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Higher microsatellite diversity in *Plasmodium vivax* than in sympatric *Plasmodium falciparum* populations in Pursat, western Cambodia

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Abstract

Previous microsatellite analyses of sympatric populations of *Plasmodium vivax* and *P. falciparum* in Brazil revealed higher diversity in the former species. However, it remains unclear whether regional species-specific differences in prevalence and transmission levels might account for these findings. Here, we examine sympatric populations of *P. vivax* (n = 87) and *P. falciparum* (n = 164) parasites from Pursat province, western Cambodia, where both species are similarly prevalent. Using 10 genome-wide microsatellites for *P. falciparum* and 13 for *P. vivax*, we found that the *P. vivax* population was more diverse than the sympatric *P. falciparum* population (average virtual heterozygosity [H_E], 0.87 vs. 0.66, $P = 0.003$), with more multiple-clone infections (89.6% vs. 47.6%) and larger mean number of alleles per marker (16.2 vs. 11.1, $P = 0.07$). Both populations showed significant multi-locus linkage disequilibrium suggestive of a predominantly clonal mode of parasite reproduction. The higher microsatellite diversity found in *P. vivax* isolates, compared to sympatric *P. falciparum* isolates, does not necessarily result from local differences in transmission level and may reflect differences in population history between species or increased mutation rates in *P. vivax*.

Keywords

Plasmodium vivax; *Plasmodium falciparum*; population structure; genetic diversity; microsatellites

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1. Introduction

Despite the continuous development of molecular typing methods and high-throughput platforms, microsatellites (tandem repeats of motifs of one to six nucleotides) remain among the most popular and informative genetic markers currently available for the study of eukaryotic organisms (Guichoux et al., 2011). The extensive variation found in microsatellites, which typically results in multiple alleles per locus, is mostly generated by strand-slippage events during DNA replication. Observed mutation rates (10^{-3} to 10^{-4} per locus per generation) result from the interplay between strand-slippage events and mismatch repair, which counteracts DNA slippage during replication (Schlötterer, 1998).

Microsatellites are abundant in the human malaria parasite *Plasmodium falciparum*; an average of one microsatellite locus is found every 2-3 kb of genome sequence (Su and Wellems, 1996). Microsatellites have been extensively used to compare the population structure of this species in different endemic settings, revealing striking geographic differences in the levels of genetic diversity and linkage disequilibrium (LD). Diversity was higher and LD lower in populations from holoendemic Africa (Anderson et al., 2000), whereas diversity was lower and LD higher in populations from hypoendemic South America (Anderson et al., 2000; Machado et al., 2004; Orjuela-Sánchez et al., 2009b), with intermediate patterns seen in Southeast Asia (Anderson et al., 2000; Anthony et al., 2005).

Microsatellite abundance correlates positively with genome AT content, which is extremely high in *P. falciparum* (the average AT content reaches 95% in repetitive domains) but is relatively low in the other major human malaria parasite, *P. vivax* (Tyagi et al., 2011). As a consequence, only 160 short repetitive sequences, many of them with features of classical microsatellites, have been characterized across the *P. vivax* genome (Carlton et al., 2008). Despite their relative paucity, microsatellites have provided valuable insights into the global genetic diversity of *P. vivax*, suggesting that a spectrum of population structures also exists for this species (Imwong et al., 2007; Ferreira et al., 2007; Karunaweera et al., 2008; Joy et al., 2008; Orjuela-Sánchez et al., 2009a; Van den Eede et al., 2010a, Van den Eede et al., 2010b; Gunawardena et al., 2010).

Two or more human malaria parasite species often occur in sympatry in endemic regions. Across most of the Amazon Basin and Southeast Asia, *P. falciparum* and *P. vivax* affect the same human populations and are transmitted essentially by the same vectors, but their population structure has been mostly examined separately. Two comparisons of microsatellite diversity in human malaria parasite species co-circulating in the same area in Brazil suggested that *P. vivax* infections are more diverse and comprise multiple clones more often than *P. falciparum* infections (Ferreira et al. 2007; Orjuela-Sánchez et al. 2009b). Moreover, a recent comparison of sequence diversity at the *apical membrane protein-1* (*ama-1*) locus, which codes for a major malaria vaccine-candidate antigen, in sympatric parasites from Venezuela revealed much more polymorphism in *P. vivax* than in the *P. falciparum* orthologue (Ord et al., 2008). Since *P. vivax* currently predominates in South America, differences in species-specific transmission levels might translate into differences in genetic diversity observed in these studies. To investigate the potential confounding effect of transmission levels on population structure, here we sought to compare levels of microsatellite diversity in sympatric populations of *P. vivax* and *P. falciparum* from Pursat province, western Cambodia, an area where both species are similarly prevalent.

