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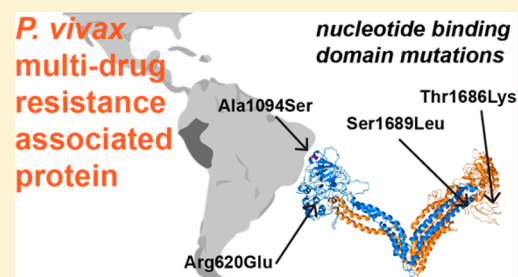
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Supporting Information

ABSTRACT: Understanding the mechanisms of drug resistance in *Plasmodium vivax*, the parasite that causes the most widespread form of human malaria, is complicated by the lack of a suitable long-term cell culture system for this parasite. In contrast to *P. falciparum*, which can be more readily manipulated in the laboratory, insights about parasite biology need to be inferred from human studies. Here we analyze the genomes of parasites within 10 human *P. vivax* infections from the Peruvian Amazon. Using next-generation sequencing we show that some *P. vivax* infections analyzed from the region are likely polyclonal. Despite their polyclonality we observe limited parasite genetic diversity by showing that three or fewer haplotypes comprise 94% of the examined genomes, suggesting the recent introduction of parasites into this geographic region. In contrast we find more than three haplotypes in putative drug-resistance genes, including the gene encoding dihydrofolate reductase-thymidylate synthase and the *P. vivax* multidrug resistance associated transporter, suggesting that resistance mutations have arisen independently. Additionally, several drug-resistance genes are located in genomic regions with evidence of increased copy number. Our data suggest that whole genome sequencing of malaria parasites from patients may provide more insight about the evolution of drug resistance than genetic linkage or association studies, especially in geographical regions with limited parasite genetic diversity.

KEYWORDS: malaria, *Plasmodium vivax*, genomics, haplotype, clone, recombination, mutation



BACKGROUND

At present there is still no long-term sustainable culture system for *Plasmodium vivax*, the parasite responsible for 47% of malaria outside of sub-Saharan Africa.¹ *P. vivax* malaria, which can be fatal and is endemic in 57 countries,¹ is primarily treated with chemotherapy. Chloroquine plus primaquine is used in many areas of the world, such as South America, where resistance is not widespread,² while artemisinin-based combination therapies (ACTs) are used in high-transmission areas, such as Papua New Guinea, where chloroquine-resistant *P. vivax* has been observed.³ Antifolates, such as the sulfadoxine-pyrimethamine combination (Fansidar), are not recommended for use because of widespread parasite resistance but have been used extensively in the past. Pyrimethamine was added to table salt in some countries, including Brazil in the 1950s.^{4,5} Although *P. vivax* alleles conferring resistance to pyrimethamine are well known^{6–9} and are found in the Amazon basin, alleles conferring resistance to chloroquine, artemisinin, and primaquine (the only therapy that prevents vivax malaria relapse) in *P. vivax* remain obscure.

To improve surveillance for drug resistance and to prevent complications from treatment with inadequate therapies it would be desirable to identify molecular markers of drug resistance. However, the lack of long-term culture methods for *P. vivax* complicates the identification of resistance genes using *in vitro* evolution or genetic crosses. On the other hand, given that there is no shortage of patients with *P. vivax* malaria, population genetic studies may provide an attractive method for mapping genes involved in drug resistance. In fact genome-wide association studies were recently used to identify loci involved in *P. falciparum* artemisinin resistance.^{10–13} Although population-based approaches are theoretically possible with *P. vivax*, to utilize these techniques it would be desirable to understand the frequency of polyclonal infections, levels of population structure, and rates of parasite inbreeding in different locations as this can affect the design of population-based studies.

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Although researchers have sought to address such questions about *P. vivax* populations using microsatellite markers and *P. vivax* DNA derived from patient infections, microsatellite markers are genetically less stable than single nucleotide variants (SNVs) and may provide overestimates of genetic diversity. Conversely, reliance on too few microsatellite markers may underestimate diversity, especially in closely related parasites.¹⁴ Here we use next-generation sequencing (NGS) to analyze the genomes of *P. vivax* parasites collected from patients in an isolated mining camp in Peru, a country that has experienced a resurgence in malaria cases over the last half a century following near elimination.^{15,16} Even with a small sample size we find evidence of intrainfection polyclonality, interinfection clonality, and inbreeding, all factors that could affect sample size calculations in genetic mapping studies. Despite a relatively homogeneous population, we nevertheless find evidence of recent evolution in known drug-resistance genes, indicating that the direct examination of genome sequences and candidate drug-resistance genes may provide insight into the genetic basis of drug resistance in this parasite.

RESULTS AND DISCUSSION

Whole Genome Sequencing Identifies Polyclonality in the Madre de Dios Region. To investigate parasite genetic diversity and clonality in the Peruvian Amazon, 10 human whole blood samples (Mdio01, Mdio03–11) were collected from *P. vivax*-infected patients presenting to a local health post in the Madre de Dios region of Southeastern Peru (Table S1). Blood was drawn from patients and filtered with CF11 cellulose powder (Whatman) to remove human white cells before DNA isolation. Microsatellite analysis was first performed at five markers to establish a baseline understanding of the clonality for each sample (Table 1), and then whole genome sequencing

Table 1. Microsatellite Repeat Size for Five Markers (bp)^a

sample	collection date	microsatellite marker				
		14.185	4.2771	6.34	12.335	7.67
Mdio01	May 09, 2012	283	85	160	170	101
Mdio03	April 21, 2012	286	80	160	164	103
Mdio04	April 26, 2012	283	85/96	160	162	101
Mdio05	April 21, 2012	270	85	160	170	101
Mdio06	May 11, 2012	283	85/98	158	160	101
Mdio07	April 23, 2012	286	98	162	N/A	101
Mdio08	May 02, 2012	283	85/98	160	160	103
Mdio09	May 14, 2012	283	80/95	134	170	99
Mdio10	May 07, 2012	270	85	158	162	101
Mdio11	April 19 2012	283	85	134	170	103

^aAlleles for each microsatellite loci are reported as PCR product size. Three to four unique genotypes are identified at each marker. All haplotypes observed are unique and nonoverlapping. bp, base pairs; N/A, not available.

was performed to further investigate the composition of the samples. Illumina paired-end sequencing yielded a mean coverage of 10.7 \times per sample with an average of 29.2% of the reads aligning to the *P. vivax* SalI reference genome. Because of the poor coverage in four of the samples, they were not included in further whole genome analysis. The six samples (Mdio03, 05, 06, 08, 10, and 11) used for subsequent analysis had a mean coverage of 16.6 \times (Table 2).

All 10 samples were used for microsatellite analysis, and 4 of these samples (Mdio04, 6, 8, and 9) had multiple microsatellite

Table 2. Whole-Genome Resequencing Statistics for 10 Patient Whole Blood Samples Analyzed^a

sample	total reads (millions)	mean coverage (\bar{x})	% reads aligned to <i>P. vivax</i> SalI	% bases covered by 5 or more reads	% bases covered by 10 or more reads
Mdio01	22.1	6.35	25.7	6.8	4.7
Mdio03	18.5	8.23	19.9	50	24
Mdio04	13.8	4.24	20.4	9.3	1.5
Mdio05	14.2	22.3	49.0	76	65
Mdio06	14.7	28.1	55.4	78	70
Mdio07	13.3	4.23	20.7	9.4	1.3
Mdio08	13.5	10.6	30.3	62	36
Mdio09	6.72	1.74	18.3	1.0	0.5
Mdio10	11.5	16.1	43.5	73	57
Mdio11	38.0	14.4	21.7	62	35
average	15.8	10.7	29.2	39	27

^aFour samples (Mdio01, Mdio04, Mdio07, and Mdio09) were excluded from genotyping studies because the *P. vivax* read coverage was low. This is most likely due to high levels of contaminating human DNA or in the case of Mdio01, contamination with *P. falciparum* DNA.

repeat sizes suggesting preliminary evidence of polyclonality in these infections (Table 1). Polyclonal infections could arise from mosquito injection of sibling parasites derived from a single meiosis into a person, from “superinfection” from multiple infectious mosquito bites, with each delivering subsequent genetically unrelated parasites, or from a combination of a relapse and a new infection. To gain further insight into the cause of polyclonality in these samples we genotyped each strain at 14 651 polymorphic sites that were confidently called in all samples (Methods, Table S2). Of these, a mean of 11 665 sites were polymorphic per sample (Table 3).

