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Population dynamics, genotypic variation and origin of *Plasmodium vivax* in Ecuador between 2016 and 2020

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Scientific knowledge belongs to humanity.

Alexandra Elbakyan

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ABBREVIATIONS LIST

Abbreviation	Meaning
CISeAL	Centro de Investigación para la Salud en América Latina
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
µl	Microliter
WHO	World Health Organization
PAHO	Pan American Health Organization
PAMAFRO	Malaria Control in Border Zones of the Andean Region
AMI	Amazon Malaria Initiative
bp	DNA “pair base”
AMA1	Apical Merozoite Antigen
AMOVA	Analysis of Molecular Variance
He	Heterozygosity
HEX	Hexachlorofluorescein
FAM	Carboxyfluorescein

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1. RESUMEN

Introducción: La epidemiología molecular permite identificar las áreas con casos de malaria en Ecuador para conocer la dinámica poblacional y la estructura poblacional de *P. vivax*. Además de caracterizar las diferentes variantes (haplotipos) que están circulando en el país, la diversidad genética, el origen y movilización por migraciones humanas de ciertos linajes de parásitos en comparación con países vecinos como Colombia y Perú. En este estudio, utilizando herramientas moleculares como los microsatélites, se buscó conocer el origen y distribución de los parásitos *P. vivax* en la Costa y Amazonía ecuatoriana con países vecinos fuera del programa de eliminación de la malaria.

Métodos: Se analizaron 69 muestras de *P. vivax* de la Costa y Amazonía ecuatoriana entre 2016 y 2020. Se realizó amplificación por PCR utilizando 9 microsatélites para identificar el tamaño alélico y establecer el número de haplotipos presentes en las muestras. Se analizó la multiclonalidad y el desequilibrio de ligamiento. También se realizó la estructura poblacional para determinar los linajes genéticos presentes dentro del país y en comparación con los países vecinos. Se realizó un AMOVA y un Pairwise-fst para observar la distancia genética entre poblaciones de parásitos.

Resultados: En Ecuador, hay mayor diversidad genética y multiclonalidad y menor desequilibrio de ligamiento en la Amazonía que en la Costa. Hubo evidencia de flujo genético y diferenciación genética moderada entre las localidades amazónicas, pero no hubo flujo genético entre la Costa y la Amazonía. Por otro lado, la Costa de Ecuador compartió linajes, y tuvo diferenciación genética baja/moderada, Chocó y Antioquia en Colombia. Pastaza y Amazonia Oriental compartieron linajes y tuvieron diferenciación genética baja/moderada con Lupuna y Cahuide en Perú.

Conclusión: Este estudio sugiere que la transmisión en la Amazonía de Ecuador ha aumentado en los últimos años y muestra la importancia de los Andes como barrera geográfica. La costa de Ecuador comparte linajes genéticos con parásitos de la costa colombiana que pueden provenir de la migración a lo largo de un corredor de la Costa del Pacífico. Del mismo modo, la Amazonía de Ecuador comparte linajes genéticos con comunidades de Perú, lo que sugiere la migración de parásitos a través de corredores fluviales.

Palabras claves: *Plasmodium vivax*, Ecuador, diversidad de haplotipos, microsatélites, multiclonalidad, flujo génico, epidemiología molecular.

2. ABSTRACT

Background: Molecular epidemiology helps to identify areas with malaria cases in Ecuador in order to understand the population dynamics and population structure of *P. vivax*. In addition to characterize the different variants (haplotypes) that are circulating in the country, genetic diversity, the origin and mobilization by human migrations of certain lineages of parasites compared to neighboring countries such as Colombia and Peru. In this study, using molecular tools such as microsatellites, the objective was to understand the origin and distribution of *P. vivax* parasites in the Ecuadorian Coast and Amazon with neighboring countries outside the malaria elimination program.

Methods: Sixty-nine samples of *P. vivax* from the Ecuadorian Coast and Amazon were analyzed between 2016 and 2020. PCR amplification was performed using 9 microsatellites to identify allele size and establish the number of haplotypes present in the samples. Multiclonality and linkage disequilibrium were analyzed. Also, population structure was performed to determine the genetic lineages present within the country, and in comparison with neighboring countries. An AMOVA and Pairwise-fst were performed to observe the genetic distance between parasite populations.

Results: In Ecuador, there is higher genetic diversity and multiclonality and less linkage disequilibrium in the Amazon than in the Coast. There was evidence of gene flow and moderate genetic differentiation between the Amazonian localities, but no gene flow between the Coast and Amazon. On the other hand, the coast of Ecuador shared lineages, and had low/moderate genetic differentiation, Choco and Antioquia in Colombia. Pastaza and East Amazon shared lineages and had low/moderate genetic differentiation with Lupuna and Cahuide in Peru.

Conclusions: This study suggest that transmission in the Amazon of Ecuador has increased in recent years and show the importance of the Andes as a geographical barrier. The coast of Ecuador shares genetic lineages with coastal Colombian parasites that may have reached Ecuador is through migration along a Pacific Coast corridor, Similarly, the Amazon of Ecuador shares genetic lineages with communities of Peru, suggesting parasite migration through fluvial corridors.

Keys words: *Plasmodium vivax,* Ecuador, haplotype, genetic diversity, microsatellites, multiclonality, gene flow, molecular epidemiology.

REVISTA

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TÍTULO

Population dynamics, genotypic variation and origin of Plasmodium vivax in Ecuador between 2016 and 2020

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Population dynamics, genotypic variation and origin of *Plasmodium vivax* in Ecuador between 2016 and 2020

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3. BACKGROUND

Worldwide, malaria has been a concern from ancient times to the present day. The incidence of malaria between 2010 and 2016 has decreased by 18% world while, with a decrease of 48% in Southeast Asia, 22% in the Americas, and 20% in Africa (37). However, in 2021 malaria cases rose to 247 million in 84 endemic countries due to the financial stagnation and medical carelessness generated by the COVID-19 pandemic compared to 245 million cases in 2020 (48). *Plasmodium* remains the most prevalent in Africa, Asian and the Americas (37).

Malaria is caused by an intracellular parasite of the genus *Plasmodium* (Family Plasmodiidae) transmitted by the bite of an infected female mosquito, genus *Anopheles* and *Nyssorhynchus* (Family Culicidae). Five species of *Plasmodium* are known to infect humans: *P. ovale*, *P. knowlesi*, *P. malariae, P. falciparum,* and *P. vivax.* Mainly, *P. vivax* and *P. falciparum* are the most common and cause the highest mortality in humans (48). There are other forms of malaria transmission, such as transfusion of infected blood (horizontal transmission) or passing from one generation to another (vertical transmission) (13). Symptoms in non-immune individuals begin 10 to 15 days after a mosquito bite. The main symptoms are fever, muscleache, headache, and chills (48). Chronic malaria generates renal, cognitive, and circulatory damage (3). Malaria primarily affects pregnant women, children, the elderly, and immunocompromised patients (48).

60 Factors that promote malaria to remain in the community and increase their range of
61 distribution are antimalarial resistance, socioeconomic lifestyle, and scarce medical
62 control in regions where malaria is endemic. In addition, asymptomatic cases should be
63 considered when silent reservoirs may acquire partial immunity and carry low parasite
64 loads (26; 48; 3). Moreover, people's mobility and migration can also influence
65 reintroduction, the expansion of the population to neighboring countries can affect the
66 frequency and dispersion of new parasite haplotypes (30).

67 *P. vivax* is the malaria parasite with the greatest geographic distribution. It produces a
68 significant impact on people's health and economy and has high proportion in South
69 and Central America, and Asia. Eighty percent of malaria cases in South America are
70 due to *P. vivax* and has the ability to maintain hypnozoite stages in the liver for a period
71 of months to years (47; 24; 44). *P. vivax* cases decreased by 8% with 20.5 million in
72 2000 to 2% with 4.9 million in 2021 (48). It is essential to carry out studies of *P. vivax*
73 due to its high prevalence and complicated biology (47).

74

75 In the Americas, malaria cases reduced by sixty percent between 2000 and 2021
76 Paraguay, Argentina, and El Salvador were certified malaria free and are removed from
77 the twenty-one countries which were within the disease elimination program. (31; 48).
78 Between 2000 and 2021 malaria deaths diminished by 64% and mortality rate by 73%
79 in America (48). On the other hand, Venezuela reported the highest number of cases in
80 2017 with 482 000 cases (48). Brazil and Colombia presented more than 79% of
81 malaria cases in this region and 71.5% of cases were due to *P. vivax* in 2021.

82 In Ecuador, malaria cases are concentrated in areas below 1,500 meters of altitude
83 such as the Amazon region and the north coast of the country; particularly, in areas
84 close to the border with Colombia and Peru, being imported cases the main difficulties
85 to achieve elimination (36).

86 Ecuador is considered one of twenty-five countries in the world to have the capacity to
87 eliminate malaria by 2025. There was a 99.5% reduction of cases between 2001 and
88 2014 (45; 37), and in 2012 558 cases were reported, out of which 478 cases
89 corresponded to *P. vivax* and 80 to *P. falciparum* (36; 39). This reduction was
90 influenced by the support of international collaborations such as the Malaria Control in
91 Border Zones of the Andean Region Program (PAMAFRO), and Amazon Malaria
92 Initiative (AMI) from USA (44). However, 618 cases were reported in 2015 and 2175
93 cases were reported in 2021 (WHO., 2022). The reasons for the increase were probably
94 the migratory activity between border towns, climatic factors such as the El Niño
95 phenomenon and, poor organization of the malaria program (39).

96

97 Molecular epidemiology focuses on genetic and environmental factors identified at the
98 molecular level for the prevention of diseases present in populations that helps obtain
99 genetic data to understand the risks involved in the distribution and frequency of malaria
100 cases (14). It focuses on population characterization and obtains information of
101 epidemiological value to carry out the program coordination for the elimination and
102 control of the disease (10). Also, genetic connectivity information can be obtained
103 between populations that circulate currently regarding ancestral populations and thus

104 observe whether residual ancestral parasite populations contribute to local transmission
105 (Veras-Arias et al., 2019).
106 To understand the population structure of *Plasmodium* it is necessary to observe the
107 diversity of haplotypes present, how the population alleles of unlinked genes combine
108 randomly generating populations of panmixia state (linkage disequilibrium) or unlinked
109 genes where there is no recombination indicating clonality in the population (linkage
110 disequilibrium) (22).
111

112 Targeting genes of interest can generate a complementary observation to understand
113 parasite microevolution and examine population adaptations with the use of markers
114 with a mutation rate that can identify recent events (10). Molecular tools have been
115 used more frequently in studies with *P. falciparum* than with *P. vivax*. Thus, more
116 detailed studies are required with *P. vivax* with molecular markers to understand its
117 population structure and lineage distribution (7).

