A Selective Sweep Driven by Pyrimethamine Treatment in Southeast Asian Malaria Parasites

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Malaria parasites (*Plasmodium falciparum*) provide an excellent system in which to study the genomic effects of strong selection in a recombining eukaryote because the rapid spread of resistance to multiple drugs during the last the past 50 years has been well documented, the full genome sequence and a microsatellite map are now available, and haplotype data can be easily generated. We examined microsatellite variation around the dihydrofolate reductase (*dhfr*) gene on chromosome 4 of *P. falciparum*. Point mutations in *dhfr* are known to be responsible for resistance to the antimalarial drug pyrimethamine, and resistance to this drug has spread rapidly in Southeast (SE) Asia after its introduction in 1970s. We genotyped 33 microsatellite markers distributed across chromosome 4 in 61 parasites from a location on the Thailand/Myanmar border. We observed minimal microsatellite length variation in a 12-kb (0.7-cM) region flanking the *dhfr* gene and diminished variation for approximately 100 kb (6 cM), indicative of a single origin of *resistant* alleles. Furthermore, we found the same or similar microsatellite haplotypes flanked *resistant dhfr* alleles sampled from 11 parasite populations in five SE Asian countries indicating recent invasion of a single lineage of *resistant dhfr* alleles in locations 2,000 km apart. Three features of these data are of especial interest. (1) Pyrimethamine resistance is generally assumed to have evolved multiple times because the genetic basis is simple and resistance can be selected easily in the laboratory. Yet our data clearly indicate a single origin of *resistant dhfr* alleles sampled over a large region of SE Asia. (2) The wide valley (~6 cM) of reduced variation around *dhfr* provides ''proof-of-principle'' that genome-wide association may be an effective way to locate genes under strong recent selection. (3) The width of the selective valley is consistent with predictions based on independent measures of recombination, mutation, and selection intensity, suggesting that we have reasonable estimates of these parameters. We conclude that scanning the malaria parasite genome for evidence of recent selection may prove an extremely effective way to locate genes underlying recently evolved traits such as drug resistance, as well as providing an opportunity to study the dynamics of selective events that have occurred recently or are currently in progress.

Introduction

When a beneficial mutation spreads through a population, flanking neutral polymorphisms are carried along (''hitchhiking''), resulting in removal of genetic variation from the chromosomal regions surrounding the selected site (Smith and Haigh 1974; Barton 2000). The spread of the selected allele also results in increased linkage disequilibrium (LD) (Tishkoff et al. 2001; Sabeti et al. 2002) with flanking markers and skews in the allele frequency spectra at loci nearby on the chromosome (Payseur, Cutter, and Nachman 2002). The size of genomic regions affected is influenced by the strength of selection, as well as the rates of recombination and mutation. There is currently great interest in using such characteristic patterns of variation to identify regions of the genome that are under selection (Kohn, Pelz, and Wayne 2000; Harr, Kauer, and Schlotterer 2002; Kim and Stephan 2002; Payseur, Cutter, and Nachman 2002; Sabeti et al. 2002; Vigouroux et al. 2002; Schlotterer 2003). However, there are rather few systems in which the efficacy of this approach can be empirically tested. Antimalarial drug resistance provides an excellent system for investigating the genomic effects of selection events in a recombining eukaryote, and the scars left in the *Plasmodium falciparum* genome by drug selection may serve as useful signposts for locating drug resistance genes (Anderson et al. 2000*a*; Wootton et al. 2002). We have good records of when new antimalarial drugs were introduced and the rate of spread of resistance, as well as estimates of recombination and mutation rates from a genetic cross (Su et al. 1999; Anderson et al. 2000*a*). Furthermore, microsatellite repeats are absurdly common in the *P. falciparum* genome, occurring on average approximately every 1 kb (Su et al. 1999; Mu et al. 2002), and haplotypes can be easily constructed using haploid blood stage parasites.

Here, we investigate effects of strong recent selection by the antimalarial drug pyrimethamine on genomic variation on chromosome 4 of *P. falciparum*. This compound is the dominant component (Watkins et al. 1997) of pyrimethamine/sulfadoxine (PS) (Fansidar™ [Roche]), an inexpensive drug that is used in countries where resistance has rendered chloroquine (CQ) ineffective. Pyrimethamine is a competitive inhibitor of dihydrofolate reductase (*dhfr*), displacing the natural folate substrate. The genetic basis of resistance to pyrimethamine is well understood (Plowe, Kublin, and Doumbo 1998). Specific point mutations in

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the active site of parasite *dhfr* (chromosome 4) alter the binding of pyrimethamine to the enzyme's active site. These mutations appear sequentially in treated populations, with the Ser→Asn mutation at codon 108 appearing first, followed by Asn→Ile (codon 51) or Cys→Arg (codon 59), and finally Ile→Leu (codon 164) (Plowe, Kublin, and Doumbo 1998). Single or doubly mutant *dhfr* have increased parasite clearance times and higher posttreatment gametocyte carriage than wild-type parasites (Mendez et al. 2002), and parasites with three or four resistance mutations are refractory to treatment (Plowe, Kublin, and Doumbo 1998). PS was introduced as the first-line antimalarial treatment on the Thailand-Myanmar border in the mid-1970s. Resistance spread to fixation in approximately 6 years (White 1992).

Since resistance spreads extremely rapidly after pyrimethamine treatment (Clyde and Shute 1957; Doumbo et al. 2000), and the mutations involved can be selected readily in the laboratory (Paget-McNicol and Saul 2001), it is generally assumed that *dhfr* mutations underlying resistance evolve multiple times in nature (but see Cortese et al. [2002]). In fact, given that infected people contain 10^{10} to 10^{12} parasites, and key point mutations in *dhfr* conferring resistance to pyrimethamine occur at frequencies as high as 2.5×10^{-9} per parasite replication in the laboratory (Paget-McNicol and Saul 2001), we might expect such mutations to arise independently in every treated malaria patient. In this case, we would expect *resistant dhfr* alleles to be associated with different alleles at flanking microsatellite loci and to see little evidence for diminished variation around *dhfr* (Doumbo et al. 2000). To test this model of resistance evolution and to investigate the use of association-based approaches to detect regions of the genome under positive selection, we examined microsatellite variation around *dhfr* in *P. falciparum* from five countries in Southeast (SE) Asia. Surprisingly, we found minimal variation around *resistant dhfr* alleles, suggesting a single origin of SE Asian pyrimethamine resistance. Furthermore, the pattern of reduced variation fits well with models based on independent measures of recombination, mutation, and selection intensity and provides an empirical demonstration of the potential to detect selected genes from patterns of genomic variation.

