2012

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BIOSCIENCE, WASHINGTON, v. 62, n. 1, supl. 1, Part 1, pp. 39-45, JAN, 2012
http://www.producao.usp.br/handle/BDPI/32567

Acta Tropica 124 (2012) 27–32

Contents lists available at SciVerse ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

The genetic diversity of *Plasmodium malariae* and *Plasmodium brasilianum* from human, simian and mosquito hosts in Brazil

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ARTICLE INFO

Article history:
Received 11 November 2011
Received in revised form 29 May 2012
Accepted 31 May 2012
Available online 15 June 2012

Keywords:
Malaria
Plasmodium malariae
Genetic diversity
Microsatellites
Brazil
Atlantic forest

ABSTRACT

Plasmodium malariae is a protozoan parasite that causes malaria in humans and is genetically indistinguishable from *Plasmodium brasilianum*, a parasite infecting New World monkeys in Central and South America. *P. malariae* has a wide and patchy global distribution in tropical and subtropical regions, being found in South America, Asia, and Africa. However, little is known regarding the genetics of these parasites and the similarity between them could be because until now there are only a very few genomic sequences available from simian *Plasmodium* species. This study presents the first molecular epidemiological data for *P. malariae* and *P. brasilianum* from Brazil obtained from different hosts and uses them to explore the genetic diversity in relation to geographical origin and hosts. By using microsatellite genotyping, we discovered that of the 14 human samples obtained from areas of the Atlantic forest, 5 different multilocus genotypes were recorded, while in a sample from an infected mosquito from the same region a different haplotype was found. We also analyzed the longitudinal change of circulating plasmodial genetic profile in two untreated non-symptomatic patients during a 12-months interval. The circulating genotypes in the two samples from the same patient presented nearly identical multilocus haplotypes (differing by a single locus). The more frequent haplotype persisted for almost 3 years in the human population. The allele Pm09-299 described previously as a genetic marker for South American *P. malariae* was not found in our samples. Of the 3 non-human primate samples from the Amazon Region, 3 different multilocus genotypes were recorded indicating a greater diversity among isolates of *P. brasilianum* compared to *P. malariae* and thus, *P. malariae* might in fact derive from *P. brasilianum* as has been proposed in recent studies. Taken together, our data show that based on the microsatellite data there is a relatively restricted polymorphism of *P. malariae* parasites as opposed to other geographic locations.

1. Introduction

The protozoan parasite *Plasmodium malariae* causes malaria in humans and is genetically indistinguishable from *Plasmodium brasilianum* which infects New World monkeys from South and Central America. This points to a recent host transfer between humans and monkeys (Escalante et al., 1995, 1998; Qari et al., 1996). *P. malariae* is widely distributed in a patchy pattern and is found throughout tropical regions in Africa, Asia and South America. Little is known about the genetics of these parasites, since studies focusing *P. malariae* and *P. brasilianum* are still scarce and the currently observed similarity between the two species may be simply due to the fact that there are only a very few genomic sequences available from simian *Plasmodium* species. Recently, six polymorphic genetic markers were described for *P. malariae* and the first molecular epidemiological data for this parasite in naturally acquired human infections from Africa and Asia were presented (Bruce et al., 2007, 2011).

In Brazil, *P. malariae* and *P. brasilianum* are encountered in the Amazon Region and also in the Extra Amazon Region (Atlantic forest regions), but in the latter malaria transmission

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0001-706X/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.actatropica.2012.05.016

is considered hypoendemic and controlled (Oliveira-Ferreira et al., 2010; Deane, 1992). The state of São Paulo is the second state that holds large remaining areas of Atlantic forest (http://mapas.sosma.org.br/dados/). This forest has many bromeliads that are an ideal environment for *Anopheles* mosquitoes of the subgenus *Kerteszia*, mainly *An. (K.) cruzii*, that use the axils of these plants as larval habitat (Forattini et al., 1996). In São Paulo state, one of the counties with higher reported incidence of human malaria cases is Juquitiba, which annually records sporadic cases of autochthonous malaria (CVE, 2008). Despite the small number of clinical cases of malaria, a significant part of the population shows serologic evidence of recent exposure to different variants of *Plasmodium vivax* or *P. malariae* (Curado et al., 1997), suggesting that asymptomatic infections with either species may be frequent. These infections apparently are often overseen because the control programs are based on passive and active search of symptomatic cases.

Our study presents the first molecular epidemiologic data for *P. malariae* and *P. brasilianum* from Brazil obtained from different hosts and uses them to explore the genetic diversity in relation to geographic origin and hosts. For this, we used microsatellite markers and analyzed infected field samples from three different host groups, namely humans, monkeys and mosquito. It is important to keep in mind that our work was not designed to show that *P. malariae* and *P. brasilianum* are close relatives, because this fact has already been documented in previous studies, but to shed a light on the genetic diversity between both species. Considering the recent expansion of *P. malariae* in human populations, the host switch between humans and monkeys might have occurred through a single transfer associated with a bottleneck, after the European colonization of the Americas in the 16th century (Escalante et al., 1995). Therefore, the direction of the host transfer can then be suggested by comparing the genetic diversity of the human and primate parasites (Tazi and Ayala, 2011). Our results indicated a greater diversity among isolates of *P. brasilianum* compared to *P. malariae* and thus, *P. malariae* might derive from *P. brasilianum* as has been proposed in recent studies.

