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Signature:

Sean Michael Griffing _____

Date

The Origin and Spread of Drug Resistant Malaria in South America

By

Sean Michael Griffing

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Population Biology, Ecology, and Evolution

_____ [Advisor's signature]

Venkatachalam Udhayakumar

Advisor

_____ [Member's signature]

Michael E. Zwick

Co-mentor

_____ [Member's signature]

Ananias A. Escalante

Committee Member

_____ [Member's signature]

Mary Galinski

Committee Member

_____ [Member's signature]

John Gimnig

Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D. Dean of the James T. Laney School of Graduate Studies

_____

Date

The Origin and Spread of Drug Resistant Malaria in South America

By

Sean M. Griffing

B.A., Oberlin College, 1999

Certificate in Science Journalism, University of California, Santa Cruz, 2002

Advisor: Udhayakumar Venkatachalam, Ph.D., Madurai-Kamaraj University, 1986

An abstract of

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Abstract

The Origin and Spread of Drug Resistant Malaria in South America

By Sean Michael Griffing

The goal of this dissertation was to show how malaria control influenced South American *Plasmodium falciparum* population structure. A total of 565 *Plasmodium falciparum* samples were obtained from from Brazil (eight sites), Peru (eight sites), and Venezuela (one site). Bolivian isolates previously sequenced for some resistant genes were also included (8 samples). We sequenced the *Plasmodium falciparum* chloroquine resistance gene (*pfcrt*), the *Plasmodium falciparum* multidrug resistance gene (*pfmdr1*), dehydrofolate reductase (*dhfr*, associated with pyrimethamine resistance) and dihydropteoroate synthase (*dhps* associated with sulphadoxine resistance). We further characterized 56 microsatellites markers around these genes and 12 neutral microsatellites located on 7 other chromosomes. For Venezuela, we observed that the chloroquine (CQ) and sulfadoxine pyrimethamine (SP) resistance were fixed and linked in multidrug resistant genotypes. Mefloquine resistance may have evolved through *pfmdr1* copy number amplification, a first for South America. Tests suggested the population was bottlenecked. In Peru, *P. falciparum* populations were restricted to five clonal lineages, after years of low malaria incidence, during malaria epidemics in the 1990s, distinctive in South America. One clonet was found on the coast and one western Amazon site, indicating the Andes were a major gene flow barrier. In the Amazon, there were four clonets distributed in varying proportions at different sites. Drug pressure influenced the selection and expansion of clonal lineages. Among isolates collected from the Peruvian Central Amazon during 2006-7, there was evidence for clonet outcrossing, contrary to our hypothesis that clonal propagation would continue. The shift from SP to artesunate combination therapy in 2001 influenced this breakdown, favoring the emergence of two major hybrid clonets. In Brazil, most parasites were moderately CQ and SP resistant in the early 1980s and highly resistant in the 1990s. We suggested that human migration within the Brazilian Amazon led to extensive admixture and outcrossing between parasite clonal lineages and populations had bottlenecked. We combined our molecular data with a historical review of malaria control and resistance to determine the relationships between the parasite populations from different countries and to examine how CQ and SP resistance may have spread throughout South America.

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CHAPTER 1

GENERAL BACKGROUND

The Burden of Malaria

Malaria is an acute febrile illness. Globally, malaria causes about 500 million episodes of febrile illness [1] and kills 700,000 to 2.7 million people each year. Greater than 50% of humanity is exposed to malaria [2]. Historically, malaria was once found in the southern United States and Europe [1] as well in the tropics and subtropics (Asia, the Americas, and sub-Saharan Africa), but now it only rarely occurs in temperate regions [3]. Malaria causes a number of clinical symptoms including anemia, cerebral malaria, pregnancy-associated complications including low birth weight, respiratory distress, and hypoglycemia among other complications[1].

Human malaria is caused by five protozoans in the phylum Apicomplexa: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi*, which differ morphologically, geographically, and in drug response [4,5]. *P. falciparum* is the most virulent. About ninety percent of malaria cases occur in Africa and are caused by *P. falciparum*. *P. vivax* is considered less virulent, but also leads to morbidity and mortality. In addition, patients may relapse later due to the activation of dormant liver forms called hypnozoites *P. vivax* is the predominant species throughout Central and South America, and Asia, the Middle East, the South Pacific and Africa (though cases are rare in the western and central portions of the continent). *P. ovale* infection is relatively rare, and can lead to relapses and thus persistent infections. *P. ovale* is found in Africa and the western Pacific. *P. malariae* can lead to asymptomatic infections for years, but also leads to renal complications. While less often reported, it is found throughout the world [1]. Human *P. knowlesi* infection was previously misidentified as *P. malariae* infection. It replicates every 24 hours, which is twice as fast as *P. falciparum*. It is typically a parasite of Old World Monkeys, however in the last few years a large

number of cases have been reported in Malaysia and may be widespread in Southeast Asia in locations with Old World monkeys. While only recently identified as a human pathogen, it has already been linked to four deaths [4].

Malaria is transmitted by female *Anopheles* mosquitoes. Infected mosquitos inject sporozoites into a host during blood meals. Uninfected mosquitos take up malaria parasite gametocytes during a blood meal and continue the cycle [3]. Sixty *Anopheles* species are known to transmit malaria [1]. Globally, the most efficient vectors for *P. falciparum* are the *An. gambiae* and *An. funestus* complexes. *Anopheles gambiae* is widespread in Africa and has the highest rate of sporozoite development [1].

Malaria species and life cycle

The *Plasmodium* life cycle shares a number of general features among all the species. After being ingested along with human blood, haploid *Plasmodium* gametocytes go through sexual reproduction in the mosquito's midgut (Figure 1.1). Macrogametocytes (female gamete precursors) become macrogametes and exflagellation of microgametocytes (male gamete precursors) leads to microgametes. The gametes fuse, undergo sexual reproduction and form a zygote, which becomes an ookinete that penetrates a cell in the midgut wall and becomes an oocyst. The parasite is only diploid during reproduction and therefore this is the only time that sexual recombination can take place. The oocyst undergoes sporogony and after rupturing produces sporozoites. These sporozoites migrate through the mosquito's hemolymph to the mosquito's salivary glands from where they can infect another host during during blood feeding. This process takes 10-18 days and a mosquito can remain infectious for 1-2 months.