Materials and Methods

Geographical Sampling

We obtained venous blood from malaria-infected patients visiting the malaria clinic in Mawker-Thai, on the Thailand-Myanmar border. We also obtained infected finger-prick blood samples from four other sites in Thailand: camps for displaced Karen people at Shoklo (November 1995 to January 1996), Maela (September 1999), Ratchaburi (October to December 1998), and Kanchanaburi (September to December 1998). In Myanmar, we collected samples from two villages (Myothugyi and Dabhine) in October and November 2000. In Laos, we collected samples from Sekong (October 1999 to June 2000) and from Phalanxay (August 2001 to September 2001). In Vietnam, we collected samples from neighboring villages Khan Phu and Khan Nan (September 1998). In Cambodia, we obtained samples from patients visiting Anlong Veng and Tropeang Prasat Health Centers (April to May 2002). Use of blood samples described in this paper was approved by the Institutional Review Board at the University of Texas Health Science Center at San Antonio and by review boards in the countries where blood samples were collected.

DNA Preparation

Parasite DNA from Mawker-Thai was prepared from venous blood by phenol/chloroform extraction of whole blood, after removal of buffy coats. Two nanograms of DNA were used in each PCR reaction. In the case of finger-prick blood samples (~50 μl of blood adsorbed and dried on a piece of filter paper) collected from other SE Asian locations, DNA was prepared from 3 mm discs removed from each blood spot using a sterile hole-punch using the Generation Card Capture Kit (Gentra Systems). Before extraction, we soaked 3 mm disks in 150 μl of DNA elution solution at 4°C overnight. Otherwise, we followed the extraction protocol suggested in the kit instructions.

Microsatellite Genotyping Methods

Primer sequences, amplification conditions, and positions of microsatellite markers in the *P. falciparum* chromosome 4 genome sequence are listed in table S1*A* of Supplementary Material online. Additional microsatellites genotyped on other chromosomes were as follows: chromosome 1: C1M38, C1M39, C1M11, C1M10, C1M4, C1M13; chromosome 2: C2M21, C2M19, C2M30, C2M33, C2M27, d_4212, d_4217, d_4227, d_4229, d_4247, d_4254, d_4265, C2M25, C2M12, C2M16, C2M17, C2M1, C2M3, C2M4, C2M6, C2M8; chromosome 3: C3M20, C3M27, C3M40, C3M86, B7M117, C3M42, C3M33, C3M81, C3M17, C3M47, C3M54, C3M43, C3M45; chromosome 12: C12M96, C12M89, C12M62, Y588M4, C12M114, C12M64, C12M105, Y588M1, C12M115, C12M81, C14M19, C12M60, Y69M2, C12M63, C12M44, Y336M1. Primer sequences and repeat array length for these loci are shown in table S1*B* of Supplementary Material online. Since repeat motif and array length are known to influence levels of microsatellite variation in *P. falciparum* (Anderson et al. 2000*b*), we used only microsatellite sequences containing uninterrupted arrays of greater than eight dinucleotide repeats in parasite line 3D7, for which full genome sequence data is available. Fluorescent end-labeled primers were purchased from Applied Biosystems, and PCR products were separated on an ABI 3100 capillary sequencer and length was scored using the Genotyper software. DNA from clone 3D7, for which the genome sequence is available, was run as a size control for all loci.

dhfr Genotyping Methods

Genotyping was performed by primer extension using the ABI PRISM SNaPshot™ Multiplex Kit (Applied Biosystems), and the products of the SNaPshot reactions were scored on an ABI 3100 capillary sequencer using

GENESCAN and GENOTYPER software (Nair et al. 2002). We genotyped *dhfr* both in high-quality parasite DNA prepared from venous blood samples from Mawker-Thai and from DNA extracted from finger-prick blood samples from 10 additional locations. For the finger-prick blood samples, we used a seminested PCR strategy to amplify the template used in the SNaPshot reactions. We used the primer 5′-TTTATATTTTCTCCTTTTTA-3′, combined with the reverse primer dhfr-r to preamplify DNA. Otherwise, genotyping was as described in Nair et al. (2002). We did not measure in vitro drug resistance phenotypes in this study. However, since the relationship between mutations within *dhfr* and both in vitro and in vivo resistance to pyrimethamine is well established (Plowe et al. 1997; Sirawaraporn et al. 1997; Plowe, Kublin, and Doumbo 1998), we refer to alleles carrying mutations in the five residues listed above as *resistant dhfr* alleles, whereas alleles with no mutations are referred to as *sensitive dhfr* alleles. To describe the different *resistant dhfr* alleles we indicate the numbers of mutations present relative to the *sensitive dhfr* allele. Throughout we write *resistant* and *sensitive dhfr* alleles in italics to emphasize that these are genotypic descriptions rather than empirically determined phenotypes.

Statistical Analysis

We measured expected heterozygosity (H_e) at each microsatellite locus as $H_e = [n/(n-1)][1 - \sum p_i^2]$, where n is the number of infections sampled and p_i is the frequency of the ith allele. We estimated the variance of H_e using a Taylor's series expansion and retaining second-order (covariance) terms, and approximate 95% confidence intervals were constructed assuming large-sample normality of the H_e estimates. We used H_e in preference to variance in repeat number because *P. falciparum* microsatellites frequently contain indels in the flanking regions or have complex repeat structure (Anderson et al. 2000*b*). Hence, inference of number of repeats from PCR product length results in frequent errors. To investigate the influence of selection on allele distributions, we used coalescent simulation (implemented using the program Bottleneck (Cornuet and Luikart 1996)) to predict H_e given the observed number of alleles under the assumption of mutation-drift equilibrium. This was done using both infinite alleles (IMM) and stepwise mutation models (SMM) of microsatellite mutation to give a range of expectations bounded by these two extreme mutation models. Differences between observed and predicted H_e were assessed using Wilcoxon's tests (Cornuet and Luikart 1996). We used F statistics to investigate levels of pairwise differentiation between parasite populations using five-amino-acid *dhfr* alleles and individual amino acid mutations within *dhfr*. Significance of F_{ST} was assessed by randomly permuting observed data sets 10^5 times using the program FSTAT (Goudet 2000), and table-wide significance levels were adjusted for multiple testing. We compared linkage disequilibrium around both *sensitive* and *resistant dhfr* alleles using extended haplotype homozygosity (EHH) (Sabeti et al. 2002), where EHH at a distance x from *dhfr* is defined as the probability that two randomly chosen haplotypes are homozygous for all microsatellites for a distance x from *dhfr*. We computed standard errors and 95% confidence intervals for this measure under a binomial model.

