Genetic diversity and population structure of Plasmodium vivax isolates from Sudan, Madagascar, French Guiana and Armenia

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ABSTRACT

Polymorphic genetic markers and especially microsatellite analysis can be used to investigate multiple aspects of the biology of Plasmodium species. In the current study, we characterized 7 polymorphic microsatellites in a total of 281 Plasmodium vivax isolates to determine the genetic diversity and population structure of P. vivax populations from Sudan, Madagascar, French Guiana, and Armenia. All four parasite populations were highly polymorphic with 3–32 alleles per locus. Mean genetic diversity values was 0.83, 0.79, 0.78 and 0.67 for Madagascar, French Guiana, Sudan, and Armenia, respectively. Significant genetic differentiation between all four populations was observed.

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1. Introduction

Malaria caused by Plasmodium vivax has been considered for many years a minor human disease compared to the deadly form caused by Plasmodium falciparum. However, P. vivax is currently the most widely distributed human malaria parasite with an estimated total of 390 million cases yearly. Mainly present outside Africa, P. vivax accounts for more than half of all malaria cases in Latin America, the Middle East, Asia and the Western Pacific. Nevertheless, P. vivax is still considered a neglected parasite and its huge socio-economic burden in endemic areas remains poorly documented (Gething et al., 2012; Battle et al., 2012).

Some threatening concerns about P. vivax malaria control are recently arising, as, for example, the emergence of P. vivax isolates with reduced susceptibility to different drugs (chloroquine, sulfadoxine/pyrimethamine and primaquine) (Hawkins et al., 2007; Baird, 2009), clinical reports of severe vivax malaria (Price et al., 2009) or observations of the capacity of this species to infect Duffy-negative reticulocytes of human populations from Africa and South America (Ryan et al., 2006; Cavasini et al., 2007; Ménard et al., 2010; Mendes et al., 2011; Wurtz et al., 2011; Woldearegi et al., 2013). Moreover, climatic changes and the associated extension of seasonal transmission areas could become a cause for concern of vivax malaria re-emergence in malaria-free areas, as recently happened in Greece (Andriopoulos et al., 2013).

Despite its public health importance, research of P. vivax biology lags far behind P. falciparum, mainly because of our inability to maintain P. vivax in continuous culture (Udomsangpetch et al., 2008). An extensive knowledge about epidemiology and genetics of P. vivax malaria is of highest importance in order to implement effective control measures in endemic areas. A step forward in this direction has been made with the completion of the whole genome project of P. vivax (Carlton et al., 2008) which has made it possible, among other issues, to identify new genetic markers such as microsatellites.

The first P. vivax polymorphic microsatellite (MS) has been described by Gomez et al. (2003). One year later, Leclerc et al. (2004a) identified other 13 MSs (di-nucleotide repeat units) from a genomic library obtained from DNA of Belem strain (adapted to Saimiri monkey), but the subsequent polymorphism analysis of
these loci in several natural parasite population revealed little variation of the MS arrays: only one locus was found polymorphic. Effectively, MS diversity is correlated with several factors including the repeat length (Sutton, 2013), and MSs identified by Leclerc et al. have very short repeat arrays that could explain their low levels of variation. Thereafter, Imwong et al. (2006) and Karunaweera et al. (2007) by scanning the whole genome sequence of P. vivax strain Salvador-1 optimized a novel set of MSs characterized by repeat units of three or four nucleotides and high polymorphism. In the last years, using different sets of highly polymorphic microsatellite markers, a number of studies of genetic variability and transmission dynamics of P. vivax isolates from different malaria endemic areas have been recently performed (Brazil-Amazonia: Ferreira et al., 2007; India/LaoPDR/Thailand/Colombia: Imwong et al., 2007; Brazil-Amazonia: Rezende et al., 2010; Peru: Van den Eede et al., 2010a; Vietnam: Van den Eede et al., 2010b; SriLanka/Myanmar/Ethiopia: Gunawardena et al., 2010; South Korea: Iwagami et al., 2012).

