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Complexity of Malaria Transmission Dynamics in the Brazilian Atlantic Forest

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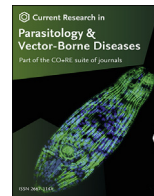
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Complexity of malaria transmission dynamics in the Brazilian Atlantic Forest



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ABSTRACT

Plasmodium malariae and *Plasmodium vivax* are protozoan parasites that can cause malaria in humans. They are genetically indistinguishable from, respectively, *Plasmodium brasilianum* and *Plasmodium simium*, i.e. parasites infecting New World non-human primates in South America. In the tropical rainforests of the Brazilian Atlantic coast, it has long been hypothesized that *P. brasilianum* and *P. simium* in platyrrhine primates originated from *P. malariae* and *P. vivax* in humans. A recent hypothesis proposed the inclusion of *Plasmodium falciparum* into the transmission dynamics between humans and non-human primates in the Brazilian Atlantic tropical rainforest. Herein, we assess the occurrence of human malaria in simians and sylvatic anophelines using field-collected samples in the Capivari-Monos Environmental Protection Area from 2015 to 2017. We first tested simian blood and anopheline samples. Two simian (*Alouatta*) blood samples (18%, $n = 11$) showed *Plasmodium cytb* DNA sequences, one for *P. vivax* and another for *P. malariae*. From a total of 9,416 anopheline females, we found 17 pools positive for *Plasmodium* species with a 18S qPCR assay. Only three showed *P. cytb* DNA sequence, one for *P. vivax* and the others for rodent malaria species (similar to *Plasmodium chabaudi* and *Plasmodium berghei*). Based on these results, we tested 25 rodent liver samples for the presence of *Plasmodium* and obtained *P. falciparum cytb* DNA sequence in a rodent (*Oligoryzomys* sp.) liver. The findings of this study indicate complex malaria transmission dynamics composed by parallel spillover-spillback of human malaria parasites, i.e. *P. malariae*, *P. vivax*, and *P. falciparum*, in the Brazilian Atlantic forest.

1. Introduction

The five agents of human malaria are vector-borne protozoans of the genus *Plasmodium*, four are considered primarily human parasites, and

one is a zoonotic parasite. The bites of *Anopheles* mosquito species transmit these malaria parasites to their vertebrate hosts, including primates. *Plasmodium falciparum* is responsible for most malaria deaths globally, reaching > 400 thousand cases worldwide in 2019, especially in

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sub-Saharan Africa (WHO, 2020). In contrast, the mortality associated with the other three species is significantly lower. *Plasmodium vivax* is the second most important because of high morbidity and high prevalence in endemic areas of Southeast Asia and Latin America (WHO, 2020). *Plasmodium ovale* and the pantropical distributed *P. malariae*, only account for a fraction of the clinical cases worldwide (Rutledge et al., 2017). These four *Plasmodium* species differ in aspects of their biology, likely because they evolved as human parasites from distinct phylogenetic lineages that are still under investigation (Escalante & Pacheco, 2019). The zoonotic malaria parasite, *Plasmodium knowlesi*, infects *Macaca* spp. in Southeast Asia and belongs to a phylogenetic clade that also includes *P. vivax* (Muehlenbein et al., 2015; Yusof et al., 2016; Yakob et al., 2018). This parasite is one of the most important causes of human malaria in Malaysia (Singh & Daneshvar, 2013). There is another causative species of zoonotic malaria, *Plasmodium cynomolgi*, which infects macaques, and has also been reported in humans (Grignard et al., 2019; Hartmeyer et al., 2019), but this species seems to be less frequent. Adding to these multiple parasites and the diversity of *Anopheles* species that can act as vectors worldwide, it is evident that the emergence of human malaria involved evolutionary processes and interspecies spillovers, which occurred in a variety of ecological settings that allow for malaria transmission (Escalante & Pacheco, 2019).

The African origin of *P. falciparum* has long been accepted (Garnham, 1966; Coatney, 1971; Escalante & Ayala, 1994). This parasite belongs to the subgenus *Laverania*, the African ape *Plasmodium* clade (Krief et al., 2010; Loy et al., 2017; Otto et al., 2018). The populations of *P. falciparum* followed the increase in the size of the human population and its global expansion out of Africa (Joy et al., 2003; Tanabe et al., 2010; Loy et al., 2017; Otto et al., 2018; Rodrigues et al., 2018). Part of this process was the colonization of the Americas, an event that likely occurred during the transatlantic slave trade 1533–1851 (Rodrigues et al., 2018).

