Plasmodium vivax malaria recurrence after radical treatment with chloroquine-primaquine standard regimen in Turbo, Colombia: Results from a prospective study.

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Running Head: Malaria recurrences by *P. vivax* in Turbo, Colombia

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Abstract

Background. *Plasmodium vivax* recurrences help maintain malaria transmission. They are caused by recrudescence, reinfection or relapse, which are not easily differentiated.

Methods. A longitudinal observational study took place in Turbo municipality, Colombia. Participants with uncomplicated *P. vivax* infection received supervised concomitantly treatment with chloroquine 25 mg/Kg and primaquine 0.25 mg/Kg/day for 14 days. Incidence of recurrence was assessed over 180 days. Samples were genotyped and origins of recurrences were established.

Results. 134 participants were enrolled between February 2012 and July 2013, and 87 were followed for 180 days in which 29 recurrences were detected. Cumulative incidence of first recurrence was 24.1% (21/87) (CI 95% 14.6 to 33.7) and 86% (18/21) of them occurred between days 51 and 110. High genetic diversity of *P. vivax* was found and 12.5% (16/128) of the infections were polyclonal. Among detected recurrences 93.1% were genotyped as genetically identical to the one from the previous episode and 65.5% (19/29) were classified as relapses.

Conclusion. Our results indicate that there is a high incidence of *P. vivax* malaria recurrence after treatment in Turbo municipality, Colombia, a large majority of which are likely relapses from the previous infection. We attribute this to the primaquine regimen currently used in Colombia, which may be insufficient to eliminate hypnozoites.

Key words: *Plasmodium vivax*; primaquine; recurrence; relapse; genotyping

Background

Plasmodium vivax has the widest global distribution of the human malaria parasites (1, 2). In the Americas, more than 60% of the annual cases of malaria are caused by *P. vivax* (2). This parasite has a dormant stage known as hypnozoite, in which it remains in the liver for an indeterminate length of time (3). Hypnozoites might be activated months after a previous episode that has been treated and cured, causing relapses (3). Relapses after treatment can cause a new clinical episode with risk of complications for the patient. Moreover, they may contribute to continue *P. vivax* transmission (4, 5).

In endemic areas, recurrent *P. vivax* infections have three origins: 1) recrudescence of parasites from blood, which could be caused by resistance to treatment, inadequate dosage or suboptimal drug absorption, 2) reinfection by inoculation of new parasites from mosquito bites, or 3) relapse by reactivation of hypnozoites from the liver (6). Diagnostic methods alone cannot distinguish among these three types of recurrence. However, a comprehensive study — including supervised treatment, individual follow-up for therapeutic efficacy, microscopic and molecular diagnosis, quantification of drugs in blood, and genetic characterization of the parasites — may allow to determine the source of the recurrence (7, 8).

Per current recommendations of the World Health Organization, the treatment for *P. vivax* infection consists of two drugs: chloroquine (CQ), a blood schizonticide against circulating parasites, and primaquine (PQ), a tissue schizontocide that clears the liver of schizonts (9).

CQ remains widely used against *P. vivax*, although treatment failures have been reported (10). In Colombia, to this date there have been no reports of CQ therapeutic failure for *P. vivax* (11). PQ is the only antimalarial commercially available to treat liver stages (12).

Previous studies have compared the efficacy of different PQ regimens in preventing *P. vivax* recurrence (13). Although the standard PQ regimen (0.25 mg/kg/day for 14 days) has a significant incidence of recurrence, it is the most commonly used worldwide (2). It was estimated that 16.2% of patients in Colombia, treated with this regimen have at least one recurrence within six months (14). Nevertheless, the standard PQ regimen yield a lower incidence of recurrences compared to regimens employing an equal total dose administered for a shorter time (13, 14).

We conducted a prospective study in an endemic region of Colombia, to determine the incidence of *P. vivax* recurrences in a six month period post-treatment in patients underwent a supervised CQ-PQ regimen. Associations between risk factors and recurrence were explored, and *P. vivax* parasites from all episodes were genotyped in order to distinguish between relapses and reinfections.

Materials and methods

Study site

This study was conducted in Turbo municipality, Antioquia department, located in northwest Colombia (8°5´42´´N, 76°44´123´´W) (Figure1). Turbo has an area of 3,055 km² and

approximately 135,967 inhabitants, 61% of whom live in the rural area. The main economic activity is banana and plantain cultivation. Malaria is endemic in Turbo, with peaks of highest transmission between February and June, according to recent statistics 80% of malaria cases in the area are *P. vivax* mono-infections (15). Over the past seven years the number of cases has been reduced significantly, from an annual parasite incidence (API) of 65 cases per 1,000 people in 2007 to 3 cases per 1,000 people in 2014 (15).

Study design

A longitudinal observational study was conducted. Participants were selected from four malaria diagnostic centers (the municipal hospital and three nearby health centers) between February 2012 and July 2013 (Figure 1). These centers account for about 20% of all *P. vivax* reported cases in the Turbo municipality.

All individuals diagnosed with *P. vivax* malaria in the diagnostic centers during the study period were invited to participate. Eligibility criteria were: mono-infection with *P. vivax*, asexual parasite count greater than 250 parasites/µl, age over 4 years, absence of general danger signs or signs of severe malaria according to the criteria adopted by World Health Organization (7, 9), negative pregnancy test, not breastfeeding, not reporting intake of antimalarials in the preceding four weeks, residence in the study area and the ability and willingness to comply with the protocol for the duration of the study.

Participants received supervised simultaneous treatment with CQ (25 mg/kg for 3 days) and PQ (0.25 mg/kg/day for 14 days) (16). Patients weighing 60 kg or over received the

maximum dose of CQ (1.5 g), while the PQ dose was adjusted for body weight. Information about demographic characteristics, current disease, history of malaria, travel to other endemic regions and use of preventive measures for malaria was collected. Participants were monitored by thick blood smears and surveillance for symptoms on days 1, 2, 3, 7, 13, 21 and 28 post-diagnosis to evaluate therapeutic response during the current episode (7). The evaluation for recurrences was carried out by monthly thick blood smear on days 60, 90, 120, 150, 180 and at any time the patient had symptoms consistent with malaria.

Recurrence was defined as a positive thick blood smear for *P. vivax* between days 29 and 180, with or without clinical symptoms. In all recurrences, participants were treated again with the same CQ-PQ regimen, and they continued the follow-up as scheduled until day 180.

For patients who did not meet one or more inclusion criteria, a sample of capillary blood was taken before treatment but no study treatment or follow-up was provided. These samples were used for malaria diagnosis and parasite genotyping; results provided information for baseline parasite genetic diversity in the study area.

All participants enrolled signed an informed consent. This study was approved by the ethics committee from School of Medicine of the University of Antioquia, Colombia. The investigators from the Centers for Disease Control and Prevention (CDC) did not engage in field study or have access to participants' personal identifying information. Therefore, their participation in this study was determined to be non-engaged after human subject review at CDC.

136

137 *Malaria diagnosis and genetic characterization*

138 Malaria diagnosis on the day of inclusion (considered Day Zero) and on each scheduled visit
139 was carried out by microscopy (17). Diagnosis was confirmed by PCR on days 0, 28 and the
140 day of recurrence. DNA samples were extracted from filter paper with blood spots by
141 saponin-chelex method (18) and they were analyzed using a nested PCR protocol described
142 by Snounou *et al* (19).

143

144 All samples determined to be *P. vivax* mono-infection by PCR were genotyped at seven
145 neutral microsatellite loci (MS) (20, 21). Microsatellites MS2 (chromosome 6), MS6
146 (chromosome 11) and MS20 (chromosome 10), previously described by Karunaweera *et al*.,
147 were amplified by simple PCR (21), while microsatellites 2.21 (chromosome 2) 3.502
148 (chromosome 3), 11.162 (chromosome 11) and 12.335 (chromosome 12), described by
149 Imwong *et al*., were amplified by semi-nested PCR (20). For each MS amplification, a
150 forward primer labeled with a fluorophore (FAM or HEX) was used. PCR products were
151 analyzed by capillary electrophoresis on an ABI Prism 3130xl sequencer (Applied
152 Biosystems) using ROX 350 (Applied Biosystems) as an internal standard. Fragment size
153 and allele determination were obtained using Genemapper v4.1 (7 (Applied Biosystems,
154 Foster City, CA). Samples for which some loci did not amplify were re-amplified twice. If
155 two or more alleles were amplified in a single locus of a given sample, and the signal from
156 the minor allele was greater than 33% of that of the predominant signal allele, the sample
157 was defined as being polyclonally infected (22).