In this context, we have undertaken a multicentric study aiming to analyze the pattern of genetic diversity in four vivax populations. For this purpose, we have characterized a total of 281 vivax isolates from Sudan, Madagascar, French Guiana and Armenia, using seven polymorphic microsatellites previously described by Karunaweera et al. (2007).

2. Material and methods

2.1. Study sites

Blood samples were obtained from P. vivax-infected patients in all age groups living in four malaria endemic countries as follows (sampling localities are shown in Supplementary Fig. 1):

1. Sudan (Africa): the fieldwork was carried out in health facilities located in Wad Medani–Gezira State (Central Sudan) and New Halfa–Kassala State (East Sudan). A total of fifty-seven P. vivax samples were collected between June and December 2007, corresponding to the period of high malaria transmission. The study sites are located in a permanent irrigated agricultural scheme, where transmission is seasonal. Wad Medani is the main town of the irrigated area of Gezira Agricultural. New Halfa is the second largest agricultural scheme in Sudan.

2. Madagascar (Africa): a total of one hundred-forty-two blood samples were collected in 2006–2007 from malaria infected individuals who came to health facilities located across Madagascar. Sixty-eight samples were from Maevatanana (n = 17) and Miandrivazo (n = 51), two endemic areas with seasonal malaria transmission in the West of Madagascar. Sixty-five samples were from low-level endemic areas in Central Madagascar: Tsiranoanomandidy (n = 50), Antananarivo (n = 10) and Moramanga (n = 5). Nine samples were collected in Southern epidemic areas (Ejeta and Ishosy). 

3. French Guiana (South America): sixty-nine blood samples were collected between 2000 and 2005 from infected patients at the peripheral health centres located around Oyapock River, along the Brazilian border. French Guiana is a hypoendemic area, where the population has limited, if any, immunity (Legrand et al., 2005).

4. Malaria occurs in foci located mainly along the Maroni River to the West and the Oyapock River to the East, which serve as natural frontiers with Surinam and Brazil, respectively.

5. Armenia (Middle East): from October 2003 to October 2004, P. vivax isolates were collected from 13 patients, registered in the endemic regions of Ararat and Yerevan. Five of these P. vivax patients acquired malaria infections in neighbouring Azerbaijan. Armenia received the WHO certificate of malaria free country in 2011, but at the time of sample collection there was a significant fluctuation of malaria cases based on climatic conditions of the various regions of the country. Ararat Valley was the most malaria affected area.

2.2. DNA extraction and microsatellite genotyping

In all study areas, finger-prick blood samples were spotted onto filter paper and DNA was extracted using Instagene matrix (BioRad™, Marnes la Coquette, France) from blood spots from Madagascar and French Guiana and using PureLink Genomic DNA Kit (Invitrogen, Life Technologies™, Carlsbad, CA) from blood spots from Sudan and Armenia, according to manufacturer’s instructions. PCR method with fluorescent end-labelled primers was used to amplify the microsatellite markers MS1, MS2, MS3, MS7, MS8, MS10 and MS20 as described by Karunaweera et al. (2007) (Supplementary Table 1). PCR products were analysed on an automated DNA sequencer Ceq8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Allele size was assigned by comparison with reference alleles for each locus, for which the exact sizes were determined previously by direct sequencing. In addition, MS amplicons with previously undetected size were also sequenced to confirm the exact size.

2.3. Population genetic analyses

The predominant allele (where multiple alleles were detected in case of multi-infection) or the single allele was used to define haplotypes (unique combinations of alleles at each locus).

Genetic diversity was assessed by the number of alleles per locus (A) and Nei’s unbiased expected heterozygosity (Hₑ) adapted to haploid data using the LIAN 3.5 software (Haubold and Hudson, 2000). Moreover, allelic richness per locus and sample, which is a measure of the number of alleles independent of sample size, was estimated using FSTAT software v2.9.3.2. (Goudet, 1995).

For isolates that were fully genotyped at all 7 loci, multilocus linkage disequilibrium (LD) was assessed using the standardized index of association (IΔ), and significance levels of the observed IΔ values were calculated by using Monte Carlo simulation, using 10,000 random data permutations by the LIAN 3.5 software. Further, a pairwise linkage disequilibrium analysis (tests between all pairs of loci in each sample) was performed with 1000 randomisations and significance level was defined as 0.05 using FSTAT.