The introduction of *P. falciparum* in the Americas required adapting to new-world *Anopheles* spp. (Molina-Cruz et al., 2016). These vector species have distinct ecological and genetic characteristics as they shared their most recent common ancestor with African vectors as long as 100 Mya ago (Foster et al., 2017). The main malarial vector in Brazil is *Anopheles (Nyssorhynchus) darlingi*, an anopheline species responsible for 99% of the reported malaria cases annually in the Amazon basin (Sallum et al., 2019). In the Atlantic tropical rainforest of Brazil, the local vector *Anopheles (Kertessia) cruzii* can transmit human malaria (Carlos et al., 2019). However, this local vector has a broader host species preference (Multini et al., 2020), ranging from humans to domestic and sylvatic animals (Medeiros-Sousa et al., 2019). In addition, larvae of this species develop in the bromeliad phytotelma in shaded or partially shaded plants inside the forest (Marques et al., 2012).

Although currently under control, the Atlantic coast of Brazil saw past epidemics of malaria caused by *P. falciparum* and *P. vivax* in the 1950s until it was eliminated from urban areas (e.g. Rio de Janeiro) in the 1970s (Ferreira & Castro, 2016). Imported cases, *P. vivax* or *P. falciparum* from endemic malaria regions such as the Amazon, frequently occur in extra-Amazonian sites where they can infect local vectors (de Pina-Costa et al., 2014). When local vectors are infected, they can generate autochthonous malaria cases, often associated with tourism or occasional activities carried out inside the forest (de Alvarenga et al., 2016). The alternative hypothesis for these autochthonous malaria cases is transmission inside the forest, which means transmission from an infected non-human primate (zoonotic malaria) (Brasil et al., 2017). Autochthonous *P. vivax* cases in humans can be caused by the genetically indistinguishable form *Plasmodium simium*, circulating in non-human primates (Brasil et al., 2017; de Alencar et al., 2018; Abreu et al., 2019). Likewise, autochthonous *P. malariae* cases can also result from circulation and transmission of *Plasmodium brasilianum* from simians to humans (Coatney, 1971; Guimarães et al., 2012). Further support for the zoonotic malaria hypothesis is the biting behavior of *An. cruzii* in the canopy where non-human primates forage and on the ground level where humans walk inside forest (Medeiros-Sousa et al., 2021). The origin of

zoonotic malaria caused by *P. simium* is a reverse zoonosis of the human parasite, i.e. spillover of *P. vivax* from human population to non-human primates during the colonization period in Brazil (de Oliveira et al., 2021). Now, the simian lineage (*P. simium*) is there circulating in nature and causing subpatent or patent infections in humans (Brasil et al., 2017). The spillback of the simian lineage is recognized as zoonotic malaria. Spillover-spillback mechanism could have hypothetically supported the persistence and adaptation of other invasive human malaria parasites arriving in the Americas, such as *P. falciparum*.

Plasmodium falciparum DNA and immunological responses were found in residents living in forested regions of São Paulo and Rio de Janeiro, suggesting its transmission among asymptomatic individuals (Maselli et al., 2014; Sallum et al., 2014; Miguel et al., 2019). A study by Laporta et al. (2015) investigated local foci of *P. falciparum* among local anophelines. Accordingly, 4.4% (21/480) of anophelines were found infected with *P. falciparum*, and most infected females (86%, 18/21) were *An. cruzii* (see Laporta et al., 2015). Finally, there is evidence of *P. falciparum* detected in Amazonian non-human primates in Brazil and Colombia (Araújo et al., 2013; Rondón et al., 2019).

The main working hypothesis tested here is that the most threatening human malaria parasite (*P. falciparum*) has a transmission cycle involving non-human primates in the Brazilian Atlantic Forest (Duarte et al., 2008; Laporta et al., 2015; Laporta, 2017; Assis et al., 2021). We tested this hypothesis using field-collected anopheline and simian blood samples in an environmental protection area (Medeiros-Sousa et al., 2019). We also tested these samples for the presence of other human malaria parasites (*P. vivax* and *P. malariae*) (Demari-Silva et al., 2020). As a complementary hypothesis, we tested for the presence of *Plasmodium* spp. in rodent liver samples considering that rodents can act as reservoirs of malaria parasites. We discuss these results in light of the *Plasmodium* spp. resilience post-elimination.

2. Materials and methods

2.1. Study area

The forest physiognomies of the Atlantic forest biome were reduced over the last century to 11–16% of their original domain that covered the entire South-to-North gradient of the Brazilian Atlantic coast (Ribeiro et al., 2009). The most extensive conserved region with these forest remnants is in the southeastern São Paulo State (Fig. 1A). Field investigations were carried out in the Capivari-Monos EPA (Environmental Protection Area) at ~800 m above sea level (Fig. 1B) (Duarte et al., 2013; Medeiros-Sousa et al., 2019). The climate in the region is classified as a tropical monsoon climate (modified Köppen AM-type classification) with excessive annual precipitation (> 2,500 mm) and dry, mild winters (Rolim et al., 2007). Field collections were carried out in sites 1–4 where few humans are present, non-human primates are abundant, and the dominance of *An. cruzii* is documented (Duarte et al., 2013; Medeiros-Sousa et al., 2019) (Fig. 1C).