2. Materials and methods

2.1 Study area and population

The 87 *P. vivax* and 164 *P. falciparum* isolates completely typed in this study were obtained directly from patients enrolled into either of two studies of genetic resistance to malaria, conducted from June to December, 2008 (ClinicalTrials.gov identifiers, NCT00341003 and NCT00663546). During this time, we screened 1875 individuals with symptoms of malaria who presented to the Sampov Meas Referral Hospital in Pursat town (population, 57,000) (12°32'N, 103°55'E), located 174 km northwest of Phnom Penh, in western Cambodia (Figure 1). Of the 634 patients with detectable parasitemia, 278 (44%), 329 (52%) and 27 (4%) were infected with *P. vivax*, *P. falciparum* and both parasite species, confirming that *P. vivax* and *P. falciparum* are similarly prevalent in this region. Blood samples from 177 and 95 patients with single-species *P. falciparum* and *P. vivax* infections, respectively, were available for this study; no mixed-species infections were typed. While these patients presented from all six districts of Pursat province (where they reside permanently), they were exposed to infectious mosquito bites in the forested areas of Veal Veng district near the Cambodia-Thailand border. This is because they live close to these areas or travel to these areas for occupational activities such as hunting, mining and logging. In western Cambodia, both *P. vivax* and *P. falciparum* are transmitted by *Anopheles dirus* and *An. minimus* (Durnez et al., 2011).

Parasitized blood samples were collected under protocols approved by the Cambodian National Ethics Committee for Health Research and the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, USA. All patients or their parents or legal guardians provided written informed consent.

2.2 Laboratory diagnosis of malaria

Giemsa-stained thick blood smears were examined by an expert microscopist. To estimate parasite density the total number of parasites per 200 white blood cells (WBCs) was multiplied by 40 to express the number of parasites per μL (assuming the patient has 8000 WBC per μL of whole blood). If no parasites were observed after counting 200 WBC, counting was continued up to 500 WBC and the total number of parasites per 500 WBC was used to calculate the parasite density. Blood samples were further examined for malaria parasites by nested PCR-based amplification of a species-specific segment of the *18S rRNA* gene of human malaria parasites (Snounou et al., 1993). DNA templates for PCR amplification were isolated from 200 μL of whole blood, using a QIAamp DNA blood mini kit (Qiagen, Valencia, CA). To increase sample volume, 10 ng of genomic DNA were submitted to whole-genome amplification (WGA) with high-fidelity multiple displacement technology (Dean et al 2002) using the REPLI-g Mini kit (Qiagen, Valencia, United States).

2.3 Microsatellite typing of *P. falciparum*

Ten single-copy microsatellites with trinucleotide repeats, which map to 6 chromosomes of *P. falciparum*, were typed. Alleles were amplified by single-stage polymerase chain reaction (PCR) with the oligonucleotide primers listed in Table 1 (Anderson et al. 1999; Su et al. 1999). PCR mixtures (15 μL) contained 3 μL of DNA template (prepared as described above), 1 unit of *Taq* polymerase, 1.5 μL 10 $\times$ buffer, 2 mM MgCl_2 , 0.1 mM of each dNTP, and 2 μM of each primer. All reagents were purchased from Fermentas (Vilnius, Lithuania), except for primers (both labeled with fluorescent dyes and unlabeled), which were supplied by Applied Biosystems (Foster City, CA). PCR cycling conditions were: 2 min, 94°C; (30 sec, 94 °C; 30 sec, 42°C; 30 sec, 40°C; 30 sec, 65°C) $\times$ 40 cycles; 5 min, 65 °C. After PCR amplification, products were pooled (TAA60+ARA2, PfG377+TA87, PfpK2+TA109, TA81+TA42, and Poly α +C2M3) according to their sizes and labels.

2.4 Microsatellite typing of *P. vivax*

Thirteen single-copy microsatellites with tri- or tetranucleotide repeats, which map to 10 chromosomes of *P. vivax*, were typed. Alleles were PCR-amplified with the oligonucleotide primers listed in Table 2 (Karunaweera et al., 2007). For each PCR reaction, 3 µl of genomic DNA (prepared as described above) were used with 2 mM MgCl₂, 2 µM of each primer, 0.1 mM of each dNTP, 1 U of recombinant *Taq* polymerase, and 1.5 µl of 10× *Taq* polymerase buffer in a final volume of 15 µl. All reagents were purchased from Fermentas (Vilnius, Lithuania), except for primers (both labeled with fluorescent dyes and unlabeled), which were supplied by Applied Biosystems (Foster City, CA). PCR cycling conditions were: 2 min, 94°C; (30 s, 94°C; 40 s, 58°C; 50 s, 72°C) ×40 cycles; 5 min, 72°C. After PCR amplification, products were pooled as follows: MS1+MS3+MS9, MS2+MS5, MS4+MS6+MS10, MS7+MS15, and MS8+MS12+MS20.