Within a given sample, many polymorphic sites had mapped reads bearing two different alleles (mixed reads, designated the major and minor alleles based on read frequency), suggesting that the infection consisted of more than one parasite clone. The NGS data showed that all samples carried mixed-allele polymorphisms, ranging from 6% of genotyped sites in Mdio3 to 28% of the sites in Mdio10, compared to the microsatellite data, which showed that only two of the six high-coverage samples were polyclonal. We additionally calculated the within-host diversity metric, F_{ws} ^{17,18} an indicator of the multiplicity of infection (MOI) that considers the relative proportions of clones within a sample as well as the genetic divergence of the sample from the local parasite population. This metric is more robust to locus-specific artifacts that may occur using microsatellite analysis to calculate MOI. The F_{ws} for the Madre de Dios isolates was between 0.46 and 0.83 (Table 3), where values ≤ 0.7 are considered highly diverse. We previously observed F_{ws} metrics between 0.97 and 0.94 for monoclonal *P. vivax* relapse infections.¹⁴ Thus, these data suggest that the Madre de Dios infections may be polyclonal, particularly Mdio10, which has the lowest F_{ws} and the greatest number of polymorphic sites in the genotyped set.

Madre de Dios *P. vivax* Genomes Are Composed of DNA from Three Hypothetical Haplotypes. Previous population studies of *P. vivax*, specifically those in the Peruvian Amazon, have revealed substantial genetic diversity among parasite populations with the vast majority of every infection examined appearing to be composed of a different haplotype using tandem repeat polymorphic markers.^{19–21} Indeed our

Table 3. Genotyping Summary^a

strain	Mdio03	Mdio05	Mdio06	Mdio08	Mdio10	Mdio11
genotyped	32 199	32 199	32 199	32 199	32 199	32 199
SNVs covered >5 reads for major allele	21 139	30 915	31 424	25 487	29 887	26 041
SNVs passing filtering criteria with unmapped contigs	15 380	15 380	15 380	15 380	15 380	15 380
genotyped sites mapping to 14 chromosomes	14 651	14 651	14 651	14 651	14 651	14 651
polymorphic sites						
single allele SNVs	8494	10 989	11 240	11 023	8868	9360
mixed allele SNVs	1016	1119	1141	1319	4132	1286
F_{ws}	0.770	0.827	0.818	0.829	0.464	0.781
SNVs in coding regions ^b						
nonsynonymous	2465	3272	3360	3274	2617	2371
synonymous	1718	1952	2298	2264	1830	1718
unique SNVs	7	2	3	1	1	0

^aSingle-nucleotide variants (SNVs) were called in the six samples with the highest mean read coverage. There were 14 651 sites in all 6 samples where a definitive base call could be made and mapped to one of the 14 chromosomal contigs. ^bMixed-allele sites are excluded from the analysis.

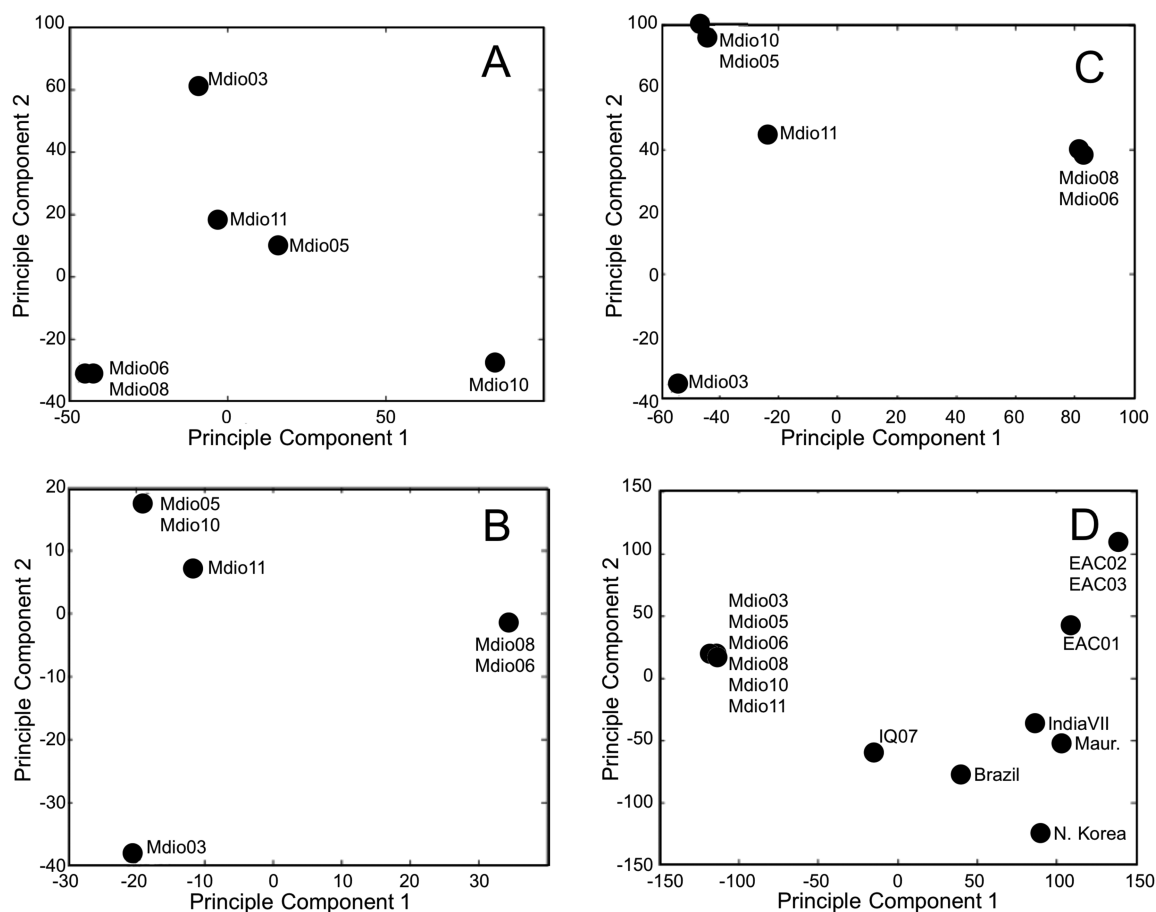


Figure 1. Principal component analysis of *P. vivax* genome sequences. (A) PCA of Madre de Dios genome sequences with all alleles in polyclonal sites included (14 651 sites). (B) PCA of genotypes excluding polyclonal sites (9499 sites). (C) PCA of major alleles with polyclonal sites included (14 651 sites). (D) PCA of Madre de Dios samples combined with worldwide isolates.^{14,22,23} EAC01, 02, 03 are from East Africa. Data is from the short read sequence archive, EAC01/03 SRA057904; North Korea I, SRP000316; Mauritania I, SRP000493; Brazil I, SRP007883; India VII, SRP007923; and IQ07, SRP003406.