2.2. Study design

The study includes samples from three sources: (i) simian blood samples; (ii) anopheline samples; and (iii) rodent liver samples. With the first two sources, we tested for the presence of human malaria, and with the latter, for the presence of any *Plasmodium* in the rodent liver. We made prospective studies for collecting and testing simian blood samples from July 2014 to June 2016 and anopheline samples from March 2015 to May 2017. After the outcomes of both studies, we made a retrospective study with rodent liver samples (collected in 2012) from June 2017 to September 2018 (Fig. 2).

Injured howler monkeys (*Alouatta clamitans*) by any cause ($n = 11$) were received from the study area and during the study period (Fig. 2). They were treated and sent back to nature by the municipal department for forestry and wildlife (DEPAVE/PMSP). Blood samples of 5 ml were

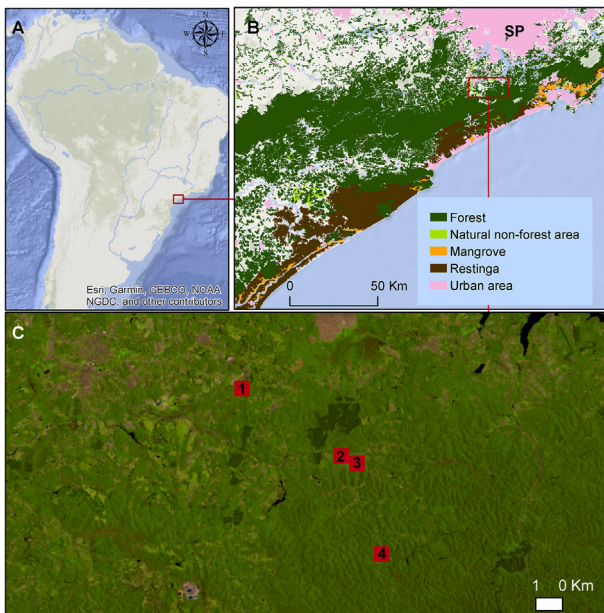


Fig. 1 Study area. **A** Atlantic tropical rainforest remnants. **B** Southeastern Atlantic Forest. **C** Field collections were conducted in Capivari-Monos EPA (−46.7, −23.9): 1, Embura village; 2, Marsilac village; 3, Transition zone; 4, Cachoeira do Marsilac (Medeiros-Sousa et al., 2019). Abbreviation: SP, São Paulo metropolitan urban area with a population of ~20 million people. Source: SOS Mata Atlântica/INPE, 2016

collected in EDTA tubes for laboratory testing. The faunal survey conducted by DEPAVE/PMSP in the study area in 2012 obtained liver tissue samples from 25 rodent species. These samples were kept stored at −80 °C before the laboratory testing (Fig. 2).

2.3. Field collections and sample DNA extraction

Anopheline sampling was performed monthly during the study period (Fig. 2). In each of the four study sites (Fig. 1C), we employed: (i) Shannon traps from 18:00 h to 22:00 h (depending on the crepuscular time); (ii) CDC light traps with CO₂ (dry ice) from 18:00 h to 6:00 h at ground level and in the canopy (10-m height); and (iii) backpack aspirator sampling on vegetation that could represent shelters for adults (20-min sampling).

Mosquito specimens were euthanized immediately before morphological identification using the keys of Forattini (2002). Non-engorged anopheline females were stored individually in isopropanol until DNA extraction. DNA was extracted in pools (a maximum of ten specimens/pool) using the Qiagen™ DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Howler monkey blood samples collected as part of the DEPAVE/PMSP 2014 and 2016 surveys were stored in −20 °C (DEPAVE/PMSP, 2012). Rodent liver tissue samples were obtained from the mammal census performed by DEPAVE/PMSP in the study area in 2012 (SVMA/PMSP, 2011). DNA extraction from blood and liver samples followed the protocol provided by the Qiagen™ DNeasy Blood and Tissue kit.

2.4. Laboratory testing

Anophelines, simian blood samples and rodent liver samples were tested for the presence of *Plasmodium* DNA using the TaqMan qPCR assay (18S rRNA gene) following Bickersmith et al. (2015). Assays to detect genus *Plasmodium*, *P. vivax* and *P. falciparum* were performed separately using the TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA). The same methodology (Bickersmith et al., 2015) was used to detect *P. malariae* but with the primers and probes described by Rougemont et al. (2004). DNA extracted from known positive *P. falciparum* samples, *P. brasilianum* blood smear, and a patient blood with *P. vivax* were used as positive controls. An aliquot of ultrapure water was used as a negative control.

