Genetic Variability of *Plasmodium vivax* in the North Coast of Peru and the Ecuadorian Amazon Basin

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Abstract. In the Peruvian North Coast (PNC), the number of *Plasmodium vivax* malaria cases increased steadily from 2007 to 2010 despite a significant decline in the overall number of cases in Peru during the same period. To better understand the transmission dynamics of *P. vivax* populations in the PNC and the neighboring Ecuadorian Amazon Basin (EAB), we studied the genetic variability and population structure of *P. vivax* in these areas. One hundred and twenty *P. vivax* isolates (58 from Piura and 37 from Tumbes in the PNC collected from 2008 to 2010 and 25 from the EAB collected in Pastaza from 2001 to 2004) were assessed by five polymorphic microsatellite markers. Genetic variability was determined by expected heterozygosity (H_e) and population structure by Bayesian inference cluster analysis. We found very low genetic diversity in the PNC ($H_e = 0-0.32$) but high genetic diversity in the EAB ($H_e = 0.43-0.70$). Population structure analysis revealed three distinct populations in the three locations. Six of 37 (16%) isolates from Tumbes had an identical haplotype to that found in Piura, suggesting unidirectional flow from Piura to Tumbes. In addition, one haplotype from Tumbes showed similarity to a haplotype found in Pastaza, suggesting that this could be an imported case from EAB. These findings strongly suggest a minimal population flow and different levels of genetic variability between these two areas divided by the Andes Mountains. This work presents molecular markers that could be used to increase our understanding of regional malaria transmission dynamics, which has implications for the development of strategies for *P. vivax* control.

INTRODUCTION

Plasmodium vivax is the most widely disseminated cause of human malaria outside of the African continent. It is estimated to result in 80 million cases annually and is associated with high morbidity. Consequently, *P. vivax* infections are a significant public health burden and have a negative impact on economic development. Approximately 80% of clinical malaria cases in South America are due to *P. vivax*.^{1,2}

Peru is a hypoendemic malaria area with low levels of transmission located in South America. In the Peruvian North Coast (PNC), the number of *P. vivax* malaria cases increased significantly between 2007 and 2010; from 1,733 (4%) to 3,922 (15%) despite a significant decline in the total number of cases from 43,467 to 26,878 in all of Peru.³ On the other hand, neighboring countries such as Ecuador reported a sustainable decrease in *P. vivax* cases since 2001 (106,641) through 2012 (558). This reduction in *P. vivax* cases was supported by economic growth and malaria control efforts by international organizations such as the Malaria Control in Border Zones of the Andean Region Program (PAMAFRO), Amazon Malaria Initiative of the United States Agency for International Development, and the Roll Back Malaria of the World Health Organization/Pan American Health Organization. However, this improvement of the economy in Ecuador created the need of new routes for exportation of agricultural products from the Amazon region to neighboring borders creating a risk of transmission of *P. vivax* from Ecuador to other countries such as Peru.^{4,5}

Population migration of humans, animal reservoirs, and vectors between the PNC and the neighboring regions such as the Ecuadorian Amazon Basin (EAB) could affect the genetic

variability, population structure, and number of cases of *P. vivax* in these regions during these periods.

Implementation of parasite control strategies for *P. vivax* requires an understanding of genetic variability as well as population structure, local epidemiology, and the dynamics of transmission caused by vectors and humans infected with this parasite.⁶⁻⁹ Genetic variability and population structure of *P. vivax* populations can be analyzed by microsatellite markers, simple sequence tandem repeats typically used in studies of population genetics in eukaryotic organisms. Studies of *P. vivax* using microsatellite markers in populations from endemic areas of South America such as the Amazon jungle of Colombia, Brazil, and Peru revealed high levels of heterogeneity ($H_e = 0.44-0.80$) and strong differentiation in *P. vivax* populations.¹⁰⁻¹⁵

In Peru, the analysis of the genetic variability and population structure of *P. vivax* populations using microsatellite markers has been described for the Peruvian Amazon Basin only.^{10,16,17} Van den Eede et al.,¹⁰ found high levels of genetic variability, highly structured populations, and strong genetic differentiation. In addition, genetic studies of *Plasmodium falciparum* populations found a clonal substructure with the presence of five different clonets in the Peruvian Amazon Basin. In contrast, a single clonet in the PNC was found. The Andes Mountains appear to act as a barrier to the flow of *P. falciparum* populations between these areas.¹⁸

To better understand the transmission dynamics and population structure of *P. vivax* populations between the PNC and the neighboring EAB, we studied *P. vivax* isolates from these areas using five polymorphic microsatellite markers.