microsatellite analysis using just four markers (excluding 4.2771 which was polyclonal) showed a unique haplotype for each of the samples tested in our small population. Because the transmission of malaria in Madre de Dios is not expected to be high, we would expect the parasites genotyped here to be more closely related. Therefore, we assessed the relatedness of the parasites comprising each infection. Principle component analysis (PCA) using the 14 651 genotyped sites showed that

the Mdio06 and Mdio08 infections were nearly identical (Figure 1A). Furthermore, although Mdio05 and Mdio10 are somewhat unrelated when considering all alleles, they are nearly indistinguishable if polyclonal sites are excluded (Figure 1B) or if only the major allele is considered for the PCA (Figure 1C). The data suggest the clones present at the highest frequency in the Mdio05 and 10 infections are nearly identical but that the minor allele clones are different. When comparing the Madre

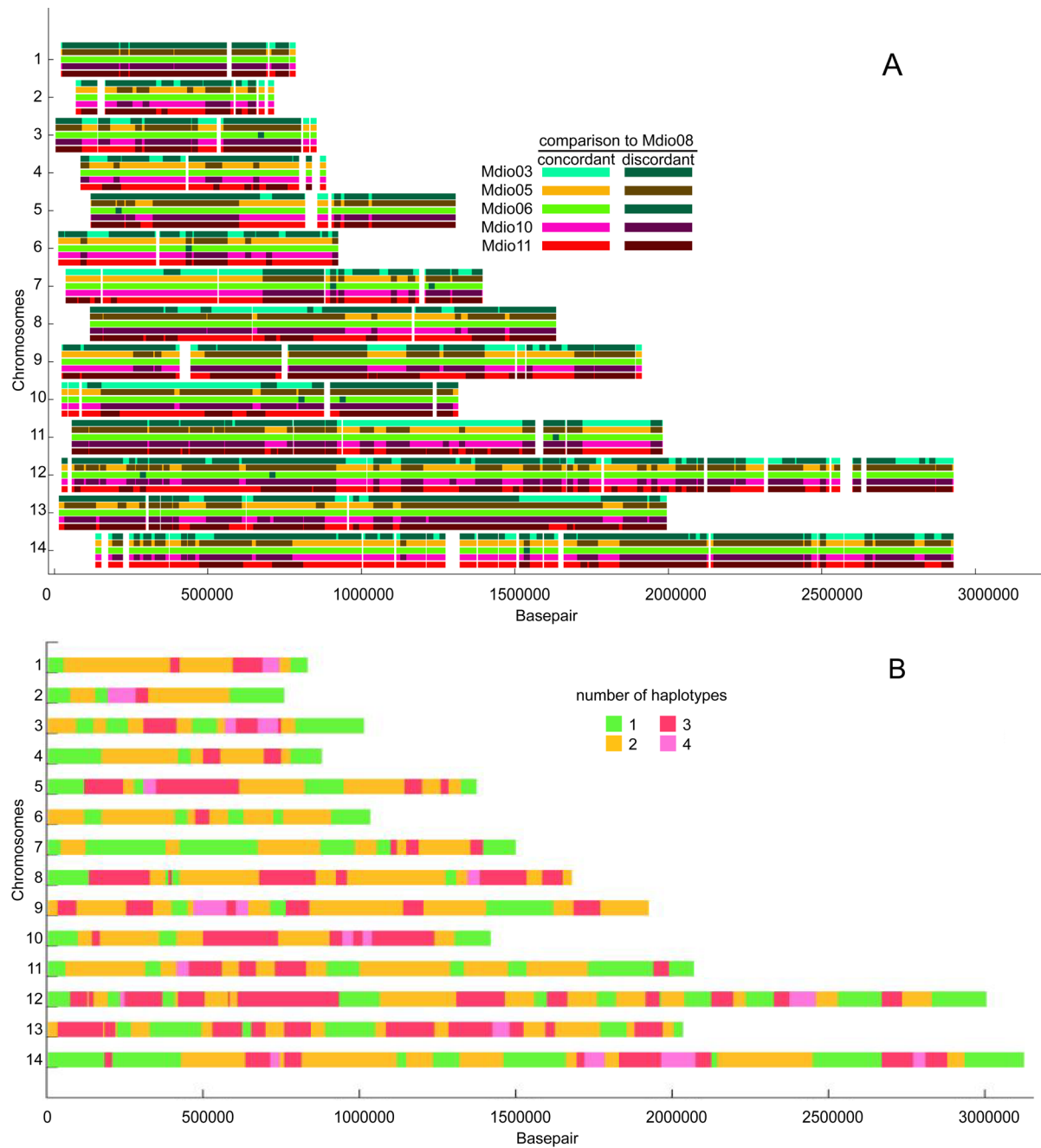


Figure 2. Similarity of the Madre de Dios isolates over the entire genome. (A) Pairwise comparisons showing regions of identity and the difference between isolates Mdio03, 05, 06, 10, and 11 compared to Mdio08 were conducted using the 9499 sites where no second allele was observed. Light colors represent the conservation of sequence; dark shading represents mismatches. (B) Number of haplotypes calculated for each region of the chromosome assessed by calculating a pairwise distance matrix for 50 000 kb overlapping windows.

de Dios population with other global *P. vivax* isolates (from Brazil, India, North Korea, Mauritania,²² Peru,²³ and East Africa¹⁴), PCA showed that all six Madre de Dios samples were very closely related, even when considering Brazil I and IQ07, both *P. vivax* isolates from the Amazon basin (Figure 1D).

Given the similarity of the population as a whole, we further sought to determine if the parasites would share chromosomal regions containing identical alleles, interspersed with unrelated regions, suggestive of sexual recombination. We performed pairwise comparisons of allele composition along each chromosome using the set of 9499 SNVs with no minor allele (Figure 2A). These data supported the PCA clustering data

showing that Mdio06 and Mdio08 and Mdio05 and Mdio10 were closely related. Additionally, of the 5071 sites that were identical within the set of 6 sequences (and the nonancestral allele), most were spatially clustered along the chromosomes, indicating linkage. For example, Mdio03, 05, 10, and 11 are virtually identical to one another on chromosome 1: Mdio10 and Mdio11 differ at only 10 of 449 genotyped sites in contrast to Mdio08 and Mdio11, which differ at 231 of the 449 sites. In contrast, Mdio08 shares the same haplotype with Mdio06, Mdio05, and Mdio11 in parts of chromosomes 4 and 6.

The data also show evidence of meiotic recombination among the haplotypes as was previously shown.¹⁴ To determine

Table 4. Coding Changes Relative to the *SaII* Reference Sequence for Known Drug-Resistance Genes^a