When the mosquito takes a blood meal from another human host, they infect them with haploid sporozoites. The sporozoites migrate to the liver where they form a schizont. They remain for 9-16 days and undergo asexual replication, with each infected hepatocyte generating tens of thousands of merozoites. This developmental stage in liver tissue is called the preerythrocytic

stage. *P. vivax* and *P. ovale* can also remain dormant in liver and cause subsequent relapses. When the schizont ruptures, merozoites infect red blood cells by attaching to their cell membranes and moving into them.

In the red blood cell, the parasite can either develop into more merozoites or, more rarely, reproductive gamete precursors called gametocytes. In the red cell, malaria parasites undergo different developmental stages starting from ring stage, to mature trophozoite, and finally schizont. When the schizont ruptures, typically 12-32 merozoites (depending on the species) are released into the blood stream and they continue the cycle by infecting more red blood cells. The synchronized, cyclical nature of this process leads to periodic fevers (every 48 hours for *P. falciparum* and *vivax*). This cycle of infection will continue unless drug treatment or natural immune response clears the infection or reduces the symptoms of disease. The parasite utilizes haemoglobin for its life cycle, but cannot degrade heme. The toxic heme is polymerized into a non-toxic form called haemozoin which is sequestered within the parasite's food vacuole. A subset of blood stage parasites develop into gametocytes and can form microgametocytes (male) or macrogametocytes (female). However, gametogenesis typically occurs 10-12 days after *P. falciparum* infection. After a mosquito becomes infected during a a blood meal, the gametocytes complete reproduction its gut and repeat the cycle [5].

Antimalarial drugs and resistance:

Chloroquine and its Action

Chloroquine (CQ) is a quinoline-ring antimalarial drug. Quinoline drugs can be classified into two subclasses, the 4-aminoquinolines (including CQ) and quinoline-4-methanol drugs (including quinine and mefloquine). Quinoline-ring antimalarial drug may interact with plasmodium's intraerythrocytic heme degradation. Chloroquine was originally synthesized in 1934 in Germany after years of work sparked by dwindling supplies of quinine during World War I. CQ became available for widespread public use after World War II [6].

CQ has the best defined drug action of the quinoline drugs [7]. It disrupts parasite intraerythrocytic heme degradation. Intraerythrocytic hemoglobin degradation occurs in *Plasmodium falciparum*'s digestive vacuole. The digestive vacuole is acidic and the parasite's cytosol is neutral. As CQ is a soluble, dibasic compound, it follows a pH gradient from the cytosol into the digestive vacuole. In the vacuole, CQ is believed to become diprotonated and also membrane impermeable, leading to its accumulation in the swelling digestive vacuole [7]. But other acidic organelles have lower levels of CQ accumulation than the digestive vacuole and this suggests the presence of drug accumulation mechanisms beyond a passive pH gradient [8].

The main alternate explanation for CQ accumulation in the digestive vacuole is interaction between CQ and hematin (Fe21-protoporphyrin IX), a dimeric, detergent-like, toxic protein produced during hemoglobin degradation. In the absence of CQ, the digestive vacuole detoxifies hematin by adding it to inert hemozoin crystals. CQ binds to either soluble (membrane-associated) or terminal hematin on the faces of hemozoin crystals. This leads to toxic levels of hematin in the parasite, possibly through peroxidation of the digestive vacuole's membrane. In addition, hematin can diffuse into the cytosol where it makes lipid membranes permeable and catalyzes the replacement of potassium with sodium or reduces the function of enzymes. CQ may also keep cytosolic hematin (typically referred to in the literature as ferriprotoporphyrin) from being degraded by glutathione [7].

Chloroquine Resistance

During the 1950s and 1960s, CQ was a lynchpin of malaria eradication programs. But by 1957, CQ resistance was noted on the Thai-Cambodian border and it rapidly spread into Thailand. CQ resistance was also noted in 1960 in Venezuela and Magdalena Valley, Colombia. It was later noted in Port Moresby, Papua New Guinea by 1976 [6]. Resistance reached East Africa in 1979 and proceeded to spread across sub-Saharan Africa [9]. Reports of resistance came from Madagascar by 1981 and it was also seen in Uganda, Sudan, and Malawi by 1983 [6]. All told, there appear to have been at least four locations from which resistance arose: two in South America, one in Papua New Guinea, and another from South East Asia from where it spread to Africa. There is evidence that there may be another two points of origin in Cambodia and the Philippines [10].

The mechanism CQ resistance was hypothesized to be generated by a number of different processes including a more acidic digestive vacuole speeding the development of hemozoin crystals, increased production of glutathione binding to heme-CQ complexes, or the efflux of hematin from the digestive vacuole. A more acidic digestive vacuole might speed the crystallization of hemozoin and thus reduce the amount of hematin available for binding with CQ. However, the support for a lower pH in the digestive vacuole of CQ-resistant malaria is mixed. The support for increased levels of glutathione ‘neutralizing’ hematin in the digestive vacuole prior to CQ contact is also mixed. The final explanation is currently thought to be the likely mechanism of resistance based on the genes associated with resistance and laboratory finds. CQ-resistant parasites release accumulated CQ 50 times faster than CQ-sensitive parasites and efflux CQ from the digestive vacuole. This action is thought to take place by way of the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) encoded protein and the protein enconded by the *P. falciparum* multidrug resistant gene 1 (*pfmdr1*; [7]). PFCRT is essential to resistance while PFMDR is helpful but not required [10].

pfcrt and *pfmdr1* polymorphisms and Drug Resistance

The *pfcrt* gene is located on chromosome 7 of *P. falciparum* and codes for a 49-kDa transmembrane protein that has been localized to the digestive vacuole's membrane and a schematic structure of this protein is illustrated in Figure 1.2. Bioinformatic analysis suggests that PFCRT is a drug-metabolite effluxer and a homodimer. The resistant alleles have point mutations that are in or around the protein's transmembrane domains and resulting in the loss of basic or hydrophobic residues. While numerous point mutations have been noted, only the K76T mutation appears to be critical for resistance [7]. The K76T mutation is thought to be near the vacuole's interior surface and to be involved in substrate specificity. PFCRT no longer repulses diprotonated CQ molecules after the removal of a positively charged lysine at this position. PFCRT may contribute to CQ resistance by allowing the diprotonated form of CQ to leak back into the cytosol in what is called the "charged drug leak hypothesis." Recent experimental studies have clearly shown that the K76T mutation allows the PFCRT to efflux CQ efficiently [11].