Positive samples for *Plasmodium* DNA using 18S qPCR were further analyzed. A nested PCR assay amplifying a ~402 bp cytochrome b (*cytb*) fragment was performed with the *cytb*-1 primers (Siregar et al., 2015) followed by sequencing for molecular identification. PCR products were purified with ExoSAP-IT PCR Product Cleanup (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and forwarded with the respective primers to a gene sequencing company (Genomic Engenharia Molecular, São Paulo, SP, Brazil). An alternative protocol for amplifying a fragment of ~424 bp mitochondrial *Plasmodium* small subunit rRNA (18S rRNA) gene, according to Siregar et al. (2015) was also employed. To generate more sequence data for phylogenetic analysis, we re-tested 15 anopheline samples confirmed positive for *P. falciparum* by Laporta et al. (2015) with the *cytb* nested PCR assay. *Plasmodium* 18S and *cytb* sequences generated in this study were deposited in the GenBank database under the accession numbers MF573323 (18S) and MF573300, MF573301, MT770753, MF476105, MT779799, MT779800, and MT779801 (*cytb*).

Because morphological identification of rodents at the species level was not possible, we applied the *cytb* protocol for species identification (Smith & Patton, 1993).

2.5. Phylogenetic analysis

The sequences generated in this study were compared with databases available using the BLAST tool (<https://blast.ncbi.nlm.nih.gov>) on GenBank (Altschul et al., 1997). A nucleotide alignment was generated using ClustalX v2.0.12 and Muscle as implemented in SeaView v4.3.5 (Gouy et al., 2010) with manual editing. The alignment was constructed with 32 *cytb* partial sequences (331 bp excluding gaps) belonging to the genus

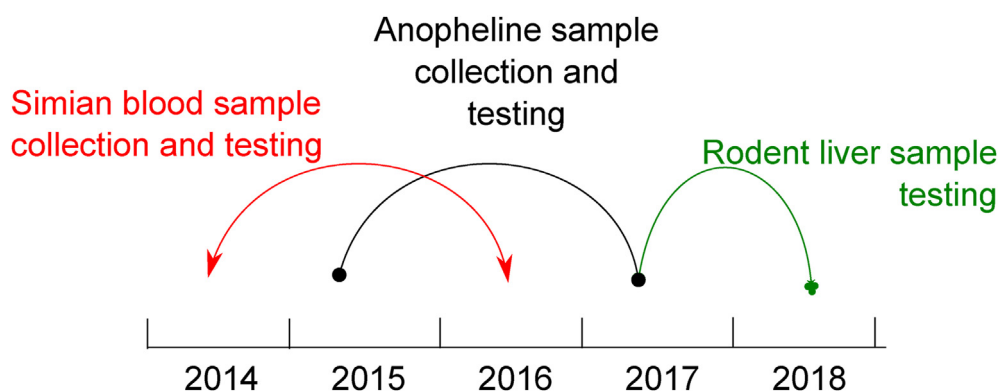


Fig. 2 Timeline of the activities carried out during the investigation

Plasmodium, including the newly generated sequences and sequences available on GenBank (Benson et al., 2013) for *Plasmodium* spp. that infect mammals (e.g. *P. falciparum*, *P. vivax*, *P. simium*, *P. malariae*, *P. knowlesi* and *Plasmodium berghei* amongst others). Sequences of species that infect birds, *Plasmodium relictum* and *Plasmodium gallinaceum*, were included as the outgroup. Sequences that showed a similarity > 95% using BLAST (Altschul et al., 1997) were included.

The phylogenetic tree was inferred based on this alignment using the Bayesian method implemented in MrBayes v3.2.6 with the default priors (Ronquist & Huelsenbeck, 2003). The general time reversible model with gamma-distributed substitution rates and a proportion of invariant sites (GTR + Γ + I) was estimated as the best-fit model of nucleotide substitution based on the lowest Bayesian Information Criterion (BIC) scores as estimated with MEGA v7.0.14 (Kumar et al., 2016). Bayesian support was inferred for the nodes in MrBayes by sampling every 1,000 generations from two independent chains lasting 2×10^6 Markov Chain Monte Carlo (MCMC) steps. Chains were assumed to have converged once the value of the potential scale reduction factor (PSRF) was between 1.00 and 1.02 and the average SD of the posterior probability was < 0.01 (Ronquist & Huelsenbeck, 2003). Then, 25% of the samples were discarded once convergence was reached as “burn-in”. GenBank accession numbers for all sequences used in the analysis are given in the phylogenetic tree.

Table 1

Testing of simian blood samples (*Alouatta guariba clamitans*) for *Plasmodium* spp., Capivari-Monos EPA, Brazilian Atlantic rainforest, 2014–2016

ID (DEPAVE)	18S <i>Plasmodium</i>	<i>cytb</i> <i>Plasmodium</i>	GenBank ID
10 (56067)	<i>P. vivax</i>	<i>P. vivax</i> ^a	MF573301
16 (63739)	<i>P. malariae</i> ^a	–	MF573323
17 (64241)	<i>P. malariae</i>	–	–
19 (65181)	–	nd	nd
20 (65218)	–	nd	nd
24 (62454)	–	nd	nd
30 (68145)	–	nd	nd
31 (68334)	–	nd	nd
36 (70954)	–	nd	nd
38 (73727)	<i>P. vivax</i>	–	–
41 (75124)	–	nd	nd

Note: *P. vivax*- and *P. malariae*-positive or negative (–) results for testing for parasite species presence in the sample.