158

Data analysis

Incidence of recurrences and risk factors

The information was reviewed in the field and validated for quality control, then entered into a database. Cumulative incidence of first recurrence was calculated using Kaplan-Meier survival analysis. Association of potential risk factors with recurrence was analyzed using Cox proportional hazards model with the extension proposed by Andersen & Gill, which allows multiple events per participant (23). Crude and adjusted hazard ratios (HR) and their 95% confidence intervals (CI 95%) were calculated. Selection of variables for calculating adjusted HR was made by biological plausibility and according to P value <0.25 in bivariate analysis (24). For quantitative variables, linear assumption of risk was evaluated, and if it was not met, the variables were dichotomized at the median or previously reported thresholds (25, 26). All analyses were carried out using Stata 11.2 software (StataCorp, College Station, TX).

Genetic diversity and genetic structure

For samples with monoclonal infection, the total number of alleles per locus and expected heterozygosity (*He*) was estimated. *He* represents the probability of finding two different alleles for a given locus on a pair of randomly selected samples from the study population (27). Additionally, the number of multilocus haplotypes (MLH) was estimated, defined as a unique combination of alleles for the 7 MS analyzed. These analyzes were conducted with GenAlEx (28) and Microsatellite toolkit applications from Microsoft Excel (29). The

181 percentage of polyclonal infection per locus and average number of alleles per locus was
182 calculated for all samples on Day Zero and day of recurrence.
183
184 Given that unknown population substructures could bias our observations, we explored
185 whether the sampled parasites could be considered as one population undergoing random
186 matting. In particular, the parasite population structure was evaluated in Structure v2.3.4,
187 which uses a Bayesian clustering approach to assign samples to one of *K* genetic groups
188 according to allele frequency per locus; the data were evaluated using values of *K* from 2 to
189 10. For each value of *K*, 10 independent runs were performed, with a burn-in period of 10,000
190 iterations followed by 100,000 iterations (30). Structure Harvester v0.6.94 program was used
191 to visualize the Structure output results (31). The most likely number of genetic groups within
192 the sample was found according to the method of Evanno *et al*. 2005 (32). These analyses
193 were complemented by inferring the MLH genealogies using the Global Optimal eBURST
194 algorithm (33), as implemented in PHYLOViZ (34). Using an extension of the goeBURST
195 rules up to n locus variants level (nLV, where n equals to the number of loci in our dataset:
196 seven), a Minimum Spanning Tree-like structure was drawn to cluster the MLH into a clonal
197 complex based on their multilocus genotypes.
198
199 *Classification of recurrences*
200 The multilocus genotype of the samples from Day Zero and the recurrence day in participants
201 with recurrence were compared. If both samples had exactly the same allele at each locus for
202 all 7 MS analyzed, i.e identical MLH, it was considered an identical recurrence. In case of a

polyclonal infection on Day Zero, the recurrence was considered to be from the same MLH when all alleles for 7 MS analyzed in the recurrence day were present on Day Zero.

For participants with identical haplotypes at zero and recurrence days, the probability that a second infection with same haplotype occurred by chance was estimated as the P(match) considering the relative frequency of each MLH in the day-zero population (35-37). The P(match) was estimated by multiplying each MLH relative frequency within each of the two genetic clusters (i.e. subpopulations) obtained by Bayesian analysis in the Day Zero sample set, by their relative frequency in the recurrence population. Polyclonal infections were excluded for this analysis, except samples from participants with a recurrence in which the haplotype frequency from the recurrence day was considered. When P(match) was <0.05, the recurrence was classified as a relapse, otherwise it was considered a reinfection by the same haplotype.

Results

Incidence of malaria recurrences by P. vivax and risk factors

Of 134 participants screened, 87 met the inclusion criteria (Figure 2). Participants included and excluded were similar in demographic characteristics and history of malaria (Table S1). All participants included in the recurrence surveillance had a negative malaria PCR result on day 28 post initial treatment. During the six month follow-up, 29 recurrences in 21 participants were detected. Seven participants had two recurrences while one had three recurrences. The remaining 66 participants (75.9%) did not develop recurrent malaria during

226 the study period (censored cases), although seven of them were lost to follow-up within the
227 six month period.
228
229 The average follow-up time was 170.34 days with a standard deviation (SD) of 3.06 days;
230 cumulative incidence to first recurrence was 24.1% at 180 days post treatment (95% CI 14.6
231 - 33.7); 86% (18/21) of the participants had their first recurrence between 51 and 110 days
232 after enrolling in the study (Figure 3). Seven participants had a second recurrence event
233 between days 118 and 177 and one presented a third recurrence on day 179.
234
235 Characteristics of participants with and without recurrence are presented on Table 1, while
236 crude and adjusted HR are shown on Table 2. After adjusting for history of malaria in the
237 preceding year, time of residence in an endemic area for longer than 5 years was the only
238 factor associated with malaria recurrence (HR = 2.5, 95% CI 1.04 to 4.87).
239
240 *Genetic diversity of P. vivax*
241 A total of 157 samples were genotyped: 128 Day Zero samples (participants included and
242 excluded from monitoring of recurrences) and 29 recurrences. 87.5% of samples from Day
243 Zero (n = 112) and 100% of the recurrences were monoclonal infections. All polyclonal
244 infections had two clones. Two samples collected on Day Zero (1.4%) did not amplify an
245 allele at one MS locus (MS20 and MS12.335) while all seven loci were successfully
246 amplified in all recurrence samples.
247

248 Average *He* was 0.721 ± 0.036 in samples from Day Zero. The average number of alleles per
249 locus was 8.71 ± 3.2 and average alleles/locus per sample was 1.04. MS20 was the most
250 polymorphic locus with *He* = 0.860, and it discriminated 43.7% of polyclonal infections.
251 (Table 3). A total of 52 MLH were detected in 112 monoclonal samples from Day Zero, of
252 which 34 (65.4%) were only present in a single sample (Figure 4). H33 and H3 MLH were
253 the most frequently detected from the Day Zero group, in 21.4% (24/112) and 9.8% (11/112)
254 of the samples, respectively.
255
256 Average *He* was 0.664 ± 0.050 in the 29 recurrence samples. Among them 13 MLH were
257 identified, of which H33 had a frequency of 24.1% and H16 of 20.7%; H54 and H55 were
258 new haplotypes not identified in samples from Day Zero and H53 was identified only in a
259 polyclonal sample from Day Zero.
260
261 In Figure 4, the monthly distribution of haplotypes detected during the study period is
262 presented. Two genetic clusters were inferred using Structure program, including the data
263 from monoclonal infections (Fig S1), and all samples had a greater than 75% probability of
264 belonging to one of the two possible genetic clusters. A total of 87 isolates (60.4%) with 46
265 haplotypes belonged to cluster 1; while 57 isolates (39.6%) with nine haplotypes belonged
266 to cluster 2. The samples were classified into these groups to estimate haplotype frequencies
267 on Day Zero and P(match). Both clusters of *P. vivax* circulated simultaneously in Turbo
268 during the study period but their geographic distribution was slightly different (Fig S2).
269 These clusters, however, could be genetically interrelated as indicated by the haplotype

network (Fig S3) with the putative primary founders in the less diverse cluster 2. Importantly, all MLH lineages were found in all localities so this source of bias did not affect our results.

Classification of recurrences by P. vivax

MLH from participants with recurrences are presented in Table 4. Of a total of 29 recurrences, 27 were identical haplotypes to those present on Day Zero (Table 4 and Table S2) to the results from the P(match) analysis for classification of recurrences indicated that 65.5% (19/29) were relapses from initial infection.

Cluster 1 contained 12 participants with recurrences and a total of 18 recurrence events (Table 4). The recurrences in two participants from this group were classified as reinfections (i.e. new infection) because the MLH from Day Zero and day of recurrence were not identical. Nevertheless, the recurrence haplotypes were highly genetically related to the infection episode at the time of inclusion in the study with differences at only two alleles (Table S2).

A group of nine participants were assigned to cluster 2 with a total of 11 recurrence events, all of which were caused by haplotypes that were identical to the previous episode/s (Table 4). There were six recurrences by the predominant haplotype (H33), which had a very high P(match), thus making it highly likely that participants were reinfected with the same parasite strain that was detected on Day Zero.