MATERIALS AND METHODS

Study areas and sample collection. One hundred and twenty-seven *P. vivax* specimens were obtained from whole blood, filter papers impregnated with whole blood, or serum

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samples of patients with malaria. These samples were analyzed by microscopy at the time of collection to confirm *P. vivax* mono-infection. Ninety-five whole blood or filter papers impregnated with whole blood samples were from the PNC; 58 from the Department of Piura collected from 2008 to 2010 at the Bellavista Health Center and 37 from the Department of Tumbes collected from 2008 to 2009 at the Zarumilla Health Center and the Pampa Grande Health Center (Figure 1).

Thirty-two serum samples were analyzed from the Province of Pastaza in the EAB, collected between 2001 and 2004 at Hospital de la IV División del Ejército "Amazonas," an Ecuadorian military hospital in the provincial capital of Puyo and at Hospital Vozandes del Oriente, a mission hospital in the nearby town of Shell (Figure 1).¹⁹ Both hospitals receive patients from throughout the region, including remote jungle villages and military outposts.

Samples from Ecuador were collected under the protocol "Etiology of Acute Undifferentiated Febrile Illness in the Amazon Basin of Ecuador." This study was approved by the research ethics committee of Hospital Vozandes del Oriente, the Pastaza province directorate of the Ecuadorean Ministry of Public Health, and the U.S. Naval Medical Research Center Institutional Review Board (Protocol NMRCD.2001.0002) in compliance with all applicable federal regulations governing the protection of human subjects.¹⁹

This study was approved by the Institutional Review Board of the U.S. Naval Medical Research Unit 6 (Protocol NMRCD: PJT.NMRCD.075) in compliance with all applicable federal regulations governing the protection of human subjects.

Laboratory methods. DNA was extracted from whole blood, filter papers impregnated with whole blood samples, or serum samples using the QIAamp DNA Blood Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The samples were analyzed by species-specific *Plasmodium* polymerase chain reaction (PCR) to reconfirm *P. vivax* mono-infection.²⁰

Five polymorphic dinucleotide microsatellite markers 14.185, 4.2771, 6.34, 12.335, and 7.67 were amplified using

the primers and PCR conditions described by Imwong et al.²¹ The PCR product sizes were analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) using GeneScan™ 350 ROX™ Size Standard (Applied Biosystems) as an internal lane size standard. Fragment sizes were determined with GeneMapper® software v4.0 (Applied Biosystems).

To determine alleles length and quantify peaks height, we used default microsatellite settings. Only the peaks greater than 100 relative fluorescence units (RFUs) were considered alleles, bands smaller than 100 RFUs were defined as background.

Population genetics were measured using the single or predominant allele (highest electrophoretic peak greater than 100 RFUs) observed at each locus to calculate allele frequencies. The predominant alleles were used to define haplotypes. Infections were defined as polyclonal if an isolate had more than one allele per locus. Only monoclonal infection samples were used for calculating genetic variability and structure population.¹⁰

Data analysis. Population genetic variability and structure population analysis were calculated using only *P. vivax* monoclonal infection to avoid the presence of artificial haplotypes by multiple alleles per locus in the same sample.

Multiclonal infections were defined as identification of more than one allele per locus per sample. Multiplicity of infection (MOI) refers to the average number of distinct parasite genotypes concurrently infecting a patient and was calculated using the number of different alleles at each locus; single infections were reported by only one allele per locus at all of the genotype loci.^{10,14,22}

We measured intra- and interpopulation diversity using the statistic expected heterozygosity (H_e) of each locus, which may be defined as the average probability that two alleles randomly obtained for each locus are different. We calculated the expected heterozygosity using $H_e = (n/[n-1])(1 - \sum p_i^2)$, where n is the number of isolates analyzed and p_i is the frequency of the i^{th} allele in the population; values for H_e range

FIGURE 1. Location of the health centers in the Peruvian North Coast and Ecuadorian Amazon Basin (Piura, Tumbes and Pastaza). Black lines reflect international borders. This figure appears in color at www.ajtmh.org.

are between 0 (null diversity) and 1. *He*, haplotypes, and allele number per locus were calculated using Arlequin software v3.1.²³