Abbreviation: ID(DEPAVE), Divisão Técnica de Medicina Veterinária e Manejo da Fauna Silvestre, São Paulo, SP, Brazil; nd, not done.

^a Sequenced sample.

Table 2

Testing of anopheline samples for *Plasmodium* spp., Capivari-Monos EPA, Brazilian Atlantic rainforest, 2015–2017

ID (pool) trap	Site	18S <i>Plasmodium</i>	<i>cytb</i> <i>Plasmodium</i>	GenBank ID	
84 (10 <i>An. cruzii</i>) Sh	4 (forest)	<i>P. vivax</i>	–	–	
85 (10 <i>An. cruzii</i>) Sh		–	<i>P. chabaudi</i>	MT770753	
87 (10 <i>An. cruzii</i>) Sh		<i>Plasmodium</i> spp.	<i>P. berghei</i>	MF476105	
92 (10 <i>An. cruzii</i>) Sh		<i>P. vivax</i>	–	–	
291 (10 <i>An. cruzii</i>) Sh		<i>P. vivax</i>	–	–	
361 (10 <i>An. cruzii</i>) Sh		<i>P. vivax</i>	<i>P. vivax</i>	MF573300	
381 (10 <i>An. cruzii</i>) Sh		<i>P. malariae</i>	–	–	
553 (10 <i>An. cruzii</i>) Sh		<i>P. malariae</i>	–	–	
386 (10 <i>An. cruzii</i>) Sh		3 (transition zone)	<i>P. vivax</i>	–	–
598 (1 <i>An. cruzii</i>) CDC _g			<i>P. malariae</i>	–	–
600 (1 <i>An. cruzii</i>) CDC _g	<i>P. falciparum</i>		–	–	
621 (1 <i>An. strodei</i>) Sh	<i>P. vivax</i> + <i>P. falciparum</i>		–	–	
638 (1 <i>An. strodei</i>) Sh	<i>P. vivax</i> + <i>P. malariae</i>		–	–	
678 (7 <i>An. strodei</i>) Sh	<i>Plasmodium</i> spp.		–	–	
599 (10 <i>An. cruzii</i>) Asp	2 (Marsilac village)		<i>P. falciparum</i>	–	–
684 (5 <i>An. cruzii</i>) CDC _c			<i>Plasmodium</i> spp.	–	–
01 (1 <i>An. evansae</i>) CDC _g			<i>P. vivax</i>	–	–
09 (1 <i>An. cruzii</i>) CDC _c			<i>P. vivax</i>	–	–

Note: *Plasmodium*-positive or negative (–) results for testing for parasite species presence in the sample.

Abbreviations: Sh, Shannon traps; CDC_g, CDC trap on the ground; CDC_c, CDC trap in the canopy; Asp, backpack aspirator.

3. Results

3.1. Simian blood samples

Out of 11 simian blood samples tested, we obtained two (18%) *P. vivax*-positive and two (18%) *P. malariae*-positive in 18S qPCR (Table 1). From the positive samples, we obtained one 18S sequence of *P. malariae* and one *cytb* sequence of *P. vivax* (Table 1).

3.2. Anopheline samples

A total of 9,416 anopheline females were collected; of these, 9,328 specimens were *An. cruzii* (> 99%), 82 *An. strodei*, 3 *Anopheles* (*Anopheles pseudotibiamaculatus*, 2 *Anopheles* (*Nyssorhynchus*) *evansae*, and 1 *Anopheles* (*Nyssorhynchus*) *albitarsis*). Seventeen pools returned *Plasmodium*-positive 18S qPCR (Table 2). Three *Plasmodium cytb* sequences were confirmed in *An. cruzii* (Table 2). The obtained *P. vivax cytb* sequence was 100% similar to the one found in howler-monkeys in the study region (Table 1). The obtained *P. berghei* and *Plasmodium chabaudi cytb* sequences (Table 2) motivated testing of rodent liver samples.

Out of 15 anopheline samples from Laporta et al. (2015), we obtained two *P. falciparum cytb* sequences, one in *An. cruzii* (GenBank: MT779800) and another in *Anopheles* (*Nyssorhynchus*) *strodei* (GenBank: MT779801).

3.3. Rodent liver samples

One rodent species (*Oligoryzomys* cf. *flavescens*) was positive for *P. falciparum* using 18S and *cytb* protocols out of 25 rodent specimens tested (Supplementary Table S1).