2. Materials and methods

2.1. Samples

Twenty samples of *P. malariae*/*P. brasilianum* genomic DNA were used in this study (Fig. 1, Tables 1 and 2). Among these, two samples were obtained from patients involved in a case of transfusion malaria: one sample (1) was obtained from a splenectomized patient (58/04) who was infected by blood transfusion and another (2) from her blood donor, an asymptomatic carrier (72/04), which probably acquired the infection in the location of Palestina, Juquitiba (Di Santi et al., 2005). From a previous project, eleven samples (two of which were collected from the same patient at different dates) were used in our study (3–11, Fig. 1A). Other available *P. malariae*/*P. brasilianum* samples were also analyzed (Fig. 1A and B): a *P. malariae* sample (12) obtained from a patient (I11) infected in Iporanga (Atlantic forest region); a *P. malariae* sample (13) obtained from a patient (RBR714) infected in Rio Branco (AC) (Brazilian Amazon Region); a *P. malariae*/*P. brasilianum* sample (M95) obtained from a pool of three *Anopheles Kerteszia cruzii* mosquitoes collected in Itanhaém (Atlantic forest region) and three *P. brasilianum* samples obtained from simian hosts captured in Abunã, approximately 200 km from Porto Velho (RO) (*Callicebus dubius*, sample P169) and Igapó-Açú, approximately 300 km from Manaus (AM) (*Callicebus caligatus*, samples P177 and P182), both located in the Brazilian Amazon. Additionally, a *P. brasilianum* strain from Peru (Peruvian III) was used as a control. In all samples, the presence of *P. malariae*/*P. brasilianum* was confirmed by two PCR protocols and sequencing: (i) a nested PCR based on the amplification of small subunit ribosomal RNA (ssrRNA) using a modification of the technique originally described by Snounou et al. (Snounou et al., 1993; Singh et al., 1999), where the first amplification reaction was modified in order to increase the sensitivity, considering samples with low parasitemia (dos Santos et al., 2009); and (ii) a nested PCR based on the amplification of the mitochondrial cytochrome b gene (cytb) (Perkins and Schall, 2002).

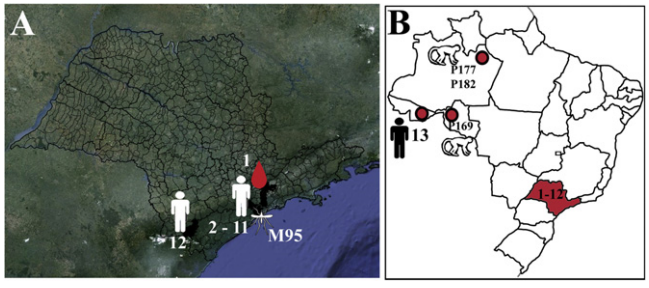

Fig. 1. Origins of Brazilian *P. malariae*/*P. brasilianum* samples used in the study. (A) From São Paulo state: human samples from a splenectomized patient who was infected by blood transfusion (1), Juquitiba (2–11) and Iporanga (12); mosquito sample from Itanhaém (M95). (B) From the Brazilian Amazon Region: human sample from Acre (13) and samples from simian hosts captured in Rondônia (P169) and Amazonas (P177 and P182).
Modified from Google5 Earth and Wikimedia Commons.

2.2. Whole-genome amplification and microsatellite typing

In order to perform a multilocus microsatellite analysis, recovered parasite material was first submitted to WGA (whole-genome amplification), a procedure that increases DNA in a nonspecific way, thus allowing analysis of natural infections with limited sample volumes. For this, 5 μl of the original sample (gDNA) was amplified using REPLI-g Mini Kits (Qiagen, Valencia, CA) according to manufacturer's instructions, yielding 50 μl of template from each reaction. To assess the extent of polymorphism we amplified four microsatellites and two minisatellites using a semi-nested PCR reaction (Bruce et al., 2007). PCR products were separated on a 310 capillary sequencer (Applied Biosystems) without dilution (except of Peruvian III and 1 samples, which were diluted 1/10). Allele sizes were measured by comparison with size standard Gene Scan 500 (Applied Biosystems). The lengths and relative abundance of peak heights in electropherograms were determined using the open-access STRand software (available at: http://www.vgl.ucdavis.edu/informatics/strand.php). In this study, the minimal detectable peak height was set to 100 arbitrary fluorescence units (FU) (Van den Eede et al., 2010), to ensure allele recognition in view of very low parasitemias found in *P. malariae* infections, especially in low transmission areas. However, our samples were analyzed in duplicate in independent experiments and the reactions showed neglectable background noise (below 10 FU).