Modeling of Selective Sweep in Mawker-Thai

We modeled the pattern of reduced microsatellite variation around *dhfr* using minor modifications of the elegant theoretical framework described in (Wiehe 1998). The reduction in variation (H_e) of a microsatellite marker after a selective event ($V_{(t1)}$) relative to variation preceding the selection ($V_{(t0)}$) was estimated as:

$$(V_{(t1)}/V_{(t0)}) = 1 - e^{(8m+2r')/s}$$

where e is the initial frequency of the *resistant* allele at $t = 0$, m is the mutation rate, s is the selection coefficient, and r' is the *effective* recombination rate between two *dhfr* and marker loci (recombination rate r adjusted by level of inbreeding). We used H_e rather than variance in repeat number to measure variation because large indels and duplications are common in *P. falciparum* microsatellite sequences (Anderson et al. 2000*b*). As a result, estimates of repeat array length are subject to considerable error.

We used a recombination rate of 5.88×10^{-4} Morgans/kb/generation (Su et al. 1999) and the microsatellite mutation rate of 1.59×10^{-4} mutations/locus/generation (95% confidence interval: 6.98×10^{-5}, 3.7×10^{-4}) (Anderson et al. 2000*a*). Both of these parameters were measured using the genetic cross between parasite lines Hb3 and Dd2 (Su et al. 1999). In *P. falciparum*, self-fertilization frequently occurs, resulting in high levels of inbreeding and reducing the effective rate of recombination (Babiker et al. 1994; Paul et al. 1995). We estimated inbreeding coefficients (F) in the parasite population from Mawker-Thai from observed levels of multiple-clone infection (measured from the microsatellite genotype data from chromosomes 1, 2, 3, and 12). Given that approximately 60% of infections contain single clones, the minimum value of F is 0.6. However, since *P. falciparum* is a hermaphrodite, a proportion of the gamete fusions that occur in mosquitoes feeding on people containing multiple clone infections are expected to be self-fertilizations, and real values of F will be 0.6 to 0.9 in this population. The effective rate of recombination (r') is given by $r' = r(1 - F)$ where r is the recombination rate and F is the inbreeding coefficient (Hill and Babiker 1995; Conway et al. 1999). We estimated e to be $1/N_e$ (i.e., initially there was only one *resistant* allele in the population), where N_e was estimated to be 10^3 to 10^5 (Anderson et al. 2000*a*). Selection coefficients (s) were estimated from the observed decline of clinical treatment success using PS in Thailand (Bunnag and Harinasuta 1987; White 1992). We inferred frequencies of *resistant* (p) and *sensitive* alleles (q) by assuming that the frequency of treatment failures is proportional to p and that $q = 1 - p$. We plotted $\ln(p/q)$ against time in generations and measured the slope to obtain s (Hartl and Dykhuizen 1981).

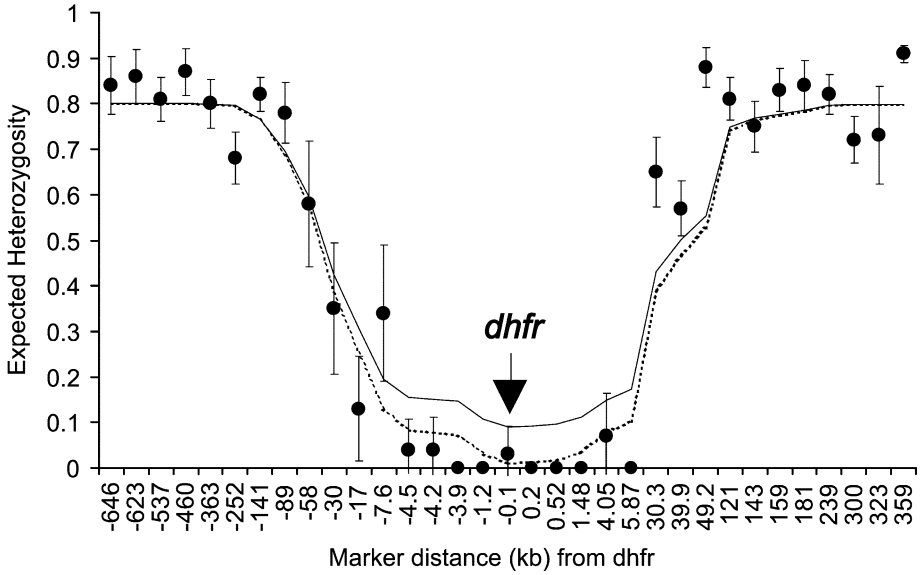

FIG. 1.—Microsatellite variability is reduced for approximately 100 kb around the *dhfr* locus on chromosome 4 on the Thailand-Myanmar border. H_e estimates are plotted with 1 SE. The distance (kb) of genotyped microsatellite markers relative to *dhfr* are shown along the *x*-axis (ranked in order across the chromosome). Details of the microsatellite markers used are shown in table S1 of Supplementary Material online. An arrow marks the position of *dhfr*. The black line shows levels of H_e predicted by a deterministic hitchhiking model (Wiehe 1998) using empirically estimated parameters ($s = 0.1$, $F = 0.8$, $r = 5.88 \times 10^{-4}$ Morgans/kb and $m = 1.59 \times 10^{-4}$/locus/generation, $e = 10^{-4}$), and H_e for all loci prior to selection was set at 0.8 (the mean of values from 56 microsatellites on chromosomes 1, 2, 3, and 12). The broken line shows predictions generated with a 10-fold lower mutation rate but otherwise the same parameter values.

Results and Discussion

Diminished Variation Around *dhfr*

We measured length variation in 33 dinucleotide microsatellite markers distributed across chromosome 4 (table S1*A* in Supplementary Material online), including 11 markers clustered in a 12-kb (0.7-cM) region around *dhfr* in 61 parasite isolates collected from Mawker-Thai on the Thailand-Myanmar border. We also examined the five codons in the *dhfr* locus that are known to be involved in resistance (16, 51, 59, 108, and 164). All *dhfr* alleles sampled from this population had between two and four mutations conferring resistance. Surprisingly, we found minimal variation in microsatellite markers for 12 kb (0.7 cM) immediately surrounding *dhfr*, and variation was reduced in a region of approximately 100 kb (6 cM) around this locus (fig. 1). In comparison, expected heterozygosity (H_e) at markers situated more than 58 kb from the 5′ end and more than 40 kb from the 3′ end of *dhfr* was high ($H_e = 0.81 \pm 0.06$) and not significantly different from H_e at 56 unlinked dinucleotide microsatellites ($H_e = 0.80 \pm 0.11$) sampled from chromosomes 1, 2, 3, and 12 and genotyped from the same collection of parasite isolates (table S1*B* in Supplementary Material online).