We evaluated population structure using STRUCTURE software v2.1. This uses an alternative Bayesian approach (using no a priori data) to infer the most likely number of populations (*K*) represented in the total sample and to measure the probability that an individual is derived from each of these *K* populations based on the allele frequencies of each locus.²⁴ The software was run with 20 replicates, with *K* varying from 1 to 10 with a burn-in of 100,000 and 500,000 iterations. For this analysis, we assumed the admixture model, which considers that each individual isolate may have ancestries from more than one of the *K* parental populations. The most probable number of populations was defined by calculating the ΔK value as described by Evanno et al.²⁵

To explore gene flow between the PNC and the EAB, we inferred two migration models using a Bayesian approach implemented in MIGRATE-N. The first model assumed no restriction gene flow between PNC and EAB (panmictic) and the second model assumed gene flow restrictions between PNC and EAB (in-island). MIGRATE-N estimates the posterior probability distribution of the migration parameters, such as mutation-scaled migration rates (*M*) and population sizes (Θ), and calculates marginal log-likelihoods (log mL) for each model using a thermodynamic integration.²⁶ The models were created considering the genetic structure and the knowledge of the people's current mobility patterns in the PNC and the EAB. Each model was run on a 1×10^5 long chain with sampling every 10 generations and a 10% burn-in under a static heating scheme with four temperature chains of 1, 1.5, 3, and 1,000,000. The log mL was used to estimate the log Bayes factors and probabilities that were subsequently used for selection of the best model. The number of immigrants per generation (N_m) was calculated as the product of *M* by Θ .^{17,27}

RESULTS

Multiplicity of infection and genetic variability in *P. vivax* isolates. Multiple-clone infections accounted for 21% of all EAB samples (seven out of 32) and the average MOI for this population was 1.05 (range: 1.0–1.13; SD: ± 0.06). On the other hand, the PNC population was composed exclusively of monoclonal infections (MOI = 1). Only monoclonal infections from the EAB and the PNC were used for genetic diversity and population structure analysis.

The number of alleles per locus ranged from one to six (Table 1). All microsatellites from isolates from the Department of Piura in the PNC were monomorphic. Two microsatellites (6.34 and 7.67) of isolates from the Department of Tumbes in the PNC were polymorphic with three alleles per locus; the

remaining microsatellites were monomorphic. In contrast, all microsatellites from the EAB were polymorphic, with three to six alleles per locus.

Eighteen haplotypes were found, one in the Department of Piura, three in the Department of Tumbes, and 15 in the Province of Pastaza (Table 2). Six of the 37 (16%) isolates from the Department of Tumbes had an identical haplotype to that found in Piura (collected 2008–2009). In addition, one haplotype collected in 2008 in Tumbes showed high similarity (80% by structure analysis) to a haplotype found in 2003 in Pastaza.

Plasmodium vivax isolates from the PNC showed very low genetic diversity; null expected heterozygosity *He* = 0 per locus was found in Piura and *He* = 0–0.32 per locus in Tumbes. In contrast, the EAB showed high values of genetic diversity with *He* = 0.43–0.70 per locus (Table 2).

Population structure. The population structure analysis identified the highest peak of ΔK = 145.4 for *K* = 3 (Figure 2). Three populations were identified; two populations in the PNC and one in the EAB. One subgroup was identified in the PNC with six out of 37 samples (16%) in the Department of Tumbes. As stated previously, this subgroup had an identical genotype to that found in Piura (Figure 3). One isolate from Tumbes had high similarity to a genotype found in Pastaza; this result is the same as that found in the haplotype analysis (Figure 3, Table 2).

Genetic flow. The gene flow was assessed using the information of the genetic structure of the parasite population from the structure analysis. From the two models tested (Panmictic and in-island), the second model was selected as the best model (log mL = –2,467.05, probability > 0.99). This model indicated that there are three distinct subpopulations with some migration flow occurring between them. This model is concordant with the results shown by structure analysis and the clear differentiation between study sites. According to this model, there is population flow between all sites, with a major contribution of immigrants from the Department of Piura to the Department of Tumbes in the PNC (N_m value of 3.434) compared with other sites. In contrast, we found lower migration flow from Pastaza in the EAB to Tumbes and Piura in the PNC (N_m values of 2.550 and 0.535, respectively). On the other hand, we found low migration flow from the Departments of Tumbes and Piura (N_m 1.377 and 2.242, respectively) to Pastaza (Table 3).