118

119 Traditional methods for population genotyping such as the presence of single nucleotide
120 polymorphism (SNPs) in the Apical Merozoite Antigen (AMA1) and other invasion
121 ligands have been used for population characterization (5). On the other hand,
122 molecular tools such as microsatellites can identify events of divergence on a recent
123 time scale from an epidemiological point of view (10). Microsatellites are short DNA
124 tandem sequences (between 1-6 bp) in non-coding regions. Besides, the number of
125 tandem repeats determines the size of a fragment known as allele (40). Microsatellites

126 have high polymorphism and are under "neutral selection", making them useful for
127 parentage identification, genetic mapping, and population genetics (10).
128
129 SNPs advantages are better inbreeding information, hybrid detection, and parentage
130 analysis, but rates of evolution may not be informative to identify genetic structures (10;
131 14). On the other hand, microsatellites advantages are information of greater allelic
132 richness, low verification bias, neutral selection, and are useful for understanding
133 evolutionary events on a recent time scale in subpopulations within a species or
134 between related species (14; 15).

135 A multiclonal infection is the coexistence of two or more clones found in a single host, in
136 ither words, it is the average of different genotypes of parasites that can be found in an
137 infected patient (14; 28). The infection of several haplotypes is due to transmission by
138 the same vector's bite that harbors several haplotypes (10). Studies of multiplicity of
139 infection have been done mostly in *P. falciparum*, but there is limited information
140 regarding *P. vivax.* It is important to know the frequency of multiclonal infections in order
141 to relate it to the intensity of disease transmission *(*28).
142

143 *P. vivax* presented twice the diversity and variability of single nucleotide polymorphisms
144 (SNPs) than *P. falciparum* (27). In addition, the genetic diversity in *P. falciparum* is
145 related to the different regional levels of endemicity, but *P. vivax* presented high genetic
146 diversity in all endemicities (2). *P. vivax* has different patterns of population structure
147 and presented high genetic diversity of microsatellite and SNPs implying a greater
148 global functional variation in population (27; 41).

149 Infected people with *P. vivax* have a higher rate of multiclonal infections than those
150 infected with *P. falciparum*, which may be the result of the accumulation of inactive
151 hypnozoites present in the liver generating multiple relapses of different haplotypes
152 (28).

153 Worldwide, in Southeast Asia, *P. vivax* presented higher diversity and allelic richness
154 than the Middle East, South America, and Africa. South and Central America showed a
155 more-structured population than Asia, the Middle East, and East Africa where there
156 seems to be a diversified structure (34). Also, African parasites are more related with
157 Middle East and Asian parasites than with South American parasites (34).

158 The population structure is limited on a regional or local scale due to genetic
159 recombination of *P. vivax* which population experiences a reduction of the effective
160 population size known as "bottleneck effect" after intervention program; in other words,
161 the number of alleles and expected heterozygosity (He) is reduced (Delgado et al.,
162 2016). Although there is a decrease in the number of cases in the Americas, there is no
163 reduction in diversity at the local level. (Delgado et al., 2016).

164

165 The presence of three genetic clusters of *P. vivax* in four countries was observed. Tight
166 clustering was seen between Central and South American isolates (6; 4). In particular,
167 Peru and Colombia clusters were less differentiated among them (6).

168 The *P. vivax* structure from four sites of the south coast of Colombia (Tierralta, Quibdo,
169 Tumaco and Buenaventura) using eight microsatellites found four genetic clusters and
170 high genetic diversity. The study suggested the existence of a malaria corridor that

171 facilitates parasite populations movement across endemic areas with high diversity (29).
172 This malaria corridor could affect other countries such as Panama which reported some
173 *P. vivax* isolates imported from Colombia through the border of the Darien jungle (4).

174 In Peru, fourteen microsatellites revealed the presence of multiclonal and monoclonal
175 infections with high genetic diversity and grouped in three clusters in 25 rural villages
176 near the Iquitos-Nauta Road (Delgado et al., 2016). The presence of clonal infections
177 was predominant in isolated areas that may favor high levels of differentiation among
178 populations than in urban areas with active gene flow (Delgado et al., 2016).

179

180 In Ecuador, Velez and collaborators (2023) used 91 isolates to understand the genetic
181 differentiation and structure population of *P. vivax* from Central Coast, West and East
182 Amazon between 2012 and 2015 using nine microsatellites (43). Several *P. vivax*
183 genetic lineages were shared between Ecuadorian regions (43). A conserved haplotype
184 was observed in the Coast that persisted over time and differed from the rest of the
185 populations (43). The study reported West Amazon as the locality with higher diversity.
186 The greatest gene flow in Ecuador (2012-2015) was found to be between East Amazon
187 and the West Amazon, as well as West Amazon and the Central Coast (43).

188 On the other hand, Ecuadorian and Peruvian parasites presented low genetic
189 differentiation between populations due to migration events (43). In addition, evidence
190 of a shared haplotype was identified between Tumbes in Peru and Pastaza in Ecuador
191 suggesting importation. Consequently, malaria control strategies should be focused on
192 sites along the border with Peru (43; 44).

193

194 There is a lack of information of the current *P. vivax* populations circulating in Ecuador.
195 For this reason, this work supplies information on molecular epidemiology that provides
196 knowledge of the genetic diversity of the parasite present in Ecuador between 2016 and
197 2020 in comparison with Ecuador (2012-2015), Colombia (2012-2013) and Peru (2012-
198 2015). Genetic characterization within a specific area helps determine information on
199 genetic connectivity between circulating ancestral lineages to identify what influences
200 local transmission and helps determine whether parasite lineages migrate from
201 neighboring locations (45).

202

203 **4. MATERIALS AND METHODS**

204 **Ethics statement**

205 This study was approved by the Human Research Ethics Committee of the Pontificia
206 Universidad Catolica del Ecuador (CEISH PUCE) through official letter ceish-517-2018
207 and authorized by the Ministry of Health of Ecuador through official letter msp-dis-2019-
208 004-o.

209 **Study sites and sample collection**

210 This study used 69 samples from Ecuador between 2016 and 2020 (Fig.1) that were
211 collected by the staff of the Ministry of Health of Ecuador (MoH) and National Institute of
212 Public Health Research (INSPI) from people who presented symptomatology and were
213 diagnosed as *Plasmodium vivax* by rapid diagnosis test or microscopy. The samples

214 came from Esmeraldas county, San Lorenzo county which were established as
215 "Esmeraldas" (N=8) and one sample from Guayas were established as " Central Coast"
216 (N=1). From the Amazon region, samples were obtained from Orellana (Aguarico
217 county), established as "East Amazon" (N=11), Pastaza presented (N= 48) samples,
218 and one from Morona Santiago (N=1) (Fig.1)

219

220 In addition, this study used 87 samples from different localities of Ecuador (2012-2015)
221 already analyzed from the study carried out by Velez and collaborators (2023) (43) in
222 order to compare Ecuador with our recent samples (2016-2020).

223 The study used the data of 168 Colombia (2012-2013) samples analyzed from the
224 Departments of Antioquia, Choco, and Cordoba from the study by Zuluaga-Idarraga
225 collaborators (2016) (50), and 927 samples from Peru (2012-2015) analyzed from the
226 localities of Santa Emilia, Lupuna and Cahuide from the study by Manrique and
227 collaborators (2019) (26).

228

229 **DNA extraction**

230 DNA was isolated from filter paper (Watmann 3MM) using the protocol provided by the
231 QIAamp genomic kit (QIAGEN Sample & Assay Technologies. Germantown, MD, USA).

232 **Microsatellites amplification and fragment size analysis**

233 The study used four microsatellites described by Imwong and collaborators (2006):
234 7.67, 8.332, 12.335, 2.21 (20). Two microsatellites were used from the study by Imwong

235 and collaborators (2007): 3.502, and 11.162 (21). These six microsatellites were
236 amplified by semi-nested PCR (Table 1). In addition, three microsatellites described by
237 Karunaweera and collaborators (2007) were used: MS2, MS6, and MS20 (23) which
238 were amplified by simple PCR (Table 1). Forward microsatellite primers had
239 fluorophores hexachlorofluorescein (HEX) or carboxyfluorescein (FAM) (50).

240 PCR products were visualized on 3% agarose gels in 1X TBE buffer, and the fragment's
241 size was compared to a 100 bp molecular ladder (Promega). Finally, the UVP
242 transilluminator was used to visualize the DNA fragments. Fragment size analysis was
243 done by Macrogen in Korea, determined by capillary electrophoresis on a 3730XL
244 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

245 The allele size in base pairs of the amplified samples was determined by Peak
246 ScannerTM v.1.0 software (17). The standard size of GS400HD and the Sizing Default-
247 PP analysis were assigned for the analysis method. The presence of more than one
248 allele at each locus was observed, so the secondary alleles were chosen to be a third of
249 height as compared to the dominant allele. In consequence, a list was created to
250 distinguish the presence of different clones in a single sample and determine
251 multiclonality of infection (Gray et al.,2013). Multiclonality was calculated by the division
252 of the total number of clones and total number of samples (Gray et al.,2013).

253

254 Statistical analysis

255 The study used Genealex 6.5.0.1 software (32) to analyze expected heterozygosity
256 (He). Values close to one correspond to high levels of diversity (11). Wright F-statistic

257 (49) was used to analyze population differentiation (Pairwise-fst) and gene flow (Nm);
258 allele frequency (Nm) was used from 10.000 permutations with confidence intervals of
259 10.000 iterations (11).

260 Analysis of Molecular Variance (AMOVA) was done in order to determine the variation
261 of individuals within and among populations. The populations were defined as the
262 localities in each period and the individuals were defined as the samples of each
263 locality.

264 **FST** indicated the probability of two identical alleles combining for the next generation,
265 **FSI** indicated the inbreeding coefficient, and **FIT** indicated the total inbreeding (35; 11).

266

267 The study determined if there was non-random association between loci in the
268 population with the online program LIAN linkage analysis version 3.7.5. (19). The
269 expected frequency distribution used Monte Carlo simulations, thus obtaining the
270 association index (I_A^S) which compares the variance (V_D) of alleles obtained between all
271 pair of haplotypes present in the population (D) with the variance expected (V_E) (18).

