Analysis of Genetic Variability of Plasmodium vivax Isolates from Different Brazilian Amazon Areas Using Tandem Repeats

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Abstract. Few genetic markers have been described to analyze populations of Plasmodium vivax. The genetic variability of P. vivax has been analyzed mainly among isolates taken from areas ranging from hyper- to holoendemic areas. These studies of genetic variability have neglected many areas with different epidemiologic profiles. The purpose of this study was to analyze the genetic variability of P. vivax isolates from four different Brazilian Amazon areas. We chose to study the five most polymorphic tandem repeats (TRs) identified so far. All TRs studied were polymorphic in at least one studied population, with a modal allele at nearly all loci. Expected heterozygosity ranged from 0.462 to 0.666 and did not correlate with the repeat array length. The genetic distances among the populations varied from 0.027 to 0.241, and did not correlate with their geographic separation. Tandem repeats identified in P. vivax isolates failed to allow geographic clustering.

INTRODUCTION

Malaria is a public health problem in over 100 countries; it causes at least 300 million cases of acute illness, and it kills more than one million people every year. Plasmodium vivax is the most widespread malaria species, and up to 40% of the world population is at risk of infection from this species. Currently, malaria caused by P. vivax is endemic in many countries of Asia, the South Pacific, North Africa, the Middle East, and South and Central America. In Brazil, 540,000 new cases of malaria were reported in the year 2006. Nearly all of them (99.5%) occurred in the Amazon states, where P. vivax was responsible for more than 70% of cases. Despite a lower mortality than Plasmodium falciparum infection, P. vivax is responsible for intense morbidity and significant economic losses. Parasite control strategies depend on an understanding of the genetic variability and population structure of the parasite and the dynamics of disease transmission. Moreover, studies of genetic variability, particularly of field isolates collected directly from patients, can provide markers to predict drug resistance, virulence, and periodic relapse. Several studies have assessed the genetic variability, population structure, and evolution of P. falciparum. In contrast, the lower virulence of P. vivax and the difficulty of propagating it continuously in culture mean that less is known about its genetic variability. Furthermore, few studies have analyzed the genetic diversity among P. vivax populations, which could lead to inferences about its population dynamics and patterns of gene flow.

Molecular markers for population studies of P. vivax have mostly been based on orthologs of P. falciparum antigen genes, such as merozoite surface antigen, erythrocyte-binding proteins, and circumsporozoite protein, which are under selection by host immunity. However, some population parameters are better inferred from neutral or nearly neutral markers, including microsatellite loci. In contrast with the ~1,000 polymorphic microsatellites described for P. falciparum, only 40 or so have been described for P. vivax. Microsatellite variability in P. vivax is strongly dependent on the repeat array length: short arrays exhibit little variation and long arrays are highly polymorphic. When repeat array length is taken into account, P. vivax microsatellites show levels of variation comparable to those in P. falciparum. However, the geographic clustering of P. vivax isolates was not detected using microsatellite loci. This may be a result of high within-population heterogeneity or to the complex phylogeny of P. vivax.

On the other hand, Feng and colleagues described 33 polymorphic tandem repeats (TRs) or minisatellites containing repeating units of five nucleotides or more. Leclerc and colleagues showed a limited variability among isolates from different areas using microsatellites and TR loci. Tandem repeat loci tend to be less variable than microsatellite loci and, for this reason, they can allow for geographic clustering of isolates. In addition, polymerase chain reaction (PCR)-amplified fragments of TRs may be separated using simple agarose gel electrophoresis and easily analyzed because of their significant variation in the number of repeats.

Thus, the aim of this study was to analyze the variability of P. vivax isolates from different endemic areas in the Amazon using the five most polymorphic TRs used by Leclerc and colleagues. Our specific objectives were to assess the genetic diversity, structure, and differentiation of P. vivax populations in relation to geographic distances between these populations and the level of malaria transmission in different areas of Brazilian Amazon. We report the results of the genetic analysis and discuss the evolutionary forces that may explain the observed patterns of diversity.