One possible explanation for this dramatic pattern is that all extant *resistant dhfr* alleles observed have a single origin and that variation around *dhfr* has been purged as a result of a single selective event. To examine this explanation further and to investigate the geographical span of the putative selective sweep, we examined infected blood samples from 10 additional sites in Thailand, Myanmar, Laos, Cambodia, and Vietnam, to give a total sample size of 583 (including those from Mawker-Thai). For these samples, we genotyped the five polymorphic sites in *dhfr*, as well as six microsatellite markers immediately flanking this gene. Blood samples containing multiple parasite genotypes occur in 25% to 40% of patients in these countries and can result in errors in constructing haplotypes (Anderson et al. 2000*a*). Such samples were excluded by inspection of microsatellite marker and SNP data, leaving 353 samples with complete data for which haplotypes could be determined unambiguously. Of these, there were 299 samples with *resistant dhfr* alleles bearing between one and four mutations and 54 samples bearing the *sensitive dhfr* allele. The most resistant populations were found on the Thai-Myanmar border, where *resistant dhfr* alleles bearing four mutations predominate and *sensitive dhfr* alleles are rare, and the lowest level of resistance was seen in Laos where more than 36% of parasites carried *sensitive dhfr* alleles and the majority of *resistant dhfr* alleles carried two mutations (fig. 2; see also table S3 in Supplementary Material online). We observed high levels of geographical differentiation, strongly suggesting local adaptation in the face of differing drug treatment history. Using five-amino-acid *dhfr* alleles, we observed pairwise F_{ST} values as high as 0.89, and maximal levels of differentiation ($F_{ST} = 1$) was seen for the Ile→Leu mutation at amino acid 164, which was absent in Laos and Vietnam but at high frequencies or fixation in Thai, Cambodia, and Myanmar populations (table 1; see also table S3 in Supplementary Material online). The patterns observed are consistent with regional treatment history. For example, in Laos chloroquine is still the first-line treatment for uncomplicated malaria (PS was introduced in 1969 as second-line treatment, but since then chloroquine has remained the predominant antimalarial drug used), which is consistent with the low levels of *resistant dhfr* alleles compared with surrounding countries.

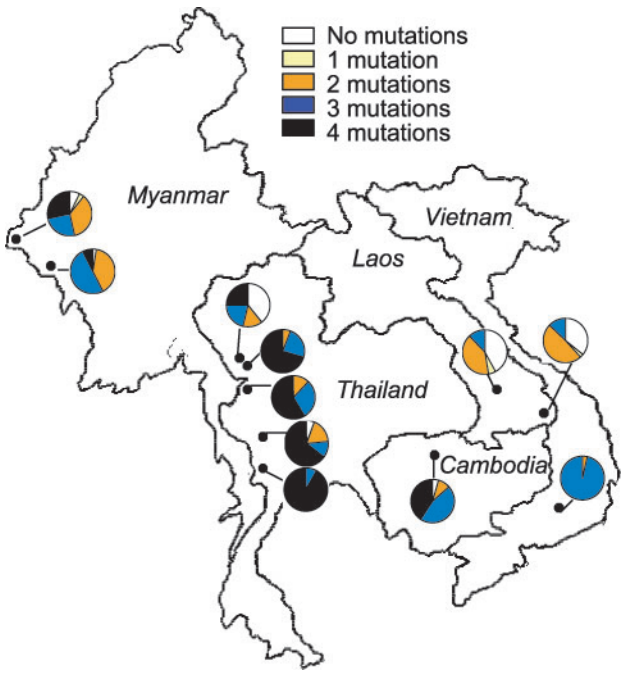

FIG. 2.—Frequencies of *resistant dhfr* alleles in parasites from 11 locations in SE Asia. From north to south, the Thailand locations are Shoklo, Maela, Mawker-Thai, Ratchaburi, and Kanchanaburi; the Laos locations are Phalanxay (southwest) and Sekong (southeast); and the Myanmar locations are Dabhine (upper) and Myothugyi (lower). Frequencies of eight *dhfr* alleles present are shown in table S2 in Supplementary Material online, and statistics describing population differentiation are shown in table 1.

Table 1
Pairwise Fixation Indices (F_{ST}) Between *dhfr* Alleles (A) and Amino Acid 164 Polymorphism (B)

A.	My_Dab	My_Myo	Th_Sho	Th_Mae	Th_Maw	Th_Kan	Th_Rat	Ca_Ang	La_Pha	La_Sek	Vi_Kha
My_Dha	0.00	0.08	0.28	0.38	0.27	0.54	0.38	0.34	0.24	0.22	0.63
My_Myo		0.00	0.12	0.18	0.08	0.34	0.15	0.13	0.13	0.11	0.54
Th_Sho			0.00	0.24	0.18	0.39	0.16	0.18	0.12	0.15	0.59
Th_Mae				0.00	0.01	0.03	0.00	0.19	0.41	0.43	0.71
Th_Maw					0.00	0.12	0.02	0.08	0.31	0.31	0.56
Th_Kan						0.00	0.08	0.36	0.53	0.56	0.89
Th_Rat							0.00	0.17	0.35	0.36	0.75
Ca_Ang								0.00	0.26	0.26	0.39
La_Pha									0.00	-0.01	0.51
La_Sek										0.00	0.54
Vi_Kha											0.00

B.	My_Dab	My_Myo	Th_Sho	Th_Mae	Th_Maw	Th_Kan	Th_Rat	Ca_Ang	La_Pha	La_Sek	Vi_Kha
My_Dha	0.00	-0.01	-0.02	0.22	0.03	0.35	0.07	0.02	0.61	0.56	0.52
My_Myo		0.00	-0.04	0.36	0.11	0.50	0.18	-0.04	0.56	0.49	0.44
Th_Sho			0.00	0.35	0.10	0.49	0.16	-0.03	0.59	0.53	0.47
Th_Mae				0.00	0.07	0.05	0.00	0.44	0.93	0.91	0.90
Th_Maw					0.00	0.20	-0.03	0.17	0.76	0.72	0.69
Th_Kan						0.00	0.18	0.58	1.00	1.00	1.00
Th_Rat							0.00	0.24	0.91	0.88	0.85
Ca_Ang								0.00	0.54	0.47	0.42
La_Pha									0.00	nd	nd
La_Sek										0.00	nd
Vi_Kha											0.00

NOTE.—Significance of observed F_{ST} values was tested by analysis of 100,000 permuted data sets. In each table, significance was adjusted for multiple statistical testing with adjusted nominal cutoffs for 5% significance set at 0.00091. Gray shading (black numbers) indicates significance at 5% level, dark-gray shading (white numbers) at the 1% level, and black shading (white numbers) at 0.1% level. Abbreviated sample location names contain first two letters of the country followed by the first three letters of the place name (see *Geographical Sampling*). Cells marked ''nd'' were invariant at the polymorphic site analyzed in both locations in the comparison.