In addition to the K76T mutation, a series of other mutations between codons 72-76 have been associated with CQ resistance in field studies. The CQ sensitive wild type parasite has the CVMNK sequence between amino acids 72-76. The most widely distributed CQ resistant parasite isolates in Southeast Asia and Africa carry CVIET genotype. In South America, SVMNT is the most common CQ resistant genotype in the Amazon region. There are two variants, namely S_{tct}VMNT, and S_{agt}VMNT, with different frequencies at different sites. CVMNT is another major genotype found in South America, especially in the coastal regions. CVMET is another genotype particularly found in Colombia. The CVIET genotype is not very common in South America and it appears it may have been introduced from Africa or Southeast Asia.

Using microsatellite markers, at least two different origins for CQ resistant genotypes in South America have been reported [12]. The CVMNT allele distributed in the coastal region appears to have originated in Colombia and this is closely related to the CVMET genotype found in Colombia. On the other hand, there is a CVMNT allele reported in the Amazon interior that

appears to share ancestry with the S_{tct}VMNT allele [12]. The S_{agt}VMNT allele is also closely related to the S_{tct}VMNT allele. It has been found that the genetic diversity around *pfcrt* in Brazil, Colombia, and Guyana was much lower than samples from Africa or Asia [13].

The distribution of CQ-resistant alleles differ across South America. S_{tct}VMNT was found in most samples in Bolivia and most of the Brazilian samples which carried highly resistant *dhfr* and *dhps* alleles. The remaining samples carried S_{agt}VMNT [14]. Another study reported that both SVMNT alleles were found in Mato Grosso, Brazil, but that S_{tct}VMNT was only found in Rondônia and Amazonas. CVIET was found in Mato Grosso as well as Manaus, Amazonas. Only CVMNT was reported on the border with Peru in Tabatinga, Amazonas. Many of the parasites carrying S_{tct}VMNT, CVMNT and a few carrying S_{agt}VMNT formed a haplotype group. It was concluded that CVIET had been introduced to Brazil within the last 20 years from Africa or Asia. The authors argued that the ancestral genotype in the Amazon region was S_{tct}VMNT and was responsible for the original sweep of drug resistance. By this hypothesis, CVMNT was a derived allele. They suggested that the CVMNT reported in Tabatinga was in fact introduced from patients recently in Peru. It was argued that S_{agt}VMNT could have originated in Mato Grosso. While their study used 15 microsatellites, they were on only 4 chromosomes (seven from chromosome 7 which carries *pfcrt*). Therefore, their conclusions are a hybrid of general population structure and the history of the *pfcrt* chromosome [15].

The *pfmdr1* gene is located on chromosome 5 and codes for a 162-kDa transmembrane protein that appears to be an ATP-binding cassette transporter (Figure 1.3). It most likely binds with CQ inside of the digestive vacuole and uses ATP to change its conformation [16] and push CQ back into the cytosol. Although originally it was believed that *pfmdr1* may be the key mediator of CQ resistance, later studies proved that this protein only played a secondary role in modulating resistance to CQ. However, it has become evident that *pfmdr1* is involved in resistance to other antimalarial drugs as further discussed in the next section. Single nucleotide mutations in *pfmdr1* are postulated to modulate drug response including N86Y, D142G, Y184F,

S1034C, N1042D, and D1246Y. N86Y has been linked to both resistance to CQ and Amodiaquine. N1042D had been linked to quinine resistance, but also lower resistance to mefloquine and artemisinin [17].

In South America, one study examined *pfmdr1* genotypes in Brazil, Colombia, and Guyana. In Brazil, the only reported genotype was N86/184F/1034C/1042D/1246Y. While this genotype predominated in Guyana (94%), there was also a 86Y/184Y/1034S/1042N/1246D genotype (6%) and these samples carried *pfcrt* CVIET. In Colombia, only one genotype was reported, which was N86/184F/1034S/1042D/1246Y. The genetic diversity of *pfmdr1* was low in South America in comparison to Africa and Asia. The *pfmdr1* haplotype for Colombia did not group with the haplotype found for Brazil and Colombia [13]. Another study that examined *pfmdr1* codon 1246 found that a Venezuelan sample from the 1980s and Peruvian samples from 1997 did not carry this mutation, while samples from Bolivia, Brazil, and Colombia did [14].

There is increasing evidence that single nucleotide polymorphisms as well as gene duplications in the *pfmdr1* gene contribute to resistance associated with several antimalarial drugs. Gene amplification appears to augment *pfmdr1*'s contribution to mefloquine resistance. *Pfmdr*1 amplification is also implicated in resistance to lumefantrine, halofantrine, quinine, and artesunate (AS) [17,18] and may decrease resistance to CQ [19]. The role of genetic amplification of *pfmdr1* in resistance to mefloquine has been well established in the Thailand and Cambodian regions [20,21,22,23,24,25,26] and, as my disseration will describe, possibly South America.

Sulfadoxine Pyrimethamine Action

Sulfadoxine Pyrimethamine (SP) is a combination of two antifolate drugs. Folate, commonly known as vitamin B9, is used by *Plasmodium spp.* to produce tetrahydrofolate, a cofactor required in single carbon transfers for "purine, pyrimidine, and amino acid biosynthetic pathways"[7]. SP causes a decrease in methionine synthesis, thymidylate levels, and glycine to serine conversion by keeping *Plasmodium falciparum* from synthesizing folate. In effect, this halts parasite DNA replication. Sulfadoxine and pyrimethamine act on different enzymes within the folate pathway and do not limit parasites from utilizing small amounts of exogenous folate [9].

Pyrimethamine and Sulfadoxine Resistance

Pyrimethamine was first used as an antimalarial drug with good results at the beginning of the 1950s but concerns about the development of resistance started at almost the same time. By 1970, US soldiers were becoming infected with pyrimethamine resistant *Plasmodium falciparum* in Vietnam [9]. Sulfadoxine is one of the sulfa drugs developed in the 1950s. Sulfadoxine showed early promised in combating *P. falciparum* in Tanzania during the 1960s. In 1959, a study showed that sulfadoxine used in combination with pyrimethamine was more effective than either drug alone [9]. Unfortunately, by 1980 resistance was seen in semi-immune people in Southeast Asia and South America and in fully susceptible people by the mid-1980s in the United States and Africa [6].