MATERIALS AND METHODS

Plasmodium vivax isolates. Blood samples were collected from 44 P. vivax–infected patients (11 individuals from each area) in different areas of the Brazilian Amazon (Figure 1): Macapá in Amapá State (AP; 00°02’20”S, 51°03’59”W), Cuiabá in Mato Grosso State (MT; 15°35’45”S, 56°05’49”W), Augusto Correa in Pará State (PA; 01°01’18”S, 46°38’06”W), and Manaus in Amazonas State (AM; 03°06’07”S, 60°01’30”W). The samples were collected from the different regions as follows: AM, May 2003; AP, November 2004; PA, October 2005; and MT, from July 2003 to July 2004. The available epidemiologic data indicated that the studied regions were areas of hypo- to mesoendemic malaria with intermittent transmission. The rate of transmission for each area was measured by the annual parasite index (API), which reflects the number of positive blood
and conditions described by Leclerc and colleagues, but for MN21, MN23, and MN25 were amplified using the primers ing to the manufacturers protocol. The five loci (MN2, MN7, DNA isolation Kit (Gentra Systems, Minneapolis, MN), accord-
was extracted from whole blood samples using the Puregene
sic analysis on Giemsa-stained blood smears. All patients
tified as variable risk. Patient infection was confirmed by micro-
scopic analysis on Giemsa-stained blood smears. All patients
were between 16 and 58 years of age (mean, 32 years).

Genomic DNA isolation and PCR amplification. The DNA
was extracted from whole blood samples using the Puregene
DNA isolation Kit (Gentra Systems, Minneapolis, MN), accord-
ing to the manufacturers protocol. The five loci (MN2, MN7,
MN21, MN23, and MN25) were amplified using the primers
and conditions described by Leclerc and colleagues, but for
ward primers were labeled with fluorescent dye (FAM). The
repeat unit ranged from 4 (MN23) to 20 nucleotides (MN25)
(Table 1). These markers were assigned to chromosome 8 of
the P. vivax SaI-1 (unfinished sequence, whole genome shotgun
sequencing project. GenBank accession no. AAKM00000000).
The markers were separated from each other at a distance of
1,727 bp (MN21/MN23) to 32,934 bp (MN2/MN7) (Table 1). The
PCR products were separated using agarose gel electrophore-
sis and the exact size of PCR products was identified using the
automated laser fluorescent (ALF) automatic DNA sequencer
(Amersham Pharmacia Biotech, Uppsala, Sweden), comparing
to the ALFexpress sizer 50–500 bp (5 fmol/μL; Amersham), and
analyzed using Allele Link software (version 1.0, Amersham).

Variability analysis. We measured allele frequencies using
the single or predominant allele at each locus from each isolate;
non-predominant alleles were recorded and used to estimate
multiplicity of infection, because all markers are single-
copy loci and blood stage malaria parasites are haploid.
The highest peak in the electropherogram traces was defined as
the predominant allele and electropherogram traces with
at least one-third the height of the predominant allele were
defined as additional alleles in a multiple-clone infection. We
measured intra-population diversity using the statistic \( H_e \),
which is the average probability that two alleles randomly
obtained for each locus are different. An analysis of molecular
variance (AMOVA) was performed to measure whether the
resulting variability could be explained by variations among
and within populations. We performed a Kruskal–Wallis test
to verify whether the differences between the \( H_e \) of each locus
for individual populations were significant.

We assessed the linkage disequilibrium (LD) for each locus
pair and each population using the Fisher exact test of LD
available in Arlequin 3.1 software with 100,000 steps in the
Markov Chain and 1,000 dememorization steps. We also used
LIAN 3.5 software to test for evidence of multilocus LD in
each population. This test compares the variance \( (V_e) \) of the
number \( D \) of alleles shared between all pairs of haplotypes
observed in the population \( (D) \) with the variance expected
when alleles are assigned randomly \( (V_e) \) as follows:
\[
P_A = \frac{V_e}{V_e - 1} (r - 1),
\]
where \( r \) is the number of loci analyzed. The \( V_e \) is derived from 100,000 simulated data sets in which alleles
were randomly reshuffled among haplotypes. Significant LD is
detected if \( V_e \) is 95% of the values derived from the reshuffled
data sets. Two analyses were done: one considering all hap-
lotypes and another with unique haplotypes to remove the
effect of the epidemic expansion of a particular haplotype on
LD in randomly mating populations.

Between-population genetic diversity was quantified using
pairwise \( F_{st} \), as implemented in Arlequin 3.1. We also estimated
the correlation among the matrices of pairwise genetic
and geographic distances using the Mantel test.