272

273 **Population Structure**

274 Haplotype networks from Ecuador's localities (2012-2015) and (2016-2020) were done
275 using NETWORK 4.6.1.3 software was used as described in Fluxus Technology Ltd.,
276 Suffolk, UK (1). The networks were used to understand how lineages are related to
277 localities (1).

278 STRUCTURE 2.3.4 software (33) was used to assume a K populations and
279 characterized the samples by a set of allelic frequency for each locus (16). The software
280 was run in fifteen iterations (K =1 to 15), with a burn-in period of 10.000 steps and
281 100.000 iterations. Subsequently, Structure Harvester online (12) was used to interpret
282 the results of K value through a standardization (K vs. Mean LnP (K)), the K value was
283 chosen where the curve was standardized in order to determine the number of
284 populations and consequently the genetic lineages were found in each locality (11).
285 According to which lineage each sample belonged to, a pie chart was made to
286 represent the frequency of each lineage in each location and to locate it on the map of
287 Ecuador corresponding to each period analyzed.

288

289 **5. RESULTS**

290 **Genetic characterization of Ecuadorian *Plasmodium vivax* between 2016 and 2020**

291

292 In order to study genetic composition of *P. vivax* in Ecuador, 69 samples were
293 genotyped by amplifying nine neutral microsatellite markers which are in eight different
294 chromosomes in samples from Ecuador between 2016 and 2020. Fifty-two of sixty-nine
295 samples were available for the use in statistical analysis. Samples missing data for
296 more than four out of nine alleles were discarded. Marker MS20 was the most diverse
297 with 13 alleles, followed by MS2 with 9 alleles, 12.335 presented 8 alleles, 2.21
298 presented 8 alleles, 3.502 had 8 alleles, 11.162 presented 7 alleles, 7.67 presented 7
299 alleles, and MS6 presented 4 alleles.

300 In the samples collected in Ecuador between 2016 and 2020, Pastaza presented higher
301 diversity (He= 0.615) than Esmeraldas (He=0.553) and East Amazon (He= 0.548)
302 (Table 2).

303 Also, East Amazon presented the highest percentage of multiclonality infection with
304 90.91% probability to find more than one locus with more than two alleles, followed by
305 Esmeraldas with 75% and Pastaza with 42.86% (Table 3).

306 Linkage Disequilibrium (I_A^S) in samples collected between 2016 and 2020 in East
307 Amazon (I_A^S= 0.0125) and Pastaza (I_A^S= 0.0974) was low in comparison to Esmeraldas
308 (I_A^S=0.5421) (Table 2).

309

310 The comparison between genetic diversity in different localities from Ecuador (2016-
311 2020) was made using Pairwise-fst. Esmeraldas and East Amazon showed high genetic
312 differentiation (fst= 0.26). Similarly, Esmeraldas and Pastaza had also high genetic
313 differentiation (fst= 0.225). On the other hand, Pastaza and East Amazon presented
314 moderate genetic differentiation (fst= 0.163) (Table 4).

315

316 We observed the relation between haplotypes established in the different localities in a
317 haplotype network. Fifty-two samples presented forty-two different haplotypes in
318 localities from Ecuador between 2016 and 2020: Pastaza had 24 haplotypes, East
319 Amazon had 9 haplotypes, Esmeraldas with 5 haplotypes, Morona Santiago had 1
320 haplotype and Central Coast had 1 haplotype, and while Pastaza and East Amazon
321 shared 2 haplotypes. Pastaza haplotypes were closely related to haplotypes in

322 Esmeraldas, Morona Santiago, and Central Coast. Haplotypes from East Amazon were
323 more related to haplotypes from Pastaza than to other regions (Fig. 2B)

324 The population structure of 52 samples from Ecuador between 2016 and 2020 predicted
325 six clusters (K=6): All samples from Esmeraldas, Guayas and Morona Santiago
326 belonged to the red cluster. East Amazon samples were divided into three differentiated
327 populations: 73% belonged to the red cluster, 18% light blue cluster, and 9% belonged
328 to the green cluster. Also, Pastaza samples were divided into three differentiated
329 populations: 68 % belonged to the green cluster, 22 % belonged to the red cluster, and
330 10% belonged to the purple cluster (Fig. 3B).

331 The presence of gene flow (Nm) in Ecuador (2016-2020) was observed between
332 Pastaza and East Amazon (Nm=1.284). On the other hand, Esmeraldas and Pastaza
333 (Nm= 0.864), as well as Esmeraldas and East Amazon (Nm= 0.710) did not present
334 gene flow (Table 6).

335

336 **Changes in population structure in Ecuador between 2012 and 2020**

337 Samples from East Amazon collected between 2012 and 2015 and analyzed by Velez
338 and collaborators (2023) (43) presented a similar diversity (He= 0.590) than samples
339 from East Amazon between 2016 and 2020 (He=0.548) (Table 2).

340 A neighbor joining network was done with samples between 2012 and 2020. Fifty-five
341 haplotypes were present in Ecuador between 2012 and 2015 in comparison with forty-
342 two haplotypes in localities between 2016 and 2020 (Fig. 2). East Amazon (2016-2020)

343 presented eleven haplotypes, seventeen haplotypes less than East Amazon (2012-
344 2015) (Fig. 2).

345 Fixation indexes by Analysis of Molecular Variance (AMOVA) were significant
346 (<0.0001). The study showed seventy-five percent of variation was between individuals
347 in each locality from Ecuador (2012-2020). On the other hand, twenty-five percent of
348 variation was found between localities in Ecuador (2012-2020). The FST value indicated
349 a high genetic differentiation (Table 5).

350 When comparing the changes of the population structure in East Amazon from 2012-
351 2015 to 2016-2020, we saw a decrease in the number of genetic lineages. Three
352 clusters were observed in samples from 2016 to 2020 in comparison five clusters
353 observed in samples collected between 2012 and 2015. The samples collected in East
354 Amazon from 2012 to 2015 were divided into yellow cluster (53%), red cluster (17%),
355 light blue (16%), purple cluster (12%) and green cluster (2%) (Fig. 3A). On the other
356 hand, the samples collected in East Amazon from 2016 to 2020 were divided into the
357 red cluster (73%), light blue (18%), and green cluster (9%) (Fig. 3B).

358 Also, the three clusters shared between 2012 and 2020 from East Amazon increased
359 their proportion. The red cluster increased from 16.33% to 72.73%, green cluster
360 increased from 2.04% to 9.09%, and light blue cluster increased from 16.33% to
361 18.18% (Fig. 4).

362

363 **Comparison of *P. vivax* populations from Ecuador and Colombia**

The comparison of genetic differentiation between samples from Ecuador (2012-2020) and Colombia (2012-2013) (50) was made using Pairwise-fst.

Ecuador (2012-2015) and Colombia (2012-2013) presented low genetic differentiation between West Amazon and Antioquia (fst= 0.106). On the other hand, moderate genetic differentiation was found between West Amazon and Choco (fst= 0.150), and between East Amazon and Antioquia (fst= 0.214). Also, high genetic differentiation was found between Central Coast and Choco (fst= 0.302), Central Coast and Antioquia (fst= 0.271), and between East Amazon and Choco (fst= 0.250).

Recent samples from Ecuador (2016-2020) and Colombia (2012-2013) presented low genetic differentiation between Esmeraldas and Antioquia (fst= 0.111), and between Pastaza and Antioquia (fst= 0.143). On the other hand, moderate genetic differentiation was found between East Amazon and Antioquia (fst= 0.166), Esmeraldas and Choco (fst= 0.187), Pastaza and Choco (fst= 0.152), and East Amazon and Choco (fst= 0.226) (Table 9).

Fixation indexes of Analysis of molecular variance (AMOVA) were significant (< 0.01). The study presented seventy-seven percent of variation between individuals in each locality from Ecuador (2012-2020) and Colombia (2012-2013). On the other hand, twenty-three percent of variation was found between localities in Ecuador (2012-2020) and Colombia (2012-2013). FST value indicated moderate genetic differentiation (FST=0.229) (Table 10).

385 The population structure in Ecuador (2012-2020) and Colombia (2012-2013) with 273
386 samples predicted five clusters (K=5). In Colombia shared three clusters with Ecuador
387 (Fig. 5: Fig. 6).

388 In Ecuador, the green cluster was present in 96.42% of Central Coast samples, 83.3%
389 of West Amazon samples, 12.5% of Esmeraldas samples, and 8.3% of East Amazon
390 samples. In Colombia, the green cluster was present in 4.8% of Antioquia samples (Fig.
391 5).

392 In Ecuador, the red cluster was present in 100% of Morona Santiago sample, 87.5% of
393 Esmeraldas samples, 86.2% of Pastaza samples, 16.6% of East Amazon samples, and
394 3.5% of Central Coast samples. In Colombia, the red cluster was present in 100% of
395 Cordoba sample, 45.4% of Choco samples, and 25.2% of Antioquia samples (Fig. 5).

396 Finally, in Ecuador the blue cluster was present in 13.7% of Pastaza samples, 8.3%of
397 West Amazon samples, and 1.6% of East Amazon samples. In Colombia, the blue
398 cluster was present in 54.5% of Choco samples and 41.4% of Antioquia samples (Fig.
399 5).

400 The presence of gene flow (Nm) between Colombia (2012-2013) and Ecuador (2012-
401 2015) was observed between: West Amazon and Antioquia (Nm=2.118), and West
402 Amazon and Choco (Nm=1.421). On the other hand, the presence of gene flow (Nm)
403 between Colombia (2012-2013) and Ecuador (2016-2020) was observed between:
404 Antioquia and Esmeraldas (Nm=2.011), Antioquia and Pastaza (Nm=1.495), Antioquia
405 and East Amazon (Nm=1.260), Choco and Esmeraldas (Nm=1.086), and Choco and
406 Pastaza (Nm=1.390) (Table 13).

407

408 **Comparison of *P. vivax* populations from Ecuador and Peru**

409 The comparison of genetic differentiation between samples from Ecuador (2012-2020)
410 and Peru (2012-2015) (26) was made using Pairwise-fst.

411 Ecuador (2012-2015) and Peru (2012-2015) presented moderate genetic differentiation
412 between East Amazon and Cahuide (fst= 0.203), and East Amazon and Santa Emilia
413 (fst= 0.180). Also, high genetic differentiation was found between Central Coast and
414 Cahuide (fst= 0.391), Central Coast and Lupuna (fst= 0.349), Central Coast and Santa
415 Emilia (fst= 0.369), and between East Amazon and Lupuna (fst=0.327) (Table 11).