Chr	Pos.	AA	ref	03	05	06	08	10	11	Br	IQ07	IndVII	Mau	NK
<i>P. vivax</i> Bifunctional Dihydrofolate Reductase-Thymidylate Synthase (<i>pvdhfr</i> , PVX_089950)														
5	964763	S58R	C	A	A	G	G	A	A	A	G	G	C	C
5	964796	Y69	T	C	C	C	C	C	C	C	C	T	T	T
5	964856	G89	T	T/G	T/G	T/G	T/G	T	T	G	G	G	G	G
5	964939	S117N	G	A	A	A	A	A	A	A	A	A	G	G
5	965106	I173L	A	A	C	A	A	C/A	A	C	A	A	A	A
5	966035	E482D	A	A/T	A	A	A	A	A	A	A	A	A	A
5	966144	L519	C	C	C	C	C	C	C	A	A	A	A	A
<i>P. vivax</i> Multidrug Resistance Gene 1 (<i>pvmdr1</i> , PVX_080100)														
10	363223	T958M	G	A	A	A	A	A	A	A	A	A	A	A
<i>P. vivax</i> Multidrug Resistance Associated Protein 1 (<i>pvmp1</i> , PVX_124085)														
14	2043012	S1689L	G	A/G	G/A	G	G	G	G	G	G	G	G	G
14	2043021	T1686K	G	T	G/T	G/T	G	G	G/T	G	G	G	G	G
14	2043859	Q1407E	G	C	C	C	C	C	G	C	G	C	C	C
14	2044798	A1094S	C	C	A	A	A	A	C	A	C	C	C	C
14	2045050	V1010M	C	T	T	T	T	T	T	T	T	T	T	T
14	2046126	V651G	A	A	A	A/C	A	C/A	A	A	A	A	A	A
14	2046219	R620Q	C	C	T	C	C	T	C	C	C	C	C	C
14	2047233	R282M	C	A	A	A	A	A	A	A	A	A	A	A
14	2047816	E88Q	C	G	G	C	C	G	C	C	G	C	G	C
Chloroquine Resistance Transporter Ortholog (<i>pvcr-t</i> , PVX_087980)														
1	330262	5' UTR	T	C	C	C	C	C	C	T	T	Y	C	C
1	331151	intron	T	C	C	C	C	C	C	C	C	C	C	C
1	331819	intron	G	G	G	A	A	G	G	G	G	G	G	G
1	332453	intron	T	C	C	C	C	C	C	C	C	C	C	C
1	332874	intron	A	C	C	C	C	C	C	C	C	C	C	C
1	333469	intron	G	G/A	G	G/A	G/A	G	G	G	G	G	G	G
1	333473	intron	G	G	G	G/T	G/T	T	T	G	G	G	G	G
1	333518	intron	A	G	G	A	A	G	G	A	G	G	G	A
1	333544	intron	T	C	C	C	C	C	C	T	C	C	C	T
1	334324	intron	A	A/T	A	T	A/T	A/T	A/T	A	A	T	A	A

^aMutations in Madre de Dios isolates identified in the 14 651 confidently called set and mutations identified in Brazil I (Br), India VII (IndVII), Mauritania I (Mau), and North Korea (NK) isolates identified from plasmodb.com were included. No single-nucleotide variants leading to coding changes were observed for *cytochrome bc1* (NC_007243), dihydropteroate synthetase (PVX_123230), gtp cyclohydrolase (PVX_123830), chloroquine resistance transporter ortholog (PVX_087980), or kelch 13 (PVX_083080) orthologue. Y, pyrimidine.

the number of founding parasites that may have contributed to the Madre de Dios breeding populations, we examined blocks of co-inherited markers (Methods). We found that 8.2 million bases (26% of the genome) were in regions that were identical across all isolates (a single haplotype). On the other hand, 43 and 25% of the genome (Figure 2B) were composed of two and three haplotypes, respectively. The remaining 6% of the genome contained four or five haplotypes. Six haplotypes (the maximum number that can be observed) were never observed. The isolated regions with more than three haplotypes typically spanned likely recombination junctions, were in regions containing known polymorphic genes, or were in genomic regions bearing signatures of a possible genome amplification event (see below). For instance, of the 10 allelic differences between Mdio10 and Mdio11 on chromosome 1, 3 are in a polymorphic gene (PVX_093645, bp 717 749 to 728 479) that contains 12 coding and noncoding substitutions. Mdio03 had the most SNVs that were present in only one strain (unique SNVs, Table 3), and it had only seven. These data suggest that two to three parasites contributed to a recombining parasite population in Madre de Dios and that differences observed within these parental genotypes are due to intrinsic mutation rates.

Exploration of Orthologs of Known Drug-Resistance Genes and Identification of Potential Novel Resistance-Associated Genes. We also examined orthologs of known drug-resistance genes in *P. falciparum* to determine if any of these genes in *P. vivax* showed evidence of selection. All *P. vivax* malaria patients are currently treated with primaquine and chloroquine in Peru (CQ 25 mg/kg, PQ 0.5 mg/kg for 7 days),²⁴ although chloroquine resistance is found in the Amazon region and contributes to treatment failure in some countries.^{25–27} The genes involved in chloroquine resistance in *P. vivax* are unknown, but good candidates include the orthologs of the *P. falciparum* genes that are involved in resistance, the chloroquine resistance transporter (*pvcr-t*, PVX_087980), and the multidrug resistance transporter (*pvmdr1*, PVX_080100) (Table 4). *Pvmdr1* (T958M) bore a single variant relative to the *SaII* reference sequence; this same allele change has arisen, presumably independently in Thailand²⁸ and in Madagascar.²⁹ We found no coding changes in *pvcr-t*, although no exonic coding changes in *pvcr-t* have been observed even in parasites resistant to chloroquine.²⁹ Nevertheless, we did find six intronic changes and one mutation in 5' UTR, placing it in the top 1% of genes with intronic changes (Table 4). Interestingly, increased expression of *pvcr-t* in

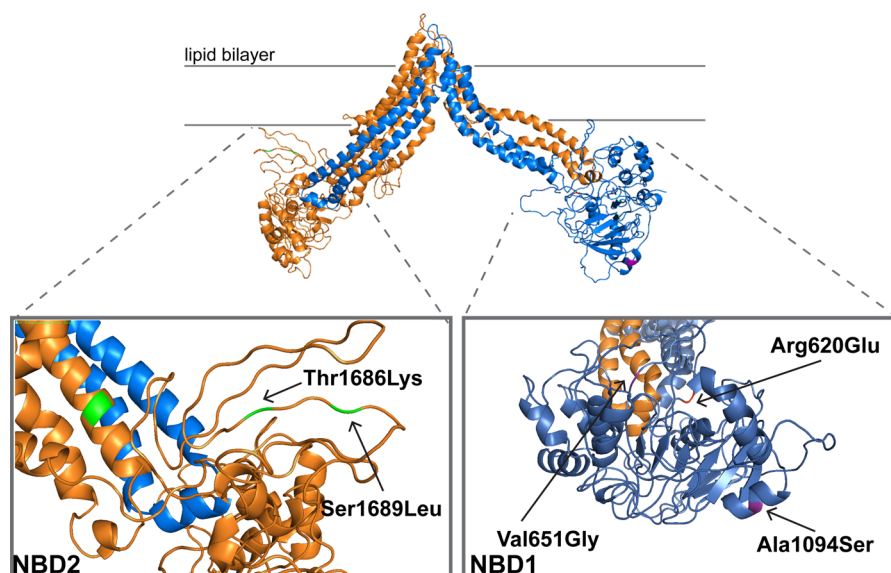


Figure 3. Homology model of PvMRP1 using *C. elegans* PGP-1 as a template. This ABC transporter protein is located in the parasite plasma membrane and has two nucleotide binding domains (NBDs) conserved between the two proteins. The locations of SNVs identified in NBD1 and NBD2 are shown in the insets. SNVs identified but not shown are in transmembrane domains.

chloroquine-resistant parasites from the Amazon has been observed.³⁰

Furthermore, we found no amino acid changes in the mitochondrially encoded gene, *pviomp3*, which encodes the cytochrome bc1 complex, the target of atovaquone in *P. falciparum*.³¹ We also found no nonsynonymous mutations relative to *SalI* in *pvkelch13* (PVX_083080), the *P. vivax* ortholog of *pfkelch13*,¹⁰ a gene conferring reduced sensitivity to artemisinin in *P. falciparum* (reviewed in ref 32) presumably because ACTs are not currently used in the Amazon region.