Molecular Targets of SP Resistance: *dhfr* and *dhps*

Pyrimethamine acts on dihydrofolate reductase (DHFR). DHFR is coded on the parasite's fourth chromosome by a single-copy gene [9] (Figure 1.4). DHFR catalyzes two different reactions, synthesizing thymidylate from deoxyuridylate and converting methylenetetrahydrofolate into dihydrofolate. There are four point mutations in DHFR that when combined confer strong resistance to pyrimethamine: S108N, N51I, I164L and C59R or C50R. The S108N mutation is predicted to cause pyrimethamine binding problems due to steric conflict with the drug's p-chlorophenyl side chain around the Cl atom. The mutation at C59R may increase the enzyme's affinity for dehydrofolate in the presence of S108N and N51I [9]. C59R and N51I may make it more difficult to access the DHFR's inhibitor site (in the presence of S108N), possibly through the interaction of polar and charged residues with protonated pyrimethamine [9]. The N51I and I164L mutations are thought to widen DHFR's binding site and lower its affinity for pyrimethamine due to the drug's rigidity. However, I164L is apparently only viable in the presence of the other three point mutations and it actually lowers SP resistance if it occurs alone [9]. While it had been thought that these point mutations occurred in a step-by-step manner, there is evidence that this is not true [27]. Drug resistant DHFR with all four mutations has cross resistance to cycloguanil, and chlorproguanil [7].

Due to the strong pyrimethamine resistance requiring four point mutations, the spread of SP resistance is complicated. Single point mutation alleles have many different haplotypes in South America, Africa, and Asia. As the number of point mutations increases, there are less unique drug resistant haplotypes. In Africa, at least two unique origins for double mutants have been found, but only one origin was found for South America and Africa. The triple mutant seen in Africa was thought to have spread from SE Asia [10]. However, recent work has shown that there are apparently multiple unique minor alleles with three and four point mutations in Africa. This suggests that the pyrimethamine resistant allele arose locally (in addition to a major 51, 59, 108 triple mutant allele spread from Southeast Asia), at least in high transmission areas like

Kenya where recombination can generate drug-resistant alleles [27]. In South America, two different triple mutant *dhfr* resistant alleles (50, 51, 108 and 51, 108 and 164) have been reported. Among them 51, 108, 164 was found in the Peruvian Amazon, Bolivia, and Southern Brazilian Amazon parts of Brazil. The triple mutant alleles found in South America have locally evolved [14].

Sulfadoxine, like other sulfa drugs, acts on dihydropteoroate synthase (DHPS). DHPS is a bifunctional enzyme and its gene is located on *Plasmodium falciparum*'s 8th chromosome (Figure 1.5). DHPS catalyzes the reaction of para-aminobenzoic acid (PABA) with dihydropteroate. Dihydropteroate, in turn, is a substrate precursor for pyrimethamine's enzyme target. Sulfadoxine stops DHPS function by mimicking PABA and creating a dead-end reaction product. The key point mutations in DHPS that contribute to sulfadoxine resistance include S436A, A437G K540E, A581G, and A613T. Amino acid 436 and 437 seem to be located near substrate and inhibitor binding locations, but the remainder of the point mutations are not. This suggests that these mutations may be located in a channel near the active site [9]. Triple and quadruple mutant *dhps* contribute to the highest levels of resistance. Three different triple mutants (S436A, A 437G and K540E; A437G, K 540E, A581G; A437G, K540N and A581G) were found in Thailand and Cambodia, while only a single triple mutant type (A437G, K540E, A581G) was seen in South America. The S436A, A 437G and K540E allele has independently evolved in the Thailand /Cambodian region while A437G, K 540E, A581G/ A437G, K540N and A581G alleles have evolved from a common ancestor in the same region [28]. The A437G, K 540E, A581G allele in South America independently evolved [14]. In Africa, only double mutant *dhps* alleles are widely reported (A437G, K540E and S436A, A437G), though recently triple mutants have been reported [29,30].

While various studies have examined *dhfr* and *dhps* throughout South American, only one has looked at both across multiple countries. The study used samples from Bolivia, Brazil, Colombia, Ecuador, Peru, and Venezuela, among other countries outside of South America. They

concluded that there were two multimutation *dhfr* alleles 50R/51I/108N and 51I/108N/164L that had a single origin in the Southern Amazon basin (possibly Mato Grosso) which had spread north-northeast. Both lineages also carried a 437G/540E/581G *dhps* allele. The lineage carrying 50R/51I/108N was found in Bolivia, Brazil and Venezuela. The lineage carrying 51I/108N/164L was found in Bolivia, Brazil, and Peru. In contrast, parasites carrying the *dhfr* single mutant 108N and wildtype *dhps* were reported in Peru, as did a Brazilian clone that was reported to have been collected around 1985. The single sample from Ecuador had wildtype *dhfr* and *dhps*. Colombian samples had some mutations in both genes, but not many [14]. A more recent study has shown that parasites collected from eastern Colombia carry *dhfr* and *dhps* double and triple mutants, while in the interior of Colombia parasites had few mutations. The authors used six microsatellties, but four were associated with these two genes. Their analysis of these markers suggests that there is migration over the Andes with coastal parasites most similar to the interior collection site closest to the Andes [31]. In summary, the origin and spread of *dhfr* and *dhps* mutants in South America has not been fully described and the results reported in this thesis will provide additional insight into their dynamics as well as underlying *P. falciparum* population structure.

The Population Structure of *Plasmodium falciparum*

Few papers have examined the global population structure of *Plasmodium falciparum*. One examined samples from Africa (Democratic Republic of Congo, Uganda, and Zimbabwe), Asia (Thailand), Oceania (Papua New Guinea), and South America (Bolivia, Brazil, and Colombia). The authors used 12 microsatellites from 7 different chromosomes. They found that mean heterozygostity was highest for African countries (0.76–0.8), intermediate for Asia (0.51–0.65), and lowest for South America (0.3–0.4). However, they suggested the low value estimated for South America could be due to insufficient sampling. While little of the variation noted in Africa was within each site ($F_{st} = 0.007$), in South America a large portion was distributed between sites ($F_{st} = 0.364$). They proposed that the effective population size of African countries was between 4,900-6,491, 1,653 for Thailiand, 2,589-2,931 for Papua New Guinea, and somewhere between 682-1,051 for South America.