416 Recent samples from Ecuador (2016-2020) and Peru (2012-2015) presented moderate
417 genetic differentiation between East Amazon and Cahuide (fst= 0.224). Also, high
418 genetic differentiation was found between Esmeraldas and Cahuide (fst= 0.357),
419 Esmeraldas and Lupuna (fst=0.384), Esmeraldas and Santa Emilia (fst= 0.348), and
420 Pastaza and Cahuide (fst= 0.271), Pastaza and Lupuna (fst= 0.259), Pastaza and
421 Santa Emilia (fst= 0.264), East Amazon and Lupuna (fst= 0.374), and between East
422 Amazon and Santa Emilia (fst= 0.329) (Table 11).

423 Fixation indexes of Analysis of molecular variance (AMOVA) were significant (< 0.01).
424 The study showed seventy-three percent of variation between individuals in each
425 locality from Ecuador (2012-2020) and Peru (2012-2015). On the other hand, twenty-
426 seven percent of variation between localities in Ecuador (2012-2020) and Peru (2012-
427 2015). The FST value indicated a high genetic differentiation (FST=0.269) (Table 12).

428

429 The population structure in Ecuador (2012-2020) and Peru (2012-2015) using 927
430 samples predicted seven genetic clusters (K=7). Peru shared six clusters with Ecuador
431 (Fig. 7: Fig. 8).

432 In Ecuador, the orange cluster was present in 82.1% of Central Coast samples, 75% of
433 Esmeraldas samples, 66.6% of West Amazon samples, 33.3% of Pastaza samples, and
434 13.3% of East Amazon samples. In Peru, the orange cluster was present in 14.09% of
435 Lupuna samples, 5.8% of Cahuide samples, and 9.02% of Santa Emilia samples (Fig.
436 7).

437 The green cluster in Ecuador was present in 50% of East Amazon samples, 16.6% of
438 West Amazon samples, 14.2% of Central Coast samples, and 11.1% of Pastaza
439 samples. In Peru, the green cluster was present in 34.2% of Cahuide samples, 3.2% of
440 Lupuna samples, and 3% of Santa Emilia samples (Fig. 7).

441 The blue cluster in Ecuador was present in 26.6% of East Amazon samples, and 12.5%
442 of Esmeraldas samples. In Peru, the blue cluster was present in 22.8% of Cahuide
443 samples, 5.6% of Lupuna samples, and 0.75% of Santa Emilia samples (Fig. 7).

444 In Ecuador, the red cluster was present in 12.5% of Esmeraldas samples, and 3.3% of
445 East Amazon samples. In Peru, the red cluster was present in and 67.6% of Santa
446 Emilia samples, 9.7% of Lupuna samples, and 3.1% of Cahuide samples (Fig. 7).

447 The blue light cluster in Ecuador was present in the Morona Santiago sample, 55.5% of
448 Pastaza samples, 8.3% of West Amazon samples, 6.6% of East Amazon samples, and
449 3.5% of Central Coast samples. In Peru, the blue light cluster was present in 3.1% of
450 Cahuide samples, and 1.6% of Lupuna samples (Fig. 7).

451 Finally, the yellow cluster in Ecuador was present in 8.3% of West Amazon samples.
452 Peru the yellow cluster was present in 55.2% of Lupuna samples, 6.7% of Santa Emilia
453 samples, and 0.6% of Cahuide samples (Fig. 7).

454

455 The presence of gene flow (Nm) between Ecuador (2012-2015) and Peru (2012-2015)
456 was observed between East Amazon and Santa Emilia (Nm=1.142). On the other hand,
457 no gene flow was identified between Ecuador (2016-2020) and Peru (2012-2015)
458 (Nm<1) (Table 14).

459

460 **6. DISCUSSION**

461 In Ecuador, malaria cases are reported on the Coast and Amazonia (45). This study
462 aimed to understand the population dynamics and population structure of *P. vivax* and
463 how the data obtained can help with the elimination of malaria in the Americas.

464 In this study, nine microsatellites were used for population characterization of *P. vivax* in
465 Ecuador: 7.67, 8.332, 12.335, 2.21, 3.502, 11.162, MS2, MS6, and MS20 (20; 21; 23)
466 (Table 1). Some of these microsatellites were employed in previous studies in
467 neighboring countries such as Colombia with 7 microsatellites (12,335, 2,21, 3,502, 11.
468 162, MS2, MS6, and MS20) (50) and Peru with 4 microsatellites (3,502, 11,162, MS6
469 and MS20) (26).

470 When analyzing Ecuador samples (2016-2020), we found a median heterozygosity of
471 He= 0.553. Pastaza was the locality with higher diversity followed by Esmeraldas and

472 East Amazon (Table 2). A previous study reported that East Amazon between 2012 and
473 2015 had medium diversity (He=0.559) (43) that is closer to our East Amazon value
474 (He=0.548) (Table 2). On the other hand, in Iquitos-Mazan Peruvian Amazon there was
475 He=0.66 (42) that was close to the Pastaza heterocigozity value (He= 0.615) (Table 2).
476 Therefore, there higher heterozygosity was found in the Amazon region than in the
477 Ecuadorian coast even though the values were close to each other. We assume that in
478 the Amazon there is a higher recombination between haplotypes that contributes to the
479 increase of *P. vivax* genetic diversity in these endemic localities (46). In addition, the
480 Amazon samples in this study were collected from localities close to the Peruvian
481 border, which can imply an increase in diversity (Figure 1).

482 A previous study from Colombia presented very high genetic diversity with a mean of
483 He= 0.978 from four localities: Buenaventura, Tierralta, Quibdo, and Tumaco (29). In
484 our study, we presented high genetic diversity with an average of He=0.616 that is lower
485 than the values reported by Pacheco and collaborators (2019) (29) (Table 7). Also,
486 Zuluaga-Idarraga and collaborators (2016) showed high diversity (He= 0.721) in Turbo-
487 Colombia (50). On the other hand, Peru presented high diversity of heterozygosity from
488 twenty-five peruvian rural villages from the Amazon along the Iquitos-Nauta, four
489 villages southwest Iquitos Road (11), around Tambo San Juan de Munich was close to
490 our diversity value with an average of He=0.562 (Table 8).

491 Clonal cases may support high genetic differentiation between parasites from isolated
492 areas, while areas where human migration is usual can generate cases of multiclonality
493 in which more than two haplotypes are found in a host (11). The greatest percentage of

494 multiclonal infections was observed in the Ecuadorian Amazon. However, in our study
495 we reported that East Amazon (2016-2020) was the locality with more multiclonal
496 infections (90.91%), followed by Esmeraldas (75%) and Pastaza (42.86%) (Table 3).
497 Velez and collaborators (2023) reported a 41% of multiclonality in the samples in
498 Ecuador (2012-2015) and Central Coast was the locality with more multiclonal infections
499 (44.4%), followed by West Amazon (30%) and East Amazon (14.29%) (43). Thus, an
500 evident increase was observed in the East Amazon, which implies that there is greater
501 transmission of *P. vivax* through the Amazonian side.

502 In Ecuador, linkage disequilibrium (I_A^S) was medium (I_A^S=0.23), which implies that there
503 is recombination (Table 2). Especially, Pastaza reported one of the lowest values of
504 linkage disequilibrium implying high genetic diversity. Our Pastaza LD (I_A^S=0.0974) is
505 close to Iquitos LD value (I_A^S=0.08) and East Amazon (I_A^S=0.0125) value close to
506 Peruvian villages southwest Iquitos city, such as Cahuide, with low LD value (I_A^S= 0.01)
507 (11) (Table 2). On the other hand, Esmeraldas showed the highest value (I_A^S=0.542)
508 indicating linkage disequilibrium that alleles are inherited from one generation to another
509 and less recombination occurs in this area, this is close to Tierralta, Colombia value
510 (I_A^S=0.36) reported by Pacheco and collaborators (2019) (29) (Table 2). Therefore, there
511 is higher recombination in the samples from the Ecuadorian Amazon than on the Coast
512 due to the higher transmission of variants in these locations with higher cases of
513 multiclonality.

514 In our study no gene flow was found between the Amazon and the Coast region and
515 there was high genetic differentiation between the two regions. This could be explained

516 because the Andes mountain range represents a geographic barrier that generates
517 isolation. On the other hand, there was gene flow and moderate genetic differentiation
518 between the two localities of the Ecuadorian Amazon due to the geographical proximity
519 between localities and the absence of important geographical barriers (Table 4; Table
520 6). On the other hand, Velez and collaborators (2023) found gene flow with high genetic
521 differentiation between the Amazon and the Central Coast of Amazon, with little
522 interbreeding (43). Therefore, there is the possibility of migration between the two
523 regions despite the geographic barrier. A previous study by Chaparro and collaborator
524 (2017) presented malaria cases in Risaralda Department which in the Andean region of
525 Colombia (9). These cases are imported from the Coast Department of Choco (9).

526

527 There was change in population structure in the East Amazon of Ecuador between 2012
528 and 2020. The persistence of three out of five lineages was observed (Fig. 3). One
529 lineage prevailed in 72% of recent samples in comparison to 16.33% in 2012-2015 (Fig
530 4). There was a total of fifteen haplotypes in the East Amazon of Ecuador between 2012
531 and 2015 (43). In our study, East Amazon (2016-2020) presented only eleven
532 haplotypes with closer relation to Pastaza parasites (Fig.2). Most individuals are related
533 to each other, regardless of locality, thus indicating that there is a mixing between *P.*
534 *vivax* populations without geographic boundaries. On the other hand, Peru showed
535 eighty-seven haplotypes from Loreto (11) indicating a possible introduction from
536 Peruvian localities to Ecuador due to their higher variation of haplotypes and higher
537 number of cases.

538

539 Analysis of Molecular Variance (AMOVA) in Ecuador (2012-2020) and neighbor
540 countries, Colombia (2012-2013) and Peru (2012-2015), indicated that the highest
541 source of variation was among individuals of each locality than among localities (Table
542 5; Table 10; Table 12). The variation within localities could be explained because of the
543 coexistence of different haplotypes due to the gene flow or genetic drift. Also, it could be
544 due to a possible mutability of microsatellites or an overestimate of the different
545 haplotypes number (11).