Discussion

In this study we found that 24.1% (21/87) of *P. vivax*-infected participants had at least one recurrence within 180 days of treatment with a standard CQ-PQ regimen, despite receiving full treatment and being monitored. Additionally, 8% (7/87) of participants had three or more episodes within six months. Moreover, it is highly unlikely that these recurrences were recrudescence by therapeutic failure to CQ, since all participants had a negative PCR for malaria on day 28 post-treatment. Results from similar studies in other parts of the world reported a cumulative incidence of recurrence between 0% and 13.5% after treatment with 0.25 mg/kg/day PQ for 14 days and follow-up time between 180 and 210 days, which is much lower than what was found in this study (38-43).

A similar study carried out in two endemic regions of Colombia (2003 – 2004), reported cumulative incidence of 16.2% for a first recurrence of *P. vivax* during six months after treatment with the same regimen as in the present study and only 1.5% of the participants had more than one recurrence event (14). Although the difference in cumulative incidence of recurrence is seemingly little between these two studies, recurrences in the previous study are more likely to have been cases of reinfection, rather than relapses, since API in that study was much higher than the present one (30 vs 3) (15, 44). In addition, a maximum PQ dose of 210 mg was used in the previous study, so participants weighing more than 60 kg body weight received a lower dose than recommended; in the present study, all participants were dosed by body weight to avoid relapse due to under-dosing, as previously reported in other studies (26, 45, 46).

316

317 *P. vivax* recurrences may have an impact on patient wellbeing via clinical symptoms and the
318 risk of complicated malaria. In this study, participants presented clinical symptoms in all
319 episodes of malaria, although recurrent episodes were not always febrile. Moreover, *P. vivax*
320 gametocytes were present at recurrence, which may have contributed to ongoing transmission
321 (4).

322

323 Potential risk factors for *P. vivax* recurrence were explored. Duration of residence of more
324 than 5 years in this endemic area was associated with an increased risk of recurrence, even
325 after adjusting for malaria history in the last year. Although this result seems to contradict
326 previous reports (25), our interpretation is that a longer time of exposure to malaria, and
327 therefore a greater probability of having had previous episodes, may increase the likelihood
328 of hosting dormant hypnozoites. Importantly, in this study a sample size was not considered
329 to assess risk factors associated with recurrence; which limited precision in estimates of risk
330 factors for recurrence.

331

332 This study included the 74% of *P. vivax* malaria reported cases in four diagnostic centers
333 from Turbo municipality during the study period, so the parasite sample genotyped (n=157)
334 can be considered a reliable representation of the *P. vivax* strains responsible for symptomatic
335 infections in the study area. The detection of 52 haplotypes in 112 Day Zero samples, and
336 the high percentage of unique haplotypes (65.4%) shows high genetic diversity, consistent
337 with other reports from South America using *P. vivax* microsatellites (37, 47-50). Overall,
338 the percentage of samples with polyclonal infection was 12.5%, which is similar to reports

339 from other populations in Colombia, Venezuela and Peru (47, 50, 51). The genetic diversity
340 of the selected markers indicates that they have enough resolution to detect differences
341 among samples of *P. vivax* in Turbo.
342
343 About 93.1% of recurrences analyzed were caused by MLH that were identical to those of
344 the first episode (Day Zero sample). This percentage is higher than reported in other studies
345 that used the same PQ supervised regimen, similar length of follow-up and genotyping
346 approach of recurrences (microsatellite loci) (37, 40). Additionally, 62.1% of recurrences
347 were classified as relapses according to established criteria based on estimation of P(match)
348 within each genetic cluster, which could indicate a problem with efficacy of the PQ regimen
349 used. The criteria used in this study for classification of recurrences were strict, and
350 additional information collected during follow-up — such as travel to other endemic areas,
351 family members with malaria in the same household, and use of preventive measures for
352 malaria — supported this interpretation.
353
354 Several aspects of this study support the finding that at least six out of ten *P. vivax* recurrences
355 in Turbo, Colombia, are caused by relapses: A PQ regimen with low incidence of recurrences
356 reported worldwide was used. CQ-PQ treatment was supervised to completion. PQ daily dose
357 was adjusted for body weight, even in patients over 60 kg. No recrudescence was confirmed
358 by negative *P. vivax* PCR on day 28. Remarkably, most recurrences were caused by
359 genetically identical parasites to the previous episode. Given the high genetic diversity of *P.*
360 *vivax* in the study area, the probability of reinfection with an identical MLH in this area is
361 extremely low. Most recurrences occurred between 51 and 110 days of a previous episode,

362 which agrees with previously reported timing for *P. vivax* relapses from South America,
363 characterized by short latency time (3). API was low during the study period and therefore
364 the probability of reinfection was also low. Most participants with recurrences had not visited
365 another endemic area during follow-up, and they had not family members infected with
366 malaria. There were recurrences in participants who resided in urban areas where currently
367 there is no malaria transmission and therefore it was not possible for the participants to
368 acquire a new infection.
369
370 The high incidence of relapses found in this study was possibly caused by the current standard
371 dose of PQ used in Colombia being insufficient to eliminate hypnozoites. It was recently
372 reported that PQ metabolism by the cytochrome P450 2D family of enzymes is required for
373 antimalarial activity in humans and lower CYP 2D6 enzyme activity, as in the poor-
374 metabolizer phenotype, could compromise its radical curative efficacy (52). It is not clear if
375 any such variation in the genetic background of this population could account for this
376 observation. In addition, it has been shown that when using higher doses of PQ such as 0.5
377 mg/Kg for 14 days as recommended by the CDC (53), lower incidence of recurrences are
378 reported (between 1.9% and 6.6% during 180 - 365 days) (43, 54, 55). However, no direct
379 comparison had been made of PQ regimen 0.5 mg/Kg for 14 days with the standard regimen
380 in malaria endemic countries (9). Further studies addressing this subject and evaluating the
381 optimal dose and type of treatment are required, as well as to establish a minimum effective
382 concentration of PQ in order to define therapeutic failure.
383

For elimination of malaria by *P. vivax*, surveillance of recurrences is necessary, including genotyping and monitoring of different factors associated with it. This type of approach not only allows for the classification of recurrence as reinfection or relapse, but opens the prospect of identifying PQ tolerance markers, evaluating the efficacy and safety of therapeutic PQ regimens with optimal doses for different epidemiological contexts, and advancing the search for new agents against liver hypnozoites.

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590 **Figures legends**

591

592 Figure 1. Map of study area. Participants were recruited in hospital of Turbo located in urban
593 center and three health centers in the periphery (El Dos, Tres and Currulao).

594

595 Figure 2. Flowchart of enrolled participants, under supervised treatment with chloroquine
596 and primaquine.

597

598 Figure 3. Survival curve Kaplan-Meier for participants included in monitoring of malaria
599 recurrences by *Plasmodium vivax*.

600

601 Figure 4. Monthly distribution of multilocus haplotypes of *Plasmodium vivax* in Turbo-
602 Colombia identified during the study period. Vertical axis are number of cases and horizontal
603 axis are dates (year - month). Monthly frequency of haplotypes in monoclonal samples from
604 Day Zero (n=115) (a) and recurrence day (n=29) (b), each color represents a different
605 haplotype.

606

607 **Tables**

608

609 Table 1. Characteristics of participants enrolled for monitoring of malaria recurrences by

610 *Plasmodium vivax*.