An unrooted neighbor-joining tree showed that the African samples clustered together. Asia and Oceania formed another cluster. Bolivia and Brazil closely clustered together but Colombia was more distantly connected. There was significant linkage disequilibrium in areas of low transmission (the Thai-Burmese border and all of the South American countries). They noted strong differentiation between the three South American countries and concluded that admixture might be common (particularly in Bolivia). Bolivia and Brazil had allele frequency distributions that suggested there may have been recent population bottlenecks. In Brazil and Bolivia there were 6 haplotypes that occurred two to four times. In Colombia, there were five hapolotypes that occurred at least seven times within the host population and the authors suggested there may have been inbreeding [32].

Another study that examined the global population structure of *Plasmodium falciparum* was actually focused on understanding the global origins of *pfcrt* resistant alleles (n=87) in Africa, Asia, and South America. The authors examined a limited number of samples (2 for the Pacific Coast and 15 for the interior of South America), but used an extensive set of

microsatellites that spanned all 14 parasite chromosomes. The South American parasites had very significant allele sharing across all of them, while Asia had somewhat less allele sharing, and Africa much less. They concluded that SVMNT (which was similar to a Peruvian CVMNT allele) in the interior, and CVMET (which was similar to a Ecuadorian CVMNT allele) on the coast had independently evolved [12].

In South America, a regional study which deserves mention studied the population structure of *P. falciparum* in the Brazilian Amazon. It used samples collected across Brazil in the states of Acre, Amapá, Pará, and Rondônia. This study found significant linkage disequilibrium among the 10 microsatellites they assayed on 6 chromosomes. A site in Pará and a site in Acre showed strong LD, while a site in Amapá showed moderate LD, and sites in Rondônia and Pará showed little linkage disequilibrium. F_{st} varied between 0.05 and 0.30. The sites that appeared most similar, with the lowest $F_{st,}$ were Marabá, Pará and Serra do Navio, Amapá. Using the most appropriate model, none of the sites showed indications of epidemic expansion or bottlenecks. Collection sites were highly differentiated but did not follow an isolation by distance model [33].

In general, there appear to be South American clonal lineages, as indicated by the limited genetic diversity and linkage disequilibrium reported in the literature [31,32,34,35,36,37,38,39,40,41]. It has been argued that *P. faliciparum* is not panmictic and generally propagates clonally through self-fertilization. Such clonal lineages will be described as clonets in this dissertation. Clonets are a group of isolates which are genetically identical for a given set of genetic markers in a basically clonal species. Their common ancestor could be a few weeks or hundreds of years old. The term is used to stress how the kind of ‘clone’ identified in molecular epidemiology is in fact dependent on the markers chosen; that is, pathogens that appear clonal by one set of markers may in fact be heterogeneous when better markers are chosen [42,43]. However, recent sequencing of the genomes of 14 isolates collected in Peru suggest that in at least one region these clonets may actually be clones [41].

Numerous South American studies have noted limited genetic diversity and linkage disequilibrium in various countries and using various markers. South American parasites have limited genetic variation at microsatellite loci and genes including antigen-coding loci and the *var* gene family [31,32,34,35,36,37,38,39,40,41]. Generally speaking, studies used too few markers, sampled too few locations, or used too few samples to draw general conclusions regarding South American *P. falciparum* population structure. In addition, studies that touched upon the origin and spread of drug resistant alleles either ignored underlying population structure or ignored the potential epistatic selection caused by resistance to other drugs. Finally, few studies choose to examine the history of South American malaria at any depth and therefore may have missed historical events which could have influenced their interpretation of the origin and spread of drug resistance.

The goal of this dissertation was to show how malaria control influenced South American *Plasmodium falciparum* population structure. In addition, an attempt was made to understand the origin and spread of drug resistant *pfcrt*, *pfmdr1*, *dhfr* and *dhps* alleles in South American regions.

I hypothesized that CQ and SP resistance arose multiple times and spread throughout South America, instead of arising only once for each resistant allele. I also hypothesized that the Andes acted as a geographic barrier, thereby explaining why samples from Colombia appeared to be different from the rest of the continent in multiple studies, and tested this hypothesis by extensively sampling samples collected on either side of the Andes in Peru. Furthermore, I tested the hypothesis that the genetic diversity in South America and the reported strong linkage disequilibrium would influence the development and spread of multidrug resistant parasite lineages by examining samples for stable multiallelic patterns of drug resistant alleles over time, which could indicate that resistance accrued over time in particular lineages, and mapping their distributions. I also used these factors to understand how different lineages of *pfcrt*, *pfmdr1*, *dhfr*, or *dhps* were related.

To test the general hypthosis that resistance arose multiple times and spread, I also conducted an extensive literature review to define the history of South American malaria and its treatment, as well as previous molecular studies. Based on this research, we tested the following hypotheses regarding how resistance originated and spread in South America:

1) that highly resistant *dhfr* alleles spread from the southern Amazon basin
2) that highly resistant *dhps* alleles spread throughout the Amazon basin with highly resistant *dhfr*
3) that CVMNT or CVMET arose once on the Pacific Coast and that SVMNT arose once in the interior around 1960
3) that there may be regional variation in *pfmdr1* that is influenced by geographic location

To test these hypotheses I examined all of the genes known to contribute to CQ and SP resistance (*dhfr, dhps, pfcrt, pfmdr1*) and numerous surrounding microsatellites (~59 loci, depending on the study) from many samples collected in Bolivia, Brazil, Peru, and Venezuela. These microsatellites neighbor drug resistant alleles and tmay have been influenced by strong natural selection arising as a consequence of malaria control. Therefore, we also examined neutral microsatellites from 10 out of 14 *P. falciparum* chromosomes that were ostensibly not hitchhiking with alleles under positive selection to define neutral *P.falciparum*'s population structure.