3.4. Phylogenetic analyses

The phylogenetic relationships between the *cytb* sequences generated in this study ($n = 7$) and those available on GenBank are illustrated in Fig. 3. Sequences MF573300 (from *An. cruzii*) and MF573301 (from *A. clamitans*) were placed in a clade containing sequences of *P. vivax* and *P. simium* (Fig. 3). Sequences MT770753 and MF476105 obtained from *An. cruzii* clustered in clades containing sequences of *P. chabaudi* and *P. berghei*, respectively. Sequences MT779800 (from *An. cruzii*), MT779801 (from *An. strodei*), and MT779799 (from *O. flavescens*) were placed together in a clade containing sequences of *P. falciparum* (Fig. 3). Although the alignment comprised only 331 bp, most of the nodes of interest had high posterior probability values supporting the identification of the parasites found in this study.

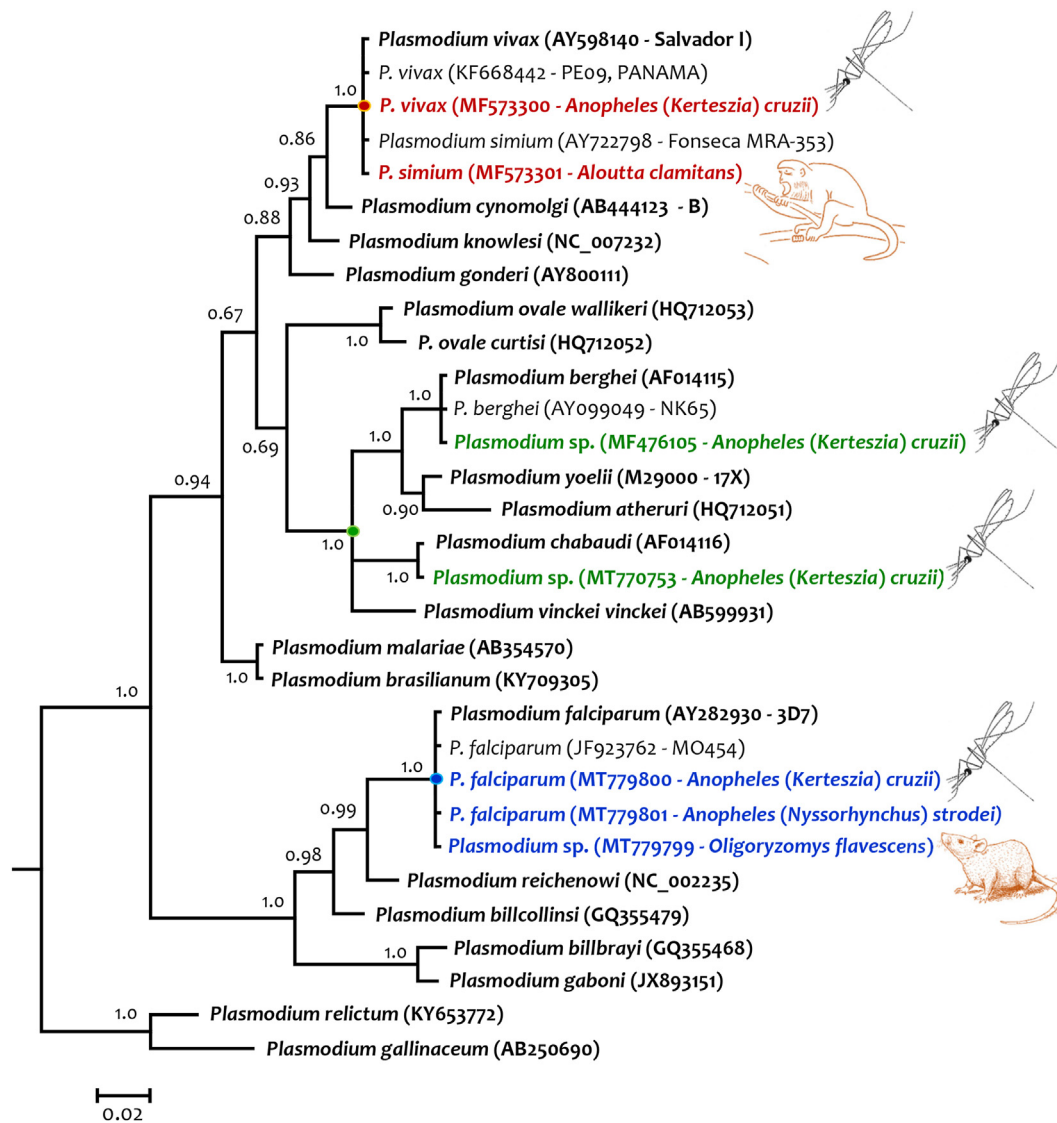


Fig. 3 A Bayesian phylogenetic hypothesis of *Plasmodium* spp. parasites infecting mammals and mosquitoes from the Capivari-Monos EPA, Brazil. We constructed a phylogenetic tree based on partial sequences of the *cytb* gene (31 sequences; 331 bp excluding gaps). Posterior probability values are shown above or below the branches. *Plasmodium relictum* and *Plasmodium gallinaceum* were used as the outgroup. In addition to parasite and host names, lineage identification (if available) and GenBank accession numbers are provided in parentheses for all sequences used in the analysis. Sequences colored in red and blue show similarities with human malaria parasites, *P. vivax* and *P. falciparum*, respectively. Sequences colored in green represent rodent malaria parasites, similar to *P. berghei* and *P. chabaudi*. The reference sequences in the clades containing colored sequences are highlighted in bold

4. Discussion

Our results indicate that *P. vivax*, *P. malariae* and *P. falciparum* are transmitted among non-human primates, primarily by *An. cruzii*, in the Atlantic tropical rainforest of Brazil. In particular, consistent with other studies, we found *P. vivax* and *P. malariae* in anopheline vector species and non-human primates (Duarte et al., 2008, 2013; Abreu et al., 2019). However, the presence of *P. falciparum* in *An. cruzii* in the sylvatic transmission cycle remains puzzling.

Our previous findings of the presence of *P. falciparum* in a sylvatic transmission cycle detected in *An. cruzii* and *An. strodei* (see Laporta et al., 2015) add to the current finding of *P. falciparum* in a rodent liver (Fig. 3). The presence of *P. falciparum* DNA in a rodent liver sample may represent an unsuccessful infection stopped at the liver stage because rodents are likely dead-end hosts for human malaria pathogens (Laporta et al., 2013). Although *P. falciparum* is considered eliminated from the Atlantic Forest, there seems to be transmission undetected by the traditional vector-borne disease surveillance methods. The lack of detection can be