A number of coding changes were observed in the *P. vivax* multidrug resistance associated protein (*pvmrp1*, PVX_124085), which bore nine nonsynonymous SNVs and two synonymous SNVs (Table 4). Position 1466K variants are associated with antifolate resistance in *P. falciparum*,³³ and the disruption of this gene in *P. falciparum* leads to increased primaquine sensitivity.³⁴ *Pvmrp1* was previously noted because of an unexpectedly high number of coding variants observed in another South American *P. vivax* patient isolate.²³ A homology model using the *Caenorhabditis elegans* multidrug resistance protein, PGP-1,³⁵ shows several of the mutations observed in the Madre de Dios population occurring in structurally conserved regions of the protein including the nucleic acid binding domain in the intracellular space (Figure 3).

Although antifolates are no longer recommended in Peru we found that coding mutations in the antifolate pathway were common, with all isolates carrying the S117N and S58R dihydrofolate reductase (*dhfr*) mutations, known in combination to confer high levels of resistance to pyrimethamine in *P. vivax* (Table 4).^{8,36} Notably, we found two independent S58R nucleotide transversions (C → A, C → G). Triallelic mutations are a feature shared by only 19 other genes, including the apical merozoite antigen (*ama-1*; PVX_092275) (Table S3), suggesting selection pressure on this nucleotide position. A subset (Mdio05 and Mdio10) also carried the I173L allele associated with treatment failure.³⁶ Given how different these strains are from those in other countries (Figure 1D), it is expected that these mutations arose independently and were not imported. We found there were no novel variants in *dihydropteroate*

synthetase (*dhps*; PVX_123230) or *GTP cyclohydrolase* (*gtpch*; PVX_123830), other genes in the dihydrofolate biosynthesis pathway.

It has been shown, on the basis of mapping study experiences with chloroquine- and pyrimethamine-resistant *P. falciparum* field isolates, that patterns of linkage disequilibrium could be used to detect genes conferring drug resistance.^{37,38} These studies have indicated that genes involved in antifolate and chloroquine resistance have arisen infrequently and have likely been transmitted through populations by selective sweeps.^{37,38} In contrast to what was observed for *P. falciparum*, the region around *dhfr* on chromosome 5 (*pvdhfr*, bp 964 590 to 966 464) did not show strong evidence of linkage disequilibrium within the Madre de Dios parasite genomes (an 80 kb surrounding region showing two haplotypes is displayed in Figure 4), suggesting that mutant *pvdhfr* has arisen more than once.

Evolution of Other Genes. To further identify genes under selection and potential genes not known to be involved in drug resistance we identified genes with a high nonsynonymous substitution to synonymous substitution ratio (K_a/K_s). Of the top 20 genes with high K_a/K_s , 18 are hypothetical, reflecting the lack of information about the interaction of *P. vivax* with the human immune system. Those genes with functional annotations typically played possible roles in immune evasion, including members of the *msp3* multigene family (Table S4), as well as PVX_092625 which encodes an uncharacterized protein, the ortholog of a possible *P. falciparum* vaccine candidate, predicted to be surface expressed.³⁹ No statistically significant K_a/K_s ratios were observed for orthologs of known *P. falciparum* drug-resistance genes (Table S5). The majority of genes (3773 out of 5507 queried), on the other hand, had no nonsynonymous variants in any of the isolates, reflecting the overall lack of diversity in the population.

Genome Regions around *pvmrp1* and Genes of the Folate Biosynthetic Pathway Show Evidence of Gene Amplification. Changes in the gene copy number have been shown to or are predicted to confer drug resistance in laboratory-evolved *P. falciparum* clones⁴⁰ and *P. falciparum* field isolates⁴¹ and are relatively common. We thus looked for

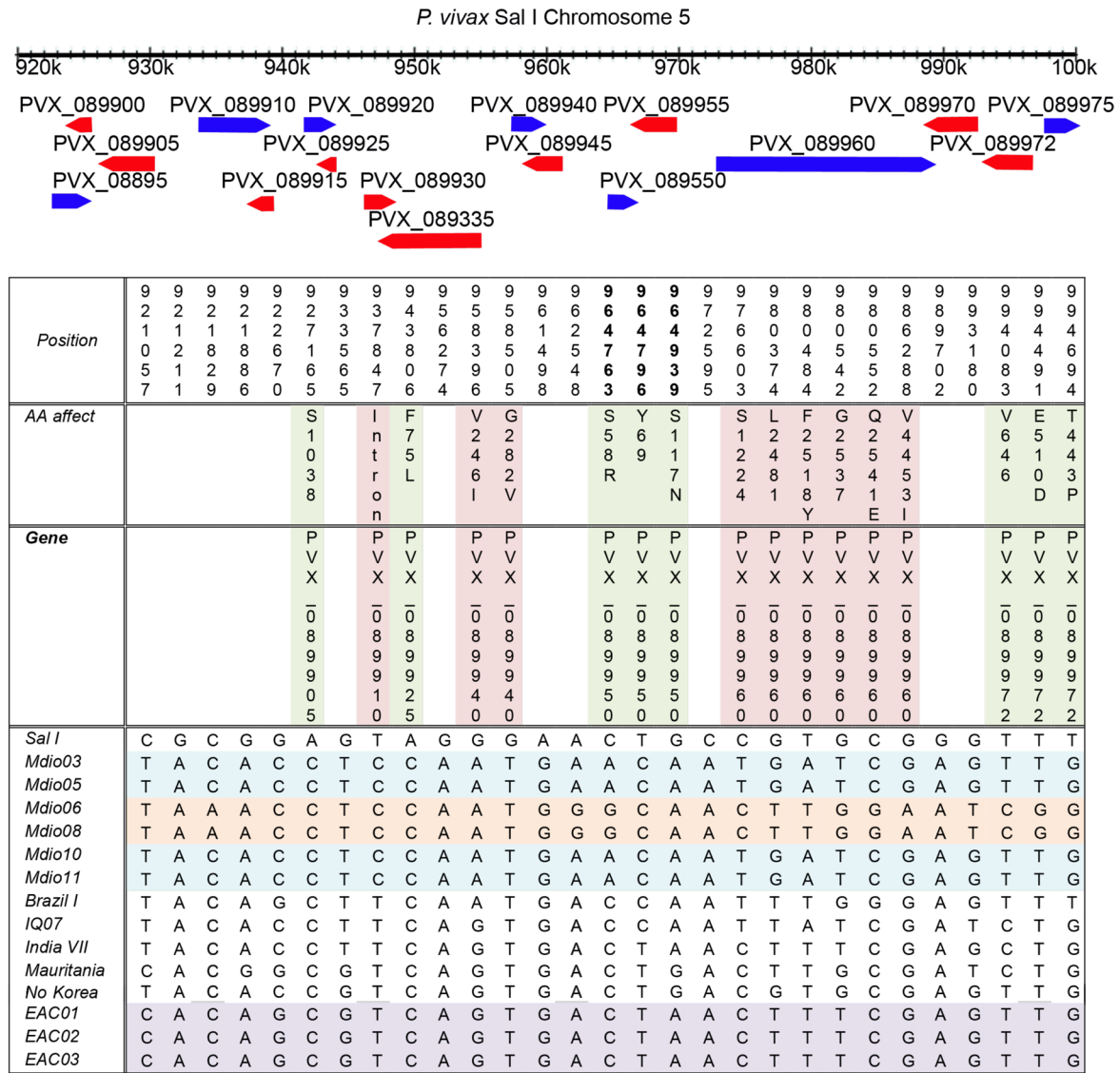


Figure 4. Chromosome 5 alleles surrounding *P. vivax* *dhfr*. Genotyped sites with no minor alleles in the Madre de Dios set were used to avoid possible phasing errors. Positions in bold are *dhfr*. Red and green alternate shading denote open reading frames. Blue, orange, and purple shading indicate identical haplotypes in this 80 kb region on chromosome 5.

potential increased read coverage across the genome in the Madre de Dios *P. vivax* sequences using an automated CNV-calling algorithm.⁴² On average, 2.8% of the genome was potentially amplified and 1.5% showed evidence of a deletion event (Figure 5A). Deleted regions typically encoded members of multigene families. For example, a 100 kb region on chromosome 12 encodes members of the PfamD family, early transcribed membrane proteins, and exported proteins. An ~200 kb region on chromosome 3 was deleted in Mdio5, Mdio6, and Mdio10 and encoded ~15 VIR proteins, 7 plasmodium exported proteins, a PfamD family protein member, and an early transcribed member protein as well as “hypothetical” proteins.