	RECURRENCE (n=21)	NO RECURRENCE (n=66)	*P* value[a]
Sex male; n (%)	14 (66.7)	37 (56.1)	0.209
Age, years; median (IQR)	25 (17.0 – 35.5)	29 (15.7 – 45.2)	0.258
Body mass index, kg/m^2; median (IQR)	23.0 (20.2 – 26.2)	22.7 (20.0 – 24.8)	0.659
Occupation; n (%)			
Farmer	8 (38.1)	19 (29.7)	0.616
Housewife	5 (23.8)	16 (25.0)	
Student	5 (23.8)	18 (28.1)	
Other job	3 (14.3)	11 (17.2)	
Likely source of infection within Turbo; n (%)	17 (80.9)	60 (90.9)	0.182
Rural residence; n (%)	14 (66.7)	41 (62.1)	0.486
Parasitemia Day Zero, parasites/µL; median (IQR)	4,480 (2280 – 7080)	4,940 (2100 -9590)	0.797
Number of days with symptoms before diagnosis; median (IQR)	5 (4 – 6.5)	5 (3 – 7)	0.210
Time of residence in endemic region, years; median (IQR)	12 (2.5 – 20)	4 (1 – 12)	0.043
Symptomatic malaria in last year; n (%)	8 (38.1)	14 (21.1)	0.390
Number of malaria episodes in last year; median (IQR)	1 (1 – 1.7)	1 (1- 2)	0.390
Last episode of symptomatic malaria; n (%)			
1 to 2 months	5 (25.0)	3 (4.8)	0.882
3 to 6 months	2 (10.0)	10 (15.9)	
7 to 12 months	1 (5.0)	1 (1.6)	
> 12 months	5 (25.0)	25 (39.7)	

No history of malaria	7 (35.0)	24 (38.1)	
CQ dosage, mg/kg; median (IQR)	24.2 (22.3 – 26.1)	23.9 (22.1 – 26.4)	0.387
PQ dosage, mg/kg; median (IQR)	3.6 (3.4 – 4.1)	3.7 (3.5 – 4.1)	0.500
Travel to other endemic region during follow-up; n (%)	7 (35.0)	19 (37.2)	0.954
Using bed-net during follow-up; n (%)			
Always	12 (60.5)	33 (61.9)	
Never	5 (25.0)	9 (16.7)	0.776
Some months	3 (15.0)	12 (22.2)	
Using insecticides to spray house during follow-up; n (%)	14 (70.0)	25 (48.1)	0.286

611

612 IQR, interquartile range

613 [a] Cox regression, Andersen-Gill extension

614 Table 2. Potential risk factors for *Plasmodium vivax* malaria recurrence during six month follow-up.

	Crude HR[a]	95% CI	Adjusted HR[b]	95% CI
Sex				
Female	1			
Male	1.66	0.75 – 3.69	1.59	0.71 – 3.56
Age				
< 18 years	1		1	
≥ 18 years	1.69	0.73 – 3.94	1.52	0.66 – 3.51
Time of residence in endemic region				
≤ 5 years	1		1	
> 5 years	2.19	1.00 – 4.77	2.25	1.04 – 4.87
Number of days with symptoms before diagnosis				
< 5 days	1		1	
≥ 5 days	0.79	0.38 – 1.66	0.85	0.40 – 1.80
Parasitemia Day Zero				
< 4,920 parasites/μL	1		1	
≥ 4,920 parasites/μL	1.11	0.50 – 2.44	1.19	0.54 – 2.61
Symptomatic malaria in last year				
No	1		1	

Yes	1.41	0.65 – 3.07	1.50	0.70 – 3.25
Place of residence				
Urban	1		1	
Rural	1.33	0.60 – 2.93	1.39	0.63 – 3.09
Travel to other endemic region during follow-up				
No	1		1	
Yes	0.98	0.45 – 2.11	1.02	0.48 – 2.14
Using bed-net during follow-up				
Always	1		1	
Never	1.32	0.52 – 3.36	1.11	0.41 – 3.00
Some months	0.75	0.26 – 2.17	0.79	0.29 – 2.16
Using insecticides to spray house during follow-up				
Yes	1		1	
No	0.64	0.29 – 1.45	0.83	0.34 – 2.03

615

616 [a] Cox regression, Andersen-Gill extension

617 [b] Cox regression, Andersen-Gill extension. Adjusted for symptomatic malaria in last year and time of residence in endemic region > 5 years.

618 HZ, Hazard ratio. CI, confidence interval

619 Table 3. Genetic diversity of *Plasmodium vivax* per locus from Day Zero samples.

LOCUS	Total number of alleles*	Allele size range (bp)	Expected heterozygosity (*He*) [a]	Polyclonal samples (%)	Average alleles/locus/sample
MS2	11	181 - 251	0.791	5.47	1.05
MS6	6	211 - 249	0.746	3.13	1.03
MS20	15	206 - 263	0.860	5.51	1.06
2.21	7	83 - 115	0.644	3.91	1.04
3.502	7	133 - 199	0.768	7.03	1.07
11.162	8	181 - 243	0.643	2.34	1.02
12.335	7	160 - 179	0.598	3.94	1.04

620

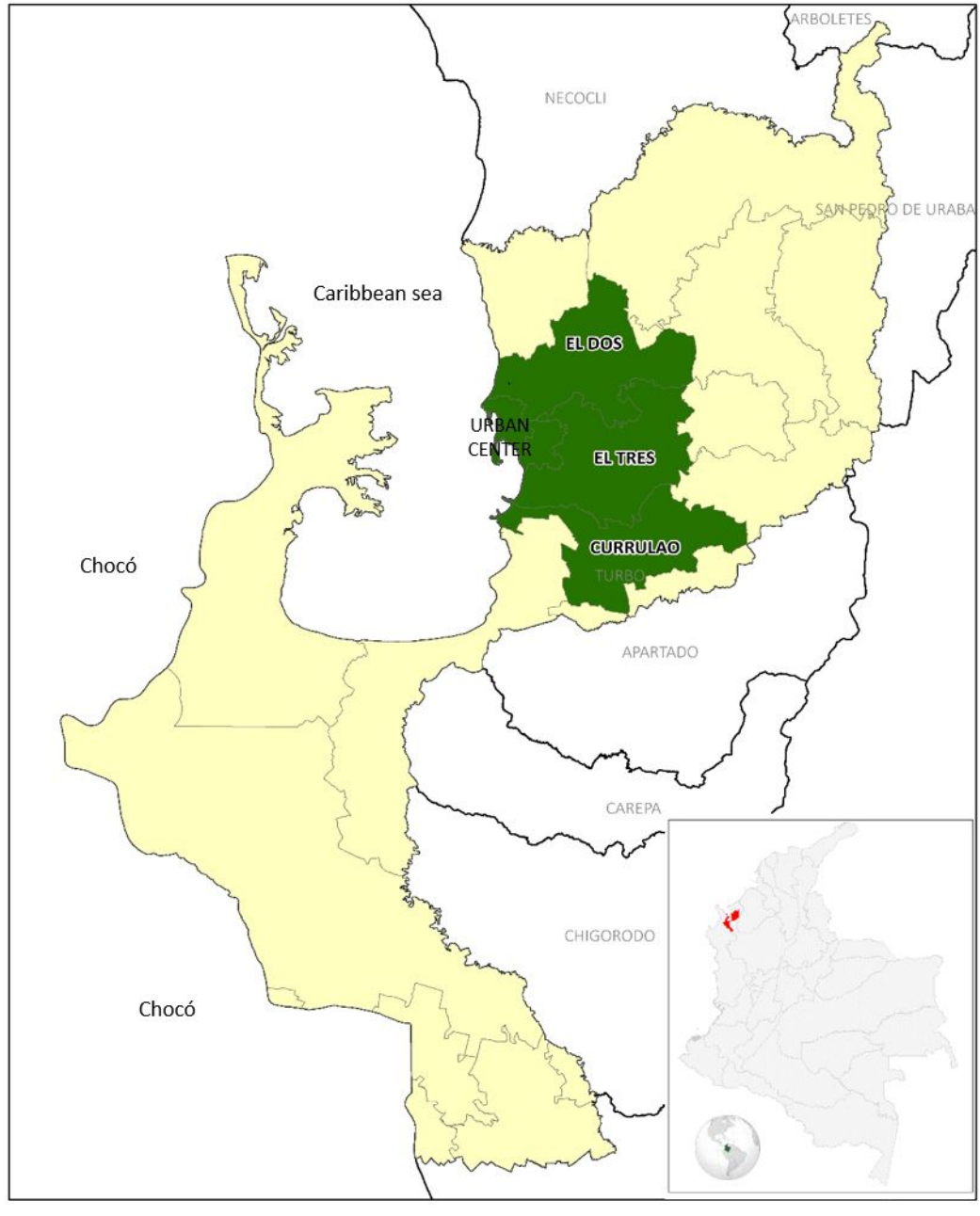
621 [a] Only from monoclonal samples (n=112). *He* represents probability of finding two different
622 alleles for a given locus on a pair of isolated randomly selected from the study population
623 ($He = n/(n\text{-}1)(1\text{-}\sum p_i^{\,2})$), where p is *i-th* frequency allele and n is sample size.

Table 4. Multilocus haplotypes in 21 participants with recurrences by *Plasmodium vivax* during six months.