2.5 Data analysis

Fragment size was measured on an ABI310 (Applied Biosystems) DNA sequencer. GeneScan 500-ROX (Applied Biosystems) was used as an internal size standard. The relative abundance of alleles (peak heights in electropherograms) was determined using STRand software (<http://www.vgl.ucdavis.edu/informatics/strand.php>). Major and minor microsatellite alleles coexisting in the same infection can theoretically be differentiated in this way, but minor alleles may be confused with artifacts such as stutter peaks or unspecific PCR amplification products. Stutter peaks are often produced by DNA strand slippage during PCR at intervals corresponding to nucleotide repeat sizes (de Valk et al., 2007). We scored two alleles at a locus when the minor peak was more than one-third the height of the predominant peak and its height exceeded 200 arbitrary fluorescence units (Havryliuk et al., 2008). Infections were considered to contain multiple clones if one or more loci showed more than one allele (Anderson et al. 2000). The proportions of multiple-clone infections were compared with standard χ^2 tests with Yates correction. Multilocus haplotypes were defined as unique combinations of alleles at each locus analyzed; only the predominant alleles were considered for haplotype assignment in multiple-clone infections (Anderson et al. 1999).

We used the virtual heterozygosity estimate (H_E) as a measure of overall genetic diversity: $H_E = [n/(n-1)][1 - \sum p_i^2]$, where n is the number of isolates analyzed and p_i is the frequency of the i -th allele in the population. H_E gives the average probability that a pair of alleles randomly obtained from the population is different. Virtual heterozygosity ranges between 0 and 1. Haplotype diversity (H_d) was calculated in a similar way, giving the average probability (ranging between 0 and 1) that a pair of haplotypes randomly obtained from the population is different. The number of alleles per locus and virtual heterozygosity estimates were compared between species using standard Mann-Whitney U tests.

The standardized index of association (F_A) was used to test for evidence of overall multilocus LD in parasite populations. This test compares the variance (V_D) of the number of alleles shared between all pairs of haplotypes observed in the population (D) with the variance expected under random association of alleles (V_E) as follows: $F_A = (V_D/V_E - 1) / (r - 1)$, where r is the number of loci analyzed. V_E is derived from 10,000 simulated data sets in which alleles were randomly reshuffled among haplotypes. Significant LD is detected if V_D is greater than 95% of the values derived from the reshuffled data sets. Data were analyzed with LIAN 3.5 software (Haubold and Hudson, 2000) available at: <http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=lian&referer=pubmlst.org>. Unique haplotypes were analyzed separately to distinguish between clonal and “epidemic” population structures of parasites (Smith et al. 1993).

Finally, we compared the allele frequency distribution of *P. vivax* and *P. falciparum* microsatellites as a test for recent population bottlenecks (Luikart et al., 1988). Alleles at low frequencies (e.g., below 0.1) tend to become less abundant after a major population bottleneck. The typically L-shaped distribution observed in populations at mutation-drift equilibrium, indicating the predominance of rare alleles, tends to become flattened as rare alleles are lost as a result of the bottleneck. Frequency distributions were compared with a standard χ^2 test.

3. Results and discussion

3.1 Genetic diversity and linkage disequilibrium

We were able to genotype completely the vast majority of available parasite samples available (87 of 95 [91.5%] of *P. vivax* samples and 164 of 177 [92.6%] of *P. falciparum* samples). The *P. vivax* population in Pursat province was more diverse than the sympatric *P. falciparum* population, with more multiple-clone infections ($\chi^2 = 41.05$, 1 degree of freedom, $P < 0.00001$) and greater number of alleles per locus ($P = 0.07$, Mann-Whitney U test; Table 3). H_E estimates ranged between 0.882 and 0.345 for *P. falciparum* microsatellites (Table 1) and between 0.947 and 0.740 for *P. vivax* microsatellites (Table 2). The difference in average H_E estimates between these species is statistically significant ($P = 0.003$, Mann-Whitney U test).

