5'-CAAACTTGGATGCAAATGA-3' Dn 5'-AAACACACACATGAACACA-3' Nest-2: Up Cy5-5'-TCCAGAGGAATAAAAAATAATA-3'	94°C 2 min; 94°C 30 sec, 52°C 30 sec, 60°C 30 sec (25x) 94°C 2 min; 94°C 30 sec, 56°C 30 sec, 60°C 30 sec (25x)
9B12 (7)	Nest-1: Up 5'-GGGTGGAATATATAAGGG-3' Dn 5'-AATGATACAATGGGATTTAC-3' Nest-2: Up Cy5-5'-ATATATTCCAGTATGTTCGC-3'	94°C 2 min; 94°C 30 sec, 52°C 30 sec, 60°C 30 sec (25x) Same as for nest-1
2H4 (7)	Nest-1: Up 5'-AAATTGTGGTTCACATAGCA-3' Dn 5'-CGAGAACGCAAAGGTGCC-3' Nest-2: Up Cy5-5'-ATCTTTAAGTTCAATCTGGA-3'	94°C 2 min; 94°C 30 sec, 52°C 30 sec, 60°C 30 sec (25x) Same as for nest-1

956456 (5)	Nest-1: Up 5'-TTTTTGTTTGTTCGGAAG-3' Dn 5'- TTACTCAAGGTATTTATAGGATA-3' Nest-2: Dn Cy5-5'-CAAGGTATTTATAGGATATTTGA-3'	94°C 2 min; 94°C 30 sec, 52°C 30 sec, 60°C 30 sec (35x) Same as for nest-1
957861 (5)	Nest-1: Up 5'-TTTTTGTCATTGTGTAAATA-3' Dn 5'-TTCTGCTCTTTACCCATC-3' Nest-2: Dn Cy5-5'-CAACACAAAATCAAATAAAA-3'	Same as above Same as above
962445 (5)	Nest-1: Up 5'-ACTCTTGTCCGTTATATTGA-3' Dn 5'-ATCACACAATTGGTAAACTTT-3' Nest-2: Dn Cy5-5'-AAAAAGGAAGAAGGAAAAA-3'	Same as above Same as above
966096 (5)	Nest-1: Up 5'-AATGTAAATATTTTTAGGGTAGA-3' Dn 5'-CCATATTTTTTCCTTTTGT-3' Nest-2: Dn Cy5-5'-TTTTTCCTTTTGTAATATGG-3'	Same as above Same as above
PfPK2 (12)	Nest 1: Up 5'-CTTTCATCGATACTACGA-3' Dn 5'-CCTCAGACTGAAATGCAT-3' Nest 2: Dn Cy5-5'-AAAGAAGGAACAAGCAGA-3'	94°C 2 min ; 94°C 30 sec, 50°C 30 sec, 72°C 30 sec (25x) Same as for nest-1

[a] Chromosome 7 (GenBank accession #AF030694), [b] Chromosome 5 (GenBank accession #NC_004326), [c] Chromosome 12 (GenBank accession #X63648)

All reactions were performed using a Peltier Thermal Cycler, PTC-225 (MJ Research, Watertown, MA) as previously described (34, 35). Optimization of amplification of MS was performed by varying the $MgSO_4$ concentration between 5.5-8.5 mM in the PCR buffer. The PCR products were mixed 3:1 (vol/vol) with denaturing loading dye buffer (formamide 10 ml, bromophenol blue 10 mg, 0.5 M EDTA [pH 8.0] 200 ml) and denatured at 95^{o}C for 10 min. The denatured products were then run on a 6% denaturing polyacrylamide gel (6.3 M urea/32% formamide) for 3 h in a Gibco BRL sequencing apparatus (model S2, Gibco BRL Life Technologies) at 1900 V. The Cy5-labeled amplicons were visualized on the Storm 860 scanner using ImageQuant software v5.2 (Molecular Dynamics, Sunnyvale, CA). MS alleles were designated by numbers corresponding to their relative positions on the gel. Multiple-allele infections were defined as those in which at least one of the seven or nine loci contained >1 allele. We used "creating a line graph and quantitating" function of the ImageQuant software to distinguish single- vs. mixed-allele infections. In this analysis, to be "truly" a mixed-allele infection, the graph peak height of the other (or minor) allele must be at least 16.6% of the graph peak height of the predominant allele. In our previous (35) as well as present study, we used much more stringent 33% (16.6% × 2) as the cut-off to designate other allele, and scored multiple alleles per locus only if minor graph peaks were $\geq$33% of the height of the predominant graph peak present for each locus. MS genotyping revealed that 6.7%, 8.4%, and 12% of all samples carried multiple alleles at *pfcrt*, *pfmdr1*, and PfPK2 loci, respectively (Asia, 5%, 5.5%, and 7.6%; Africa, 15%, 18.5%, and 25.8%; South America, <1%, 2.9%, and 3%). No sample carried more than two alleles at any MS locus. However, we did not estimate the mean number of parasite clones per sample. *Pfcrt* and *pfmdr1* allelic haplotypes were constructed using the predominant allele observed at each MS locus.