In contrast to what is found in *P. falciparum*^{43,44} and *P. vivax* in Thailand⁴⁵ we found little evidence of increased read coverage near *pvmr1* on chromosome 10 (361 701–366 095) nor could we find evidence of an amplification event near *pvcrt-0* (chr1:330 260 to 334 540). On the other hand, the region around *pvmrp1* (chr14:2 042 042–2 048 077) in Mdio06, Mdio08, and Mdio05 has increased read coverage suggestive

of a copy number variant in that region (Figure 5B). Although amplification of this gene in *P. vivax* or *P. falciparum* patient isolates have not been reported, *P. falciparum* parasites exposed to atovaquone in the laboratory also acquired a CNV that encompassed this gene,⁴⁶ providing some evidence that extra copies provide an adaptive advantage.

Our data showed that *pvdhfr* is contained within a region with significantly increased read coverage in Mdio08 (Figure 5C), and manual inspection also showed increased read coverage in Mdio06. In addition, we also found that four isolates had significantly increased read coverage around the *P. vivax* dihydrofolate synthase gene (*pvdhfs*; PVX_116635), which encodes the protein upstream of dihydrofolate reductase in the folate biosynthesis pathway and converts 7,8-dihydropterolate, and L-glutamate to 7,8-dihydrofolate through the hydrolysis of ATP. Recombination events surrounding smaller amplified regions (e.g., 2 kb) were conclusively observed upstream and downstream of *pvdhfr*, suggesting that the genome may have harbored a large 30–40 kb amplification at one time, but the amplified region may have been partially

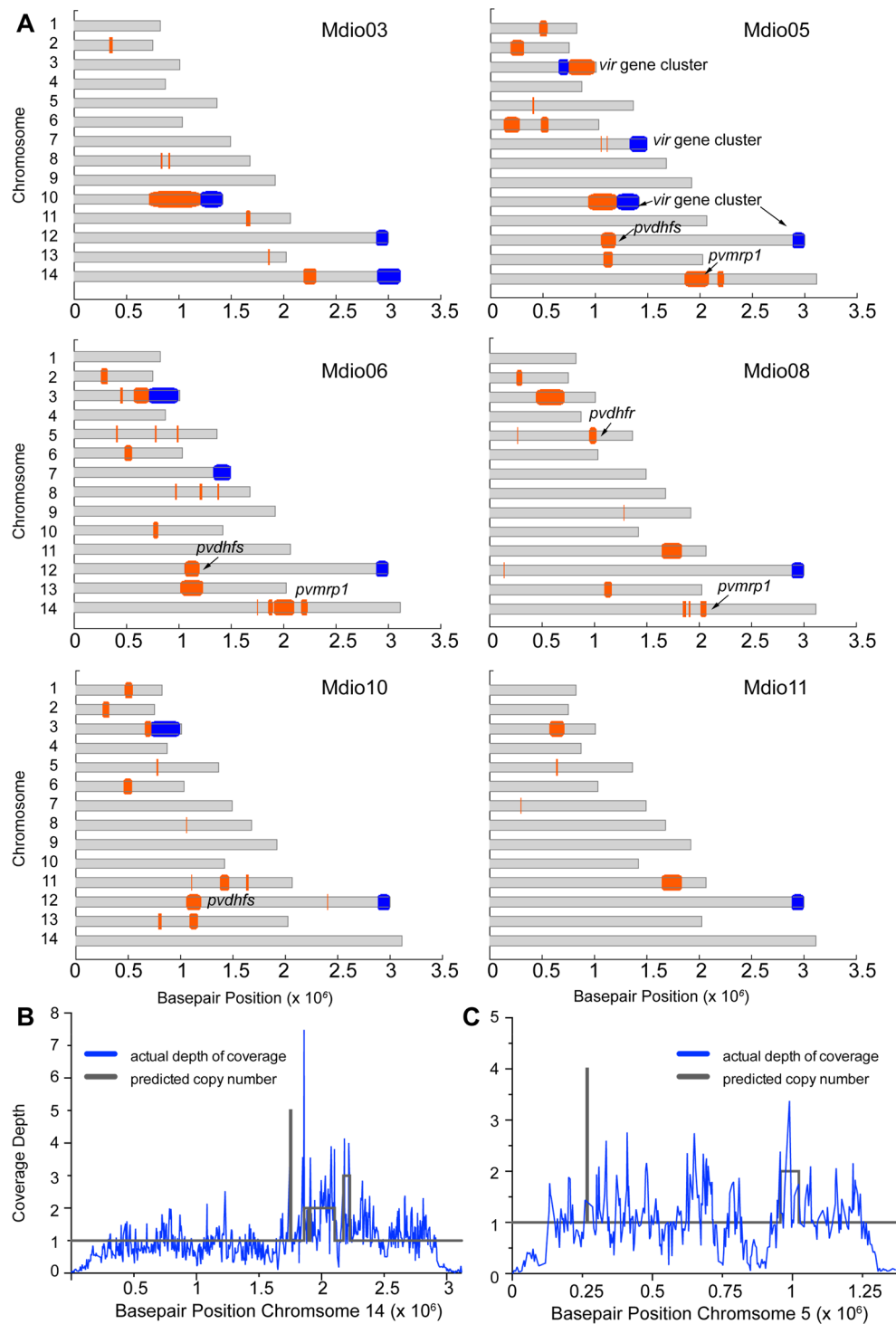


Figure 5. Copy number variants identified in Madre de Dios isolates using a systematic calling algorithm. (A) Distribution of increased (red) and decreased (blue) read coverage in all isolates. Sensitivity is based on overall read coverage for an isolate, and regions may have been missed in isolates with overall lower sequencing depth (e.g., Mdio03). (B) Normalized coverage depth and predicted copy number for Mdio06 chromosome 14 showing the amplification of *pvmrp1*. (C) Normalized coverage depth and predicted copy number for Mdio08 chromosome 5, highlighting amplification in *pvdhfr*.

lost as antifolate drug pressure was removed. Sequencing to a higher depth of coverage or the use of long read sequencing technology such as PacBio could provide more resolution. Further evidence for *pvdhfs* amplification as well as *dhfr* SNVs is suggestive of selective pressure from the past indiscriminate use of antifolates in the 1950s and 1960s.^{4,5}

Amplified regions with no clearly known drug-resistance genes were also observed (Table S6) and could represent novel resistance markers. Examples of such amplified regions include chromosome 1:473 919–541 139 bp and a number of regions on chromosome 3 that were observed in all but Mdio03. Although sequencing coverage is too low to establish exact boundaries of these events, one possibly interesting gene that

was potentially amplified in this region is PVX_000585, which is predicted to encode a homologue of the *E. coli* multidrug transporter, *EmrE*. This membrane protein exchanges small aromatic drugs such as ethidium bromide and methyl viologen (paraquat) across the membrane in *E. coli*⁴⁷ and belongs to the same transporter class as *pfcr*.⁴⁸ Orthologs are missing from most eukaryotes but are found in other apicomplexan parasites such as *Toxoplasma gondii* and *Babesia bovis* (OrthoMCL DB v.5.0). Further work will be needed to establish whether these changes are significant.