For linkage disequilibrium in LIAN program, the measure of disequilibrium is followed by testing the null hypothesis of linkage equilibrium. P-values derived from the parametric as well as from the Monte Carlo process and the 5% critical value calculated from the parametric approach.

Genetic differentiation between populations was estimated using pairwise unbiased estimators of F-statistics with no assumption of Hardy–Weinberg equilibrium by FSTAT. According to the FSTAT program (Goudet, 1995), the pairwise significance test of differentiation (FST) after standard Bonferroni correction is estimated for each pair corresponding to significance at the 5% nominal level. Moreover a Factorial Discriminant Analysis (FDA) was applied on fully genotyped individuals, whether 212 in total for the 4 populations sampled, to find discriminant variables (MS microsatellite loci) which differentiate well the groups of individuals using XLSTAT (XLSTAT, 2014). A circle with vectors of MS indicate the correlations between variables and factors of the FDA. Furthermore, a Factorial Analysis (FA) was carried out to assess the statistical differentiation between fully genotyped haplotypes of the P. vivax samples during successive years (2000–2003) in French Guiana and genetic differentiation among three localities sampled in Madagascar using XLSTAT (XLSTAT, 2014).
2.4. Ethic statement

The informed consent of each patient or an adult guardian of children enrolled in this study was obtained at the moment of blood collection. For all blood samples, identifying information have been removed so that data cannot be linked or re-linked with identifiable human subjects, making anonymous each sample processed in the present study.

3. Results

Total number of *P. vivax* infected blood samples successfully analyzed by PCR genotyping was 57 from Sudan, 142 from Madagascar, 69 from French Guiana and 13 from Armenia (Supplementary Table 2). Among them, complete data set for all 7 MS loci were obtained for 54 isolates (94.7%) from Sudan, for 93 isolates (65.4%) from Madagascar, 55 isolates (79.7%) from French Guiana and 10 isolates (76.9%) from Armenia (Table 1).

### 3.1. Allelic distribution and heterozygosities

The seven microsatellite loci surveyed were polymorphic in all stocks. The number of alleles observed per locus ranged from 3 (for locus MS1 in Armenian isolates) to 32 (for locus MS8 in Sudanese isolates). The total number of alleles detected reached 163 for overall loci. Unbiased expected heterozygosity (H$_e$ ± SD) was lower in Armenia (H$_e$ = 0.67 ± 0.11) compared to Madagascar (H$_e$ = 0.83 ± 0.08), French Guiana (H$_e$ = 0.79 ± 0.12) and Sudan (H$_e$ = 0.78 ± 0.17) samples (Supplementary Table 2). Differences of pairwise combinations of heterozygosities per locus between the four populations were all not significant using the Wilcoxon signed rank test with continuity correction two sided, except for the pairwise combination of Armenia-Madagascar heterozygosities (P-value = 0.02).

Considering the subset of 212 fully genotyped isolates (Table 1), 205 haplotypes were identified. The number of alleles per locus varied from 7 (total alleles for locus MS3) to 53 (total alleles for locus MS8). All four populations displayed a variable number of exclusive alleles (i.e. not present in the each of other analyzed populations), and specifically we observed 28, 22 and 12 exclusive alleles in Sudan, Madagascar and French Guiana, respectively and only 2 in Armenian isolates.

Sudan and Madagascar isolates displayed the highest number of shared alleles (n = 52). In addition, Madagascar shared 50 alleles with French Guiana parasite population while the latter shared 40 alleles with the Sudan population. Otherwise, high genetic diversity was confirmed by the high values of H$_e$, particularly for the Madagascar population (Table 1). Significant differences of pairwise allelic richness were detected using Wilcoxon test: French Guiana and Armenia (P-value = 0.01), French Guiana and Madagascar (P-value = 0.03), Sudan and Armenia (P-value = 0.03) and Madagascar and Armenia (P = 0.01).