ID SAMPLE	Time from Day Zero to first recurrence	Time from Day Zero to second recurrence	Comparison Day Zero vs recurrence day	Haplotype code	Cluster	P(match)[a]	Classification[b]	Residence zone	Last episode of malaria before study	Travel to other endemic region during follow-up	Family member with malaria at time of recurrence
1014	54		Identical	6	2	0.0019	Relapse	Rural	more 12 months	No	No
1015	68	154	Identical	33[c]	2	0.5146	Reinfection	Rural	never	No	No
1018	173		Identical	33	2	0.5146	Reinfection	Rural	7-12 months	No	No
1022	94		Identical	25	1	0.0008	Relapse	Rural	3-6 months	No	No
1034	103	170	Identical	39[c]	1	0.0019	Relapse	Urban	never	Si	No
1050	180		Identical	33	2	0.5146	Reinfection	Rural	1-2 months	Si	No
1051	90		Identical	33	2	0.5146	Reinfection	Rural	never	No	No
1052	81	154	Identical	48*	1	0.0019	Relapse	Rural	more 12 months	Si	No
1055	110		Identical	25	1	0.0008	Relapse	Urban	more 12 months	Si	No
1057	91		Identical	33	2	0.5146	Reinfection	Rural	never	No	No
1063	108		Different	13 (Day Zero) - 54 (R1)	1		Reinfection	Rural	more 12 months	No	No
1065	72	142	Identical	3[c]	2	0.0572	Reinfection	Rural	more 12 months	No	No
1066	80		Identical	28	2	0.0075	Relapse	Urban	never	Si	No
1075	179		Different	10 (Day Zero) - 55 (R1)	1		Reinfection	Urban	1-2 months	Si	No
1089	75	145	Identical	26[c]	1	0.0002	Relapse	Urban	1-2 months	No	No

1094	69		Identical	33	2	0.5146	Reinfection	Rural	3-6 months	No	No
1098	76		Identical	53	1	0.0002	Relapse	Rural	1-2 months	No	No
1101[d]	58	118	Identical	16[c]	1	0.0034	Relapse	Rural	more 12 months	No	Yes
1102	99		Identical	16	1	0.0034	Relapse	Rural	never	No	No
1103	57	177	Identical	16[c]	1	0.0034	Relapse	Rural	never	No	Yes
1111	51		Identical	17	1	0.0002	Relapse	Urban	1-2 months	No	No

[a] Probability of finding by chance an identical haplotype in different episodes for a participant. [b] Relapse when P (match) is less than 0.05 and reinfection when greater or equal to 0.05. [c] Second recurrence with haplotype identical to previous two episodes in the study. [d] Third recurrence day 179 with haplotype 16, identical to previous three episodes in the study.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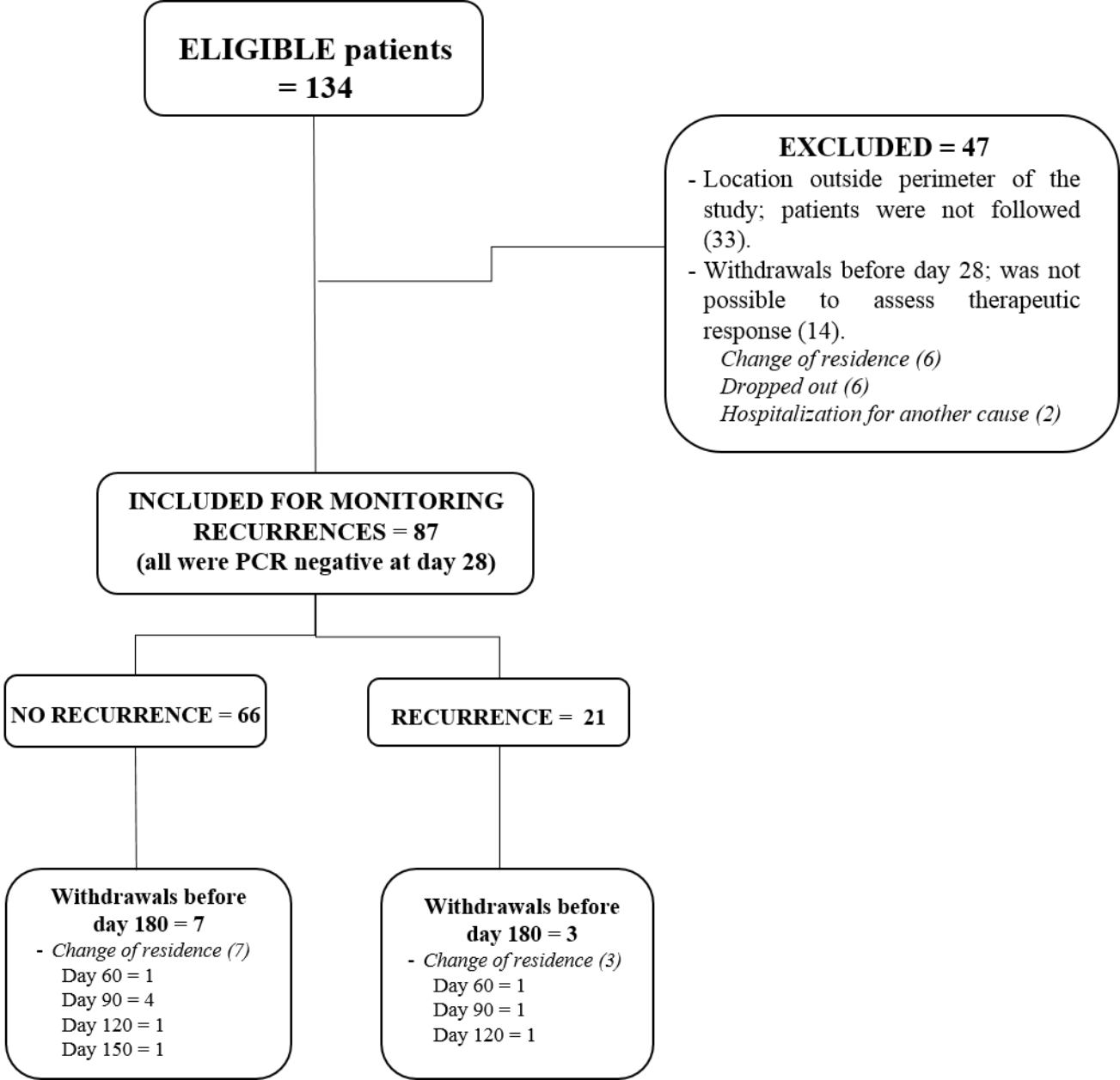
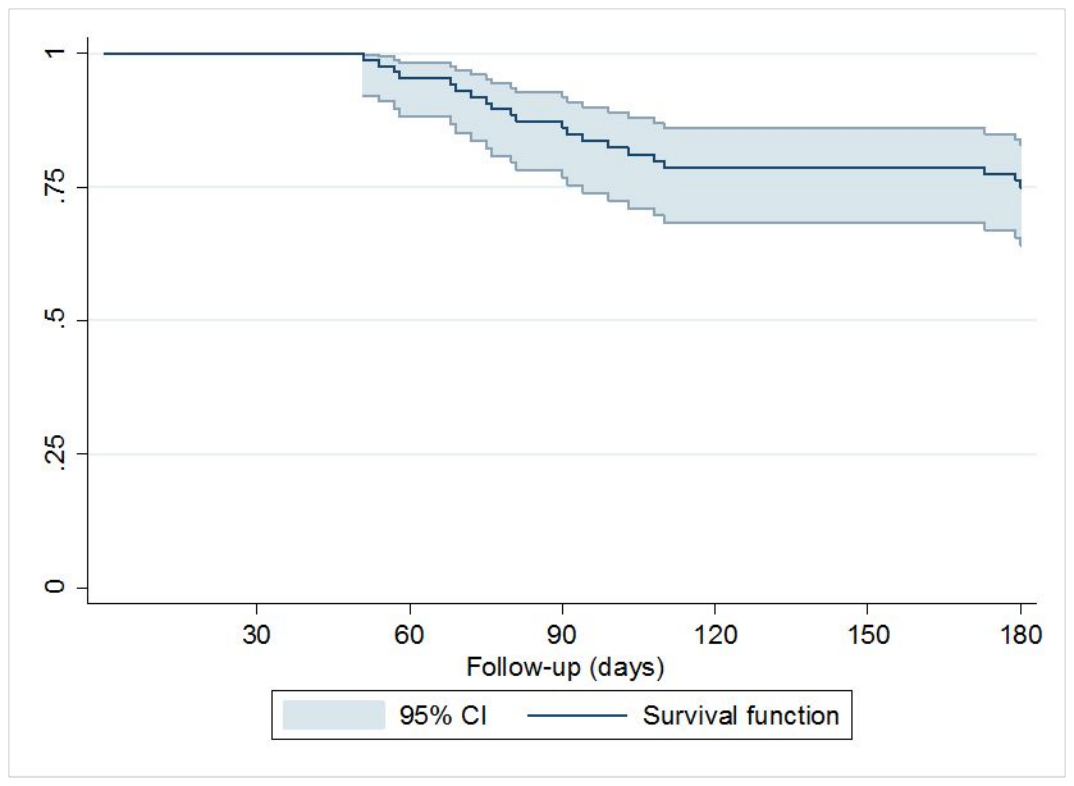

