Genome-wide variation and identification of vaccine targets in the *Plasmodium falciparum* genome

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One goal in sequencing the *Plasmodium falciparum* genome, the agent of the most lethal form of malaria, is to discover vaccine and drug targets¹. However, identifying those targets in a genome in which ~60% of genes have unknown functions is an enormous challenge. Because the majority of known malaria antigens and drug-resistant genes are highly polymorphic and under various selective pressures^{2–6}, genome-wide analysis for signatures of selection may lead to discovery of new vaccine and drug candidates. Here we surveyed 3,539 *P. falciparum* genes (~65% of the predicted genes) for polymorphisms and identified various highly polymorphic loci and genes, some of which encode new antigens that we confirmed using human immune sera. Our collections of genome-wide SNPs (~65% nonsynonymous) and polymorphic microsatellites and indels provide a high-resolution map (one marker per ~4 kb) for mapping parasite traits and studying parasite populations. In addition, we report new antigens, providing urgently needed vaccine candidates for disease control.

Genetic mapping using genetic crosses is a powerful approach for identifying genes underlying various traits of parasites^{7–10}. However, high costs and laborious procedures have prevented widespread application of genetic crosses for genetic studies in *P. falciparum*. Association mapping using field isolates is an alternative, but this approach requires typing large numbers of genetic markers and parasite isolates because of the complexity of the parasite population structure and highly variable recombination rates¹¹. A genetic map consisting of ~800 microsatellite markers has been constructed for *P. falciparum*¹²; however, compared with the methods recently developed for large-scale SNP typing^{13–16}, microsatellite typing is relatively laborious. A genome-wide map with high-density SNP markers will greatly facilitate our ability to identify important genes underlying parasite traits and address important biological questions pertaining to the genome of *P. falciparum*. Unfortunately, a high-density SNP map is currently available only for chromosome 3 (ref. 11).

We searched for polymorphisms in the *P. falciparum* genome by amplifying and sequencing 3,539 predicted genes or fragments

(~19% of each isolate genome; 91% coding sequence) from four cloned isolates (Dd2, Hb3, D10 and 7G8) (Table 1 and Supplementary Table 1 online). After aligning the DNA sequences to the 3D7 genome sequence¹, we identified 3,918 well-validated SNPs, giving a genome-wide average of one SNP per ~5.9 kb DNA (Table 1 and Fig. 1). Of the genes surveyed, approximately half (54.3%) had one or more SNPs (Supplementary Fig. 1 online); the majority (~65%) of SNPs were nonsynonymous (nsSNP; Table 1). The predominance of nsSNPs is probably due to codon bias and high frequencies of nonsynonymous sites in the parasite genome^{17,18}. The estimates of the genome-wide average population mutation rate, $4N\mu$ (Watterson's θ), where N is the effective population size and μ is the per-nucleotide mutation rate, is 5.05×10^{-4} , and the estimate of average pairwise nucleotide diversity (π) is 4.83×10^{-4} (Table 1), similar to previous reports¹¹. Although these diversity values are lower than those from many model organisms, this could be due to our sequencing of mostly coding regions and our high stringency of SNP calling (see Methods).

We resequenced ~45 kb of DNA covering 183 known SNPs on chromosome 3 from 99 worldwide isolates¹¹, 108 of which are common SNPs (minor allele frequency ≥ 0.05). We discovered 185 new SNPs, 29 of which were common SNPs (Supplementary Table 2 online). Because only ~22% of the 202 kb sequenced for the five isolates was resequenced for the 99 isolates, we would expect to miss 132 (29/0.22) common SNPs in five isolates. This indicates that our survey of five isolates captures ~45% of common SNPs (108/(132 + 108) = 0.45) relative to the worldwide sample of 99 isolates and gives us a frequency of one common SNP per 842 bp (202 kb/240) and a global genome-wide expectation of > 27,000 common SNPs, as estimated based on SNPs largely from single-copy nontelomeric genes.

Microsatellites are also abundant in the genome, averaging one polymorphic microsatellite per 1.3 genes (Fig. 1 and Table 1). The true frequency of polymorphic microsatellites throughout the genome would probably be much higher if more noncoding regions were assayed. Combining polymorphic microsatellites and SNPs, our data constitute a map with an average of one polymorphic marker per ~3.6 kb for the *P. falciparum* genome (Fig. 1), providing a powerful tool for genetic studies of the parasite.