We attempted to assess the population structure of samples
using Structure 2.1 software. This uses Bayesian approaches

![Figure 1. Map of Brazil showing the four locations where blood was collected: Macapá (Amapá State, AP), Manaus (Amazonas State, AM), Cuiabá (Mato Grosso State, MT), and Augusto Correa (Pará state, PA).](image-url)
to infer the most likely number of populations or clusters (K) represented in the total sample, and then measure the probability that individual parasites come from each of these K populations. The parameters set to run the software were an admixture model and linkage model that assumed dependent allele frequencies. For each K value, we ran the software three times, each one with 100,000 burn-ins and 1,000,000 iterations. The K values ranged from 2 to 8.

RESULTS

Five TR loci were selected to analyze 44 isolates of *P. vivax* taken from four different endemic areas in the Brazilian Amazon; their characteristics are shown in Table 1. The TR markers used in this study map to chromosome 8, and they include non-coding loci (MN7, MN21, and MN25), a kinase domain locus (MN2), a protein containing a hypothetical protein locus (MN23), and a protein containing a kinase domain locus (MN2). The number of alleles per locus ranged from 2 to 9, with a modal allele in most cases (frequency ≥ 0.5) (Table 2). For all the TR loci studied, isolates were polymorphic in at least one population, and only MN2 was monomorphic in isolates from the PA population. We used a Kruskal–Wallis test to verify whether the difference between the heterozygosity expected (H_e) for each locus was significant (Table 3). We noted no difference between the loci, indicating that the length of the repeating unit of each locus did not correlate with its variability. Multiplicity of infection was estimated at 27% and 66% in the MT and AM populations, respectively. However, we did not find any individual infected with more than one haplotype in the PA and AP populations.

The comparative analysis of the four Amazon populations studied showed that all of them were highly variable, with H_e ranging from 0.46182 to 0.66600. In addition, when AMOVA was performed, we found that approximately 84% of the diversity detected was a result of variation within each population. Only 16% of the diversity could be explained by variation among populations.

The F_{st} values between populations (Table 3) were between 0.02 and 0.24, and they did not correlate with geographic distance between populations (Mantel Test, Z = 0.031, P = 0.295). The AM and AP populations could not be differentiated, and all of the populations diverged significantly (P ≤ 0.05) from the PA population. This F_{st} distribution and the derived estimations for the number of effective migrants (M = Nm) suggest that gene flow is higher among the AM and AP populations.

The LD was estimated using the Fisher test. The number of steps in the Markov Chain used in the analysis allowed that the results converged. We found LD to vary as a function of population and loci pairs, but these results should be interpreted with caution given our small sample sizes. Analysis of LD for the loci pairs in all isolates showed that six of 10 possible pair combinations showed high LD and did not correlate with the physical distance between the marker loci (Figure 2). Presence of marker loci in LD varied among populations. In the AP population, the loci pairs MN7/MN21, MN2/MN23, and MN21/MN25 showed high LD; in the MT population, the loci pairs MN7/MN23 and MN2/MN27 showed high LD; in the AM population, only the loci pair MN7/MN25 showed LD. Linkage disequilibrium was not found in the PA population. When LD analysis was performed with AP and AM as one population, because they both had low F_{st} values, we noted only the locus pair MN7/MN21 in LD. The AP and AM populations showed significantly higher multilocus LD. However, when the analysis was carried out only on the unique haplotype infections, only the AP population showed significant LD (Table 4).