ELIGIBLE patients
= 134
EXCLUDED = 47
- Location outside perimeter of the study; patients were not followed (33).
- Withdrawals before day 28; was not possible to assess therapeutic response (14).
Change of residence (6)
Dropped out (6)
Hospitalization for another cause (2)
INCLUDED FOR MONITORING RECURRENCES = 87
(all were PCR negative at day 28)
NO RECURRENCE = 66
RECURRENCE = 21
Withdrawals before day 180 = 7
- Change of residence (7)
Day 60 = 1
Day 90 = 4
Day 120 = 1
Day 150 = 1
Withdrawals before day 180 = 3
- Change of residence (3)
Day 60 = 1
Day 90 = 1
Day 120 = 1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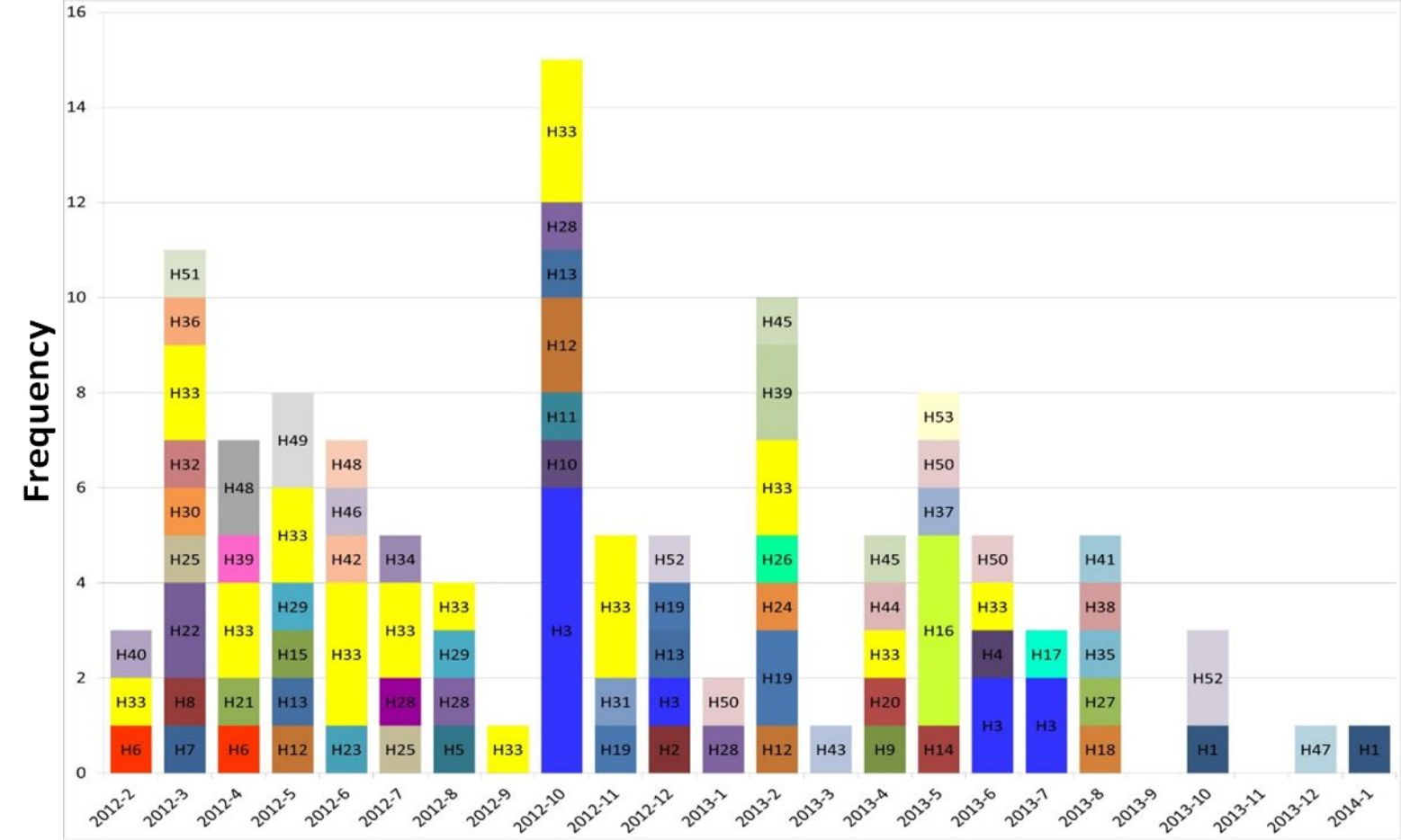
Follow-up (days)
95% CI
Survival function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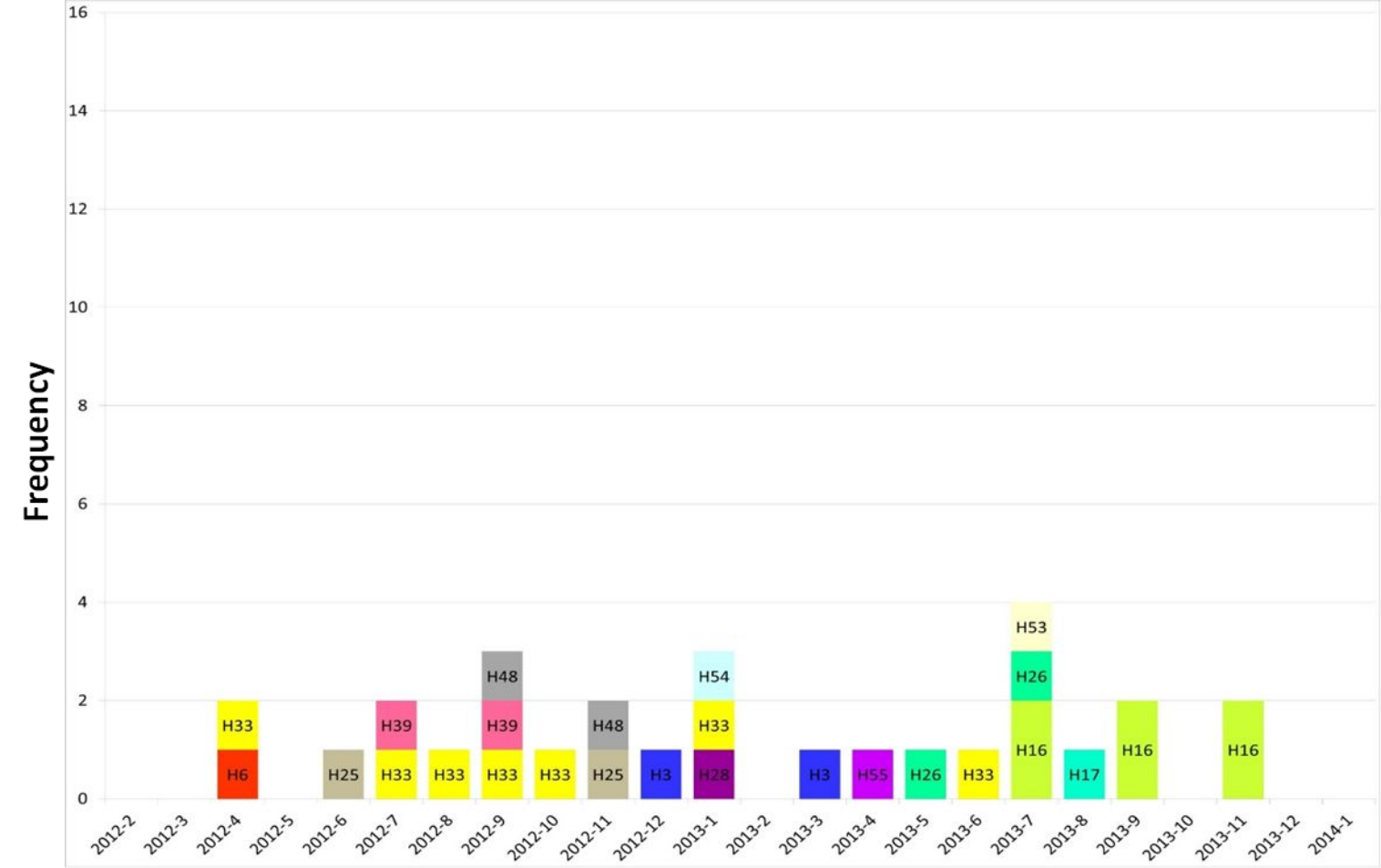
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TABLE S1 Characteristics of participants included and excluded for monitoring recurrences.