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Table 1 Summary of sequenced genes and diversity among five *P. falciparum* isolates

Chr.	Bp sequenced ^a	Percentage sequenced	Genes sequenced ^b	Genes with SNPs	Number of SNPs	Genes with >4 SNPs	sSNPs	nsSNPs	ncSNPs	MS	θ (10^{-4})	π (10^{-4})	Rec. events
1	81,256	12.6	73 (155)	44	120	3	35	81	4	62	6.21	6.18	18
2	136,980	14.5	115 (224)	70	134	3	48	82	4	70	6.71	6.52	12
3	203,069	19.1	203 (245)	108	229	7	60	138	31	165	4.79	4.44	24
4	128,567	10.7	114 (249)	59	120	3	32	86	2	71	4.91	5.10	16
5	280,845	20.9	230 (330)	118	240	7	68	156	16	172	3.98	4.7	41
6	302,182	21.9	234 (319)	130	258	7	78	162	18	198	4.36	4.21	28
7	267,968	19.8	206 (297)	139	450	28	147	272	31	186	8.27	9.55	66
8	282,518	21.4	209 (299)	125	277	11	80	188	9	191	4.84	4.51	23
9	344,478	22.3	277 (366)	153	287	7	89	187	11	237	3.98	4.85	23
10	326,926	19.3	263 (405)	143	268	6	80	179	9	180	4.41	4.30	27
11	387,591	19.0	311 (516)	167	365	7	97	244	24	239	4.28	4.21	47
12	457,373	16.6	367 (533)	190	343	8	114	213	16	263	3.38	3.72	30
13	539,419	19.2	469 (687)	221	407	10	89	293	25	270	3.43	4.11	36
14	510,063	15.5	468 (776)	253	420	7	133	279	8	244	4.12	4.33	35
Total	4,249,816	18.6	3,539 (5,401)	1,920	3,918	114	1,150	2,560	208	2,548	4.83	5.05	426

Chr, chromosome; sSNP, synonymous SNPs; nsSNP, nonsynonymous SNPs; ncSNP, noncoding SNPs; MS polymorphic microsatellites/indels; θ , average population mutation rate; π , average pairwise nucleotide diversity; rec. events, recombination events detected within each chromosome.

^aTotal bp sequenced for each of four isolates. ^bNumbers in parentheses are the number of predicted genes in 3D7.

SNPs are not distributed evenly across chromosomes; rather, some regions have consecutive genes without any SNPs, and other DNA segments have consecutive genes with multiple SNPs (Fig. 1). The percentage of genes with SNPs varies from chromosome to chromosome, ranging from 47.1% (chromosome 13) to 67.5% (chromosome 7) (Table 1). The numbers of SNPs per gene differs more than twofold between chromosomes, averaging 0.85–2.08 SNPs per sequenced gene, with large chromosomes having fewer SNPs per gene (Table 1 and Supplementary Table 1). Indeed, excluding chromosome 7, there is a negative correlation between chromosome size and the number of SNPs per gene (Supplementary Fig. 2 online). Comparison of the average θ values from genes at chromosome ends ($\sim 15\%$ of the sequenced genes from each chromosome end) with genes in the remainder of the chromosome showed significantly higher θ values for genes at chromosome ends ($P = 0.0001$, Wilcoxon signed rank test; similarly, $P = 0.0001$ if we compared and tested ten genes from each end). These results suggest that a generally higher level of polymorphism at chromosome ends may contribute to this negative correlation,

because these regions take up a relatively larger proportion of the small chromosomes.

With these genome-wide markers, we estimated the number of recombination events using methods described previously¹¹. We detected recombination events at relatively high frequencies (Table 1); they were distributed nonuniformly both within and among chromosomes, clustering in subtelomeric regions, as previously described for a larger sample on chromosome 3 (ref. 11). Understanding the patterns and rates of recombination is of critical importance for genetic studies, particularly association mapping.

Genes encoding surface antigens, cell adhesion molecules and proteins involved in drug interactions are mostly polymorphic (Supplementary Fig. 3 online). The antigen group has a high ratio of nonsynonymous pairwise differences per nonsynonymous site (pN) relative to synonymous pairwise differences per synonymous site (pS) (pN/pS = 5.8), suggestive of balancing, diversifying or partial directional selection. Additionally, estimates of Tajima's D , a measure of the frequency distribution of alleles, across chromosomes also identified some genes that show an excess of

Figure 1 Physical maps showing distribution of polymorphic sites across 14 *P. falciparum* chromosomes. Vertical bars represent SNPs (black, nonsynonymous substitutions; red, synonymous substitutions) or microsatellites (blue, under the horizontal lines). Only one nonsynonymous SNP and one synonymous SNP are presented if there was more than one SNP in a gene (noncoding SNPs were grouped with synonymous SNPs). Most of the chromosomal ends (green vertical bars) were excluded because of gene families such as *var*, *rifin* and *stevor*.