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Figure 1.1 The lifecycle of *Plasmodium*

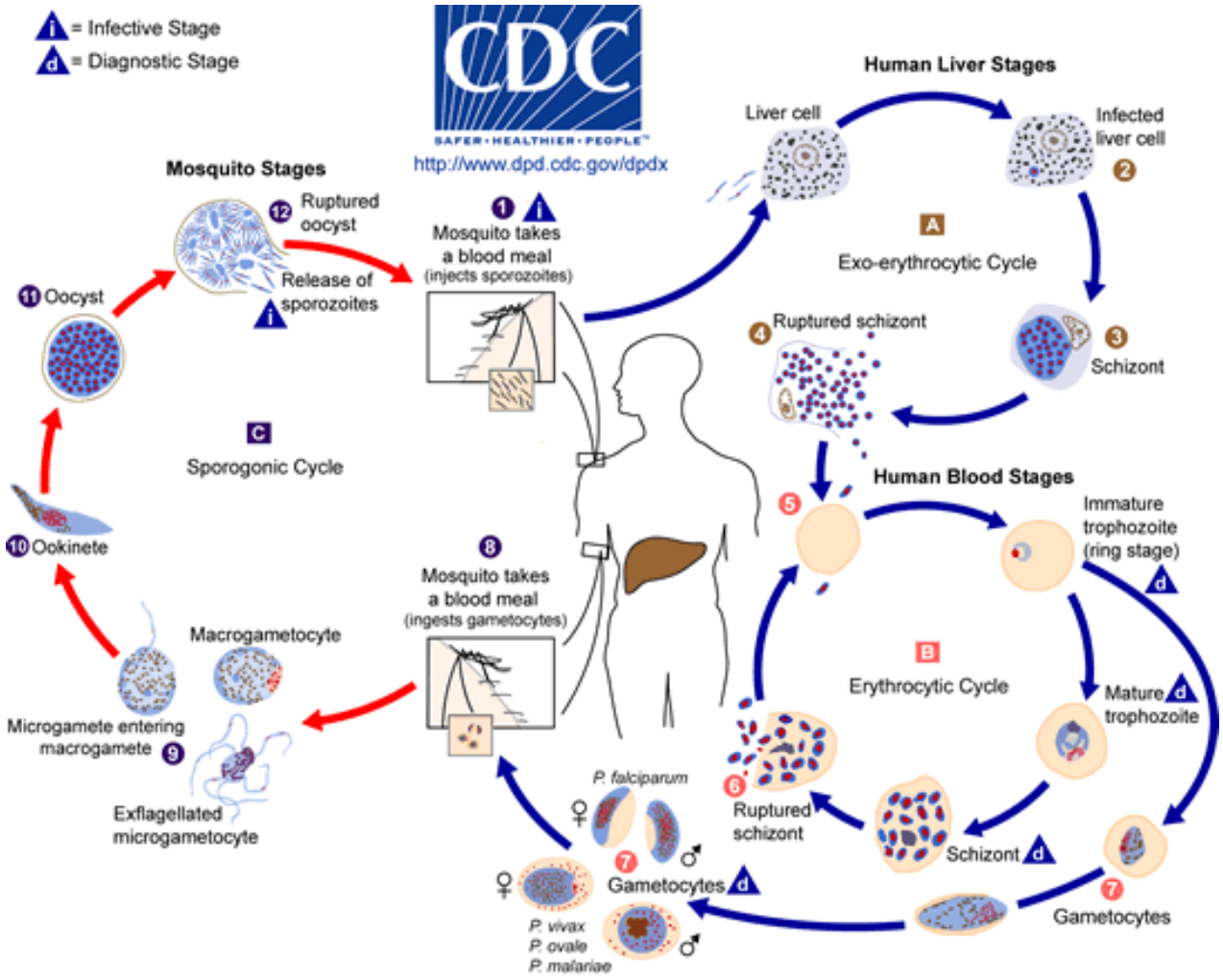
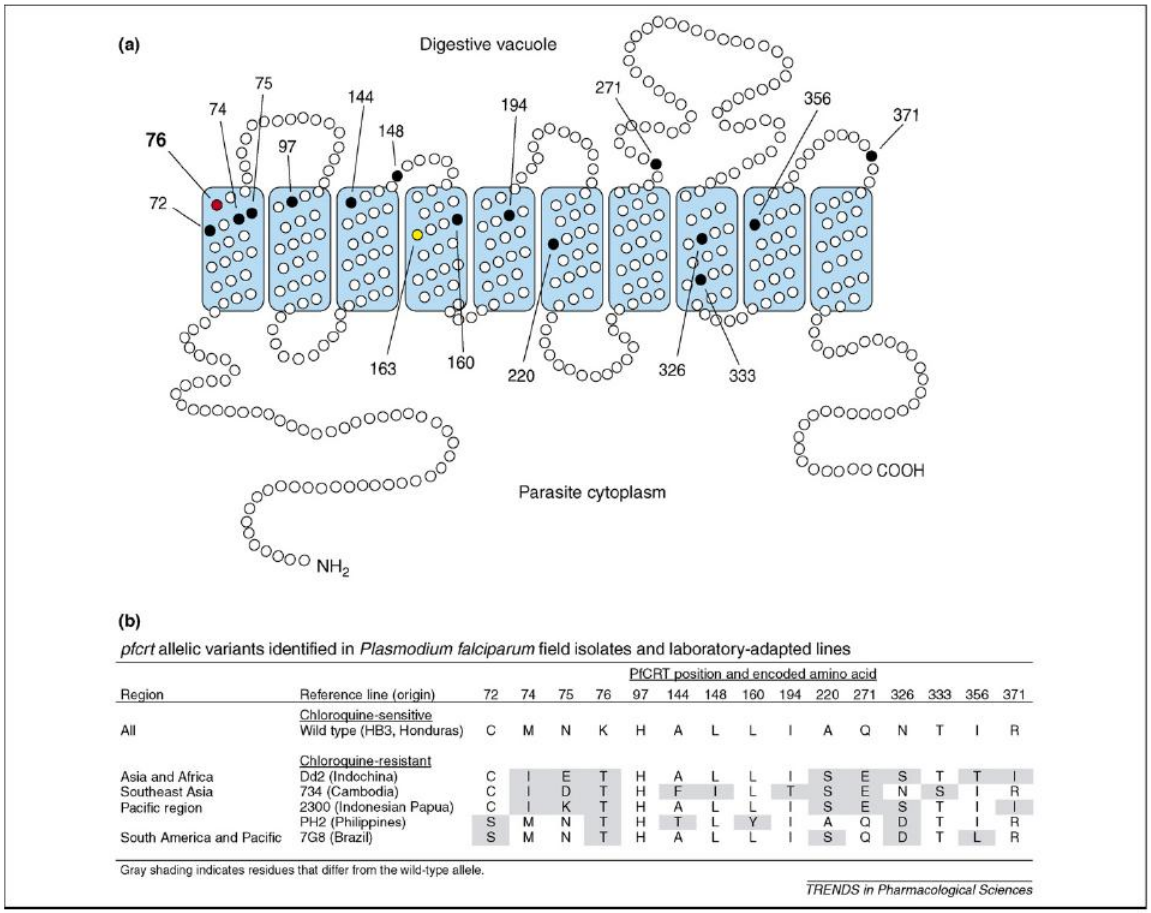