Evidence for Selection of a Single Lineage of Resistant Alleles in SE Asia

The chromosome-wide data collected from parasites in Mawker-Thai provide suggestive evidence for selection around *dhfr*. However, the absence of parasites bearing *sensitive dhfr* alleles in this population limits the strength of this conclusion. We found both *sensitive* and *resistant dhfr* alleles among the samples collected from other SE Asian locations. Hence, we could directly compare patterns of microsatellite variation flanking both classes of alleles. These data provide striking evidence that extant *resistant dhfr* alleles have been subject to strong selection and indicate a single origin of resistance in these five countries. Markers flanking *resistant dhfr* alleles show reduced diversity, elevated linkage disequilibrium (LD), and skewed allele frequency distributions relative to markers flanking *sensitive dhfr* alleles. Resistant chromosomes were significantly less variable than sensitive chromosomes at each of the six loci (fig. 3*A*). Similarly, LD, measured using EHH (Sabeti et al. 2002) was significantly greater in flanking regions of *resistant dhfr* alleles than around *sensitive dhfr* alleles, suggesting that these alleles are much younger (fig. 3*B*). Allele distributions provide additional evidence for a recent selection of resistance alleles. Microsatellites flanking *resistant dhfr* showed a single predominant allele and occasional rare variants, indicative of a recent bottleneck (Cornuet and Luikart 1996), whereas loci flanking *sensitive dhfr* alleles showed a more even allele frequency distribution. Given the number of alleles observed at each locus, microsatellite H_e on chromosomes carrying *resistant dhfr* alleles was significantly lower than expectations, assuming both infinite alleles (IAM) and stepwise mutation models (SMM) of microsatellite mutation (Wilcoxon test, $P < 0.016$ for both IAM and SMM). In comparison, chromosomes carrying *sensitive dhfr* alleles showed no significant deviation from equilibrium expectations (Wilcoxon test, $P < 0.92$ [IAM]; $P < 0.28$ [SMM]) (fig. 3*A*). These tests for skewed allele distributions and H_e deficiency were also significant when we analyzed only Laos, where the majority of *sensitive dhfr* alleles were found ($P < 0.016$ for both IAM and SMM).

Examination of six-locus microsatellite haplotypes flanking *resistant dhfr* alleles provides strong evidence for a single origin of these alleles. Of the 299 chromosomes bearing *resistant dhfr* examined, 238 had identical six-locus haplotypes surrounding *dhfr*, and of the remaining 61, 47 differed at only one of the six markers, seven differed at two loci, and seven differed at three or more

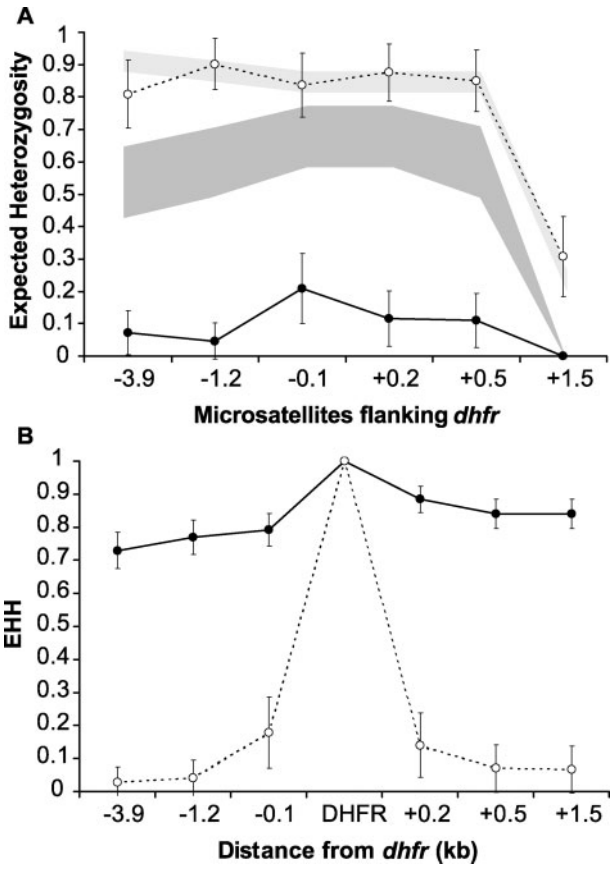

FIG. 3.—Microsatellites flanking *resistant dhfr* alleles show reduced variation, skewed allele frequency distributions, and greater LD than those flanking *sensitive dhfr* alleles. (*A*) Observed and predicted H_e of microsatellite markers flanking *resistant dhfr* (filled dots) and *sensitive dhfr* alleles (open dots) from five SE Asian Countries. The distance (kb) of microsatellite markers relative to *dhfr* are shown along the *x*-axis. Confidence intervals of 95% are shown for the observed H_e measures. The upper and lower bounds of the dark-gray shaded area show the predicted H_e around *resistant dhfr* alleles, assuming mutation/drift equilibrium and stepwise mutation (SMM) or infinites alleles model (IMM), respectively. Predicted H_e was estimated using coalescent simulation based on the observed numbers of alleles and sample size. The light-gray shaded area shows equivalent predictions for microsatellites flanking *sensitive dhfr* alleles. (*B*) LD, measured by EHH (Sabeti et al. 2002), in sensitive (open dots) and resistant (filled dots) chromosomes from SE Asia. Confidence intervals of 95% are shown for the observed EHH values. We obtained very similar graphs when this analysis was restricted to samples from Laos or Shoklo, demonstrating that the dramatic differences in the combined sample are not an artifact of population structure.

of the loci (fig. 4). In comparison, in 54 chromosomes examined bearing *sensitive dhfr* alleles, we found 49 unique six-locus haplotypes. A particularly interesting feature of the data is that seven different *resistant dhfr* alleles, carrying different combinations of 51, 59, 108, and 164 codon mutations, are associated with identical background microsatellite haplotypes. This clearly demonstrates that new mutations have arisen sequentially on the same chromosomal lineage (Plowe et al. 1997; Sirawaraporn et al. 1997).