2.3. Data analysis

Arlequin 3.0 software was used to haplotype identification and analysis of molecular variance (AMOVA) (Excoffier et al., 2005). The genetic diversity of the human and non-human parasite populations was examined by calculating the virtual heterozygosity (H_E) of each group, defined as $H_E = [n/(n-1)][1 - \Sigma p^2 i]$, where n is the number of isolates analyzed and pi is the frequency of the ith allele in the population. Virtual heterozygosity may be defined, in this context, as the average probability that a pair of alleles randomly obtained from the population is different. Virtual heterozygosity

Table 1
Human samples used in this study.

Sample	Collection date (dd/mm/yyyy)	Source	Origin
1	22/09/2004	Patient 58/04	São Paulo, SP (Blood transfusion from Patient 72/04)
2	08/12/2004	Patient 72/04	Juquitiba, SP, Atlantic Forest, BR
3	19/08/2005	Patient PPP23	Juquitiba, SP, Atlantic Forest, BR
4	17/02/2006	Patient PPP16	Juquitiba, SP, Atlantic Forest, BR
5a	23/06/2005	Patient	Juquitiba, SP, Atlantic Forest, BR
5b	30/03/2006	PPP62	Juquitiba, SP, Atlantic Forest, BR
6a	19/04/2006	Patient	Juquitiba, SP, Atlantic Forest, BR
6b	24/05/2007	PPP190	Juquitiba, SP, Atlantic Forest, BR
7	10/08/2006	Patient PPP68	Juquitiba, SP, Atlantic Forest, BR
8	24/05/2007	Patient PPP194	Juquitiba, SP, Atlantic Forest, BR
9	27/06/2007	Patient PPP222	Juquitiba, SP, Atlantic Forest, BR
10	30/08/2007	Patient PPP157	Juquitiba, SP, Atlantic Forest, BR
11	19/04/2007	Patient PPP328	Juquitiba, SP, Atlantic Forest, BR
12	xx/01/2002	Patient I11	Iporanga, SP, Atlantic Forest, BR
13	09/07/2004	Patient RBR714	Rio Branco, AC, Amazon Region, BR

SP = São Paulo state; AC = Acre state; BR = Brazil.

Table 2
Non-human samples used in this study.

Sample	Collection date (dd/mm/yyyy)	Source	Origin
M95	26/05/2010	*Anopheles (K.) cruzii*	Itanhaém, SP, Atlantic Forest, BR
P169	31/03/2010	*Callicebus dubius*	Porto Velho, RO, Amazon Region, BR
P177	06/02/2011	*Callicebus caligatus*	Manaus, AM, Amazon Region, BR
P182	07/02/2011	*Callicebus caligatus*	Manaus, AM, Amazon Region, BR
Peru III	xx/xx/1987	*Saimiri sciureus peruviensis*	Iquitos, Peru
Peru I[a]	xx/xx/1983	*Aotus vociferans*	Iquitos, Peru

SP = São Paulo state; RO = Rondônia state; AM = Amazonas state; BR = Brazil.
[a] from Bruce et al., 2007.

ranges between 0 and 1, with high values (i.e. those close to 1) reflecting high genetic diversity levels of that population.

The standardized index of association (I_A^S) was used to test for evidence of overall multilocus linkage disequilibrium in each parasite population (human and non-human). Data were analyzed with LIAN 3.0 software (Haubold and Hudson, 2000).

We applied the Bayesian clustering model implemented in the STRUCTURE 2.1 software to test whether microsatellite haplotypes clustered according to the host of collection of the isolates (Pritchard et al., 2000). We run the program with seven different *K* values (from 2 to 8) and computed the posterior probability for each *K* (Evanno et al., 2005). Here we show the clustering patterns obtained with *K* value = 2 (human and non-human group) associated with the strongest statistical support.

We used BOTTLENECK 1.2.02 software (Luikart and Cornuet, 1998) to verify whether the population had suffered a population reduction, such as a bottleneck or a founder effect.

2.4. Ethical considerations

Approval of the study protocol was obtained from the Ethical Review Board of the Institute of Biomedical Sciences of the University of São Paulo, Brazil (CEPSH 749/2006 and 065/2008). All procedures adopted in this work were approved in a full compliance with specific federal permits issued by the Brazilian Ministry of Environment (SISBIO, process numbers 18861-3 and 24319-3).