### 3.2. Linkage disequilibrium

For the four studied populations, the analysis of linkage disequilibrium was estimated using the multilocus index of association ($I^2_S$) test (Table 2). Overall, linkage disequilibrium was observed in five pairs of loci in Armenian population (data not shown), resulting in a highly significant $I^2_S$ value. In fact, in Armenia 2 multilocus genotypes were identified more than once in our samples, that is the same multilocus genotype was found in 4 isolates within 8 of autochthonous cases, whereas a second identical genotype was found in 2 isolates from imported cases from Azerbaijan.

Furthermore, significant $I^2_S$ values were found in French Guiana and Sudan, where identical genotypes were found in some samples. No linkage disequilibrium was observed in isolates from Madagascar (Table 2).

### 3.3. Genetic divergence among sites

The inter-population differentiation, calculated by F$_ST$ estimators was significantly different from 0, with the highest value found between Armenian and French Guianan populations (F$_ST$ = 0.143). Malagasy and Sudanese populations showed the lowest differentiation (F$_ST$ = 0.042) (Table 2).

Overall F$_ST$ is highly significant (0.071 ± 0.012), probably due to the large geographical distance between the respective study areas.

### 3.4. Temporal and spatial analyses

Genetic diversity and $I^2_S$ indexes were also calculated for four subpopulations from French Guiana (temporal data) and for three subpopulations from Madagascar (spatial data) (Table 3). Specifically, French Guiana isolates were divided in four different groups based on the year of collection (i.e.: year 2000; 2001; 2002; 2003), except for 3 isolates collected between 2004 and 2005, which were not included in the analyses. Madagascar isolates were grouped in three subpopulations according to the site of collection (i.e.: Southern Madagascar, Central Madagascar, Western Madagascar). In our analyses, no haplotypes were shared between subpopulations, and likewise, for each of examined groups, the level of genetic diversity was high and concurring with mean H$_e$ value of the respective country (Table 3). However, when linkage disequilibrium was tested for the distinct subpopulations, highly significant linkage disequilibrium was found in a subpopulation in French Guiana (year 2002: $I^2_S$ = 0.140, P < 0.001) (Table 3).

Factorial Discriminant Analysis (FDA) was carried out to assess the statistical differentiation between *P. vivax* isolates from the

### Table 1

<table>
<thead>
<tr>
<th>Locus</th>
<th>Sudan (n = 54)</th>
<th>Madagascar (n = 93)</th>
<th>F. Guiana (n = 55)</th>
<th>Armenia (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>Rs</td>
<td>H$_e$</td>
<td>A</td>
</tr>
<tr>
<td>MS1</td>
<td>7</td>
<td>5.5</td>
<td>0.73</td>
<td>7</td>
</tr>
<tr>
<td>MS2</td>
<td>13</td>
<td>7.8</td>
<td>0.85</td>
<td>18</td>
</tr>
<tr>
<td>MS3</td>
<td>6</td>
<td>5.2</td>
<td>0.78</td>
<td>6</td>
</tr>
<tr>
<td>MS7</td>
<td>3</td>
<td>2.9</td>
<td>0.45</td>
<td>10</td>
</tr>
<tr>
<td>MS8</td>
<td>32</td>
<td>14.9</td>
<td>0.98</td>
<td>25</td>
</tr>
<tr>
<td>MS10</td>
<td>11</td>
<td>6.7</td>
<td>0.75</td>
<td>18</td>
</tr>
<tr>
<td>MS20</td>
<td>17</td>
<td>11.1</td>
<td>0.94</td>
<td>17</td>
</tr>
<tr>
<td>mean</td>
<td>12.7</td>
<td>7.7</td>
<td>0.78</td>
<td>14.4</td>
</tr>
<tr>
<td>s.d.</td>
<td>9.7</td>
<td>4.0</td>
<td>0.17</td>
<td>6.9</td>
</tr>
</tbody>
</table>