The molecular data demonstrate a rapid spread of a single lineage of *resistant dhfr* alleles in five countries. Why do we not see multiple origins, as the epidemiological data would suggest? We suggest two possible explanations. (1) The initial Ser→Asn mutation at codon 108 may occur multiple times, but sequential bottlenecks resulting from selection of additional mutations at codons 51, 59, and 164 will result in progressive winnowing of numbers of selected haplotypes. Under this model, we would expect to see alleles bearing Asn-108 associated with a variety of genetic backgrounds, whereas parasites bearing *resistant dhfr* alleles with multiple mutations would have fewer associated haplotypes. Our data provide some support for this model. Only five of 299 resistant *dhfr* alleles examined contained the Ser→Asn mutation at codon 108 in isolation. Yet, three of these five are associated with a divergent microsatellite haplotype differing at multiple loci (fig. 4). Hence, the Ser→Asn change at codon 108 has occurred multiple times, but parasites bearing more than one mutation in *dhfr* occur on a common genetic background. This model is also strongly supported by a recent study in which two-microsatellite markers just 5′ of *dhfr* were genotyped from South American parasites (Cortese et al. 2002). In this study, multiple two-locus haplotypes were associated with *sensitive dhfr* alleles and with *resistant dhfr* alleles bearing the 108N and 51I mutation. However, the range of haplotypes associated with *resistant dhfr* alleles bearing three mutations was considerably reduced. (2) Compensatory mutations (Schrag, Perrot, and Levin 1997) elsewhere in the parasite genome may be required in addition to changes in *dhfr*. Because mutations in *dhfr* result in changes in the shape of the active site, they also result in weaker binding of the natural substrate (folate) and may therefore carry a fitness cost. For example, fitness costs of the Ile→Leu mutation at codon 164 are strongly supported by work on enzyme kinetics of *resistant dhfr* alleles and expression of *P. falciparum dhfr* in yeast (Sirawaraporn et al. 1997; Cortese and Plowe 1998). Additional compensatory mutations (Schrag, Perrot, and Levin 1997) elsewhere in the parasite genome may therefore be required to limit the deleterious effects of mutations in *dhfr*. Because concurrent mutations in two different genes occur at reduced frequency, this would help to explain the rarity with which resistance has evolved. This model is compatible with the data and is also consistent with the persistence of high frequencies of *resistant dhfr* alleles bearing multiple mutations over 15 years since PS was abandoned as antimalarial treatment in Thailand. If present, we expect that such compensatory mutations are likely to be situated close to *dhfr* on chromosome 4, because recombination would rapidly break down linkage disequilibrium between *dhfr* and physically unlinked compensatory changes (Levin, Perrot, and Walker 2000).

Width of the Selective Sweep

The valley of reduced variation around *dhfr* spans approximately 100 kb in the Mawker-Thai parasite population (fig. 1). The valley of reduced variation around *resistant dhfr* alleles appears not to be symmetrical as suggested by simulation studies (Kim and Stephan 2002). *dhfr* diversity is restored to background levels 50 kb from the 3′ end of the gene, whereas to the 5′ markers 58 kb distant

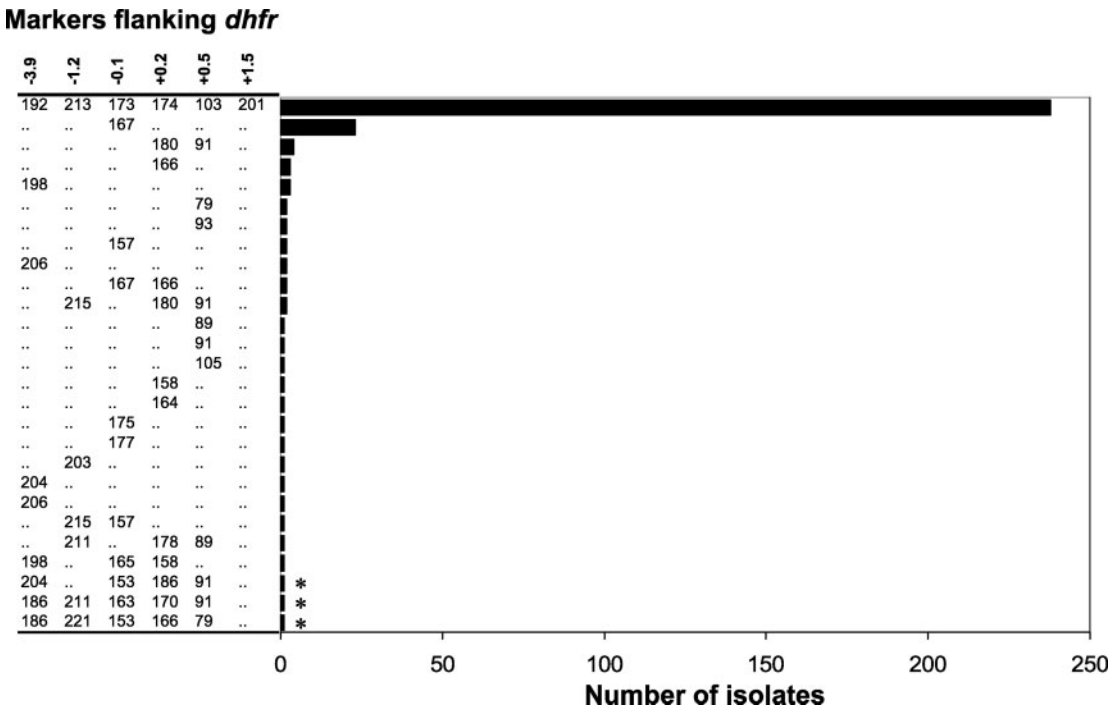

FIG. 4.—A single predominant microsatellite haplotype is associated with resistant *dhfr* alleles from five SE Asian countries. The column titles show the distance (kb) of microsatellites from *dhfr*, while microsatellite allele lengths (bp) at these loci are shown below. Dots indicate identical allele lengths to the predominant haplotype. Details of microsatellite markers are shown in table S1of Supplementary Material online. The bar chart indicates the abundance of different haplotypes. Asterisks mark six-locus microsatellite haplotypes flanking *resistant dhfr* alleles bearing a single Ser→Asn mutation at codon 108.