546 Colombia (2012-2013) and Ecuador (2012-2020), had low/moderate genetic
547 differentiation between Esmeraldas (2016-2020) and the two Colombian Departments,
548 Antioquia and Choco with presence of gene flow (Table 9; Table 13). In our study, we
549 observed two lineages that are shared between the two countries indicating that there
550 are possible migration routes along the Pacific Coast (Fig. 6B).

551 A Pacific Coast corridor facilitates the transmission of malaria infections taking into
552 account that 80% of malaria cases from Colombia occur in this region (8). In a previous
553 study, it was shown that San Lorenzo had higher *P. falciparum* diversity because it
554 borders Colombia where activity is common and are related with activities such as
555 mining and palm oil agriculture (45). Border populations, such as Mataje and Ricaurte,
556 located in San Lorenzo county are sources of human migrations where Colombians and
557 Ecuadorians cross the border several times (37).

558

559 On the other hand, we observed gene flow and low/moderate genetic differentiation
560 between Pastaza (2016-2020) and Antioquia (2012-2013), East Amazon (2016-2020)
561 and Antioquia (2012-2013), Pastaza (2016-2020) and Choco (2012-2013) (Table 9;
562 Table 13). However, there is not direct mobilization between the coast of Colombia and
563 the Amazon of Ecuador and there is considerable geographical distance between the
564 two localities, in addition to geographical barriers such as the Andes mountain range. A
565 possible explanation for the low differentiation between localities is that infected people
566 can hold hypnozoites of *P. vivax* (up to eight months) which could give enough time for
567 the long distance mobilization of people (47). People infected could have crossed the
568 Andes through routes of lowland to highland transition area such as routes that connect
569 the province of Esmeraldas with Santo Domingo and Pichincha to the provinces of the
570 Ecuadorian Amazon such as Napo and Sucumbios. Another possible reason for the low
571 differentiation is that Amazon parasites from Ecuador could share a common ancestor
572 with Colombia and by recombination events some alleles could have been maintained
573 over time. The fact that there is low genetic differentiation between Ecuador and
574 Colombia indicates that they shared alleles between the two localities that persisted
575 through time despite genetic recombination during in the sexual cycle inside the
576 mosquitoes (38). One limitation that our study has is that we used seven microsatellites
577 to compare between the parasites of Colombia and Ecuador and that data for some
578 samples was incomplete for some markers which could result in low resolution in our
579 analyses because the Structure software grouped the samples without data into clusters
580 with genetic similarity for the available alleles.

581

582 Peru (2012-2015) and Ecuador (2012-2020) presented moderate genetic differentiation
583 between East Amazon (2012-2015) and Cahuide, and between East Amazon (2012-
584 2015) and Santa Emilia with the presence of gene flow (Table 11; Table 14). On the
585 other hand, moderate genetic differentiation was found between East Amazon (2016-
586 2020) and Cahuide, and between Pastaza (2016-2020) and Lupuna, indicating that
587 there are possible migration routes through the Amazon basin, even though gene flow
588 values are low and close to one for recent samples (Table 14) (26).

589 Velez and collaborators (2023) observed the genes mobilization from Peru to the East
590 Amazon, which revealed that Ecuador parasites shared a common ancestor with Peru
591 (43). Also, there is a connection between parasites from Ecuador and Peru, they are
592 related by their population structure shared by three out of seven lineages (Fig. 7).

593 The transmission of malaria between Ecuador and Peru is through fluvial routes
594 (Gunderson et al., 2020). Rivers form a network which connects region of Loreto to the
595 Amazon basin of Ecuador exhibits a gradient of transmission with intensity (Chuquiyauri
596 et al., 2012), this transmission of malaria is an influence from Peru to communities
597 connected with Ecuador by this network. People use the river connections to travel to
598 short and long distances to communities. In addition, the Amazon of Ecuador and Peru
599 also share commercial ties across the border, including fishing, oil, and mining
600 extraction activities (26). The localities of Cahuide and Lupuna are connected to the
601 Amazon River through the following secondary rivers such as Natay River and Itaya
602 River (26). The Amazon River also connects with the Ecuadorian localities by rivers

603 such as Napo, Pastaza and Curaray rivers, implying a connection between these
604 parasite populations in the two countries (25).

605

606 One limitation that our study had is that we used four microsatellites to compare
607 between the parasites of Peru and Ecuador. The data for some samples of Peru and
608 Ecuador was incomplete for some markers which could give very low resolution in our
609 analysis. Structure software established these samples with no data as part of a lineage
610 which shared the same allele size of some markers between samples from the
611 Ecuadorian Coast and Amazon region.

612

613 ## 7. CONCLUSION

614 In summary, recent samples of *P. vivax* in Ecuador show higher diversity in the
615 Amazon basin (Pastaza and East Amazon) than the coast. In this work, no gene flow
616 was detected between the Amazon and coast locations because of the Andes
617 geographical barrier. Nevertheless, there is a genetic connection and sharing of genetic
618 clusters between parasites of the two Amazonian locations. Compared to previous
619 years, a differentiation of the population structure was observed in East Amazon with
620 persistence of only three lineages between 2012 and 2020. In addition, we found a
621 higher number of multiclonal infections in recent samples, thus suggesting a greater of
622 transmission in this locality than in previous years.

623 The origin of the parasites in the Ecuadorian coast is possibly due to the migration from
624 neighboring countries, such as Colombia through the Pacific Coast corridor and Peru
625 through the Amazon rivers corridors.

626 The *P. vivax* diversity present in Ecuador could be product of migration and
627 recombination between haplotypes circulating through the localities. Molecular
628 epidemiology and population characterization help determine the origins of the parasite
629 with the objective of preventing the circulation of new haplotypes and reach the local
630 elimination of malaria.

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9. FIGURES

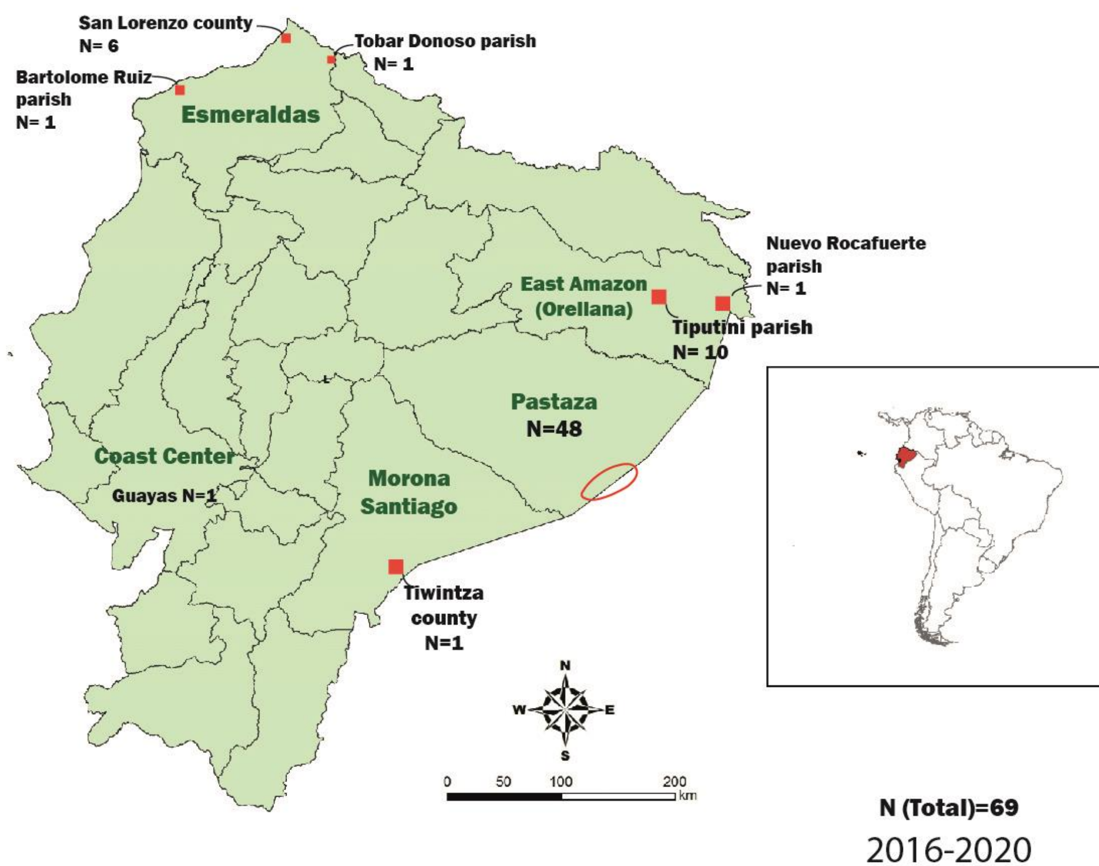

Figure 1. Number of samples (N) collected in Ecuador between 2016 and 2020. In the Coastal Region, 9 confirmed samples of *P.vivax* were collected. In the Amazon Region, 60 samples of P. vivax were collected.