n: sample size, s.d: standard deviation.
This analysis was undertaken using XLSTAT package. The data for the FDA were the fully genotyped haplotypes (Armenia \( n = 10 \), French Guiana \( n = 55 \), Madagascar \( n = 93 \), Sudan \( n = 54 \)). The first plan of the FDA explains 96% (F1 73% and F2 23%) of the total inertia. Fig. 1 of the results shows largely overlapping distributions (centroids and ellipses of the 95% C.I.) of the genetic variability of \( P. \) vivax among sites with a higher scattering for the Sudan isolates. However the differentiation among populations is significant (Test of Bartlett, \( p \)-values < 0.001 for both F1 and F2 axes). MS2 and MS8 are the most informative loci for the differentiation of the Sudan isolates as attested by the circle of correlation variables/factors in Fig. 1. Additionally, Factorial Analysis (FA) was applied on multilocus genotypes to estimate the genetic variation of temporal data in French Guiana (from 2000 to 2003) and spatial data in Madagascar (Southern, Western and Central regions) (Fig. 2). The first plan of the FA showed that all isolates displayed a low inter-individual divergence with ellipses overlapping. The temporal and spatial differentiations are not significant among \( P. \) vivax isolates sampled.

### 4. Discussion

This study was undertaken to investigate the population genetic structure using 7 microsatellite loci (MS) in \( P. \) vivax populations from endemic areas belonging to three different continents, i.e. America, Africa and Asia. These endemic areas are characterized by various patterns of malaria transmission, from very low to relatively high. In Armenia, efficacious epidemic control interventions

<table>
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<th>Table 2</th>
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<tbody>
<tr>
<td>Linkage disequilibrium (LD) estimated by the standardized index of association (( I_A^0 )) with ( p )-value at 0.05 using LIAN and estimated pairwise ( F_{ST} ) among populations using FSTAT for fully genotyped ( Plasmodium ) vivax isolates.</td>
</tr>
<tr>
<td>Country</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Sudan</td>
</tr>
<tr>
<td>Madagascar</td>
</tr>
<tr>
<td>French Guiana</td>
</tr>
<tr>
<td>Armenia</td>
</tr>
</tbody>
</table>

* Significant \( p \)-value after standard Bonferroni correction at the 5% nominal level.

<table>
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<th>Table 3</th>
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<tr>
<td>Genetic diversity and linkage disequilibrium of ( Plasmodium ) vivax populations from French Guiana and Madagascar according to local temporal and geographical data using LIAN.</td>
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<tr>
<td>Country</td>
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<tr>
<td>F. Guiana 2000</td>
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<td>F. Guiana 2002</td>
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<td>F. Guiana 2003</td>
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<td>Southern Madag</td>
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<td>Central Madag</td>
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<td>Western Madag</td>
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* Significance level < 0.05.

**Fig. 1.** First plan (96% of the total inertia) of the Factorial Discriminant Analysis performed on microsatellite haplotypes of four \( Plasmodium \) vivax populations (Sudan \( n_{SUD} = 54 \) in black, Madagascar \( n_{MAD} = 93 \) in red, French Guiana \( n_{GUI} = 55 \) in green, Armenia \( n_{ARM} = 10 \) in blue) with ellipse confidence and centroids per population. The correlations between the seven microsatellite loci (MS) and the two factors of the FDA are shown on top right of the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
led to the elimination of the local malaria cases since 2006. In Sudan, malaria transmission is seasonal and particularly extended in agriculture schemes areas. French Guiana is characterized by a permanent transmission along the main rivers. In Madagascar, a variety of epidemiological patterns coexist. Previous similar studies demonstrated that the genetic variability of vivax populations cannot be affected by the level of transmission, conversely to what happens in P. falciparum populations (Arnott et al., 2012).

We show here that the level of genetic diversity, estimated by the number of haplotypes and the allelic richness, is remarkably high in all four P. vivax populations and the mean $H_e$ values are very similar among the investigated countries, except for Armenia (Supplementary Table 2 and Table 1). In fact, in this country the smallest sample size and the lowest $H_e$ values were recorded. However, at the time of sample collection (2003–2004) the total number of registered vivax malaria cases (imported + autochthonous) were 65 (Davidyants, 2011), hence our sampling represented 10% of vivax cases registered in 2003 and 25% of vivax cases circulating in Armenia in 2004, including all the 6 indigenous cases registered in 2004.