still show reduced variation. However, denser marker spacing and information on baseline levels of marker heterozygosity are needed to better characterize sweep asymmetry. Another selective sweep has recently been documented around the chloroquine resistance transporter (*pfcrt*) locus on chromosome 7 of *P. falciparum* (Wootton et al. 2002). The valley of reduced variation around *pfcrt* is larger (>200 kb) than that observed around *dhfr*. Heterogeneity in recombination rate across the genome may explain some of the difference observed. Time since selection is also likely to be important. The pyrimethamine selection in Thailand was imposed between 1976 and 1989 (approximately 90 parasite generations ago, assuming a 2-month generation time), whereas many of the African samples surveyed in Wootton et al. (2002) are from populations in which CQ alleles conferring resistance are currently in the process of spreading. Hence, there has been less time for recombination to break down associations between selected point mutations and flanking microsatellites in the case of the CQ sweep. Regardless of the reasons for the differences between the two sweeps, the valleys of reduced variation are quite large in both the CQ (>200 kb) and pyrimethamine (~100 kb) associated selective events. If we assume similar rates of recombination across the genome, markers spaced at 50 kb (~every 3 cM) intervals should be sufficient to identify regions in which selection has removed variation around loci underlying resistance to other important antimalarial drugs. Hence antimalarial drug resistance appears to be an unusual trait in that genes may be located by genome-wide association with a relatively low density of genetic markers (Anderson et al. 2000*a*; Wootton et al. 2002). Encouraged by these empirical results from regions around known drug resistance loci, we are currently conducting a genome scan to locate unknown genes underlying resistance to other important antimalarial drugs such as mefloquine, quinine, and artemisinin. In this case, we will compare heterozygosity, allelic skew, and LD in parasites isolates showing high and low levels of in vitro drug resistance to locate genome regions under drug selection (Wootton et al. 2002).

We expect that both recombination rate and selection intensity will play key roles in determining the size of genomic regions affected by selection. Microsatellite mutation is expected to be of lesser importance because the selective events have occurred very recently. The recombination rate of *P. falciparum* has been measured in a genetic cross and is approximately 50 times greater than in the human genome. At first sight it is surprising that variation is reduced over such a distance from *dhfr*. The high rate of inbreeding in *P. falciparum* populations may help to explain this (Babiker et al. 1994; Paul et al. 1995; Anderson et al. 2000*a*). Malaria parasites are hermaphroditic protozoans: asexual mitotic division of haploid stages occurs in the bloodstream, and fusion of gametes and meiosis occurs in the mosquito midgut. Hence, if a mosquito feeds on an infected person containing male and female sexual stages of a single genotype, the meiotic products will result from self-fertilization. The level of inbreeding is therefore dependent on the proportion of people bearing parasites of multiple clones and varies considerably between populations, depending on levels of transmission (Anderson et al. 2000*a*). In the Mawker-Thai population approximately

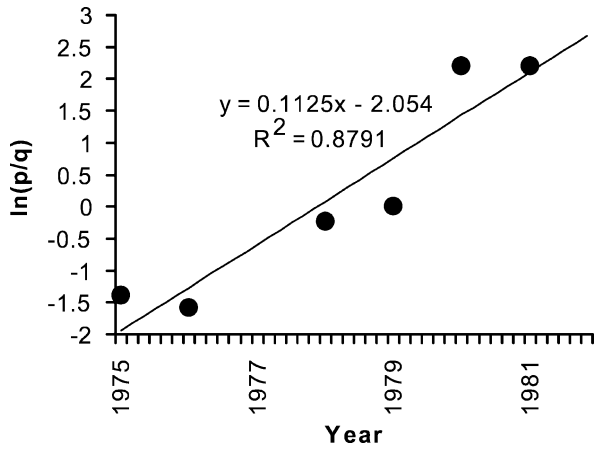

FIG. 5.—Estimation of selection coefficient (s) driving pyrimethamine resistance. p is the frequency of *resistant dhfr* alleles inferred from treatment failure rate data, and q ($=1-p$) is the inferred frequency of *sensitive dhfr* alleles. The x-axis shows the generation number (assuming six generations per year). The slope gives an estimate of $s=0.1125$. Failure rates may not provide a perfect estimate of *resistant dhfr* allele frequency. However, so long as the failure rate is proportional to the frequency of *resistant dhfr* alleles (p), estimates of s can be made. Furthermore, since failure rates may also be influenced by resistance to the partner drug sulfadoxine, these estimates should be viewed as upper bounds to selection coefficients for pyrimethamine. Data from (Bunnag and Harinasuta 1987).

60% of patients carried infections consisting of a single clone, suggesting a minimal inbreeding coefficient of approximately 0.6. In reality, the inbreeding coefficient in this location is probably much higher (0.6 to 0.9) because a proportion of matings that occur in mosquitoes feeding on multiply infected patients will involve self-fertilization. As a consequence of inbreeding, the effective recombination rate is considerably lower than the actual recombination rate measured in the genetic cross.

The variation in levels of inbreeding (and effective recombination rate) among *P. falciparum* populations may provide a valuable tool for genetic mapping and suggests a two-step strategy. First, a genome screen could be conducted in a locality where parasites are highly inbred and have a low effective recombination rate. In such a situation, selection will purge variation across a large region of the genome, allowing identification of the chromosomal regions involved with relatively low-density marker coverage. To fine-scale map the genes involved, these genomic regions could be then investigated in more detail in parasite populations with low levels of inbreeding (higher effective recombination rate). In such populations, the valleys of reduced variation are expected to be much narrower, providing more precise localization of genes targeted by selection.

The strength of selection will also determine the size of genomic regions affected by drug selection. In the selection event driven by CQ treatment on chromosome 7, selection intensity was estimated indirectly from the pattern of LD between markers flanking *pfcrt* (Wootton et al. 2002). Values of between 0.1 and 0.7 were consistent with the patterns observed. We were able to estimate selection intensity for *resistant dhfr* alleles more directly using the decline in therapeutic efficacy of PS in Thailand. A selection coefficient of 0.11 is consistent with this data if we assume a 2-month generation time for *P. falciparum* in Thailand (fig. 5). The selection coefficient driving the spread of *resistant dhfr* alleles is high. However, coefficients of comparable magnitude have been recorded in many studies of adaptive traits in natural populations of other organisms (Endler 1986). As such, drug selection of malaria parasites may also provide a useful model system for understanding genomic effects of hitchhiking in natural systems where the history of selection is poorly known (Harr, Kauer, and Schlotterer 2002).