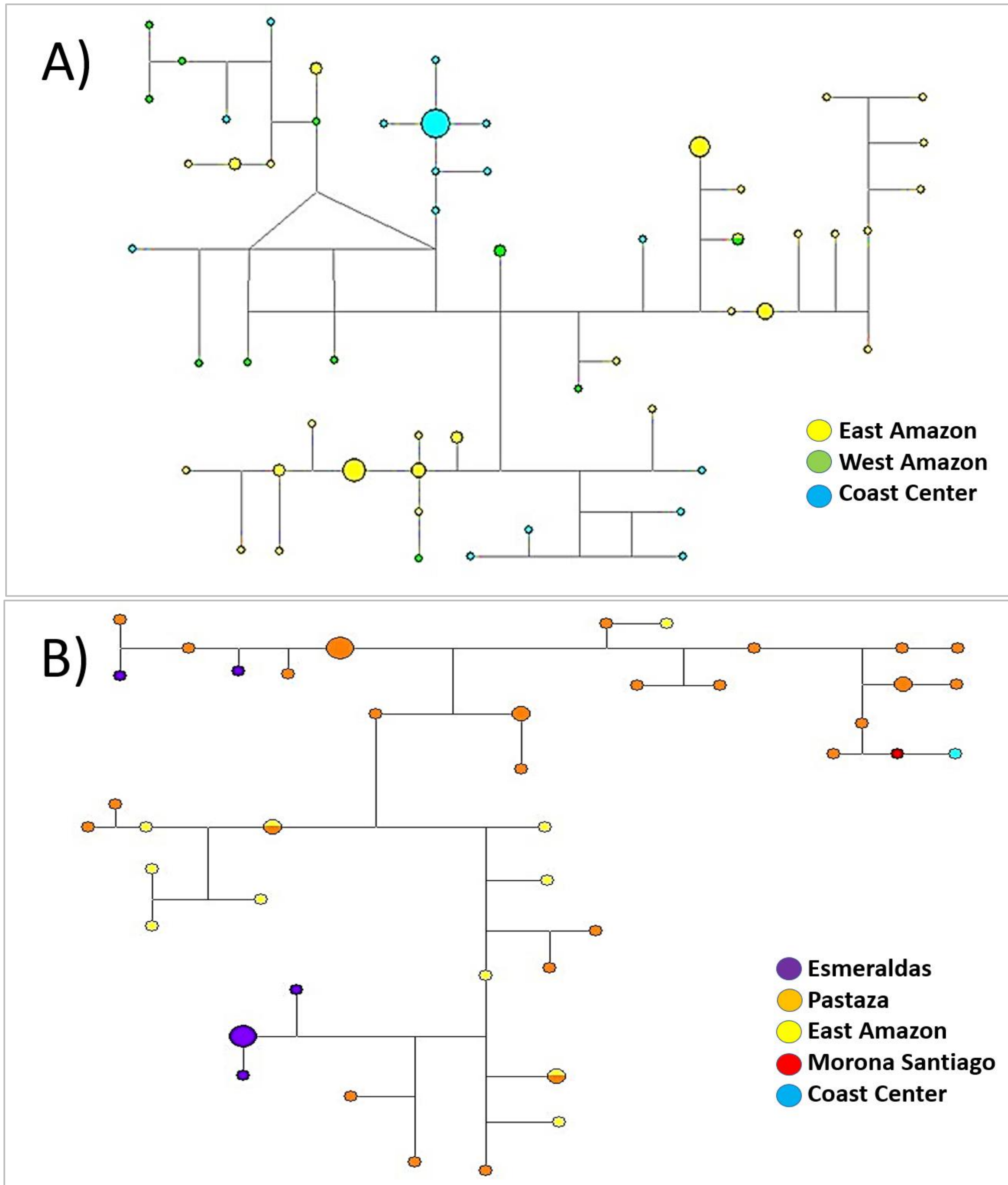

Figure 2. Haplotype network in Ecuador (2012-2020). A) Haplotype network with samples from 3 localities in Ecuador (2012-2015) (43). B) Haplotype network with samples from 5 localities in Ecuador (2016-2020).

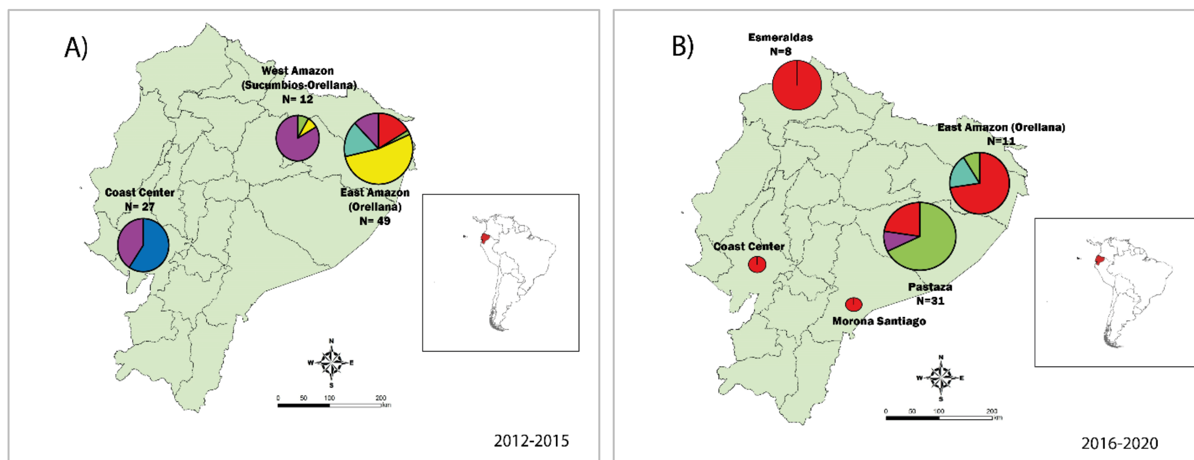

Figure 3. Lineage frequency of *P. vivax* presented in localities of Ecuador (2012-2020). A) Lineages frequency of *P. vivax* presented in localities of Ecuador (2012-2015)* for six eventual populations (K=6). B) Lineages frequency of *P. vivax* presented in localities of Ecuador (2016-2020) for six eventual populations (k=6).

*** Includes samples from Velez and collaborators (2023).**

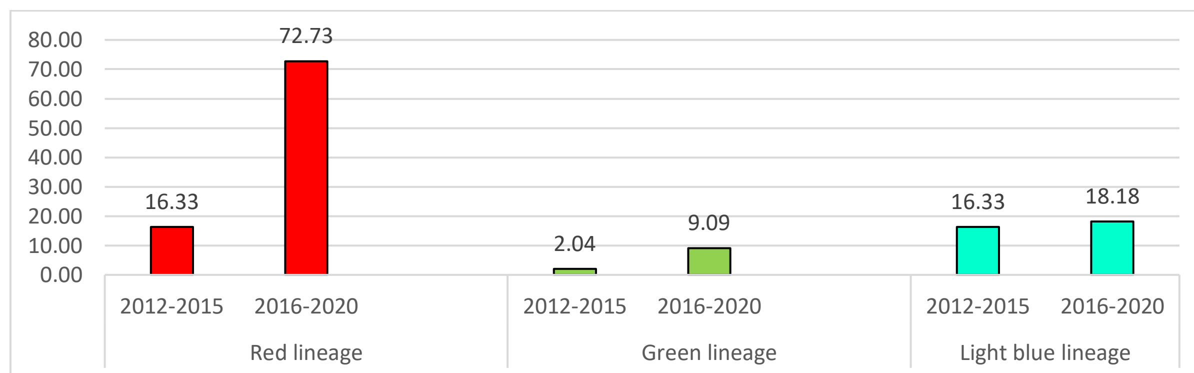

Figure 4. Comparison of 3 lineages frequency presented in the East Amazon with samples from the period 2012-2020*.

*** Includes samples from Velez and collaborators (2023).**

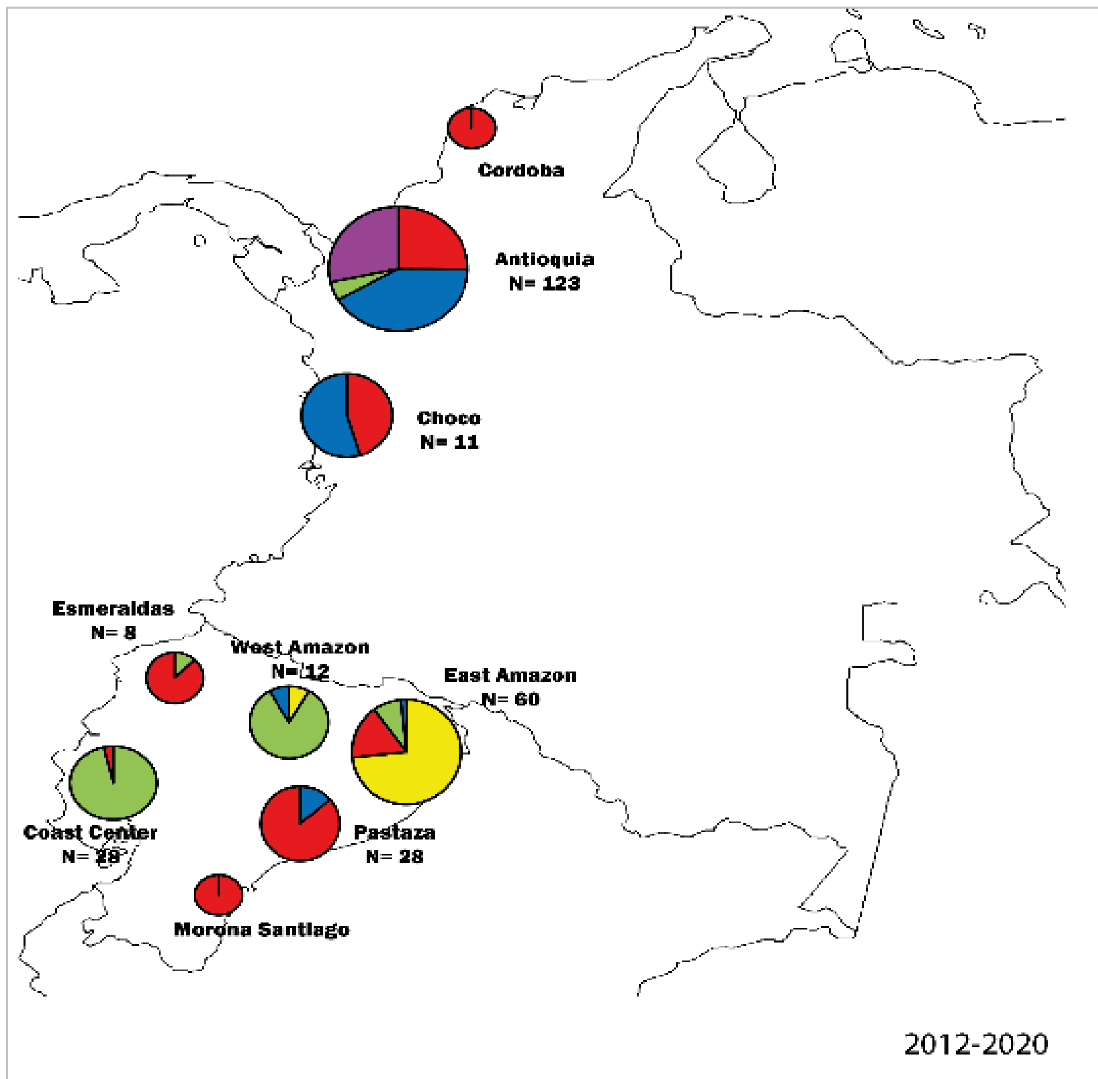

Figure 5. Lineage frequency between Ecuador (2012-2020)* and Colombia (2012-2013) (50) for five eventual populations (K=5).