CONCLUSIONS

Our data highlight potential challenges with using genetic mapping methods to identify genes involved in antimalarial drug resistance. These include inbred populations, which can skew the true diversity of the population if diversity is measured by only a few markers; polyclonal infections, which can affect SNV calling and haplotype inference; and the appearance of de novo mutations which may or may not be associated with resistance in an otherwise homogeneous genetic background. In addition, genome amplifications, which may or may not be correctly mapped to their correct genome location, are likely to be common. All of these features may impact the study design.

On the other hand, our studies demonstrate that insight into the genetic basis of drug resistance can be gained simply by examining the genomes of parasites using high-resolution methods. This method was previously used to identify alleles associated with resistance to the antibiotic clindamycin in *P. falciparum* isolates from Peru⁴⁹ and GTP cyclohydrolase gene amplification events in many laboratory and field isolates. The GTP cyclohydrolase event was hypothesized to confer resistance to antifolate drugs because of its presence in the folate biosynthetic pathway^{50,51} and was eventually shown to help parasites survive exposure to antifolate drugs by increasing drug flux through the pathway in the presence of mutations in *pfdhfr* that cause a loss of fitness.⁵² In our study we identify known and novel mutations potentially involved in drug resistance. Relatively uncharacterized genes that could be further tested for association with resistance to currently used therapies (primaquine and chloroquine) include *pvmrp1* as well as PVX_000585, the *P. vivax* homologue of the *Escherichia coli* multidrug resistance gene, *EmrE*.

Genes involved in resistance to small molecules are now routinely found in *P. falciparum* using laboratory evolution and whole genome sequencing.⁵³ Identification of these genes allows for monitoring of the emergence of drug resistance in parasite populations. Additionally, knowledge of resistance genes can aid in the design of novel molecules that are effective at targeting the known target in the presence of these known population variants. The identification of resistance genes using laboratory evolution relies on *in vitro* culture systems and would be challenging for *P. vivax*. On the other hand, because parasites in a region such as Madre de Dios could effectively be clonal, such isolated parasite populations might offer opportunities to directly observe the effects of drug exposure on the population. Alleles that appeared in the population only after a new therapy was introduced might be readily identified in an inbred parasite population such as this. Thus, archiving parasite material before and after the introduction of a new therapy could eventually give valuable insight into the evolution of drug resistance in the field.

Our studies also have implications for the design of clinical trials aiming to test novel radical cure drugs that might replace

primaquine, the only licensed drug that can eliminate dormant *P. vivax* and *P. ovale* hypnozoites. Although initial clinical trials might be performed with travelers or soldiers returning from endemic regions, groups that would not be expected to be reinfected, larger patient studies in endemic regions could be complex—if a patient returned with an infection that was genotypically very similar to his original infection, then one might assume that the drug candidate was not effective at preventing relapse when in fact the patient had actually been reinfected. Thus, estimates of the number of parasite haplotypes circulating in a region would be needed before trials could be effectively designed. This method would be particularly suited for an area where transmission is low and thus less parasite diversity is expected. In regions where diversity is high and patients are infected with multiple strains, it may prove more difficult to observe mutations related to drug pressure.

METHODS

Sample Collection. This study was approved by the Human Subjects Protection Program of the University of California San Diego, the Institutional Review Board of NAMRU-6, Lima, Peru and the Directorate of Health, Madre de Dios, Peru. Peripheral blood samples for next-generation sequencing were collected from patients with self-reported fever and microscopy-confirmed *P. vivax* malaria. Patients presented at the Peruvian Ministry of Health facility of Bajo Puquiri, locally called Delta-1, in the Madre de Dios department, Peru. This location is approximately 8 h from the capital city of Puerto Maldonado, over difficult terrain. *P. vivax* mono-infection was later confirmed by PCR as previously described.⁵⁴ Blood was collected using EDTA-containing Vacutainer tubes. Tubes were centrifuged at 1000g to remove plasma, and the packed red blood cells were resuspended in a 2× volume of sterile phosphate-buffered saline (1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 137 mM NaCl, 2.7 nM KCl). Within 1 h of collection diluted blood was filtered through CF11 cellulose powder (Whatman) to remove white blood cells.⁵⁵ Eluted blood was pelleted by centrifugation at 1000g for 10 min and stored at −20 °C for a maximum period of 2 to 3 weeks before being shipped in batches to the NAMRU-6 laboratory in a liquid-nitrogen dry shipper.

Microsatellite Analysis. *P. vivax* microsatellite characterization was conducted using the following previously published loci: 14.185, 12.335, 7.67, 6.34, 4.2771, and 3.35.^{56,57} Microsatellites were amplified with primers previously described,⁵⁶ and fluorescently labeled PCR products were analyzed on an Applied Biosystems Prism 3130xl Avant Genetic Analyzer (Applied Biosystems). A minimum peak height of 200 fluorescent units was used to define amplification products.

Whole Genome Sequencing. Genomic DNA was isolated from frozen whole blood samples using a DNeasy blood and tissue kit (Qiagen), and whole genome paired end-sequencing libraries were created using the NEBNext DNA library prep kit (New England Biolabs). DNA was sheared using Covaris adaptive focused acoustics and size selected on an agarose gel. DNA libraries were clustered and run on an Illumina HiSeq2000 (Illumina, Inc., San Diego) according to the manufacturer's instructions. Base calls were made using Consensus Assessment of Sequence and Variation v. 1.8.2 (Illumina). Sequencing reads in fastq format are available on the National Center for Biotechnology Information's short read archive (PRJNA272364). Reads were aligned to the *P. vivax*

SaI reference (v. 10.0) using the Burrows-Wheeler aligner with the default options.^{58,59} Picard was used to remove PCR duplicates and evaluate the sequencing quality.

Single nucleotide variants (SNVs) were called simultaneously in the Madre de Dios set of 10 using the Genome Analysis Toolkit Unified Genotyper tool. Variant sites within SNV clusters (cluster window = 10, SNP cluster = 3) and with low mapping quality ($MQ < 50$) were filtered out. Additionally, variant sites with a combined depth of coverage of less than 20 reads ($DP < 20$) were excluded. A total of 32 199 high-quality SNVs were identified and genotyped in each of the 10 samples. Because we were interested in high-quality SNVs that would allow us to determine relatedness of the strains, we further filtered the variants. Overall read coverage was low in Mdio01, Mdio04, Mdio07, and Mdio09; therefore, these samples were excluded from further analysis. For the samples included, Mdio03, Mdio05, Mdio06, Mdio08, Mdio10, and Mdio11, sites were filtered if they did not have callable alleles in all of these samples or a read coverage of < 5 . Additional custom filtering was designed to retain sites where we were confident of the zygosity. Homozygous SNVs were called if $> 80\%$ of the reads were the major allele, and heterozygous SNVs were called if 50–80% of the reads represented the major allele. SNVs with more than one alternate allele were also excluded. This resulted in 15 380 confidently callable sites for determining clonality located on both contigs and chromosomes. SNVs identified in contigs were further removed to yield a high-quality set of 14 651 genotyped sites (Table 4).

Diversity and Relatedness Analyses. To characterize within-host diversity we used the F_{ws} statistic^{18,55} ($F_{ws} = 1 - (H_w/H_s)$, where H_w is the within-host heterozygosity and H_s is the population-level heterozygosity), using the 14 651 genotyped sites. The principle component analysis was performed using the Matlab (R2012b, The Mathworks) princomp command. Calculation of the ratio of the non-synonymous substitution rate to the synonymous substitution rate (K_a/K_s) was done using the modified Yang–Neilsen algorithm.⁶⁰ Haplotype blocks were calculated using a set of 4430 SNVs with no minor allele and which were variant in one Madre de Dios isolate relative to the others. The number of haplotypes present was calculated for 50 kb windows (moving over each chromosome in 5 kb steps) by calculating the pairwise distance matrix for all sample pairs within the window. The pairwise distance estimate, d , was calculated as $d = n_d/n_c$, where n_d is the number of nonidentical sites and n_c is the number of sites compared. Values for the distance matrix that were less than 5×10^{-5} were considered to be similar haplotype blocks between samples.