The critical parameters determining the extent of hitchhiking with resistance genes are the rates of recombination and mutation and the strength of selection. We used estimates of these parameters and an analytical framework (Wiehe 1998) to model the expected shape and dimensions of the pyrimethamine selective sweep in Mawker-Thai. The rate of spread of *resistant dhfr* alleles estimated using the observed decline in PS therapeutic efficacy (fig. 5) and is consistent with selection coefficients (s) of approximately 0.1 (see above). Similarly, we have estimates of both recombination rate ($r = 5.88 \times 10^{-4}$ Morgans/kb/generation) (Su et al. 1999) and microsatellite mutation rate ($\mu = 1.59 \times 10^{-4}$/generation) (Anderson et al. 2000*a*) from the HB3/Dd2 genetic cross and local inbreeding rates can be estimated from the proportion of blood samples containing multiple malaria clones ($F = 0.6$ to 0.9). The model fits well to the observed data (fig. 1), giving us considerable confidence in our estimated parameter values for *P. falciparum*. However, the estimated mutation rates appear to be too high, perhaps indicating that the mean values derived from the cross are elevated by a few highly mutable loci (fig. 1). The good fit of the data to the model suggests we can make predictions about the size of genomic regions influenced by selection in *Plasmodium*, and thus our ability to detect these regions by genome scans. Despite the high base recombination rate of *Plasmodium*, variation may be reduced for more than 95 kb around selected alleles at a resistance gene when $s > 0.05$ and F (inbreeding coefficient) > 0.8 (fig. 6).

It should be noted that the models developed by Wiehe (1998) examine the reduction in variance in microsatellite allele length after a selective event. We have used H_e rather than variance. As such, the appropriateness of our approach depends on reduction in variance being closely related to reduction in H_e. Simulations show that H_e at flanking microsatellite markers will be restored slower than variance in repeat number after a selective event (Thomas Wiehe, personal communication). Hence, our modeled lines represent an upper bound to H_e after a sweep and will be conservative in predicting amount of H_e removed by selection and the size of the chromosomal region affected. Given the uncertainties about mode of microsatellite evolution, a theoretical framework for directly examining the influence of selective events on H_e, assuming both infinite alleles (IAM) and stepwise models (SMM) of microsatellite evolution, would be extremely useful.

Implications for Malaria Parasite Biology

Removal of variation in genes linked to drug resistance loci is likely to have important influence on malaria parasite

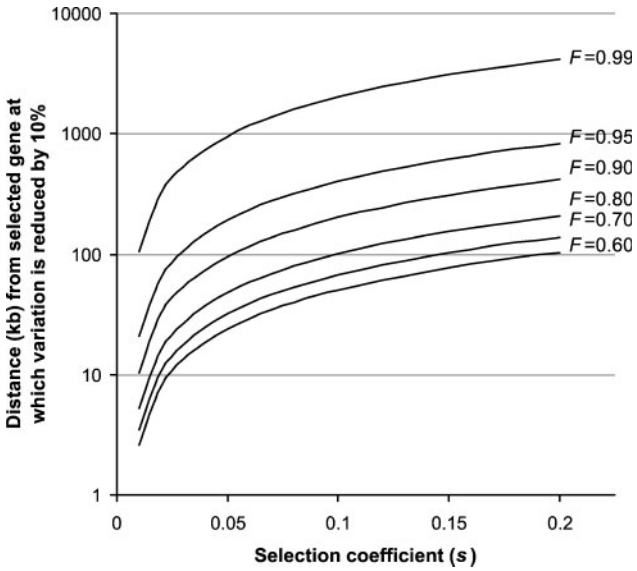

FIG. 6.—Effect of realistic parameter values for selection (s) in *P. falciparum* and the inbreeding coefficient (F) on our ability to detect drug resistance genes by association. We used independent measured estimates of m and r. The y-axis shows the size of genomic regions in which variation is reduced, such that marker variation after selection is 90% of that preceding selection ($V_{(t1)}/V_{(t0)} = 0.9$). We assume a population size of 10^4, giving a starting allele frequency (ε) of $1/10^4$. Inbreeding levels have a large influence on variation since they reduce the effective recombination rate. When $F = 0.8$ and $s = 0.1$, variation is reduced for 100 kb from selected loci, whereas when $F = 0.99$, variation may be reduced over whole chromosomes when $s > 0.05$. Ten percent reduction (from $H_e = 0.8$ to 0.7) in marker diversity between drug-resistant and drug-sensitive parasites should be detectable ($P < 0.05$) with 80% power with a sample size of approximately 300 in each group (Fleiss 1981).

biology. Many antigen genes in *P. falciparum* show extreme levels of nonsynonymous mutation, and allelic distributions characteristic of balancing or frequency-dependent selection (Hughes and Hughes 1995; Polley and Conway 2001; Volkman et al. 2002). Purging of variation from antigens due to directional selection on neighboring drug resistance genes may therefore reduce mean fitness of parasites in the face of immune selection (Hill-Robertson interference [Hill and Robertson 1966]). In the approximately 100-kb region around *dhfr* in which variation is reduced, there are 22 predicted genes that are likely to have reduced variation resulting from selection on *dhfr*. In the countries studied here, the effects of selection are localized to a region encompassing approximately 8% of chromosome 4. In parasite populations where self-fertilization predominates and levels of recombination are consequently much lower such as those in South America (Anderson et al. 2000*a*), drug selection may result in genome-wide reduction in genome variation (fig. 6). Indeed, recurrent selection by chloroquine and PS (Fansidar™) may contribute to the genome-wide reduction in variation observed in South American parasites.

PS treatment costs are low and it is increasingly being used in Africa in regions where chloroquine has ceased to be useful. Furthermore, in SE Asian countries such as Myanmar and Laos, PS is still sometimes used. A problem with PS is that it does not kill gametocytes, the sexual stages required for mosquito transmission. This is thought to promote rapid spread of parasites bearing resistance mutations (Mendez et al. 2002). Combinations of PS with artemisinin derivatives that have strong gametocytocidal action (Price et al. 1996) have been suggested as a means to prolong the life of PS, and large-scale clinical trials are currently taking place in Africa to test these combinations (Olliaro, Taylor, and Rigal 2001). The surprising finding that extant *resistant dhfr* alleles have a single common ancestor in the SE Asian countries surveyed suggests that viable *dhfr* mutants capable of spread may evolve rather rarely. If so, treatment using PS in combination with ''transmission blocking'' drugs such as artemisinin may be especially valuable in reducing the rate of resistance evolution (White et al. 1999). It will be of great interest to see if similar patterns of *dhfr* resistance evolution are observed in sub-Saharan Africa, where the consequences of pyrimethamine resistance are likely to be the most severe.

Note Added in Proof

Roper et al. (2003) genotyped three microsatellites flanking *dhfr* in *P. falciparum* sampled from sites in South Africa and Tanzania separated by 4,000 km. They found evidence for three independent origins of *resistant dhfr* alleles, suggesting similar patterns of resistance evolution in both Sub-Saharan Africa and SE Asia.

Supplementary Material

Table S1*A*, table S1*B*, and table S3 are available online at the journal's Web site.

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