Table 2 Highly polymorphic genes that are potential immune or drug targets

Gene	Annotation	Number of SNPs	θ	TM	Signal peptide
PFB0985c	HP	3	0.016941	3	None
PF11_0344 ^{a,b,c,d}	AMA-1	48	0.012327	1	aa 1–24
MAL7P1.6 ^{b,d}	HP	15	0.008922	None	aa 1–21
MAL7P1.43 ^a	Rifin	15	0.006630	None	None
PFA0655w ^{a,b}	Surfin	32	0.005796	None	None
PF13_0201 ^{a,b}	CSP2	18	0.005729	None	aa 1–25
PFB0105c ^{b,c}	HP	7	0.005554	1	None
MAL7P1.65 ^{b,c,d}	HP	14	0.004462	2	None
PFB0935w ^{a,b,d}	CLAG2	9	0.004458	None	aa 1–24
MAL7P1.66 ^{b,d}	HP	13	0.004315	None	None
MAL7P1.35 ^{b,d}	Cell division cycle ATPase	13	0.004254	None	None
MAL7P1.58 ^{b,c,d}	HP	6	0.004248	2	aa 1–20
PFI0690c	HP	13	0.004211	None	None
PFE1615c	HP	3	0.004138	None	None
PF08_0005 ^{a,b}	Tryptophan/threonine-rich antigen	9	0.003702	1	aa 1–21
PF07_0044 ^d	HP	3	0.003664	None	None
PF14_0445	HP	2	0.003569	None	None
PFE0815w ^b	tRNA pseudouridine synthases	10	0.003550	None	aa 1–24
PF11_0109	HP	3	0.003478	None	None
PF14_0040 ^d	HP	2	0.003478	None	aa 1–19
PFD1120c	Integral membrane protein	2	0.003466	1	aa 1–28
MAL13P1.268 ^b	HP	7	0.003432	None	aa 1–17
PFD0310w	Sexual stage-specific protein	2	0.003429	1	aa 1–25
PFA0665w ^a	EBA-like	8	0.003339	None	None
PF14_0759	HP	2	0.003265	1	None
MAL7P1.59 ^{b,d}	HP	5	0.003252	None	None
PFL0070c ^d	HP	11	0.003212	None	aa 5–24
MAL6P1.248	Histone h3	2	0.003200	None	None
PF08_0012 ^{b,c}	HP	10	0.003198	None	None
PF07_0128 ^{a,b,c}	EBA-175	10	0.003196	1	aa 1–21
PFE0565w ^b	HP	7	0.003164	1	aa 1–24
PFD0110w ^{a,b,d}	Reticulocyte binding protein	8	0.003132	None	aa 1–19
PF14_0467 ^{b,d}	HP	6	0.003006	1	aa 1–24
PFI0085c ^d	HP	2	0.002963	2	None
MAL7P1.34 ^d	Cell division cycle ATPase	4	0.002949	4	None
PFL1410c	ABC transporter	9	0.002878	11	None
PF08_0001	HP	3	0.002851	None	aa 1–17
PFI0165c ^b	DEAD/DEAH box helicase	9	0.002846	None	None
PFD0980w ^b	Holo-acyl-synthase	7	0.002843	None	None
PF14_0019	HP	4	0.002832	None	None
PF08_0024 ^{b,c}	HP	5	0.002800	None	aa 1–20
PF14_0260 ^d	HP	7	0.002786	12	None
PF07_0042 ^{b,d}	Lysophospholipase	9	0.002785	None	None
PF11780w	HP	8	0.002779	1	None
PF07_0124 ^b	HP	10	0.002741	None	None
PF11_0211 ^d	HP	7	0.002701	None	None
PF13_0275 ^{b,c}	HP	5	0.002667	2	None
MAL6P1.231 ^{b,d}	Long-chain fatty-acid Co-A ligase	8	0.002663	7	None
PF13_0304 ^b	Elongation factor 1 alpha	7	0.002646	None	None
PFL2225w	Myosin A tail domain	2	0.002637	None	None
PF14_0014	HP	4	0.002588	1	aa 1–21
PF08_0130	WD repeat protein	8	0.002569	None	None
PF11_0144	HP	5	0.002564	None	None
PF13_0198 ^{a,b,c}	Rh2	9	0.002564	1	None
PF07_0035 ^d	CG1	8	0.002540	None	aa 1–20
MAL13P1.290	High mobility-group protein 4	2	0.002533	None	None

All genes have θ values greater than the mean θ (+ 2 s.d.) from 1,920 genes having one or more SNPs. Annotations are based on those in PlasmoDB. HP, hypothetical proteins; TM, number of transmembrane domains in each gene; aa, amino acids (indicates which residues represent a signal peptide).