*** Includes samples from Velez and collaborators (2023).**

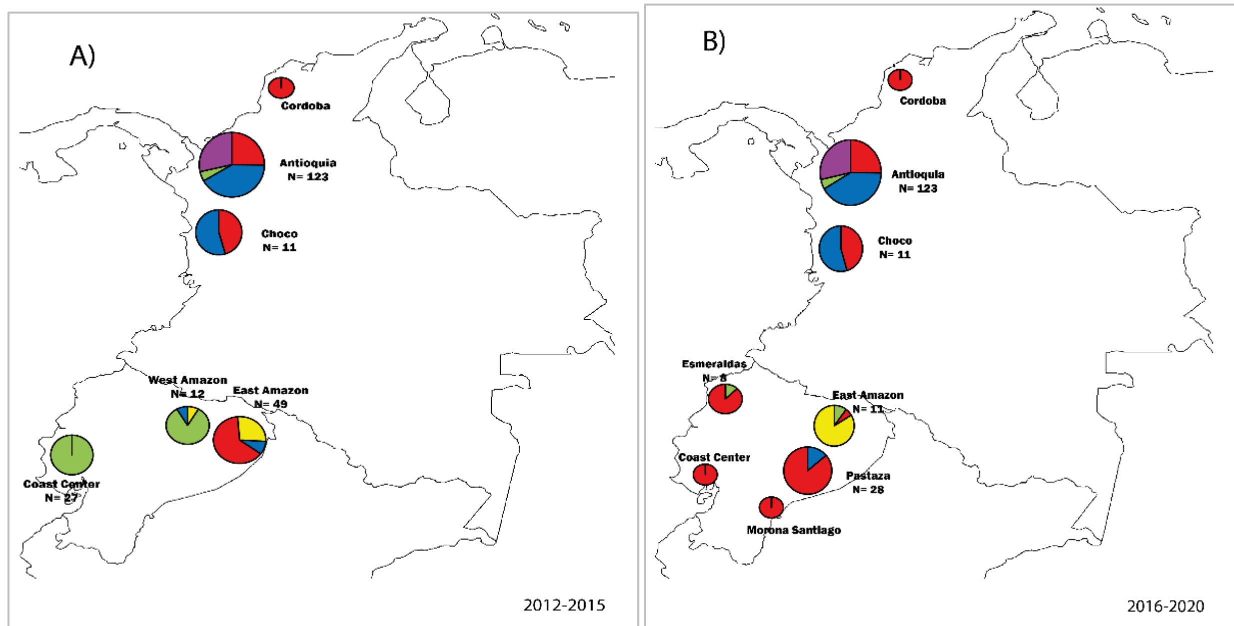

Figure 6. Lineage frequency for each period ecuadorian period (2012-2020)* and Colombia (2012-2013) (50). A) Lineage frequency between Ecuador (2012-2015)* and Colombia for 5 eventual populations (k=5). B) Lineage frequency between Ecuador (2016-2020) and Colombia for five eventual populations (k=5).

*** Includes samples from Velez and collaborators (2023).**

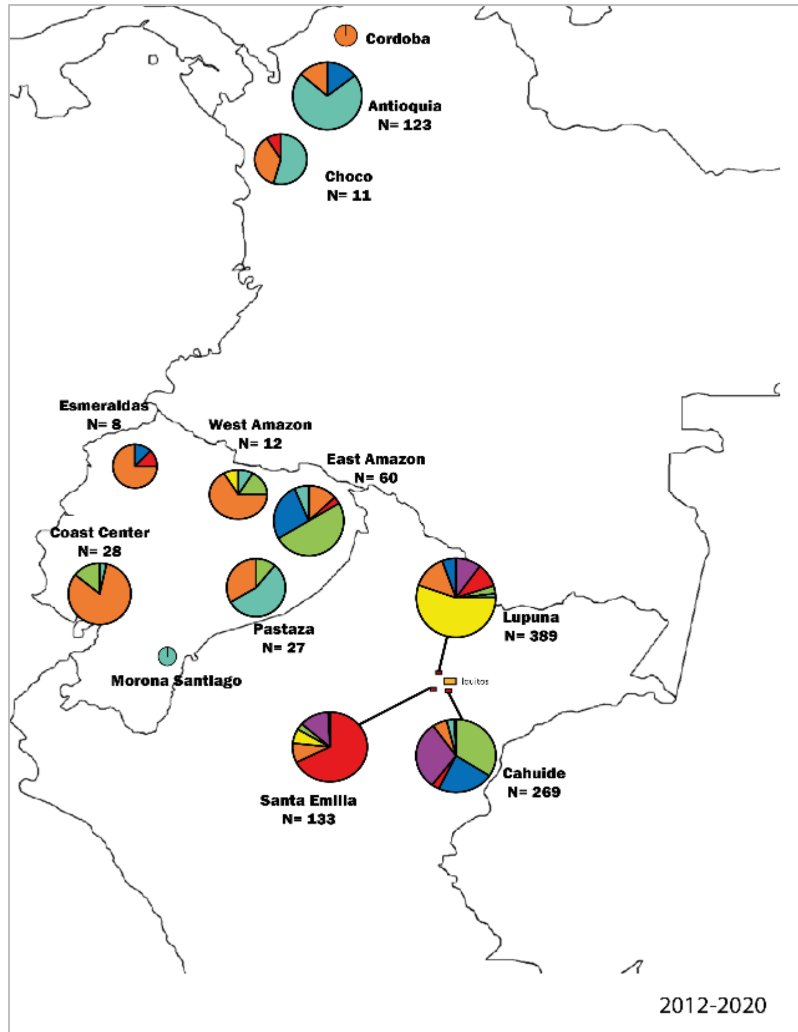

Figure 7. Lineage frequency between Ecuador (2012-2020)*, Colombia (2012-2013) (50), and Peru (2012-2015) (26) for seven eventual populations (k=7).
*** Includes samples from Velez and collaborators (2023).**

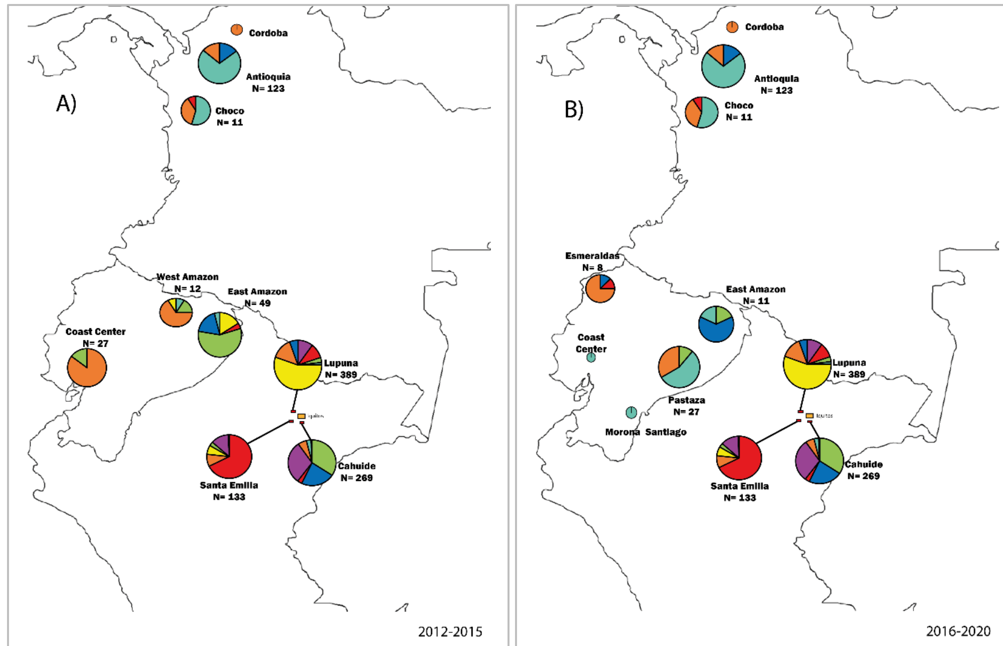

Figure 8. Lineage frequency for each ecuadorian period (2012-2020)*, Colombia (2012- 2013), and Peru (2012-2015). A) Lineage frequency between Ecuador (2012-2015)*, Colombia, and Peru for seven eventual populations (k=7). B) Lineage frequency between Ecuador (2016-2020), Colombia, and for seven possible populations (k=7).

*** Includes samples from Velez and collaborators (2023).**

10. TABLES

Table 1. Primers used in this study.

Microsatellite	Chr	Motif	Min	Max	External Forward primer	Reverse primer	Internal Forward primer	References
7.67	7	AT	100	134	ATGAGGTTTTCACGTTGTTC	AAAATGCACCTCTTTCATTC	FAM-AACAAATTGTGGGTAGATGC	(20)
8.332	8	AT	222	260	CTTCTAAGCGTGAGCAGTTT	CGAATTTTATAGGGGGAGAC	HEX-GAGAAGGTAACCCCAAAGAG	(20)
12.335	12	AT	155	197	TTAGTTCCAGCAAAACCTTC	TTATAACCTTCGGGGTTTTT	HEX-AAAAATGGAGACATGGAAGA	(20)
2.21	2	AC	91	129	GTGGGGTTGTTTAGCTTGT	TAACCCTCTATCGCTCTCAC	HEX-GTGCCATCTGCTCAAATC	(20)
3.502	3	AACGGATG	128	265	CCATGGACAACGGGTTAG	TCCTACTCAGGGGGAATACT	HEX-GTGGACCGATGGACCTAT	(21)
11.162	11	ATAC	172	228	GTAGGAACACGCCACGTT	TAAATGACACTTTGGCTTCC	FAM-TTTGTTAGGAGATCCGTCTG	(21)
MS2	6	(TAAA)2TATA(TAAA) 6TATA(TAAA)19	182	226	HEX-GAGCTAGCCAAAGGTTCAACA	CTGTCTTTGGGGAG AGACTCCCTTTTC	-	(23)
MS6	11	(TCC)2(TCT)3(CCT)2 (TCC)2GCTTCT(TCC)10	213	255	FAM-GGTTCTTCGGTGATCTCTGC	CTGTCTTGGAGGAC ATCAACGGGATT	-	(23)
MS20	10	(GAA)11GAG(GAA)13	195	237	HEX-GCACAACAAATGCAAGATCC	CTGTCTTGTGGCAGT GGCTCATCTTCT	-	(23)

	(CAA)4GAA(CAA)5

Chr: Chromosome Min: Minimum DNA fragment size to be amplified Max: Maximum DNA fragment size to be amplified

Table 2. Heterozygosity and linkage disequilibrium present in Ecuador (2012-2020)*.