### Table 2

<table>
<thead>
<tr>
<th>Locus*</th>
<th>Allele†</th>
<th>Number of alleles (frequency)</th>
<th>PA</th>
<th>MT</th>
<th>AP</th>
<th>AM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN2</td>
<td>133</td>
<td>2 (22.2)</td>
<td>1 (9.1)</td>
<td>2 (25)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>152</td>
<td>6 (77.8)</td>
<td>10 (90.9)</td>
<td>3 (37.5)</td>
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<td></td>
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<tr>
<td></td>
<td>171</td>
<td></td>
<td>1 (12.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>189</td>
<td></td>
<td>2 (25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN7</td>
<td>209</td>
<td>5 (45.5)</td>
<td>9 (81.8)</td>
<td>7 (70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>220</td>
<td>6 (54.7)</td>
<td>2 (18.2)</td>
<td>3 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN21</td>
<td>254</td>
<td>7 (58.3)</td>
<td>6 (54.5)</td>
<td>5 (55.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>3 (25.0)</td>
<td>2 (18.2)</td>
<td>1 (7.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>266</td>
<td>1 (8.4)</td>
<td>2 (14.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>1 (9.1)</td>
<td>3 (21.4)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>278</td>
<td></td>
<td>3 (21.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>1 (8.4)</td>
<td>3 (27.2)</td>
<td>3 (21.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN23</td>
<td>219</td>
<td>1 (8.4)</td>
<td>5 (45.5)</td>
<td>8 (55.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>223</td>
<td>4 (33.3)</td>
<td>3 (27.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>237</td>
<td>3 (27.2)</td>
<td>1 (6.7)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>240</td>
<td>1 (8.4)</td>
<td>6 (40.0)</td>
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</tr>
<tr>
<td></td>
<td>247</td>
<td>5 (41.6)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>251</td>
<td>1 (9.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MN25</td>
<td>169</td>
<td>8 (72.7)</td>
<td>1 (9.1)</td>
<td>1 (11.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>191</td>
<td>1 (9.1)</td>
<td>1 (11.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>212</td>
<td></td>
<td>1 (11.1)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>220</td>
<td>1 (9.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>234</td>
<td>3 (27.3)</td>
<td>2 (18.2)</td>
<td>4 (44.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>241</td>
<td>1 (9.1)</td>
<td>2 (18.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>6 (54.5)</td>
<td>4 (36.4)</td>
<td>1 (11.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>261</td>
<td></td>
<td>2 (22.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>276</td>
<td>3 (27.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Locus named according to Leclerc and colleagues.†Mean size of amplicons determined using Allele Link software (version 1.0, Amersham).‡Geographic regions: PA = Para State; MT = Mato Grosso State; AP = Amapá State; AM = Amazonas State.

### Table 3

<table>
<thead>
<tr>
<th>Locus</th>
<th>MN2</th>
<th>MN7</th>
<th>MN21</th>
<th>MN23</th>
<th>MN25</th>
<th>Mean in population</th>
<th>F_{st}(X100)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>0</td>
<td>0.51</td>
<td>0.56</td>
<td>0.74</td>
<td>0.49</td>
<td>0.46 (0.248)</td>
<td></td>
</tr>
<tr>
<td>MT</td>
<td>0.47</td>
<td>0.54</td>
<td>0.56</td>
<td>0.71</td>
<td>0.69</td>
<td>0.59 (0.092)</td>
<td>20.355‡</td>
</tr>
<tr>
<td>AP</td>
<td>0.18</td>
<td>0.33</td>
<td>0.65</td>
<td>0.71</td>
<td>0.82</td>
<td>0.54 (0.242)</td>
<td>19.697‡</td>
</tr>
<tr>
<td>AM</td>
<td>0.82</td>
<td>0.47</td>
<td>0.84</td>
<td>0.51</td>
<td>0.69</td>
<td>0.67 (0.153)</td>
<td>24.160‡</td>
</tr>
<tr>
<td>Mean at locus</td>
<td>0.37 (0.310)</td>
<td>0.46 (0.083)</td>
<td>0.65 (0.111)</td>
<td>0.67 (0.092)</td>
<td>0.67 (0.117)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*H_e expected heterozygosity and F_{st} were calculated using Arlequin 3.1 software.†Mean size of amplicons determined using Allele Link software (version 1.0, Amersham).‡Statistically significant (P < 0.005).
It was not possible to cluster the samples using Structure 2.1 (data not shown). This was probably because of the limited number of markers used and the LD between the markers. Consequently, little independent information was available for the software to use for clustering.

**DISCUSSION**

All molecular markers that were previously reported to be polymorphic in populations from different countries (Azerbaijan, Ethiopia, Turkey, Venezuela, and Thailand) have also turned out to be polymorphic in Brazilian Amazonic populations. As reported by Leclerc and colleagues, we observed a predominant allele at almost all loci, yet at only one locus was the same allele prevalent in all geographic areas studied. We detected in our isolates 68% of the alleles previously described.