Both parasite populations showed highly significant multilocus LD. We found 151 unique 10-locus haplotypes in 164 *P. falciparum* isolates (one haplotype was shared by 2 parasites, two haplotypes were shared by 4 parasites and one haplotype was shared by 5 parasites), giving an H_d estimate of 0.998). The LD for *P. falciparum* remained significant when each haplotype was counted once (Table 3), indicating that recent epidemic expansions of particular lineages (Smith *et al.* 1993) do not account for the linkage observed. Given that wrong assignment of predominant haplotypes in multiple-clone infections may generate falsely recombinant haplotypes, we repeated the LD analysis considering only the 86 single-clone *P. falciparum* infections, obtaining an even greater I_A^S estimate (0.068, $P = 0.001$). Each of the 87 *P. vivax* isolates displayed a unique 13-locus haplotype ($H_d = 1$). The significant LD suggests that local populations of parasites of both species have relatively low rates of effective meiotic recombination, consistent with a predominantly clonal mode of reproduction that has also been found in other *P. falciparum* and *P. vivax* populations from Southeast Asia (Anderson et al., 2000; Anthony et al., 2005; Imwong et al., 2007; Van den Eede et al., 2010a) and South America (Machado et al., 2004; Ferreira et al., 2007; Orjuela-Sánchez et al., 2009a; Orjuela-Sánchez et al., 2009b; Van Den Eede et al., 2010b). Alternatively, natural selection on some of these microsatellite loci or neighboring sites may have originated nonrandom allele associations.

3.2 Allele frequency distribution and recent population history

Major demographic events, such as a severe reduction in population size in the recent past (e.g. as a consequence of drug pressure) provide a possible explanation for the reduced diversity in *P. falciparum* from Cambodia. Population genetics theory predicts that a major population bottleneck within the past few generations would have resulted in a loss of rare alleles, distorting the overall distribution of microsatellite allele frequencies (Luikart et al., 1998). In fact, we found a significant difference in the frequency distributions of alleles from each species ($\chi^2 = 25.6$, 8 degrees of freedom, $P = 0.001$), although both distributions were clearly L-shaped (Figure 2). Rare alleles (those found at frequencies below 0.1) predominated in both populations, but were less abundant in *P. falciparum* (71.4% of all alleles) than in *P. vivax* (81.0% of all alleles). The overall *P. falciparum* allele frequency distribution is slightly shifted to the right; 8 (7.1%) *P. falciparum* alleles, but none of *P.*

vivax, were found at frequencies above 0.4. In conclusion, although we found no support for a major recent bottleneck in the *P. falciparum* population from Pursat, we did find a trend towards some loss of rare alleles in this population. The observed between-species difference in allele frequency distributions may result from demographic events, but natural selection on some of our microsatellite loci may also play a role. In fact, more *P. vivax* than *P. falciparum* microsatellites (9 vs. 4) map to protein-coding sequences, although none of them is located in genes encoding major surface antigens or drug-resistance traits that are expected to be under strong selective pressure (Ferreira et al., 2007).

3.3 Multiplicity of infection

Multiple clones present in the same parasite isolate (*i. e.*, multiple-clone infections) were detected when more than one allele was found in one or more single-copy microsatellites analyzed for each species (Table 3). However, the number of markers with more than one allele detected in multiple-clone infections varied widely (between 1 and 8 for *P. falciparum* and between 1 and 12 for *P. vivax*). Note that most (38 of 78, 61.5%) multiple-clone *P. falciparum* infections had a single microsatellite (out of 10 typed) with more than one allele, suggesting that a fairly number of markers are required to find multiple-clone *P. falciparum* infections in western Cambodia. In contrast, 74.4% (58 of 78) of all multiple-clone *P. vivax* infections had three or more (out of 13) markers with more than one allele (Figure 3), suggesting that a reduced set of markers would be able to diagnose all or nearly all multiple-clone *P. vivax* infections in the study site. A more conservative approach to define multiple-clone infections would be to consider only those with more than one allele at two or more microsatellite loci. Applying this criterion would result in an even greater disparity in the proportion of multiple-clone infections between *P. vivax* (81.6%) and *P. falciparum* (24.3%), which is clearly statistically significant ($\chi^2 = 73.15$, 1 degree of freedom, $P < 0.00001$).