	Na	Ne	He	I_A^S
Coast Center 2012-2015	4.000	2.121	0.507	0.4221
West Amazon 2012-2015	4.333	3.002	0.625	0.1012
East Amazon 2012-2015	5.222	2.687	0.590	0.227
Esmeraldas 2016-2020	3.444	2.388	0.553	0.5421
East Amazon 2016-2020	3.778	2.825	0.548	0.0125
Pastaza 2016-2020	5.111	3.127	0.615	0.0974

*** Includes samples from Velez and collaborators (2023)**
Na: Number of alleles
Ne: Effective number of alleles
He: Heterozygosity
I_A^S: Linkage disequilibrium

Table 3. Multiclonal infections in Ecuador (2016-2020).

	Number of samples	Number of samples with multiple infection	MCI (%)	MCI at>1 loci (%)
East Amazon	11	11	100	90.91
Esmeraldas	8	6	100	75
Pastaza	28	24	85.71	42.86

MCI (%) is the percentage of infections with at least 1 locus showing more than 1 allele
MCI>1 (%) indicates the proportion of infections with >1 locus showing more than 2 alleles

Table 4. Genetic differentiation (Pairwise-fst) between locations in Ecuador (2012-2020) *.

Coast Center 2012-2015	West Amazon 2012-2015	East Amazon 2012-2015	Esmeraldas 2016-2020	Pastaza 2016-2020	East Amazon 2016-2020	
0.000						**Coast Center 2012-2015**
0.194	0.000					**West Amazon 2012-2015**
0.295	0.173	0.000				**East Amazon 2012-2015**
0.349	0.206	0.250	0.000			**Esmeraldas 2016-2020**
0.306	0.171	0.243	0.225	0.000		**Pastaza 2016-2020**
0.349	0.199	0.221	0.260	0.163	0.000	**East Amazon 2016-2020**

*** Includes samples from Velez and collaborators (2023).**

Table 5. Analysis of molecular variance (AMOVA) of Ecuador 2012-2020 *.

	Source	df	SS	Est. Var.	%	Fixation indices	Value	P-value
1 group: 6 Populations	**Among Pops**	5	225.932	0.953	25%	**FST**	0.250	0.0001
	Among Indiv	129	737.964	2.860	75%	**FIS**	1.000	0.0001
	Within Indiv	135	0.000	0.000	0%	**FIT**	1.000	0.0001
	Total	269	963.896	3.813	100%			

*** Includes samples from Velez and collaborators (2023)**

Table 6. Gene flow presented between localities in Ecuador (2012-2020)*.

Coast Center 2012-2015	West Amazon 2012-2015	East Amazon 2012-2015	Esmeraldas 2016-2020	Pastaza 2016-2020	East Amazon 2016-2020	
0.000						**Coast Center 2012-2015**
1.038	0.000					**West Amazon 2012-2015**
0.599	1.197	0.000				**East Amazon 2012-2015**
0.467	0.966	0.749	0.000			**Esmeraldas 2016-2020**
0.566	1.216	0.780	0.864	0.000		**Pastaza 2016-2020**
0.467	1.007	0.880	0.710	1.284	0.000	**East Amazon 2016-2020**

*** Includes samples from Velez and collaborators (2023).**

Table 7. Heterozygosity presented between Ecuador (2012-2020)* and Colombia (2012-2013).

	Na	Ne	He
Coast Center 2012-2015	4.000	2.123	0.509
West Amazon 2012-2015	4.571	3.165	0.637
East Amazon 2012-2015	5.000	2.600	0.587
Esmeraldas 2016-2020	3.571	2.401	0.558
East Amazon 2016-2020	3.857	2.976	0.561
Pastaza 2016-2020	5.571	3.439	0.652
Antioquia 2012-2013	5.428	4.256	0.743
Choco 2012-2013	7.857	3.645	0.683

*** Includes samples from Velez and collaborators (2023)**
Na: Number of alleles
Ne: Effective number of alleles
He: Heterozygosity

Table 8. Heterozygosity presented between Ecuador (2012-2020)* and Peru (2012-2015).

	Na	Ne	He
Coast Center 2012-2015	3.750	2.014	0.472
West Amazon 2012-2015	4.750	3.291	0.674
East Amazon 2012-2015	4.500	2.301	0.547
Esmeraldas 2016-2020	3.250	2.059	0.508
East Amazon 2016-2020	3.250	2.259	0.438
Pastaza 2016-2020	6.250	3.868	0.676
Cahuide 2012-2015	7.5	2.449	0.579
Lupuna 2012-2015	7.75	2.371	0.573
Santa Emilia 2012-2015	6	2.785	0.594

*** Includes samples from Velez and collaborators (2023)**
Na: Number of alleles
Ne: Effective number of alleles
He: Heterozygosity

Table 9. Genetic differentiation (Pairwise-fst) between Ecuador (2012-2020)* and Colombia (2012-2013).

Coast Center 2012-2015	West Amazon 2012-2015	East Amazon 2012-2015	Esmeraldas 2016-2020	Pastaza 2016-2020	East Amazon 2016-2020	
0.000						**Coast Center 2012-2015**
0.211	0.000					**West Amazon 2012-2015**
0.333	0.204	0.000				**East Amazon 2012-2015**
0.375	0.221	0.298	0.000			**Esmeraldas 2016-2020**
0.299	0.174	0.252	0.223	0.000		**Pastaza 2016-2020**
0.376	0.242	0.248	0.276	0.168	0.000	**East Amazon 2016-2020**
0.271	0.106	0.214	0.111	0.143	0.166	**Antioquia**
0.302	0.150	0.250	0.187	0.152	0.226	**Choco**

*** Includes samples from Velez and collaborators (2023)**

Table 10. Analysis of molecular variance (AMOVA) between Ecuador (2012-2020)* and Colombia (2012-2013).

	Source	df	SS	Est. Var.	%	Fixation indices	Value	P-value
1 group: 8 Populations	**Among Pops**	7	306.457	0.696	23%	**FST**	0.229	0.01
	Among Indiv	261	1223.438	2.344	77%	**FIS**	1.000	0.01
	Within Indiv	269	0.000	0.000	0%	**FIT**	1.000	0.01
	Total	537	1529.896	3.040	100%			

*** Includes samples from Velez and collaborators (2023)**

Table 11. Genetic differentiation (Pairwise-fst) between Ecuador (2012-2020)* and Peru (2012-2015).

Coast Center 2012-2015	West Amazon 2012-2015	East Amazon 2012-2015	Esmeraldas 2016-2020	Pastaza 2016-2020	East Amazon 2016-2020	
0.000						**Coast Center 2012-2015**
0.204	0.000					**West Amazon 2012-2015**
0.363	0.204	0.000				**East Amazon 2012-2015**
0.427	0.261	0.327	0.000			**Esmeraldas 2016-2020**
0.283	0.142	0.298	0.202	0.000		**Pastaza 2016-2020**
0.447	0.285	0.337	0.346	0.238	0.000	**East Amazon 2016-2020**
0.391	0.273	0.203	0.357	0.271	0.224	**Cahuide**
0.349	0.258	0.327	0.384	0.259	0.374	**Lupuna**
0.369	0.280	0.180	0.348	0.264	0.329	**Santa Emilia**

*** Includes samples from Velez and collaborators (2023)**

Table 12. Analysis of molecular variance (AMOVA) between Ecuador (2012-2020)* and Peru (2012-2015).

	Source	df	SS	Est. Var.	%	Fixation indices	Value	P-value
1 group: 9 Populations	**Among Pops**	8	427.070	0.435	27%	**FST**	0.269	0.01
	Among Indiv	601	1418.704	1.180	73%	**FIS**	1.000	0.01
	Within Indiv	610	0.000	0.000	0%	**FIT**	1.000	0.01
	Total	1219	1845.774	1.615	100%			

*** Includes samples from Velez and collaborators (2023)**

Table 13. Gene flow presented between localities in Ecuador (2012-2020)* and Colombia (2012-2013) (50).

Coast Center 2012-2015	West Amazon 2012-2015	East Amazon 2012-2015	Esmeraldas 2016-2020	Pastaza 2016-2020	East Amazon 2016-2020	
0.000						**Coast Center 2012-2015**
0.936	0.000					**West Amazon 2012-2015**
0.500	0.975	0.000				**East Amazon 2012-2015**
0.416	0.880	0.589	0.000			**Esmeraldas 2016-2020**
0.586	1.190	0.743	0.873	0.000		**Pastaza 2016-2020**
0.414	0.785	0.757	0.656	1.234	0.000	**East Amazon 2016-2020**
0.674	2.118	0.916	2.011	1.495	1.260	**Antioquia**
0.579	1.421	0.750	1.086	1.390	0.857	**Choco**

*** Includes samples from Velez and collaborators (2023)**

Table 14. Gene flow presented between localities in Ecuador (2012-2020)* and Peru (2012-2015) (26).

Coast Center 2012-2015	West Amazon 2012-2015	East Amazon 2012-2015	Esmeraldas 2016-2020	Pastaza 2016-2020	East Amazon 2016-2020	
0.000						**Coast Center 2012-2015**
0.975	0.000					**West Amazon 2012-2015**
0.439	0.976	0.000				**East Amazon 2012-2015**
0.335	0.707	0.514	0.000			**Esmeraldas 2016-2020**
0.634	1.510	0.589	0.985	0.000		**Pastaza 2016-2020**
0.309	0.627	0.492	0.473	0.801	0.000	**East Amazon 2016-2020**
0.390	0.667	0.984	0.450	0.672	0.867	**Cahuide**
0.466	0.719	0.515	0.401	0.716	0.418	**Lupuna**
0.427	0.642	1.142	0.468	0.698	0.510	**Santa Emilia**

*** Includes samples from Velez and collaborators (2023)**

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee on Human Beings of Pontificia Universidad Católica del Ecuador through official letter ceish-517-2018 and authorized by the Ministry of Health of Ecuador through official letter msp-dis-2019-004-o.

Consent for publication

Not applicable

Availability of data and materials

Data analyzed in this study is available in CISeAL in charge of PhD. Fabián Sáenz.

Competing interests

The authors declare that they have no competing interests.

Funding

Pontificia Universidad Católica del Ecuador.

Author's contribution

EC performed the experiments; EC and FES designed the study and wrote the manuscript.

Acknowledgements

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11. SUBMISSION GUIDELINE

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Acknowledgements

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University site

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FTP site

Doe, J: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999.

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