3. Results and discussion

In order to standardize the *P. malariae* microsatellite characterization in our laboratory, we initially analyzed the sample 1 which was from a splenectomized patient with higher parasitemia. The results obtained after PCR amplification of the 6 markers are shown in Table 3. In this work, we include the results of samples for which all 6 loci were available (except for samples 6b and 13, where one and three markers were not amplified, respectively). In addition to the samples shown in Table 1 and 2, many other *P. malariae* samples were also tested but gave negative results in the PCR amplification of the microsatellite markers. These samples, although *P. malariae* positive by nested PCR of the ssrRNA (which is repetitive), probably had too small amounts of target DNA, considering that these microsatellite markers are single copy loci. It is important to note that the WGA technique was useful only for samples with higher parasitemias (1 and Peruvian III). Therefore, it was not possible to amplify all 6 loci in some samples without using the original gDNA sample to complete the analysis. Nevertheless, the definition of the haplotype in some samples was incomplete and unfortunately, these experiments could not be repeated due to insufficient sample amounts. Amplification of some loci and not others from the same sample is not an unusual result since differences in the efficiency of performance of primers used in the analysis is possible. Importantly, the PCR sensitivity threshold has to be taken in account. This limit has been maximized by concentrating the sample prior to DNA extraction (by a saponin lysis), using a nested PCR protocol and detecting the product by capillary electrophoresis with fluorophores. Therefore, we assume that mixed clone infections were not missed. Moreover, these samples have been studied previously for other targets such as ribosomal and mitochondrial genes and we found only unique sequences for each locus (data not shown).

From the 14 Brazilian human samples from the Atlantic forest (1–12), 5 different multilocus genotypes were recorded (Table 3): haplotype 1 (185/342/288/107/220/331) was found in four samples; haplotype 2 (185/342/277/107/220/331) in three samples; haplotype 3 (185/342/277/107/222/331) in two samples; haplotype 4 (185/342/288/107/222/331) in four samples; haplotype 5 (185/342/288/107/Nd/331) in one sample. Haplotype 6 (185/Nd/277/107/Nd/Nd) was found in the Amazon Region sample (13). Haplotype 7 (181/325/277/107/222/331) was found only in the mosquito sample (M95). From the 4 samples obtained from simian hosts, 4 different multilocus genotypes were recorded

Table 3
Microsatellite markers from the samples shown in Table 1.

Group	Sample	Pm02	Pm09	Pm11	Pm25	Pm34	Pm47	Haplotype
Human	1	185	342	288	107	220	331	1
	2	185	342	288	107	220	331	1
	3	185	342	277	107	220	331	2
	4	185	342	288	107	220	331	1
	5a	185	342	277	107	222	331	3
	5b	185	342	288	107	222	331	4
	6a	185	342	288	107	222	331	4
	6b	185	342	288	107	Nd[a]	331	5
	7	185	342	277	107	222	331	3
	8	185	342	288	107	222	331	4
	9	185	342	288	107	222	331	4
	10	185	342	277	107	220	331	2
	11	185	342	288	107	220	331	1
	12	185	342	277	107	220	331	2
	13	185	Nd[a]	277	107	Nd[a]	Nd[a]	6
Non-human	M95	181	325	277	107	222	331	7
	P169	185	325	288	107	222	331	8
	P177	177	325	288	100	220	331	9
	P182	185	325	277	107	210	331	10
	Peruvian III	185	265	277	107	220	331	11
	Peruvian I[b]	181	299	288	107	220	331	12

[a] Nd means that no readable signal was obtained after at least two repetitions.
[b] From Bruce et al., 2007.

(Table 3): haplotype 8 (185/325/288/107/222/331), haplotype 9 (177/325/288/100/220/331), haplotype 10 (185/325/277/107/210/331) and haplotype 11 (185/265/277/107/220/331). Multiple alleles were not detected in our samples.

When we tested a mosquito sample, we expected that a recent genetic recombination may have occurred (zygote formation and subsequent meiosis and onset of sporogony). However, only one allele per locus was detected even in this sample, indicating that the examined loci are relatively stable although obviously general data for the stability of loci in *P. malariae*/*brasilianum* are lacking. On the other hand, the chance for seeing mixed-clone infections in mosquitoes is low even for highly endemic regions (Razakandrainibe et al., 2005). In this sense, the observation of an apparently single clone infection in the mosquito sample may be expected.

Using geographic information system to locate the human samples in the region of Juquitiba, we found that there are clearly two groups (data not shown), which are distant about 3 km. However, the five haplotypes obtained in this population were randomly distributed in both groups. This lack of population sub-structuring has also been demonstrated in villages separated by much larger distances (Bruce et al., 2011). Considering that the flying range of *An. (Ker.) cruzii* is about 1 km (Ferreira et al., 1969), in our case, the admixture is likely to be attributable to human movement.