Increasing the number of markers examined theoretically improves our ability to detect multiple-clone infections (*i. e.*, to find at least one marker with more than one allele) (Havryliuk and Ferreira, 2009). Differences in the number of microsatellites examined might account, at least in part, for the between-species differences in multiplicity of infection in Pursat, since more markers were used for *P. vivax* typing. To examine the minimum number of markers required to estimate the prevalence of multiple-clone infections for different species, we first ranked microsatellite loci according to their H_E estimates (shown in Tables 1 and 2). When only the marker with the highest H_E value was used (Poly- α for *P. falciparum* and MS10 for *P. vivax*), it detected 23 out of 78 *P. falciparum* multiple-clone infections diagnosed with the complete 10-marker set (29.5% of the total) and 30 out of 78 *P. vivax* multiple-clone infections diagnosed with the complete 13-marker set (38.5% of the total). By combining this marker with the one with the second largest H_E (PfPk2 for *P. falciparum* and MS8 for *P. vivax*), we detected 43 *P. falciparum* and 47 *P. vivax* multiple-clone infections.

The remaining microsatellites were then added one at time to the panel of markers, in order of decreasing H_E (Orjuela-Sánchez et al., 2009b). Sixty-four *P. falciparum* and 74 *P. vivax* multiple-clone infections would have been diagnosed with a restricted panel comprising only the six microsatellite loci with the highest H_E values, resulting in estimates of prevalence of multiple-clone infections of 85.1% for *P. vivax* and 42.1% for *P. falciparum*.

Microsatellite panels with equal number of markers for each species would always detect a higher prevalence of multiple-clone infections among *P. vivax* isolates, compared to *P. falciparum* isolates from the same region of Cambodia (Figure 4). Ten microsatellites would suffice for diagnosing all 78 multiple-clone *P. vivax* infections. We conclude that our ability to detect multiple-clone infections in *P. vivax* is influenced by the greater diversity in

microsatellite markers in this species, but not by the fact that a larger number of markers were typed.

The high prevalence of multiple-clone *P. vivax* infections in Cambodia is far from surprising. In fact, most populations of this species so far examined show high clonal diversity, which would allow for frequent mating between genetically unrelated gametocytes found in the same mosquito blood meal, favoring outbreeding during the sexual reproduction of parasites in the vector. Hypnozoites, the dormant liver stages, provide a biologic basis for increased multiplicity of infection in *P. vivax*, compared to non-relapsing species such as *P. falciparum*. If super-infection with a different clone occurs weeks or months after the primary infection, genetically diverse strains are likely to accumulate in the same patient. If the dormant stages awaken at the time the second brood of parasites concludes its schizogony in the liver, genetically diverse strains are likely to be found in the bloodstream and their gametocytes may be taken up by the same vector (Havryliuk and Ferreira, 2009). Alternatively, several populations of dormant stages may be reactivated at once by an external stimulus, such as an unrelated systemic febrile illness (White, 2011).

Malaria parasites display sexual reproduction during their life cycles in the mosquito vector, but effective recombination rates may be quite variable among populations. In areas where most human hosts harbor a single parasite clone, selfing is common, since only genetically identical male and female gametes are taken up by mosquitoes during a single blood meal. This situation would maintain linkage disequilibrium among physically unlinked loci during several generations. Where multiple-clone infections are common in humans, gametes from different clones are often available in the same bloodmeal; they may fuse and originate detectable recombination events (outbreeding), which break down preexisting associations between physically unlinked loci and generate new multilocus haplotypes. In human *P. vivax* infections, virtually all patients have mature gametocytes at the time of diagnosis (Lima et al., 2012), suggesting that all circulating clones are potentially able to produce gametocytes early during the course of infection.

Paradoxically, however, frequent multiple-clone infections do not disrupt the clonal structure observed in Cambodia and other *P. vivax* populations from hypo- to mesoendemic regions (Imwong et al., 2007; Ferreira et al., 2007; Orjuela-Sánchez et al., 2009a; Gunawardena et al., 2010; Van den Eede et al., 2010b). A possible explanation is that the proportions of mature gametocytes produced by each genetically distinct clone in multiple-clone infections may be so different (say, 95% vs. 5%) that selfing remains the most likely outcome when two gametes taken at random fuse in the mosquito vector. Furthermore, the maintenance of high inbreeding rates, leading to strong LD, does not translate into reduced levels of genetic diversity in *P. vivax*. If there is so much inbreeding, how so many microsatellite haplotypes are generated? Our results suggest that microsatellite repeats do not require frequent meiotic recombination for their diversification, creating novel alleles and haplotypes; mitotic recombination events may play a major role in generating these new alleles. Alternatively, LD may not necessarily imply a predominantly clonal mode of parasite propagation, but might result, at least in part, from strong natural selection on some of the microsatellite markers used. However, none of them map to either coding sequences or genome regions that are expected to be under such an intense selective pressure.