explained by low prevalence and focal circulation in a “post-elimination” phase. Subpatent or undetectable malaria transmission appears to be common in South America, e.g. Molina Gómez et al. (2017); Manrique et al. (2019). Exploring the possibility that *P. falciparum* remains undetected in these areas of Brazil is a matter that requires active surveillance and further investigations.

We can speculate that *P. falciparum*-infected (likely asymptomatic) humans could infect local vectors. Consistent with this scenario, a recent cross-sectional study carried out on humans living on the border of the Atlantic tropical rainforest region of Rio de Janeiro identified *P. falciparum* (0.3%), *P. vivax* (0.6%) and *P. malariae* (1.9%) in humans with malaria (Miguel et al., 2019). These authors also reported positive serological testing for *P. falciparum* (3.5%), *P. vivax* (7.7%) and *P. malariae* (30.9%). All thick blood smears were negative, indicating that the individuals had submicroscopic, asymptomatic infections. Overall, people who entered the forest were more likely to exhibit reactive serology (Miguel et al., 2019). These data corroborate evidence found in Espírito Santo and São Paulo states (Curado et al., 2006; Duarte et al.,

2006; Cerutti et al., 2007). Thus, our findings are consistent with a scenario where infected asymptomatic individuals may be entering the forest environment frequently. However, it is difficult to detect these infectious individuals without carrying out a longitudinal study.

Alternatively, our findings are also consistent with non-human primates being competent hosts for *P. falciparum* (see Duarte et al., 2008; Monteiro et al., 2020). *Plasmodium falciparum* DNA was detected in two fecal samples of red howler monkeys (*Alouatta seniculus*) from Colombia (Rondón et al., 2019) and one *Alouatta guariba clamitans* from the Atlantic tropical rainforest (Duarte et al., 2008). Still, testing this hypothesis is logistically complicated even when using fecal samples (Rondón et al., 2019) because of the likely low prevalence of *P. falciparum* parasites in non-human primates (Duarte et al., 2008), if any. Although explaining negative results is particularly difficult, a case could be made that the complexities of detecting parasites with low frequency may explain why other studies have failed to detect *P. falciparum* in Brazilian non-human primates (Abreu et al., 2019). Altogether, the available data indicate a continuous forest cycle involving *P. falciparum*-infected zoophilic mosquitoes and that such event occurs with low frequency. Whether it results from asymptomatic human patients or non-human primates cannot be determined in this study. Nevertheless, a small fraction of the mosquito population is responsible for this residual transmission, challenging investigations. Longitudinal, long-term studies will be necessary to uncover the mechanisms that can maintain this putative silenced transmission. Whatever scenario is sustaining transmission, a *P. falciparum* malaria case at the forest border does not fit the case definition of imported malaria based on travel history (de Pina-Costa et al., 2014; Lorenz et al., 2015).