The two samples of *P. malariae* obtained from a blood donor (2) and her recipient (1) involved in a previously reported case of transfusion malaria (Di Santi et al., 2005), showed the same haplotype. This provides further evidence that both were infected with the same strain, as would be expected. Assuming that an absence in polymorphisms in microsatellites reflects stability also in other loci, it seems that the same *P. malariae* haplotype can persist for almost 3 years in a population (see samples 2 and 11). We also analyzed the longitudinal change of circulating plasmodial genetic profile in two untreated non-symptomatic patients (5 and 6) during a long time interval (12 months). The circulating genotypes in the two samples from the same patient present nearly identical multilocus haplotypes (differing by a single locus).

The allele Pm09-299 described by other authors as a genetic marker for South American *P. malariae* (Bruce et al., 2007) was not found in our samples. Another interesting finding is that the Pm09 marker was described with sizes ranging from 281 to 351 bp (Bruce et al., 2011), however, this marker showed a novel allele size of 265 bp in the Peruvian III strain. To confirm this result, this new allele was cloned and sequenced (GenBank HQ677907). This sequence showed two deleted repeat units when compared to the original *P. brasilianum* microsatellite Pm09 sequence (Colombian I/NYU strain) (GenBank DQ787849) (Bruce et al., 2007). The strain of *P. brasilianum* designed Peruvian III was also compared to Peruvian I, already analyzed by other authors (Bruce et al., 2007). Three markers were found in different sizes compared to those described (Table 3). In addition to Pm09, we obtained differences in markers Pm02 and Pm11. Although the two strains had been isolated from monkeys of Iquitos, Peruvian I was obtained in 1983 from *Aotus vociferans* (Collins et al., 1985), while Peruvian III was obtained from *Saimiri sciureus peruviensis* in 1987 (Collins et al., 1990).

It is important to note that Pm34 is a dinucleotide microsatellite marker [$(CA)_n$] and therefore the estimates of allele frequency for this locus are often confounded by overlapping "shadow" bands (Murray et al., 1993). In this case, in addition to the main PCR product band, shadow bands can be seen 2 bp below the main band. The presence of these shadow bands is especially troublesome if the alleles differ by 2 bp. Thus, the alleles Pm34-220 and Pm34-222 could be considered the same allele. Also, it has been suggested that microsatellite diversity correlates positively repeat array length, as the rate of strand-slippage events that create diversity increases exponentially with repeat array lengths (Imwong et al., 2006). In fact in our work the only marker monomorphic for all samples was Pm47, which was described as a $(CAATT)^7$ (Bruce et al., 2007), representing very few repeats and would not be expected to be variable. However, the other five markers presented from two to four alleles per locus in our limited population and could be considered good genotyping markers to study the genetic diversity of these parasites.

Allele frequencies were calculated as a proportion of the total number of alleles detected for each locus using Brazilian *P. malariae* human samples showed in Table 1 and were compared with those found in a previous study where samples from Malawi, Gambia and Thailand were analyzed (Bruce et al., 2007). Although the number of samples has been lower in our study (15 versus 52), allelic diversity was more restricted in the Brazilian samples. Different genotypes were recorded in 39 of 52 samples from Malawi/Thailand/Gambia (Bruce et al., 2007) and 6 of 15 human samples from Brazil (this

study). The proportion of different genotypes was significantly higher in non-Brazilian samples compared to Brazilian samples ($x^2 = 6.47$, $P < 0.05$). Pm02-185 was the only allele found in this locus in our human samples while Pm02-181 was found in the mosquito sample, but these were also the most frequent alleles in Asian and African samples. Similarly, Pm09-342 was the only allele found in this locus in our human samples while Pm09-325 was found in the mosquito sample. Although absent in our human population, the latter was found in more than 80% of the Asian and African samples and in all the Brazilian simian samples (this study). Pm11-288 was found more frequently and Pm11-277 was the second most frequent, but these are also the most frequent alleles in Asian and African samples in an inverse proportion. Pm34-220 and Pm34-222 were found in the same frequencies in Brazilian isolates and are the second and third most frequent in Asian and African samples. Pm25-107 and Pm47-331, monomorphic in our human population, were the most frequent and the third most frequent in Asian and African samples. This distribution remained unchanged in the recent study carried out only with samples from Malawi (Bruce et al., 2011).

When AMOVA was performed, we found 54.89% of the diversity within groups (human and non-human) and 45.11% among groups. However, diversity indexes in the studied populations range from 0.2218 ± 0.0668 (human group) to 0.4095 ± 0.0447 (non-human group) (0.3211 ± 0.0518 mean H_E). In human samples, other authors have obtained values between 0.236 and 0.811 (Bruce et al., 2007) or 0.192 and 0.849 (Bruce et al., 2011), depending on the *P. malariae* microsatellite locus. Interestingly, we found higher heterozygosity at locus Pm09, which was the one with lowest heterozygosity values in the other two studies (Bruce et al., 2007, 2011).