^aKnown surface proteins and/or antigens. ^bGenes expressed in a cell-free expression system and detected by antibodies to the His tag. ^cGenes that encode proteins recognized by pooled human immune sera. ^dGenes located in the loci with consecutive polymorphic genes in **Supplementary Table 3**.

Figure 2 Immunoblots of candidate antigens expressed *in vitro*. Separated proteins were probed with (a) pooled human immune sera from affected individuals, (b) pooled human nonimmune sera or (c) antibodies to the His tag. Eleven of the fourteen proteins (except PFB0105c, PF080012, and PFE1600w) were clearly recognized by human immune sera but not by nonimmune sera. PFB0105c seems to cross-react with nonimmune sera. PF080012 and PFE1600w showed low expression, and we did not detect any bands by the immune sera (acting as negative controls). EBA-175, Rh2, AMA-1, and MSP-1 are known antigens (positive controls). Genes PF11-0034, PFI-0170w, PFE1600w, and PFL1358c also have multiple SNPs, but their θ values are not high enough to be included in **Table 2**. Gene identifications are as in PlasmoDB. Arrowheads indicate specific bands recognized by human immune sera.

diversity indicative of balancing selection, such as *eba-175*, which has been shown to be under strong balancing selection (**Supplementary Table 1**)¹⁹.

Chromosomal regions flanking many *var* clusters are more polymorphic than the genome-wide average (**Supplementary Fig. 4** and **Supplementary Table 1** online). In addition to many subtelomeric *var* genes, three of the four internal *var* clusters in the 3D7 genome (on chromosomes 7, 8, and 12) are flanked by five or more consecutive polymorphic genes, with some extending ~100 kb from the core *var* cluster, particularly the two clusters on chromosomes 7 and 8 (**Supplementary Fig. 4**). On one side of the chromosome 7 internal *var* locus, elevated polymorphism extended over 200 kb to the chloroquine-resistance transporter gene (*pfcr*). Indeed, genes flanking the *var* loci on chromosomes 7 (25 genes) and 8 (14 genes) have significantly higher θ values than the average values for their chromosome (**Table 1**, $P < 0.0001$ for chromosome 7 and $P < 0.001$ for chromosome 8, Wilcoxon signed rank test). The *var* genes encode a family of variant antigens called PfEMP1 that are important for immune invasion and disease pathogenesis^{20–22} and that may be under strong balancing selection from the host immune response²³, thus maintaining more variation than expected under neutrality. Genes flanking *var* clusters may have correlated evolutionary histories with the *var* genes, preserving diverse alleles linked to each unique *var* haplotype. Therefore, a peak of elevated nucleotide diversity surrounding a selected target is one of the signatures of balancing selection^{24–26}. Some *var* clusters, however, do not have obvious elevated polymorphism in the flanking regions, which could be due to the absence of *var* genes at a specific location in some parasites, or it could be that some *var* clusters are subject to lesser selective pressures (perhaps expressed less frequently). If this is true, vaccine development based on *var* genes should probably give emphasis to *var* genes that are under strong selection.

Signatures of selection can be exploited to identify genes encoding new antigens or drug targets. We searched for genomic regions with consecutive polymorphic genes or peaks of polymorphism (indicative

of balancing selection) that may harbor genes encoding antigens. Indeed, approximately 40% of the 83 loci with five or more consecutive polymorphic genes contain genes encoding known antigens (**Supplementary Table 3** and **Supplementary Fig. 4** online). These results suggest that most of the parasite antigens are under selection from the host immune system. Further investigation of the other 37 loci (**Supplementary Table 3**) with unknown genes may lead to some new vaccine candidates. **Table 2** lists 56 highly polymorphic genes having θ values 2 s.d. higher than the mean θ value for 1,920 genes with one or more SNP. Although more than half of these genes encode proteins of unknown function, ~18% of them (10) are known antigens (**Table 2**). Two genes encode proteins involved in lipid metabolism, one of which is a known drug target in other organisms²⁷. According to the annotation in PlasmoDB²⁸, 32 of these genes (57%) have one or more predicted transmembrane domains (38%) and/or a signal peptide (36%), whereas only approximately 11%

and 31% of the genes in the genome have a predicted signal peptide or transmembrane domain, respectively. The higher proportion of proteins with signal peptides and transmembrane domains suggests potential membrane and/or surface localization that may be recognized by the host immune system.