Our results increase the body of evidence supporting that humans introduced malarial parasites to the native non-human primate species that are now maintaining a forest transmission cycle in the Brazilian Atlantic Forest. Transference of human parasites to animals has been reported for other parasitic diseases (e.g. *Cryptosporidium hominis*, *P. ovale wallikeri*, strongyloid nematodes) (Estrada-Peña et al., 2014; Hasegawa et al., 2014; Mapua et al., 2018; Pafco et al., 2019). Reverse zoonosis of human *P. vivax* into simian *P. simium* in the past Brazilian colonization (de Oliveira et al., 2021) is the result of spillover of the human parasite to New World monkeys from European or African people who arrived at the Americas during the colonization period. The spillover of the simian lineage (*P. simium*) is recognized as zoonotic malaria (Brasil et al., 2017), an alternative hypothesis for malaria infections in the Atlantic Forest opposite to the classical case definition based on imported malaria vectorized by the local vector. Likewise, human *P. malariae* may have been affected by the same process in South America. Further supporting evidence of a complex human-non-human primate cycle is a higher genetic diversity found in the Brazilian Amazon and Atlantic Forest populations of *P. brasilianum* vs *P. malariae* (Guimarães et al., 2012; Lalremruata et al., 2015). This pattern suggests that the *P. brasilianum* populations could be the source of at least part of the *P. malariae* cases. It is worth noting that *P. malariae* has a broader distribution than *P. vivax*. Indeed, *P. malariae* has been detected in approximately 31 species of New World monkeys (de Alvarenga et al., 2017; Erkenwick et al., 2017; Rondón et al., 2019) from Costa Rica to Brazil. This broad host and geographic ranges are unique among primate malaria parasites. Reverse zoonosis of *P. malariae* in South America may have occurred before the colonization period because spillover of the simian lineage (*P. brasilianum*) has been considered to explain zoonotic malaria by *P. malariae* in humans across the continent and not only in the Atlantic Forest (Rondón et al., 2019).

In the case of the parasites similar to African rodent malaria agents (*P. chabaudi* and *P. berghei*) obtained from the *An. cruzii* pools, we can only speculate that this could be an unknown rodent parasite in South America whose relationship with the African species needs to be explored. Interestingly, we observed 18% of blood meals being taken from rodents by *An. cruzii* (68 samples with rodent DNA/373 engorged

females) in the study area (Evangelista et al., unpublished observations). Evidence of a rodent-specific *Plasmodium* species has been previously found, i.e. *Plasmodium* spp. in capybaras (dos Santos et al., 2009). More data are needed to understand what these rodent parasites are.

Another layer of complexity in malaria dynamics is the anopheline vector composition in the Atlantic Forest. Although *An. cruzii* is the dominant vector, we observed *An. strodei* and *An. evansae*, together with *An. cruzii*, infected with human malaria on the forest edges. This diversity in the vector composition was previously observed (Duarte et al., 2013; Laporta et al., 2015). While *An. cruzii* is sylvatic, the other vectors can survive man-made changes in the natural ecosystem and proliferate in an anthropic environment (Forattini & Massad, 1998). The role of local vectors as “bridge vectors” of malaria parasites in the human environment cannot be neglected.

Overall, malaria transmission dynamics in the Brazilian Atlantic rainforest is consistent with a mosaic of cycles involving human malaria parasites being transmitted among local non-human primates. Such dynamics is maintained by vectors feeding upon a broad range of vertebrate hosts, in this case, the dominant vector *An. cruzii* combined with local vectors. Thus, the vector host range seems crucial to explain the proposed spillover-spillback process.

5. Conclusions

We tested the hypothesis of the transmission cycle of human malaria parasites (*P. vivax*, *P. malariae* and *P. falciparum*) involving non-human primates and anophelines in the Brazilian Atlantic Forest. Although the role of long-lasting asymptomatic infections in humans cannot be ruled out, particularly in the case of the *P. falciparum* infections, these results yield additional evidence indicating that non-human primates could act as reservoirs for human malaria. The evidence is clearer for *P. vivax* and *P. malariae*. These parasites have been found in both non-human primates and the dominant vector (*An. cruzii*). Furthermore, outbreaks that have been reported (Brasil et al., 2017) are more likely the result of spillback. Assessing whether spillbacks constitute significant risk for the reintroduction of malaria into the human population, particularly in urban areas, is a matter that requires longitudinal studies and scaling up molecular surveillance on the forest edges, the human-non-human primate interface.

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Ethical approval

All procedures were authorized by the Federal Environment Institute (ICMBio; n. 47812-4). Laboratory testing from simian blood and rodent liver samples were approved by the Tropical Medicine Institute Ethics Committee on the use of animals in research (IMTUSP; n. 260108–012.028/2013 and n. 000421A).

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Data availability

The sequences generated in this study were deposited in the GenBank database under the accession numbers MF573323 (18S) and MF573300, MF573301, MT770753, MF476105, MT779799, MT779800 and MT779801 (*cytb*).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crvbd.2021.100032>.

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