Significant multilocus linkage disequilibrium (LD) was found in the isolates from human ($I_A^S = 0.2005$, $P < 0.05$) and non-human origin ($I_A^S = 0.0375$, $P < 0.05$), with a mean of $I_A^S = 0.1336$, ($P < 0.05$). The higher LD value obtained for the human group may have some explanations. The parasites from this population may have expanded more recently than the parasites from the non-human population and few mutations could arise and accumulate during this shorter time frame, although a bottleneck effect was not detected in human and non-human groups. This hypothesis agrees with the view that *P. malariae* has derived from *P. brasilianum* (Tazi and Ayala, 2011). Furthermore, the lower transmission rate of the Atlantic forest samples compared to the Amazon Region samples may also explain the higher LD value found in the human group, since the chances of recombination in this area are certainly low. Lastly, this difference of LD values between human and non-human parasite populations can be related to the vector. Although *An. cruzii* is the principal vector for human and non-human populations in the Atlantic forest (Deane et al., 1970), in the Amazon Region, *An. darlingi* is the major vector for human parasites (Oliveira-Ferreira et al., 2010) but is unlikely to be involved in the transmission of *P. brasilianum* (Lourenço-de-Oliveira & Luz, 1996). In *P. vivax*, different parasite variants were better adapted to a specific vector than others (Joy et al., 2008), but unfortunately, there is no similar study for *P. malariae* or *P. brasilianum*. Moreover, the chromosomal position of the *P. malariae* markers is not known making it not possible to compare physical distance and linkage disequilibrium.

The population structure was evaluated using the Bayesian model-based approach implemented in STRUCTURE software, with the most likely $K = 2$. Fig. 2 shows the clustering patterns obtained of *P. malariae*/*P. brasilianum* isolates into human and non-human origins. We represent in the bars the proportion of these two inferred genetic subpopulations (represented by colors) for each of the host populations. Each host population showed heterogeneity with one distinct predominant genetic subpopulation.

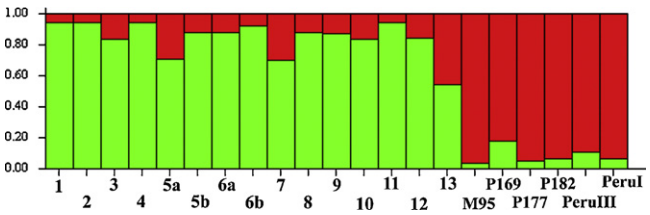

Fig. 2. Population structure of *Plasmodium malariae*/*Plasmodium brasilianum* plotted in a single line according to host (Human or Non-human) inferred from microsatellite typing of 21 isolates at $K = 2$. Isolates are numbered as described in Tables 1 and 2.

The high genetic identity found between *P. malariae* from humans and *P. brasilianum* from primates suggests the occurrence of recent transfers between hosts (Escalante et al., 1995, 1998; Qari et al., 1996), but there is no evidence of the transfer direction human/monkey. Although studies that focus on these species are rare, a recent study indicate that *P. brasilianum* strains seem to be more divergent than *P. malariae* and thus, *P. malariae* might in fact derive from *P. brasilianum* (Tazi and Ayala, 2011). Here we also found a greater diversity among isolates of *P. brasilianum*, however, one of the markers was monomorphic and the number of samples was limited. Moreover, this hypothesis of transfer of the parasite from New World monkeys to humans may not be plausible, since the characteristic symptoms of *P. malariae* malaria, like quartan fever, were specifically recorded in Greece by Hippocrates in the 4th century B.C. and were also known in Roman times in Italy (Boyd, 1949; Russell, 1955), this is, before any migration of humans from the Old World to the New World. Thus, *P. brasilianum* would have naturally adapted to grow in monkeys following human settlement of South America within the last 500 years (Collins and Jeffery, 2007). By all terms, it is impossible to differentiate infections of *P. malariae* from infections of *P. brasilianum* because they may, in fact, be one and the same exhibiting "host polymorphism" (Escalante and Ayala, 1994).

It may be assumed that diversity and multiplicity of *P. falciparum* infection increase as transmission frequency increases (reviewed by Babiker and Walliker, 1997). We detected low allelic diversity in the Brazilian isolates that reflects a very low transmission rate. It is known that *P. malariae* has a reduced growth rate (Gilles and Warrell, 1993) and the low parasitemias are possibly caused by its preference for mature erythrocytes (Garnham, 1966). *P. malariae* is also known to produce infections which are frequently non-symptomatic and long-lasting (up to 40 years in the absence of re-infection) (Vinetz et al., 1998). It is also possible that the infection with exclusively *P. malariae* explains the very low transmission rates found in the study area. In other locations where *P. malariae* occurs in co-infections mainly with *P. falciparum*, *P. malariae* developed means of increased transmission efficiency in the presence of a co-infecting parasite of another species (Bruce et al., 2011).