We next expressed the 56 genes in **Table 2** plus 52 genes that have five or more SNPs and encode a predicted signal peptide and/or transmembrane domains (**Supplementary Table 4** online) using an *E. coli* cell-free rapid expression system. Expression of proteins was verified via protein blot using antibodies to the His tags incorporated into the C terminus of the expressed proteins and detected using pooled human immune sera. Eleven of the 65 expressed proteins were recognized by pooled human immune sera but not by pooled nonimmune sera (**Fig. 2**); seven of these represented previously unknown antigens that require further evaluation as potential vaccine candidates.

This study identifies thousands of well-validated SNPs and polymorphic microsatellites for mapping genes that may be important in drug resistance, parasite development and disease pathogenesis. Developing high-density markers and high-throughput methods for genotyping large numbers of parasites is critical for mapping genes associated with malaria phenotypes, particularly in high-transmission populations where limited linkage disequilibrium exists. A high-throughput array-based genotyping method is being developed for use in the malaria community. The genome-wide data also show that the *P. falciparum* genome is highly polymorphic, with at least one polymorphic site per 0.5 kb in only five isolates. Additionally, this work shows that a genome-wide survey for polymorphisms and signatures of selections is a valuable approach for identifying antigens, which should lead to the identification of many new antigens as potential vaccine targets. Our study also suggests that different *var* clusters may be under variable immune selective pressures that should be taken into consideration when designing a *var*-based vaccine. Further characterization of the proteins encoded by these genes may lead to new vaccines, which are urgently needed to combat this deadly disease.

METHODS

Parasites and DNA amplification and sequencing. DNA sequences of the 3D7 parasite were downloaded from PlasmoDB. Primers for PCR (Supplementary Table 1) and DNA sequencing were designed from predicted ORFs larger than 400 bp (~1.5 kb was sequenced for large genes, excluding the well-known gene families *var*, *stevor* and *rifin*) using a proprietary primer selection software (Visual Basic script). Primers for sequencing both strands of DNA (18–25 bp, with four to seven G/Cs and spaced ~400 bp apart) were automatically selected and commercially synthesized. Genomic DNA from cultured parasites Dd2 (Thailand), HB3 (Honduras), 7G8 (Brazil) and D10 (Papua New Guinea) were amplified and sequenced as described previously¹⁸. Direct sequencing of PCR products eliminates artificial polymorphism frequently introduced when cloning AT-rich DNA into bacteria.

Sequence alignment and analysis. A Java package was written to process raw sequence data, including trimming and aligning DNA sequences using Phred/Phrap and Sequencher 4.5 (GeneCodes). The 3D7 genomic sequence and the corresponding annotated coding sequences from PlasmoDB 5.0 (both sets of chromosomal flat files dated 2002 and 2005) provided the gene annotations, including annotation of coding and noncoding regions and gene ontology classifications. The program also mapped alignment files to the 3D7 genome and characterized any variation found in the alignment as SNPs or indels or microsatellites. A set of scripts was then used to calculate summary statistics of diversity (θ , π and Tajima's D values). All SNPs and microsatellites were confirmed by visually inspecting chromatogram traces of all potential polymorphisms. All alignments of indels and microsatellites were manually adjusted to minimize mismatches and size polymorphism. SNPs in repetitive regions were not called, because misalignments may create artificial SNPs. The number of recombination events throughout the genome was estimated using the nonparametric methodology of ref. 29 as described previously¹¹.

Protein expression and blotting. Cell-free expression of proteins was performed using a rapid translation system (Roche Diagnostics) according to the manufacturer's instruction. Proteins expressed in 50 μ l of an *E. coli* cell-free expression system were enriched using paramagnetic precharged nickel particles (Promega), separated on 4%–12% polyacrylamide gels, transferred to PVDF membrane and detected using antibodies to the His tag or pooled human antisera from villagers of Mali.

Accession codes. SNPs have been deposited at NCBI dbSNP database (accession codes 65654288–65658180) and also at PlasmoDB (v5.2).

URLs. PlasmoDB: <http://www.plasmodb.org/>. Phred/Phrap: <http://www.phrap.org/>.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

J.M.: DNA amplification, sequencing and data analysis; J.D.: DNA amplification and sequencing; K.S.: primer design; K.M.M. and J.K.: software and database development and data analysis; G.A.T.M.: manuscript preparation; P.A.: software and database development, data analysis and manuscript preparation; X.-z.S.: project design, data analysis and manuscript preparation.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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