Moreover, $H_e$ values (0.67 in Supplementary Table 2; 0.63 in Table 1) found in Armenian population were high if we take into consideration the low level of malaria endemicity in this country. In effect, these values were higher than what calculated in South Korea (10 MS loci analyzed in 87 isolates, mean $H_e = 0.43$ (Iwagami et al., 2012), and at the same time slightly lower of the $H_e$ detected in India (9 MS loci, 67 isolates, mean $H_e = 0.72$) (Imwong et al., 2007), considering the high level of malaria endemicity within these countries.

Our hypothesis is that isolates introduced from the neighboring Azerbaijan ($N = 5$ of 13) may have contributed to increase the level of heterozygosity of the Armenian vivax population, taking into account that a study based on the analysis of polymorphic genes showed an extensive genetic variability of P. vivax isolates circulating in Azerbaijan (Leclerc et al., 2004b).

Globally, our findings are in line with what has been already observed in previous studies carried out in some endemic areas of Asia (India, Thailand, Myanmar, Laos and PNG) (Imwong et al., 2007; Gunawardena et al., 2010; Koepflı et al., 2013) and South America (Colombia and Brazil) (Imwong et al., 2007; Ferreira et al., 2007; Rezende et al., 2010) demonstrating a high level of genetic diversity of vivax populations independently of the intensity of malaria transmission.

When we analyzed the LD, as expected, the higher $I^A$ value ($0.54$, $P < 0.001$) was found in Armenia, where the population has a more homogenous structure probably mainly to the presence of “plasmodial clones”, in fact six over the total ten analyzed isolates shared two multilocus genotypes. Similarly, a significant $I^E$ value ($0.033$, $P < 0.01$) was obtained by analyzing all haplotypes from Sudanese isolates (N = 54). Moreover, when only unique haplotypes from Sudan and Armenia populations were considered for assessment of LD indexes, significant values were also observed: Armenia, 6 isolates, $I^E$ 0.244, $P < 0.01$; Sudan, 51 isolates, $I^E$ 0.021, $P < 0.05$. The co-occurrence of high $H_e$ value and significant LD found in Sudan was already observed in other endemic areas (Gunawardena et al., 2010). Significant $I^E$ value was found also in French Guiana ($P < 0.05$), imputable to the LD value calculated in the subgroup of isolates collected in year 2002 (Table 3), even though the limited epidemiological data we have do not allow to us any speculation about the presence of linkage disequilibrium in this particular subgroup.

The vivax isolates from Madagascar showed no evidence of LD, probably due to the fact that, even in low endemic setting, the large migratory flux of people from India, South-East Asia (Indonesia and Malaysia) and Africa could contribute to maintain a high rate of recombination in the Malagasy vivax population.

Concerning the genetic differentiation between the studied populations, $F_{ST}$ values seem to be proportional to the geographical distance of the 4 sampling areas, classically named “isolation by distance”. P. vivax populations from French Guiana and Armenia showed the highest value of $F_{ST}$ while the lowest-one was between Sudan and Madagascar. Furthermore, no significant spatial and temporal differentiations (FA) were detected in Madagascar and French Guiana samples, respectively.

Fig. 2. First plan (18% of the total inertia) of the Factorial Analysis carried out on temporal samples of French Guiana (nGUI 2000 = 15 in blue, nGUI 2001 = 14 in green, nGUI 2002 = 12 in red, nGUI 2003 = 11 in azur) with dashed confidence ellipses and spatial samples of Madagascar (nMAD South = 7 in violet, nMAD Central = 37 in black, nMAD West = 19 in brown) with solid confidence ellipses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
5. Conclusions

In our study, we notice a high heterogeneity in each of the studied P. vivax populations.

These findings are consistent with those reported in previous studies carried out in other endemic areas. Globally, in the four countries belonging to three considered continents, we observed a comparable levels of genetic diversity that seem to be not really affected by the sample size, the length of sampling period and sampling areas.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.07.029.

References


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