Figure courtesy of the Centers for Disease Control and Prevention

Figure 1.2 The predicted structure of *pfcrt*



(b)

pfcrt allelic variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

		PfCRT position and encoded amino acid														
Region	Reference line (origin)	72	74	75	76	97	144	148	160	194	220	271	326	333	356	371
	Chloroquine-sensitive															
All	Wild type (HB3, Honduras)	C	M	N	K	H	A	L	L	I	A	Q	N	T	I	R
	Chloroquine-resistant															
Asia and Africa	Dd2 (Indochina)	C	I	E	T	H	A	L	L	I	S	E	S	T	T	I
Southeast Asia	734 (Cambodia)	C	I	D	T	H	F	I	L	T	S	E	N	S	I	R
Pacific region	2300 (Indonesian Papua)	C	I	K	T	H	A	L	L	I	S	E	S	T	I	I
	PH2 (Philippines)	S	M	N	T	H	T	L	Y	I	A	Q	D	T	I	R
South America and Pacific	7G8 (Brazil)	S	M	N	T	H	A	L	L	I	S	Q	D	T	L	R

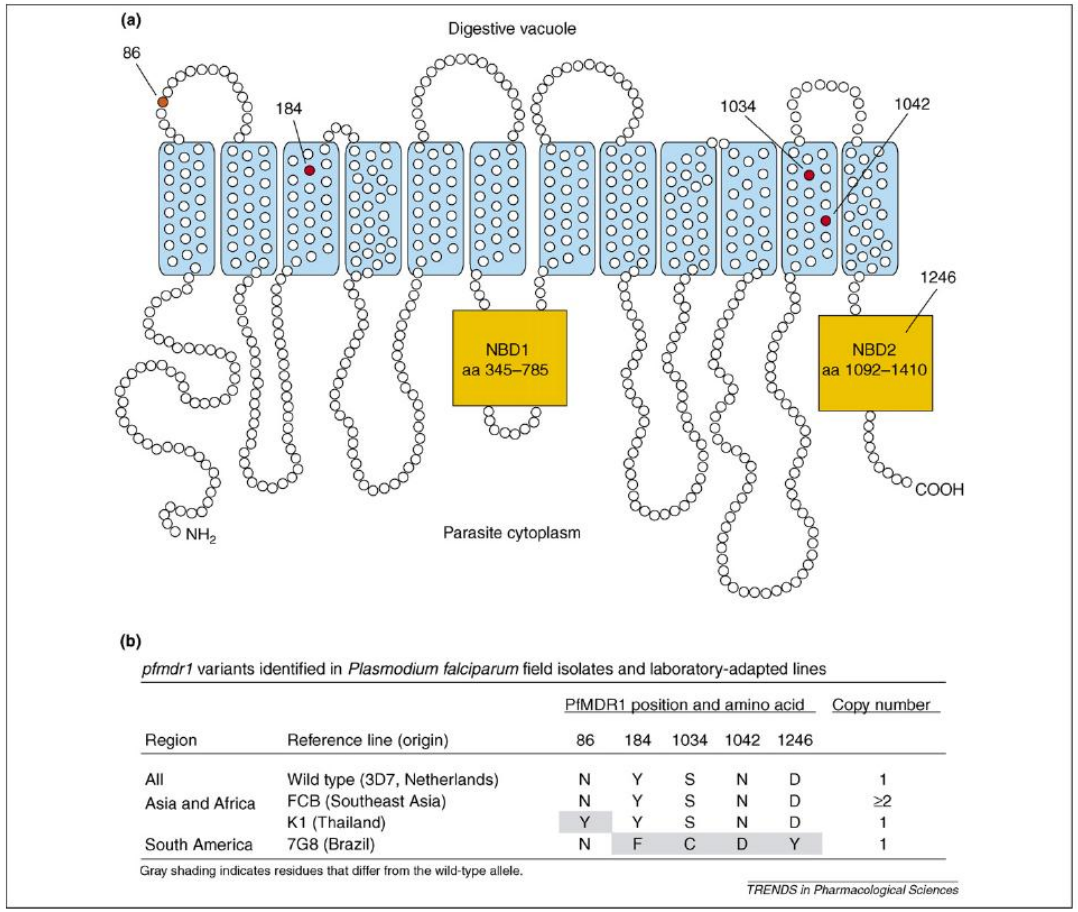
Gray shading indicates residues that differ from the wild-type allele.

TRENDS in Pharmacological Sciences

Figure 1. Predicted structure and representative haplotypes of *P. falciparum* chloroquine resistance transporter. **(a)** PfCRT is predicted to have ten transmembrane domains, with its N and C termini located on the cytoplasmic side of the digestive vacuole membrane (adapted, with permission, from Ref. [20]). Mutations identified in *pfcrt* cDNA sequences from CQR lines (black circles), the crucial K76T mutation common to all CQR strains (red) and the S163R mutation identified in amantadine- and halofantrine-resistant parasites (yellow circle) [24] are indicated. **(b)** Representative *pfcrt* haplotypes.

From [19]

Figure 1.3 The predicted structure of *pfmdr1*



(b)

pfmdr1 variants identified in *Plasmodium falciparum* field isolates and laboratory-adapted lines

		PfMDR1 position and amino acid					Copy number
Region	Reference line (origin)	86	184	1034	1042	1246	
All	Wild type (3D7, Netherlands)	N	Y	S	N	D	1
Asia and Africa	FCB (Southeast Asia)	N	Y	S	N	D	≥2
	K1 (Thailand)	Y	Y	S	N	D	1
South America	7G8 (Brazil)	N	F	C	D	Y	1

Gray shading indicates residues that differ from the wild-type allele.

TRENDS in Pharmacological Sciences

Figure 3. Predicted structure and genetic polymorphisms in *P. falciparum* multidrug resistance-1. (**a**) PfMDR1 has two homologous halves, each with six predicted transmembrane domains and a nucleotide-binding pocket [56]. The nucleotide-binding domains (NBD1 and NBD2; orange boxes) are each formed by large cytoplasmic domains. Polymorphic amino acids found in the K1 allele (N86Y) and the 7G8 allele (Y184F, S1034C, N1042D and D1246Y) are indicated. The D1246Y mutation is located in the predicted NBD2. (**b**) Representative haplotypes, including the one most commonly associated with amplification of *pfmdr1* copy number.