	INCLUDED (n=87)	EXCLUDED (n=47)
Sex; n (%)		
Male	51 (58.6)	23 (48.9)
Female	36 (41.4)	24 (51.1)
Age in years (median and IQR)	29 (16 - 39)	21 (15 – 32)
Body weight (median and IQR)	60 (50 - 67)	58 (45 – 69)
Occupation; n (%)		
Farmer	27 (31.8)	10 (21.7)
Housewife	21 (24.7)	14 (30.4)
Student	23 (27.1)	10 (21.7)
Other job	14 (16.5)	12 (26.1)
Likely place of infection; n (%)		
Turbo	77 (88.5)	36 (76.6)
Outside Turbo	10 (115)	11 (23.4)
Parasitemia Day Zero, parasites/μ**L;** median (IQR)	4,920 (2,200 – 8,840)	4,960 (2,120 – 10,720)
Number of days with symptoms before diagnosis; median (IQR)	5 (4 – 7)	5 (4 – 8)
Place of residence; n (%)		
Rural	55 (63.2)	32 (68.1)
Urban	32 (36.8)	15 (31.9)

Time of residence in endemic region; median (IQR)	5.0 (1.0 – 15.0)	5.0 (1.0 – 15.7)
Symptomatic malaria in last year; n (%)		
Yes	22 (25.3)	17 (37.0)
No	65 (74.7)	29 (63.0)
Number of malaria episodes in last year; median (IQR)	1 (1 – 2)	1 (1 – 2)
Last episode of symptomatic malaria; n (%)		
1 to 2 months	8 (9.6)	5 (13.5)
3 to 6 months	12 (14.5)	9 (24.3)
7 to 12 months	2 (2.41)	1 (2.7)
> 12 months	30 (36.4)	11 (29.7)
No history of malaria	31 (37.5)	11 (29.7)
Health system affiliation regime; n (%)		
Contributory	3 (3.7)	1 (2.2)
Subsidized	74 (91.4)	40 (88.9)
Unaffiliated	4 (4.9)	4 (8.9)

IQR, interquartile range

TABLE S2 Multilocus haplotypes from participants with malaria recurrences by *Plasmodium vivax* during six months.

Code sample	Date sample collection	Follow-up day	MS2	MS6	MS20	MS2.21	MS3.502	MS11.162	MS12.335
1014	14/02/2012	0	181	249	209	105	159	181	165
	09/04/2012	54 (R1)	181	249	209	105	159	181	165
1015	21/02/2012	0	214	249	215	105	167	181	162
	28/04/2012	68 (R1)	214	249	215	105	167	181	162
	23/07/2012	154 (R2)	214	249	215	105	167	181	162
1018	03/03/2012	0	214	249	215	105	167	181	162
	23/08/2012	173 (R1)	214	249	215	105	167	181	162
1022	26/03/2012	0	206	249	221	105	142	181	179
	29/06/2012	94 (R1)	206	249	221	105	142	181	179
1034	13/04/2012	0	214-239	239-249	209-224	83-105	142-151	181-185	162-165
	25/07/2012	103 (R1)	214	249	224	83	151	185	162
	30/09/2012	170 (R2)	214	249	224	83	151	185	162
1050	22/06/2012	0	214	249	215	105	167	181	162
	10/01/2013	180 (R1)	214	249	215	105	167	181	162
1051	25/06/2012	0	214	249	215	105	167	181	162
	25/09/2012	90 (R1)	214	249	215	105	167	181	162
1052	26/06/2012	0	239	246	209	105	151	181	165
	15/09/2012	81 (R1)	239	246	209	105	151	181	165

	27/11/2012	154 (R2)	239	246	209	105	151	181	165
1055	14/07/2012	0	206	249	221	105	142	181	179
	01/11/2012	110 (R1)	206	249	221	105	142	181	179
1057	14/07/2012	0	214	249	215	105	167	181	162
	13/10/2012	91 (R1)	214	249	215	105	167	181	162
1063	03/10/2012	0	189	243	212	103	167	197	162
	18/01/2013	108 (R1)	189	243	212	103	167	201	162
	17/10/2012	0	181	227	209	105	159	201	162
1065	28/12/2012	72 (R1)	181	227	209	105	159	201	162
	08/03/2013	142 (R2)	181	227	209	105	159	201	162
1066	17/10/2012	0	214	227	218	107	167	201	162
	05/01/2013	80 (R1)	214	227	218	107	167	201	162
1075	31/10/2012	0	189	239	236	105	151	201	165
	28/04/2013	179 (R1)	218	239	236	105	151	201	162
	16/02/2013	0	209	211	209	107	133	193	171
1089	02/05/2013	75 (R1)	209	211	209	107	133	193	171
	11/07/2013	145 (R2)	209	211	209	107	133	193	171
1094	13/04/2013	0	214	249	215	105	167	181	162
	21/06/2013	69 (R1)	214	249	215	105	167	181	162
1098	06/05/2013	0	206	211	206-240	107	167	181	162
	21/07/2013	76 (R1)	206	211	206	107	167	181	162

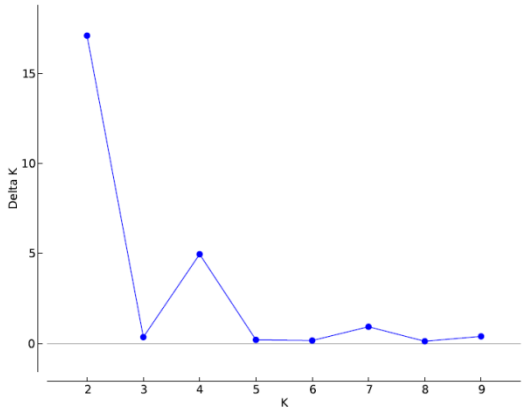
1101	28/05/2013	0	206	211	240	107	167	181	162
	25/07/2013	58 (R1)	206	211	240	107	167	181	162
	23/09/2013	118 (R2)	206	211	240	107	167	181	162
	23/11/2013	179 (R3)	206	211	240	107	167	181	162
1102	29/05/2013	0	206	211	206-240	107	167	181	162
	05/09/2013	99 (R1)	206	211	240	107	167	181	162
1103	29/05/2013	0	206	211	240	107	167	181	162
	25/07/2013	57 (R1)	206	211	240	107	167	181	162
	22/11/2013	177 (R2)	206	211	240	107	167	181	162
1111	08/07/2013	0	206	239	212	97	159	201	160
	28/08/2013	51 (R1)	206	239	212	97	159	201	160

R1, first recurrence

R2, second recurrence

R3, third recurrence

a)



b)

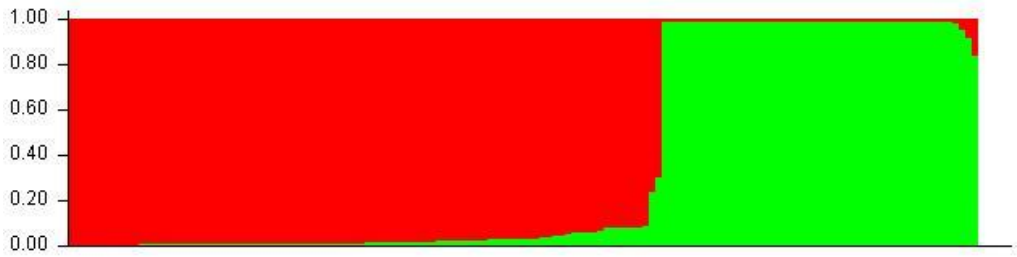


FIG S1 Population structure analysis of *Plasmodium vivax* monoclonal samples from Day Zero and recurrence day using Structure 2.3.4 program. a) Probability of assignment of all samples to each of K populations previously established. b) Bar graph for K = 2, each sample is represented by a vertical bar divided into K colors. Each color represents a cluster and segment size shows probability of a sample of belonging to certain cluster, 87 isolates belong to cluster 1 (red) and 57 to cluster 2 (green).