3.4 Number of genetic markers and haplotype diversity

Increasing the number of markers examined may also improve our ability to score more multilocus haplotypes and inflate our estimates of haplotype diversity. Therefore, it is essential to define the minimal number of markers that would accurately estimate the overall genetic diversity when comparing malaria parasite populations and species. Figure 5 shows that the highest possible number of *P. vivax* multilocus haplotypes (87 haplotypes in 87

isolates, corresponding to $H_d = 1$) would have been scored with a rather restricted panel consisting of only four microsatellite loci with the highest H_E values (MS2, MS5, MS8, and MS10). In contrast, nine of the ten microsatellites would be required to score all *P. falciparum* haplotypes (151 haplotypes in 164 isolates). We thus conclude that the greater haplotype diversity found in the *P. vivax* population from Cambodia, when compared with sympatric *P. falciparum* isolates, does not result from the use of a larger number of genetic markers to characterize the former species; in fact, we would have obtained identical results with only four informative markers.

3.5 Conclusions

This is the first study comparing the population structure of sympatric *P. vivax* and *P. falciparum* isolates in Southeast Asia. We found higher microsatellite diversity in *P. vivax*, supported by a greater average virtual heterozygosity and a larger mean number of alleles per marker. Moreover, we identified more multiple-clone infections among *P. vivax* than *P. falciparum* isolates. These between-species differences seem to result neither from differences in the prevalence of each species in the study site nor from the use of a greater number of microsatellite markers to characterize the *P. vivax* population (since similar differences would have been observed by using the same number of markers for each species).

Most studies have shown extensive diversity in di-, tri- and tetranucleotide microsatellites in *P. vivax* populations from areas of low (Sri Lanka, Mexico and Brazil), intermediate (Myanmar, Vietnam and Cambodia) and high (Papua New Guinea) levels of malaria transmission (Imwong et al., 2006; Imwong et al., 2007; Ferreira et al., 2007; Karunaweera et al., 2008; Joy et al., 2008; Orjuela-Sánchez et al., 2009a; Van den Eede et al., 2010a, Van den Eede et al., 2010b; Gunawardena et al., 2010; Koepfli et al., 2011; but see also Leclerc et al., 2004 and Bruce et al., 2006). Therefore, although microsatellite repeats are relatively rare across the genome of *P. vivax*, the vast majority of those so far characterized are highly polymorphic.

It remains unclear whether (and, if so, why) microsatellites are intrinsically more diverse in *P. vivax* than in sympatric populations of *P. falciparum*. Our results might have originated from a bias in the selection of microsatellite-type repeats used for typing parasites of each species, which may not reflect a genome-wide pattern. However, a recent analysis of six whole-genome sequences of each species has consistently revealed significantly more diversity not only in microsatellites but also in single-nucleotide polymorphisms of *P. vivax* compared to *P. falciparum* (Neafsey et al., 2012).

Several factors might explain the disparity in microsatellite diversity observed in the two major human malaria parasite species. Historical demography is one of them. Cambodia is close to the putative region of origin of *P. vivax* (Cornejo and Escalante, 2006), where genetic diversity is expected to be highest for this species. In contrast, part of the *P. falciparum* diversity originally seen in Africa may have been lost as parasite populations migrated to Southeast Asia (Tanabe et al., 2010). Nevertheless, a recent comparison of whole-genome sequences of isolates of both species from different continents suggests that the greater diversity in *P. vivax* is not a geographically restricted phenomenon (Neafsey et al., 2012). Alternatively, more recent demographic events, such as drastic changes in the population size of *P. falciparum*, might have reduced the overall diversity of parasites from Cambodia. Partial support to this hypothesis is provided by the analysis of microsatellite allele frequency distributions (Figure 2), which is consistent with some loss of rare alleles in the *P. falciparum* population from Pursat.

Finally, repetitive sequences in *P. vivax* might show greater plasticity, perhaps as a consequence of a partially defective DNA mismatch repair. Little is currently known about DNA mismatch repair pathways in malaria parasites; however, DNA mismatch repair defects induced by mutations in MSH-2 (a homologue of a protein involved in the well-characterized methyl-directed MutHLS mismatch repair system of *Escherichia coli*) favor microsatellite instability in the rodent malaria parasite *P. berghei* (Bethke et al., 2007). A better understanding of DNA mismatch repair pathways in different malaria parasites may be required to interpret microsatellite-based comparisons between species.