DISCUSSION

In this study, we observed a low level of genetic variability and monomorphic *P. vivax* populations in the PNC. In contrast, the EAB revealed a highly heterogeneous population with polymorphic alleles, a high number of haplotypes, a high level of genetic variability, and multiple-clone infection and MOI values similar to what others have observed in *P. vivax* populations from the Amazon Basin in Peru, Colombia, and Brazil.^{10–17,28} Multiple-clone infection and MOI values in the EAB are within the range of those from other studies conducted in South America such as Brazil, Colombia, and Peru (0–70% and 1.1–3.0, respectively). Multiplicity of infection values has different genetic implications. Pacheco et al. reported a significant association between MOI values and disease severity by *P. vivax* infection in Colombia, with average values of 1.40 and 1.68 for uncomplicated and complicated cases, respectively. Low MOI value of 1.05 reported in

TABLE 1
Description of microsatellite markers per locus

Marker	Motif	Chromosome location	Size range (bp)	Dye
14.185	AT	14	262–290	VIC
4.2771	AT	4	82–100	6FAM
6.34	AC	6	136–166	PET
12.335	AT	12	155–197	VIC
7.67	AT	7	100–134	VIC

AC = adenine-cytosine; AT = adenine-thymine; bp = base pairs. Dinucleotide microsatellites described by Imwong et al.²¹

TABLE 2
Haplotypes and genetic diversity (H_e) observed in the PNC and EAB

Sites	Alleles per locus	H_e range per locus	Haplotype	Alleles per microsatellite markers				
			Id	14.185	4.2771	6.34	12.335	7.67
EAB-Pastaza	3–6	0.43–0.70	H1	272	85	144	160	101
			H2	270	87	144	162	105
			H3	270	85	140	160	101
			H4	270	85	144	164	115
			H5	272	103	149	160	101
			H6	275	85	134	160	101
			H7	275	85	144	160	101
			H8	272	103	149	162	109
			H9	275	85	149	160	101
			H10	275	85	134	166	101
			H11	270	85	144	160	101
			H12*	270	95	154	160	95
			H13	270	85	144	164	109
			H14	270	85	149	160	101
			H15	272	85	152	164	101
			H16*	270	85	154	162	98
PNC-Tumbes	1–3	0–0.32	H17	270	85	157	162	101
			H18†	270	85	149	162	105
PNC-Piura	1	0	H18†	270	85	149	162	105

EAB = Ecuadorian Amazon Basin; PNC = Peruvian North Coast.

* Haplotype shared between Pastaza and Tumbes, only 40% of microsatellites.

† Haplotype shared between Tumbes and Piura, 100% of microsatellites.

the EAB is representative of hypoendemic malaria with low level of *P. vivax* population transmission. However, results could be biased because of small sample size (EAB $N = 32$), decreasing the number of *P. vivax* cases by effective malaria control and effects of DNA quality.

The PNC is a hypoendemic malaria zone separated by the Andes Mountains from other endemic malaria sites, including the Peruvian Amazon Basin and the EAB. Evidence of this geographic isolation is found in the population structure analysis, which revealed three distinct populations corresponding to each location; two populations in the PNC and one population in the EAB. We did not find shared *P. vivax* populations between the PNC and the EAB, indicating minimal population flow between these two areas. Similarly, Griffing et al.,¹⁸ 2011 identified that *P. falciparum* population present in the PNC was monomorphic and shared no genetic similarity with populations on the other side of the Andes Mountains in the Peruvian Amazon Basin.

We observed one subpopulation (six of the 37) in the Department of Tumbes that was also identified in the Department of Piura. This suggests possible population flow between the

two neighboring departments (150 km apart), between which there are no significant geographical barriers that may obstruct the movements of *P. vivax* populations by infected humans or vectors. This intrapopulation flow in the PNC is supported by MIGRATE-N analysis; we observed a major number of *P. vivax* immigrants from Piura to Tumbes, this interaction can be explained by human population migration from South to North. Tumbes shares a border with Ecuador; this strategic location promotes high levels of commerce and agriculture, thus creating an impetus for human population movement from surrounding areas, including Piura.

We did not find shared *P. vivax* populations between the PNC and the EAB, indicating minimal population flow between these two areas. Apparently, the risk of transmission of *P. vivax* populations by new routes of communications for exportation of agricultural products from the EAB to the PNC is very low. This was supported by STRUCTURE and MIGRATE-N analysis. However, one isolate from Tumbes in the PNC showed high similarity (80%) to a haplotype found in 2003 in Pastaza, which could be an imported case to Peru from the EAB. Nevertheless, these findings strongly indicate minimal population flow and different levels of genetic variability between these two areas divided by the Andes Mountains.