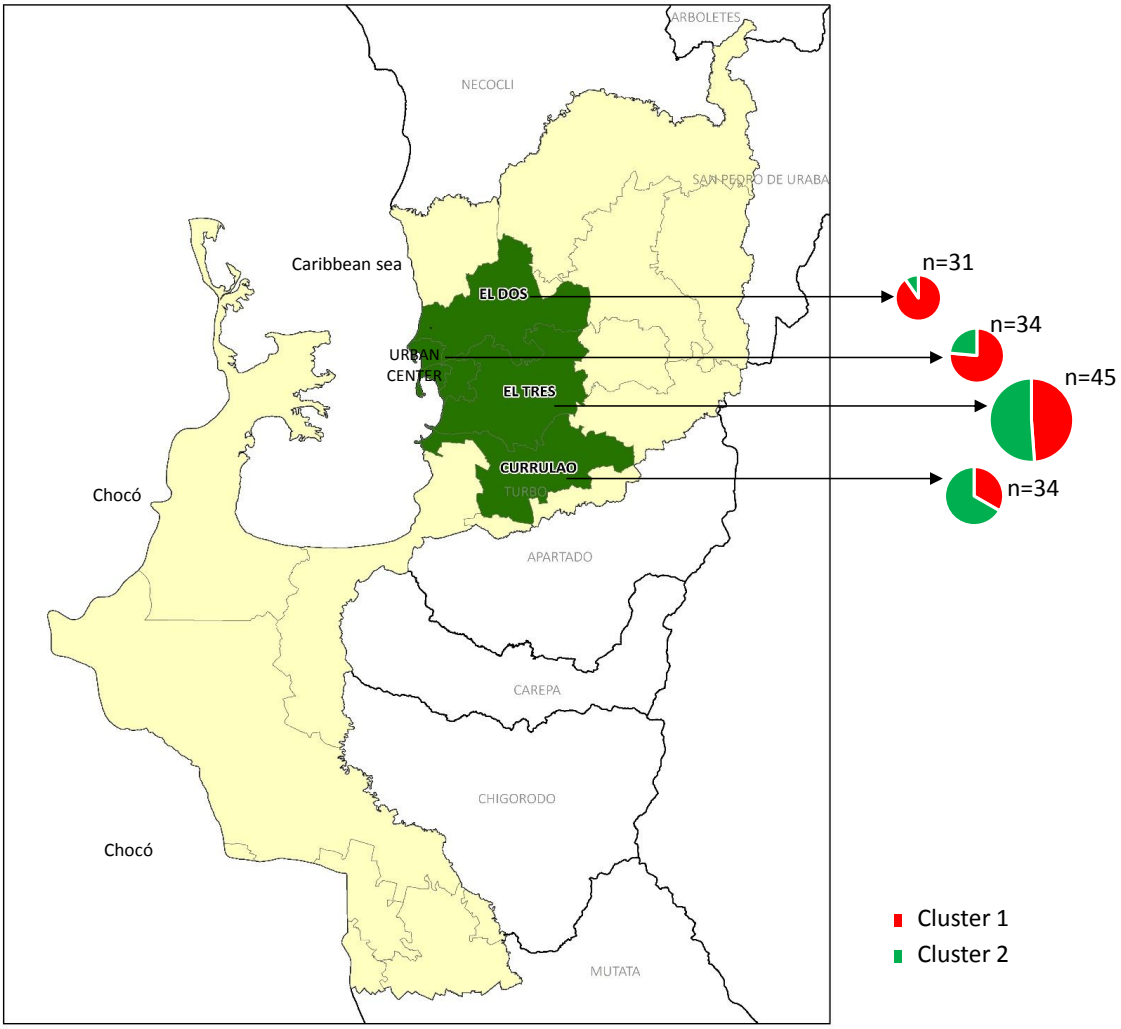
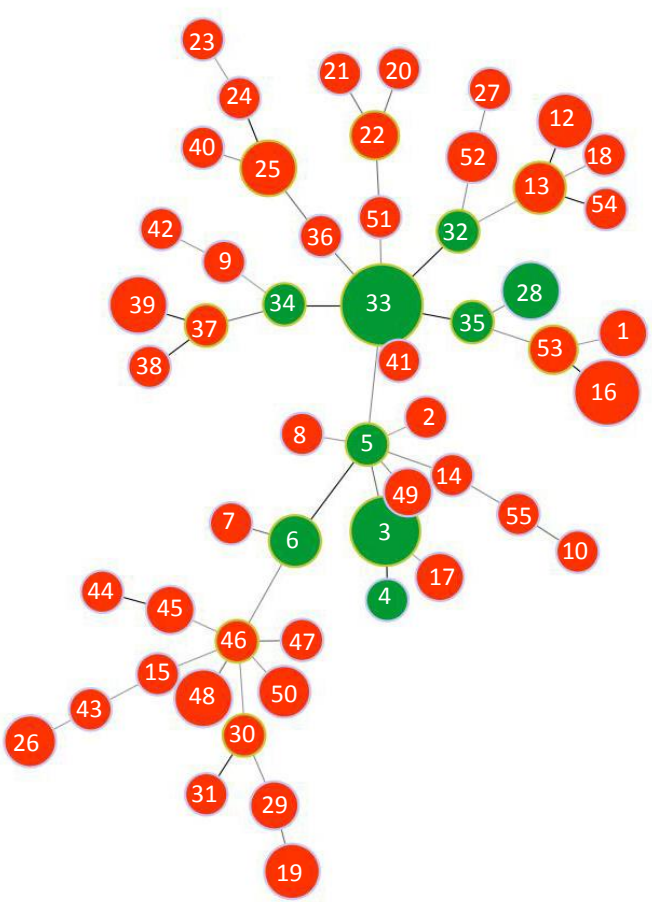
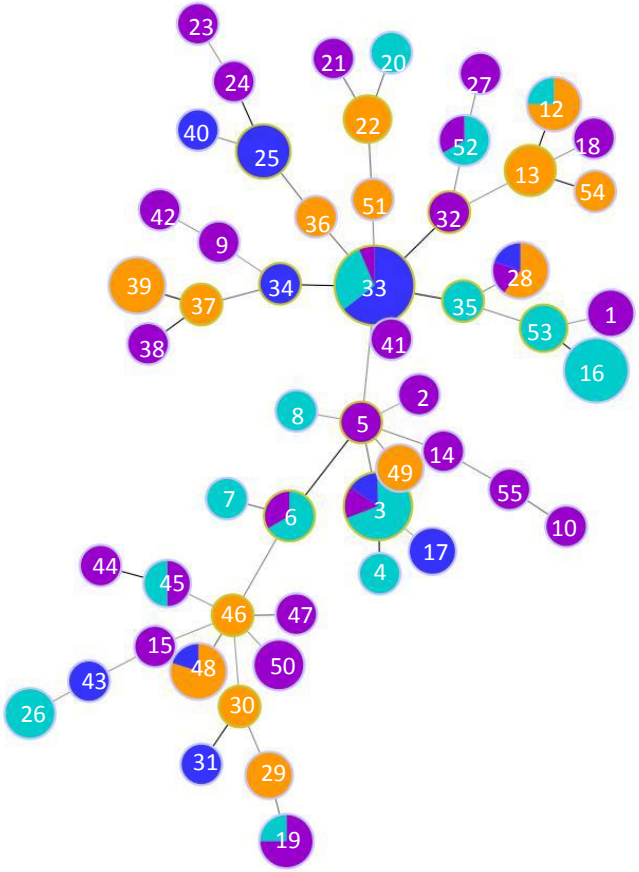


FIG S2 Geographical distribution of the two genetic clusters obtained by Bayesian analysis. The geographical location reflects the participants residence place. Each sample corresponds to individual multilocus haplotype, which are monoclonal infections of *P. vivax* detected at day Zero and recurrence day. Each color represents a genetic cluster (n = 144).



Cluster 1 (n=86, 60.14%)

Cluster 2 (n=57, 39.86%)



El Tres (n=45, 31.47%)

Urban center (n=34, 23.78%)

Currulao (n=34, 23.78%)

El Dos (n=30, 20.98%)

FIG S3 Minimum spanning tree for *Plasmodium vivax* constructed using goeBURST algorithm. The tree depicts the relationships among multilocus haplotypes (MLH) at the n locus variants level of *Plasmodium vivax* monoclonal samples from Day Zero and recurrence day (where n equals to the number of loci in our dataset: seven). Each MLH is represented by a circle with size being proportional to its frequency and the links are character differences. The color of each circle represents the cluster identified by Structure v2.3.4 (panel left) and study localities (panel right).