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Abbreviations

AMA-1	apical membrane protein-1
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil
H_d	haplotype diversity
H_E	virtual heterozygosity
I^s_A	standardized index of association
LD	linkage disequilibrium
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health, United States
PCR	polymerase chain reaction
WBC	white blood cells
WGA	whole genome amplification

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Highlights

Plasmodium vivax was hypothesized to be genetically more diverse than *P. falciparum*.

We found more microsatellite diversity in *P. vivax* than in *P. falciparum* from Cambodia.

These findings may reflect differences in population biology or genome plasticity between species.

Figure 1. Map of Southeast Asia showing the location of Cambodia, its capital (Phnom Penh), and Pursat town ($12^{\circ}32'N$, $103^{\circ}55'E$). The distance between Phnom Penh and Pursat, by road, is about 174 km.

Figure 2.

Distribution of microsatellite allele frequencies in *P. falciparum* isolates (n = 164) and *Plasmodium vivax* (n = 87) collected in Pursat province, western Cambodia. Data are presented for all loci combined (13 *P. vivax* microsatellites with a total of 211 alleles and 10 *P. falciparum* microsatellites with a total of 112 alleles). An L-shaped frequency distribution is expected for non-bottlenecked populations at mutation-drift equilibrium (Luikart et al., 1998).

Figure 3.

Frequency distribution of *Plasmodium falciparum* (A) and *P. vivax* (B) isolates collected in Pursat province, western Cambodia, according to the number of single-copy microsatellite loci with more than one allele. Data for 78 multiple-clone infections detected for each species. The maximum number of markers with >1 allele was eight for *P. falciparum* and twelve for *P. vivax*.

Figure 4.

Relationship between the number of microsatellite markers typed and the prevalence of multiple-clone infections detected in 164 *Plasmodium falciparum* and 87 *P. vivax* isolates collected in Pursat province, western Cambodia. The markers were added one by one in decreasing order of virtual heterozygosity (H_E ; see Tables 1 and 2). By combining the nine microsatellite loci with the highest H_E values, all *P. vivax* multiple-clone infection could be detected. Note that when the same number of markers for *P. falciparum* and *P. vivax* is used, a greater prevalence of multiple-clone infections is always diagnosed among *P. vivax* isolates.

Figure 5.

Relationship between the number of microsatellite markers typed and the number of genetically distinct haplotypes (unique combinations of alleles) detected in 164 *Plasmodium falciparum* and 87 *P. vivax* isolates collected in Pursat province, western Cambodia. The markers were added one by one in decreasing order of virtual heterozygosity (H_E ; see Tables 1 and 2). By combining the four microsatellite loci with the highest H_E values, we were able to distinguish all *P. vivax* haplotypes, indicating that the greater haplotype diversity in *P. vivax* than *P. falciparum* (87 different haplotypes in 87 isolates vs. 151 different haplotypes in 164 isolates) is not merely a consequence of the use of more microsatellite markers (13 vs. 10) to characterize *P. vivax* isolates.

Table 1

Sequences of oligonucleotide primers used to amplify 10 microsatellite loci of *Plasmodium falciparum* and virtual heterozygosity (H_E) and number of alleles observed in 164 isolates from western Cambodia

Name ^a	5' Label	Sequence 5'-3'	Chromosome	Virtual heterozygosity (H_E)	No. of alleles
Polya-F		AAAATATAGACGAACAGA	4	0.882	17
Polya-R	VIC	ATCAGATAATTGTTGGTA	c		
TA60-F	VIC	CTCAAAGAAAAATAATTCA	13	0.722	15
TA60-R		AAAAAGGAGGATAAATACAT			
ARA2-F	NED	GTACATATGAATCACCAA	11	0.698	10
ARA2-R		GCHH GAGTATTATTAATA			
PfG377-F		GATCTCAACGGAATTAT	12	0.587	6
PfG377-R	NED	TTATGTTGGTACCGTGT			
PfPK2-F		CTTTCATCGATACTACGA	12	0.840	20
PfPK2-R	NED	CCTCAGACTGAAATGCAT			
TA87-F	VIC	ATGGGTAAATGAGGTACA	6	0.747	11
TA87-R		ACATGTTTCATATTACTCAC			
TA109-F	FAM	TAGGGAACATCATAAGGAT	6	0.345	9
TA109-R		CCTATACCAAACATGCTAAA			
C2M3-F	NED	GGTTAATATGATCACAAAATG	2	0.508	3
C2M3-R		ATTGTTGATTCATGAAATGCA	c		
TA81-F	FAM	GAAGAAATAAGGGAAGGT	5	0.807	9
TA81-R		TTTCACACAACACAGGATT			
TA42-F	VIC	ACAAAAGGGTGGTGATTCT	5	0.466	12
TA42-R		GTATTATTACTACTACTAAAG			

^aF = forward, R = reverse. The fluorescent dyes used to label primers were VIC (Applied Biosystems proprietary "green" fluorescent dye), NED (Applied Biosystems proprietary "yellow" fluorescent dye), and 6-FAM (6-carboxyfluorescein, "blue" fluorescent dye).