Pairwise Comparisons. Pairwise comparisons were completed using either all 15 380 genotyped sites or sites where no minor allele was shared (9571 sites left). Chromosome maps were made to represent whether alternate alleles were shared between samples.

Homology Modeling. Amino acid sequences of PvMRP1 were retrieved from PlasmoDB (search ID: PVX_124085) and were cross referenced with Uniprot. A template search was conducted against the SWISS-MODEL template library, with the highest scoring template being chosen for model building. A web-based homology model was produced using the SWISS-MODEL pipeline which operates via Promod-II and Modeler platforms. The PyMOL Molecular Graphics System (version 1.3, 2010 Schrodinger, LLC.) was then used to display and label the protein structure.

CNV Analysis. Plasmodium Type Uncovering Software (Platypus)⁶¹ was used to identify regions of the genome where a significant increase or decrease in read coverage was observed. This software sensitively detects the largest changes in read signal after GC bias normalization. Changes were also observed for the various unassembled contigs (data not shown).

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00049.

Demographic characteristics of the sample population. Tri-allelic SNVs observed in the Madre de Dios population. Top 20 genes with the largest K_a/K_s ratios. K_a/K_s ratios for *P. vivax* orthologs of known *P. falciparum* drug-resistance genes. (PDF)

Genomic regions with significantly increased or decreased read coverage identified using paired-end NGS data. (XLSX)

Single nucleotide variants identified in each of the Madre de Dios whole genome sequencing samples. (XLSX)

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Author Contributions

E.A.W. and E.L.F. wrote the manuscript. E.A.W., E.L.F., A.T.B., F.G., V.C., M.A., A.A., V.B., and T.W. analyzed the data. A.T.B. prepared samples for genomic sequencing. J.F.S., M.L.S., G.C.B., K.A.E., and L.A.R. performed sample collection and microsatellite analysis. A.G.L. collected the samples and analyzed the clinical data. J.M.V. analyzed the clinical data and genetic phenotypes. All authors edited the manuscript.

Notes

The authors declare no competing financial interest.

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Supporting Information for the manuscript titled:
Next generation sequencing of Plasmodium vivax patient samples shows evidence of direct evolution in drug resistance genes

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Supplementary Tables

Demographic Characteristic	Value
Mean age	38 years
Sex	9M:1F
Previous malarial history	60%
Signs of serious infection	30% difficulty breathing
Fever present	50% > 38.5°C
Mean parasitemia	0.45%

Table S1. Demographic characteristics of the sample population, with units separate for each row. 10 patients were analyzed, with mean patient age and sex as well as previous malarial history and current clinical correlate listed.

Table S2. provided separately as excel file.

Gene	Ref	Alt	AA	Description
PVX_087665	T	A,C	D240E/ D240	hypothetical protein
PVX_085790	C	A/T	A306E/ A306V	Sperm-specific protein Don juan, putative
PVX_086125	C	A,T	P439T/P 439S	hypothetical protein, conserved
PVX_086250	C	A,G	L116M/ L116V	hypothetical protein, conserved
PVX_079910	A	G,T	V3032E/ V3032A	hypothetical protein, conserved
PVX_080525	T	C,G	S736R/S 763G	asparagine-tRNA ligase, putative
PVX_098025	G	A,C	S2340N/ S2340T	DNA-directed RNA polymerase I 190 kDa polypeptide
PVX_097695	C	G,T	G668E/ G668A	merozoite surface protein 3 alpha (MSP3a), putative
PVX_097680	G	A,T	N885K/ N885	merozoite surface protein 3 beta (MSP3b)
PVX_091820	A	C,T	C1407S/ C1407G	hypothetical protein, conserved
PVX_092275	G	A,C	R112K/ R112T	apical merozoite antigen 1
PVX_092630	T	C,G	N977H/ N977D	hypothetical protein, conserved
PVX_123760	T	A,C	D2961E/ D2961	hypothetical protein
PVX_124070	G	C,T	Q2494K /Q2494E	WD domain, G-beta repeat domain containing protein
PVX_003820	A	C,G	N813T/ N813S	serine-repeat antigen 4 (SERA), putative
PVX_111370	T	A,G	R1422W /R1422R	hypothetical protein
PVX_089950	C	A,G	S58R/S5 8R	bifunctional dihydrofolate reductase-thymidylate synthase 1
PVX_005057	A	C,T	H313P/ H313L	Vir gene

Table S3. Tri-allelic SNVs observed in the Madre de Dios population. Genes with more than two alleles coding for nonsynonymous changes were identified and are noted. Notes: REF, *P. vivax* Sall reference; Alt, alternate allele calls; AA, amino acid substitution.

Gene	Function	Ka/Ks	P-Value	SYN	NON-SYN
PVX_115420	conserved hypothetical protein	8.46	0.021	1	9
PVX_081820	conserved hypothetical protein	4.95	0.025	2	11
PVX_086195	conserved hypothetical protein	4.79	0.143	1	7
PVX_097685	merozoite surface protein 3 (MSP3)	4.38	0.202	1	13
PVX_118020	conserved hypothetical protein	4.25	0.162	1	8
PVX_087140	hypothetical protein	3.88	0.216	1	5
PVX_096325	conserved hypothetical protein	3.62	0.278	1	9
PVX_086275	conserved hypothetical protein	3.59	0.274	1	8
PVX_091745	conserved hypothetical protein	3.56	0.040	3	12
PVX_084725	conserved hypothetical protein	3.35	0.082	2	18
PVX_092625	hypothetical protein	3.26	0.419	1	7
PVX_086135	hypothetical protein	3.13	0.416	1	7
PVX_084175	hypothetical protein, conserved	3.10	0.436	1	9
PVX_090240	hypothetical protein, conserved	2.90	0.232	1	9
PVX_115350	hypothetical protein, conserved	2.89	0.217	2	9
PVX_091085	hypothetical protein, conserved	2.88	0.357	1	4
PVX_084360	Tat binding protein 1(TBP-1)-interacting protein, putative	2.83	0.399	1	6
PVX_092265	hypothetical protein, conserved	2.79	0.419	1	8
PVX_091530	hypothetical protein, conserved	2.73	0.625	1	5
PVX_124090	hypothetical protein, conserved	2.69	0.425	1	9

Table S4. Top 20 genes with the largest Ka/Ks ratios. Calculation of substitution rates was done using the modified version of the Yang-Neilsen algorithm. SYN, number of synonymous changes; NON-SYN, number of non-synonymous changes.

Gene ID	Ka/Ks	P-value	SYN	NON-SYN	annotation
PVX_080100	NA	NA	0	1	multidrug resistance protein (<i>mdr1</i>)
PVX_089950	0.373	0.248	3	4	bifunctional dihydrofolate reductase-thymidylate synthase (<i>dhfr</i>)
PVX_124085	1.02	0.933	2	9	multidrug resistance associated protein (<i>mrp1</i>)
PVX_116635	0.329	0.230	1	1	dihydrofolate synthase/folylpolyglutamate synthase (<i>dhfs</i>)
PVX_083080	0	NA	7	0	kelch protein

Table S5. Ka/Ks ratios for *P. vivax* orthologs of known *P. falciparum* drug resistance genes. Calculation of substitution rates was done using the modified version of the Yang-Neilsen algorithm. SYN, number of synonymous changes; NON-SYN, number of non-synonymous changes.

Table S6. provided separately as excel file.