Another important aspect of the dynamics of transmission in *P. vivax* population between these regions is the role of the malaria vector. The PNC and EAB have different principal malaria vectors. In the PNC and EAB the principal malaria vectors are *Anopheles albimanus* and *Anopheles darlingi*, respectively.^{29–32} A successful adaptation process between the parasite and the vector requires particular ecological and evolutionary factors; this adaptation could potentially make it more difficult to transmit *P. vivax* populations by different vector species.^{32,33}

In addition, selective pressure from treatment policies implemented in the PNC during the malaria peak caused by the “El Niño” phenomenon (1997–2000) may have produced small *P. vivax* population with little or no genetic diversity. Also, isolation by Andes Mountains may have contributed to

FIGURE 2. Estimate of the number of *Plasmodium vivax* populations by Delta K values using STRUCTURE V2.1 software. The highest peak of $\Delta K = 145.4$ for $K = 3$. This figure appears in color at www.ajtmh.org.

FIGURE 3. Population structure of *Plasmodium vivax* populations in the Peruvian North Coast (Tumbes and Piura) and Ecuadorian Amazon Basin (Pastaza) using bar plot for $K = 3$. Each sample is represented by a single vertical bar divided into K colors, where K is the number of populations assumed. Each population is represented by a color, and the length of the colored bar depicts the estimated proportion of membership of the sample in each population.

the development of a *P. vivax* population refuge, decreasing the probability of interactions with Amazonian *P. vivax* populations.¹⁸

The low genetic diversity of the *P. vivax* population we found in the PNC contrasts with the middle to high genetic diversity seen in previous studies. This information has important implications for future epidemiologic studies of malaria in this zone.^{9,16,34–36}

This study presented some limitations. First, different types of samples were collected at each study site. In the PNC, 90% of all samples were whole blood and 10% were from filter paper impregnated with whole blood. In both cases the yield of parasite DNA was optimal. However, the EAB samples isolated from serum resulted in lower yield and quality of parasite DNA, which led to shorter microsatellite peaks than in the PNC samples. This difference could impact the identification of polyclonal infections and underestimate the levels of polyclonal infections, MOI, and genetic diversity.

Second, sampling strategies and time collection were different between the PNC and EAB. Therefore, our results in terms of migration and comparison of genetic diversity might be biased because of the timing differences. In this regard, there is a need of more studies comparing the current diversity of *P. vivax* between these two regions after the decrease in cases in Ecuador.^{4,5} More studies are necessary to know the actual genetic diversity status and if this decreasing of *P. vivax* cases could affect the genetic diversity today.

Third, according to Imwong et al.,¹³ Russell et al.,⁸ Rezende et al.,²⁸ and Sutton³⁷ the repeat array length of microsatellites is directly associated with the number of alleles found in a *P. vivax* population. Therefore, genetic variability in the PNC and the EAB areas could be underestimated using these dinucleotide markers and additional markers with major repeat array length should be tested. In this study, only the marker 6.34 appears in the list of *P. vivax* genetic diversity markers recommended by Sutton. Interestingly, this marker reported

the highest level of polymorphisms in the PNC and EAB.³⁷ This evidence suggests that the actual genetic diversity could be higher and additional markers should be used for an accurate estimation and comparison of genetic data from other *P. vivax*–endemic regions. However, markers used in this study could give us a preliminary description of the genetic diversity status in these regions poorly studied.

In summary, the results obtained in this study strongly suggest a minimal population flow and different levels of genetic variability between these two areas divided by the Andes Mountains. The PNC showed an unusual *P. vivax* population isolated with very low genetic diversity compared with others *P. vivax*–endemic regions. In addition, these results could be used to increase our understanding of regional malaria transmission dynamics, new routes of transmission, imported cases, and *P. vivax* populations that could have unique clinical presentations, which are important for defining regional strategies for control and treatment of *P. vivax*.^{38–44}

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TABLE 3

Number of migrants per generation

Direction of migration	Θ values	M	N_m
Tumbes → Pastaza	1.18	1.167	1.3770
Piura → Pastaza	1.18	1.900	2.2420
Pastaza → Tumbes	0.34	7.500	2.5500
Piura → Tumbes	0.34	10.100	3.4340
Pastaza → Piura	0.22	2.433	0.5350
Tumbes → Piura	0.22	8.500	1.8700

Θ = population sizes; M = mutation-scaled migration rates; N_m = number of immigrants per generation.

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