Table 2

Sequences of oligonucleotide primers used to amplify 13 microsatellite loci of *Plasmodium vivax* and virtual heterozygosity (H_E) and number of alleles observed in 87 isolates from western Cambodia

Name ^a	5' Label	Sequence 5'-3' ^b	Chromosome	Virtual heterozygosity (H_E)	No. of alleles
MS1-F	FAM	TCAACTGTTGGAAGGGCAAT	3	0.745	8
MS1-R		ctgtcttTTGCTGCGTTTTTGTCTG	A		
MS3-F	NED	GAAGATCCTGTGGAGGAGCA	4	0.740	6
MS3-R		ctgtcttCTCCTTCGCTCCTTTCTTT	s		
MS4-F	FAM	CGATTACTGTTGACGCTGAA	6	0.830	17
MS4-R		ctgtcttCAAAGGAACATGCTCGATGA			
MS5-F	NED	CGTCCTCTATCGCGTACACA	6	0.913	16
MS5-R		ctgtcttAAAGGGAGAGGAGCGAAAAC			
MS6-F	VIC	GGTTCTTCGGTGATCTCTGC	11	0.829	13
MS6-R		ctgtcttGGAGGACATCAACGGGATT			
MS7-F	FAM	TTGCAGAAAATGCAGAGAGC	12	0.884	14
MS7-R		ctgtcttAGGGTCTTCAGCGTGTGTT		A	
MS8-F	NED	AGAGGAGGCAGAAATGCAGA	12	0.939	26
MS8-R		ctgtcttAGCCCCCTTGCCTTCTTTAT			
MS9-F	FAM	AGATGCCTACACGTTGACGA	8	0.880	17
MS9-R		ctgtcttGAAGCTGCCCCATGTGGTAAT			
MS10-F	NED	TTATCCCTGCTGGATGTGAA	13	0.947	22
MS10-R		ctgtcttTCCTTCAGGTGGGACTTGTT			
MS12-F	FAM	AATGCGCATCCTATGTCTCC	5	0.866	16
MS12-R		ctgtcttCTGCTGTTGTTGTTGCTGCT			
MS15-F	FAM	TGTTTGCAAAGGAATCCACA	5	0.891	16
MS15-R		ctgtcttCGGCCAGATGAAAAGGATAA			
MS20-F	VIC	GCACAACAAATGCAAGATCC	10	0.911	18
MS20-R		ctgtcttGTGGCAGTGGCTCATCTTCT			

^aF = forward, R = reverse. The fluorescent dyes used to label primers were 6-FAM (6-carboxyfluorescein, "blue" fluorescent dye), VIC (Applied Biosystems proprietary "green" fluorescent dye), and NED (Applied Biosystems proprietary "yellow" fluorescent dye).

^bCTGTCTT tail (lowercase letters) was added to the 5' end of the reverse primers to promote nontemplate-directed nucleotide addition to amplicons in a reproducible way (Raby et al., 2003).

Table 3

Proportions of mixed-clone infections, mean number of microsatellite alleles per locus (and standard error of the mean, SE), genetic diversity (average virtual heterozygosity [H_E] and SE) and linkage disequilibrium (standardized index of association [I_A^S]) in malaria parasite populations from Pursat, western Cambodia

Parasite species	Multiple-clone infections, %	Alleles per locus, mean $\pm$ SE (range)	H_E , mean $\pm$ SE (range)	I_A^S by infection type ^a	
				all	Those with unique haplotypes
<i>P. falciparum</i>	47.6	21.2 $\pm$ 1.6 (3-20)	0.66 $\pm$ 0.06 (0.34-0.88)	0.051 (n = 164)	0.036 (n = 151)
<i>P. vivax</i>	89.6	16.1 $\pm$ 1.5 (6-26)	0.87 $\pm$ 0.02 (0.74-0.95)	0.010 (n = 87)	0.010 (n = 87) ^b

^a All I_A^S values were significantly larger than zero ($P = 0.001$), denoting significant multilocus linkage disequilibrium. For *P. falciparum*, we recalculated I_A^S considering only the 86 single-clone infections and obtained an even greater value of 0.068 ($P = 0.001$).

^b All *Plasmodium vivax* isolates had unique haplotypes.