In summary, *P. malariae* was found practically conserved in human samples varying only in two loci for all samples analyzed independently of their geographical origin. The data may have been partial given that only one sample from the Amazon Region was available. On the other hand, in relation to the hosts, different haplotypes were found in the samples from the three different host groups. However, the lack of evidence of geographical clustering of haplotypes may be due to the available small sample number. The further analysis of Brazilian samples from different geographical origin will be important to characterize the epidemiology of Brazilian *P. malariae* isolates. It will be interesting to compare human and simian isolates of *P. malariae* and *P. brasilianum* in areas of Atlantic forest where humans and simians are subject to infection transmitted by the same anopheline vectors. This circumstance possibly

permits the frequent contact of simian derived *Plasmodium* and humans and vice versa.

Acknowledgments

We are grateful to Profª Luzia Helena Carvalho (Laboratório de Malária, Centro de Pesquisas René Rachou – FIOCRUZ) for the provision of *P. brasilianum* (Peruvian III strain) and to Ricardo Luiz Dantas Machado (Centro de Investigação de Microrganismos, Faculdade de Medicina de São José do Rio Preto – FAMERP) for the provision of *P. malariae* (RBR714 isolate). We are also grateful to Prof Luis Fabio Silveira (Museu de Zoologia da Universidade de São Paulo) for the provision of P169 sample. This research was funded by CNPq (475727/2007-0 – Edital Universal) to Karin Kirchgatter. Lilian de Oliveira Guimarães has a CAPES scholarship.

References

Babiker, H.A., Walliker, D., 1997. Current views on the population structure of *Plasmodium falciparum*: implications for control. Parasitology Today 13, 262–267.

Boyd, M.F., 1949. In: Boyd, M.F. (Ed.), Malariology, vol. 2. Saunders, Philadelphia, 3–28, 1643 pp.

Bruce, M.C., Macheso, A., Galinski, M.R., Barnwell, J.W., 2007. Characterization and application of multiple genetic markers for *Plasmodium malariae*. Parasitology 134, 637–650.

Bruce, M.C., Macheso, A., McConnachie, A., Molyneux, M.E., 2011. Comparative population structure of *Plasmodium malariae* and *Plasmodium falciparum* under different transmission settings in Malawi. Malaria Journal 10, 38.

Collins, W.E., Jeffery, G.M., 2007. Plasmodium malariae: parasite and disease. Clinical Microbiology Reviews 20, 579–592.

Collins, W.E., Ruebush 2nd, T.K., Skinner, J.C., Filipski, V.K., Broderson, J.R., Stanfill, P.S., Morris, C.L., 1990. The Peruvian III strain of *Plasmodium brasilianum* in *Saimiri sciureus boliviensis* monkeys. Journal of Parasitology 76, 676–680.

Collins, W.E., Skinner, J.C., Huong, A.Y., Broderson, J.R., Sutton, B.B., Mehaffey, P., 1985. Studies on a newly isolated strain of *Plasmodium brasilianum* in *Aotus* and *Saimiri* monkeys and different anophelines. Journal of Parasitology 71, 767–770.

Curado, I., Duarte, A.M., Lal, A.A., Oliveira, S.G., Kloetzel, J.K., 1997. Antibodies anti bloodstream and circumsporozoite antigens (*Plasmodium vivax* and *Plasmodium malariae*/*P. brasilianum*) in areas of very low malaria endemicity in Brazil. Memorias do Instituto Oswaldo Cruz 92, 235–243.

CVE (Centro de Vigilância Epidemiológica), 2008. Epidemiological situation of malaria in the State of São Paulo, 2007. BEPA, São Paulo. vol. 5, no. 56. Aug. Available on: http://www.cve.saude.sp.gov.br/agencia/bepa56.htm.

Deane, L.M., 1992. Simian malaria in Brazil. Memorias do Instituto Oswaldo Cruz 87 (S3), 1–20.

Deane, L.M., Ferreira Neto, J.A., Deane, S.P., Silveira, I.P., 1970. Anopheles (*Kerteszia*) *cruzii*, a natural vector of the monkey malaria parasites, *Plasmodium simium* and *Plasmodium brasilianum*. Transactions of the Royal Society of Tropical Medicine and Hygiene 64, 647.

Di Santi, S.M.F., Carvalho, M.E., Costa, M.J., Kirchgatter, K., Pereira, B.F., Toniolo, C.R.C., Langui, D., Marret, A.C., 2005. Malária transfusional causada por *Plasmodium malariae* transmitido por doador assintomático infectado na Mata Atlântica do Estado de São Paulo. Revista da Sociedade Brasileira de Medicina Tropical 38, 333 [abstract].

dos Santos, L.C., Curotto, S.M., de Moraes, W., Cubas, Z.S., Costa-Nascimento, M.J., de Barros Filho, I.R., Biondo, A.W., Kirchgatter, K., 2009. Detection of *Plasmodium* sp. in capybara. Veterinary Parasitology 163, 148–151.

Escalante, A.A., Ayala, F.J., 1994. Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. Proceedings of the National Academy of Sciences of the United States of America 91, 11373–11377.