Our data showed a high variability within populations, with high values of $H_h$. Similar values of $H_h$ were also reported using microsatellite analysis of *P. falciparum* isolates from different regions of Brazil. However, a study by Ferreira and colleagues analyzing a multilocus microsatellite in *P. vivax* isolates from one Brazilian population in Acre State reported slightly higher $H_h$ values than ours. This population showed a high percentage of multiple-clone infections, whereas only one population in our study showed a high multiplicity of infection: AM population, 66%. All of our populations showed a similar level of within-population variability. The population studies here were found to be structured with minimal gene flow, based on $F_{st}$ values. Our results corroborate the findings of multilocus microsatellite analysis in *P. falciparum* populations in Brazil. Moreover, our study and that of Machado and colleagues did not show a correlation between genetic and geographic distances for populations of *P. vivax* or *P. falciparum*, respectively.

Despite the fact that all the molecular markers map to the same chromosome, we did not observe a correlation between physical distance and LD. For instance, the loci MN21 and MN23 lay less than 2,000 bp away from each other, but they did not show significant LD. When we performed multilocus LD analysis, we observed a correlation between the $P_A$ value and number of loci pairs in LD in each population. If we excluded the MT population, the correlation was $r = 0.9425$. The MT population was excluded because their isolates were obtained from individuals infected in many localities. Moreover, the blood samples in this group were collected over the longest period (one year). These two factors may increase the variability in these populations. Interestingly, we did not find a negative correlation between multiplicity of infection and LD in populations, contrary to what has been observed with *P. falciparum*. This result is similar to that of Ferreira and others, and it may indicate that these markers are under some selection pressure, which would alter the pattern of LD. Nevertheless, according to the EWens–Watterson neutrality test, the loci used in this work do not suffer from selection pressure. Indeed, none of the TR markers used here map to known surface antigens likely to be under immune-mediated diversifying natural selection. A higher variability was observed at loci MN7, MN21, and MN25, which lie in non-coding regions (Table 1). However, these molecular markers may not be considered to be strictly neutral, because of their physical proximity and LD with genes encoding proteins under natural selective pressure.

In *P. falciparum*, the genetic structure of the population is strongly linked to patterns of transmission. A widely accepted model is that high levels of single infection, inbreeding, and rare recombination characterize low transmission regions with low genetic variability. In contrast, frequent mosquito inoculation, multiple infections, frequent outbreeding, and extensive recombination characterize high transmission regions with high genetic variability. A similar result was obtained in *P. falciparum* from the Amazon region, which showed a negative correlation between LD and multiplicity of infection. This correlation between genetic variability and patterns of transmission was less clear in *P. vivax* infections. For South America (Colombia), Laos, and Thailand, the isolates fit the model well, as demonstrated by Anderson and others. However, Indian isolates were found to have a low LD and low frequency of multiple-clone infections. Conversely, Ferreira and colleagues showed a high LD and high frequency of multiple-clone infection in their study of Brazilian isolates from Acre State. Here, we observed high LD in the absence of multiple infections only in one of our populations.

According to Inwong and colleagues and Russell and colleagues, the repeat array length of microsatellites is directly associated with genetic variability. The fact that we did not observe this correlation for TRs suggests that the relationship is too weak to be detected by our small number of TRs, or it may not exist for these particular markers. These molecular markers were shown to be highly polymorphic, though slightly less so than the microsatellites that have already been described. In addition, this approach allows easy analysis based only on agarose gel electrophoresis, which suggest that high-throughput processing is possible using an automatic DNA sequencer.

| Table 4 |

Linkage disequilibrium (LD) (standardized index of association, $P_A$) in all tandem repeat (TR) loci in different populations

<table>
<thead>
<tr>
<th></th>
<th>Amapa</th>
<th>Amazonas</th>
<th>Mato Grosso</th>
<th>Para</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All infections</td>
<td>0.2360*</td>
<td>0.2038†</td>
<td>0.0626</td>
<td>0.0358</td>
<td>0.0278</td>
</tr>
<tr>
<td>Infection with unique haplotype</td>
<td>0.1870* (N = 9)</td>
<td>0.1122 (N = 6)</td>
<td>−0.0007 (N = 9)</td>
<td>−0.0159 (N = 9)</td>
<td>0.0088 (N = 33)</td>
</tr>
</tbody>
</table>

* $P < 0.01$
† $P < 0.05$
However, there is no evidence for a putative mutation mechanism to explain the variability at these markers, and this makes it difficult to understand how TR diversity is maintained. Therefore, further research on this issue is necessary.

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REFERENCES

2. Health HMoBSoVi. 2007. Malaria epidemiologic situation in Brazil.