Escalante, A.A., Barrio, E., Ayala, F.J., 1995. Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. Molecular Biology and Evolution 12, 616–626.

Escalante, A.A., Freeland, D.E., Collins, W.E., Lal, A.A., 1998. The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome. Proceedings of the National Academy of Sciences of the United States of America 95, 8124–8129.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology 14, 2611–2620.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1, 47–50.

Ferreira, E., Corrêa, R.R., Tomich, A., de Sá, F.T., 1969. Flying range of *Anopheles (Kerteszia) cruzii* and *Anopheles (Kerteszia) bellator* in Guaratuba, coast line of the State of Paraná, Brazil. Revista Brasileira de Malariologia e Doencas Tropicais 21, 819–822.

Forattini, O.P., Kakitani, I., Massad, E., Marucci, D., 1996. Studies on mosquitoes (Diptera: Culicidae) and anthropic environment. 11—Biting activity and blood-seeking parity of Anopheles (Kerteszia) in south-eastern Brazil. Revista de Saude Publica 30, 107–114.

Garnham, P.C.C., 1966. Malaria Parasites and Other Haemosporidia. Blackwell, Oxford.

Gilles, H.M., Warrell, D.A., 1993. Bruce-Chwatt's Essential Malariology. Edward Arnold, London.

Haubold, B., Hudson, R.R., 2000. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage Analysis. Bioinformatics 16, 847–848.

Imwong, M., Sudimack, D., Pukrittayakamee, S., Osorio, L., Carlton, J.M., Day, N.P., White, N.J., Anderson, T.J., 2006. Microsatellite variation, repeat array length, and population history of *Plasmodium vivax*. Molecular Biology and Evolution 23, 1016–1018.

Joy, D.A., Gonzalez-Ceron, L., Carlton, J.M., Gueye, A., Fay, M., McCutchan, T.F., Su, X.Z., 2008. Local adaptation and vector-mediated population structure in *Plasmodium vivax* malaria. Molecular Biology and Evolution 25, 1245–1252.

Lourenço-de-Oliveira, R., Luz, S.L., 1996. Simian malaria at two sites in the Brazilian Amazon. II. Vertical distribution and frequency of anopheline species inside and outside the forest. Memorias do Instituto Oswaldo Cruz 91, 687–694.

Luikart, G., Cornuet, J.M., 1998. Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. Conservation Biology 12, 228–237.

Murray, V., Monchawin, C., England, P.R., 1993. The determination of the sequences present in the shadow bands of a dinucleotide repeat PCR. Nucleic Acids Research 21, 2395–2398.

Oliveira-Ferreira, J., Lacerda, M.V., Brasil, P., Ladislau, J.L., Tauil, P.L., Daniel-Ribeiro, C.T., 2010. Malaria in Brazil: an overview. Malaria Journal 9, 115.

Perkins, S.L., Schall, J.J., 2002. A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. Journal of Parasitology 88, 972–978.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Qari, S.H., Shi, Y.P., Pieniazek, N.J., Collins, W.E., Lal, A.A., 1996. Phylogenetic relationship among the malaria parasites based on small subunit rRNA gene sequences: monophyletic nature of the human malaria parasite, *Plasmodium falciparum*. Molecular Phylogenetics and Evolution 6, 157–165.

Razakandrainibe, F.G., Durand, P., Koella, J.C., De Meeüs, T., Rousset, F., Ayala, F.J., Renaud, F., 2005. Clonal population structure of the malaria agent *Plasmodium falciparum* in high-infection regions. Proceedings of the National Academy of Sciences of the United States of America 102, 17388–17393.

Russell, P.F., 1955. Man's Mastery of Malaria. Oxford, London, 308 pp.

Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Shukri Abdullah, M., Abdul Rahman, H., 1999. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. American Journal of Tropical Medicine and Hygiene 60, 687–692.

Snounou, G., Viriyakosol, S., Zhu, X.P., Jarra, W., Pinheiro, L., Rosário, V.E., Thaithong, S., Brown, K.N., 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Molecular and Biochemical Parasitology 61, 315–320.

Tazi, L., Ayala, F.J., 2011. Unresolved direction of host transfer of *Plasmodium vivax* v. *P. simium* and *P. malariae* v. *P. brasilianum*. Infection, Genetics and Evolution 11, 209–221.

Van den Eede, P., Erhart, A., Van der Auwera, G., Van Overmeir, C., Thang, N.D., Hung le, X., Anné, J., D'Alessandro, U., 2010. High complexity of *Plasmodium vivax* infections in symptomatic patients from a rural community in central Vietnam detected by microsatellite genotyping. American Journal of Tropical Medicine and Hygiene 82, 223–227.

Vinetz, J.M., Li, J., McCutchan, T.F., Kaslow, D.C., 1998. Plasmodium malariae infection in an asymptomatic 74-year-old Greek woman with splenomegaly. New England Journal of Medicine 338, 367–371.