From [19]

Figure 1.4 The active site of *dhfr*

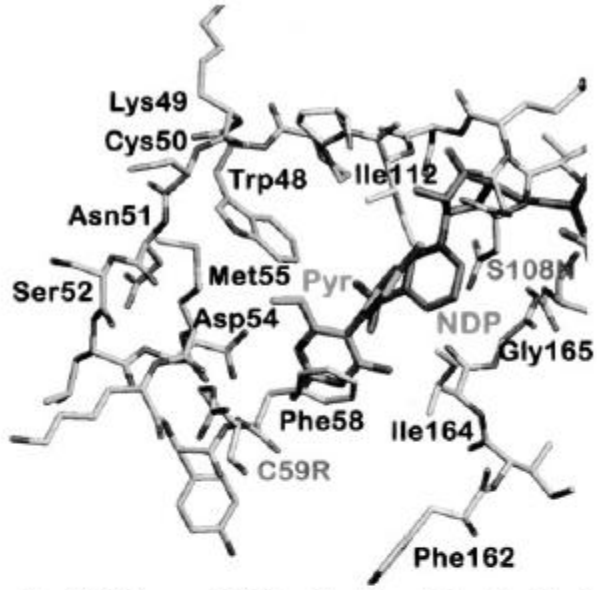


FIG. 2. DHFR enzyme-inhibitor interactions at the active site. The DHFR double mutant, S108N/C59R, complexed with pyrimethamine. Reproduced with permission of the authors (Yuvaniyama et al., 2003).

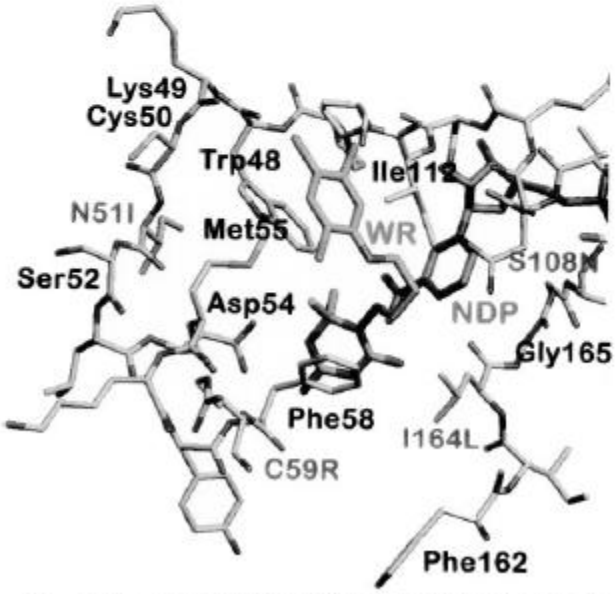
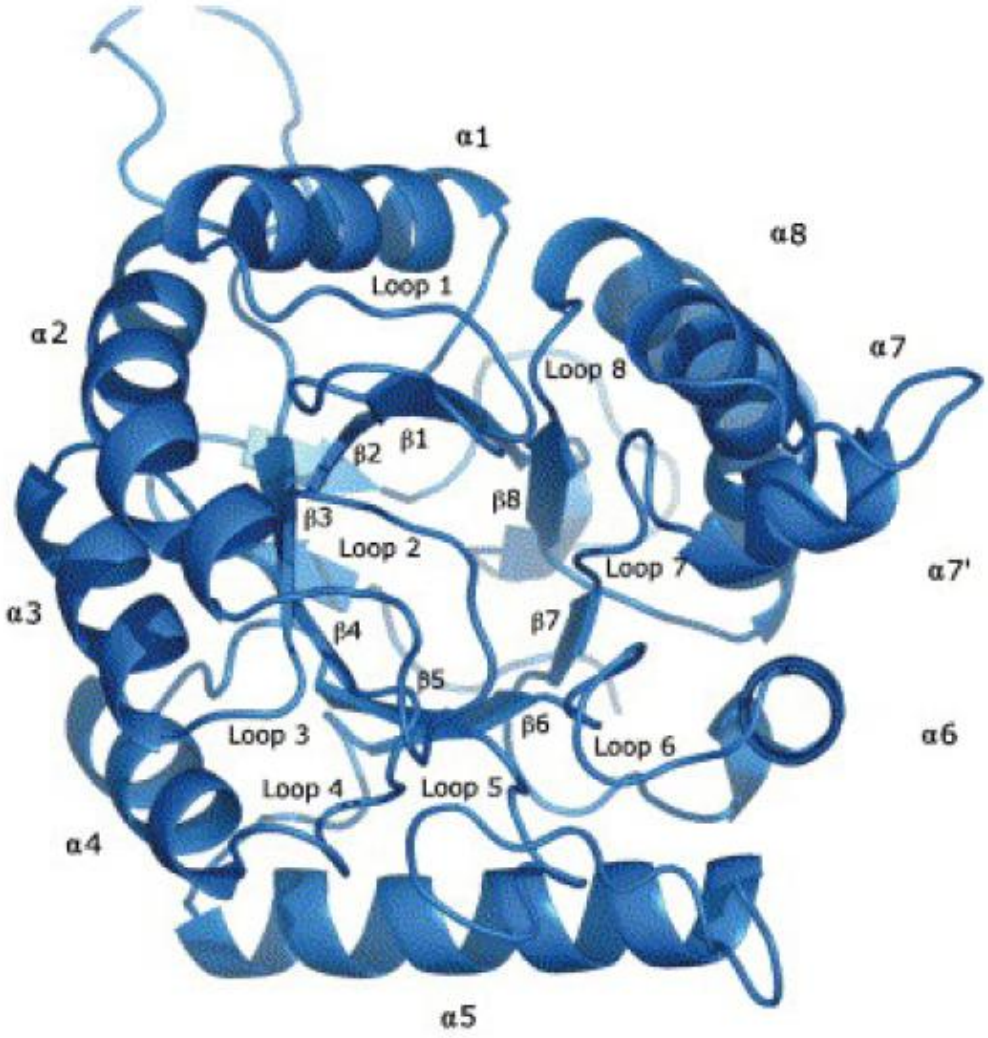


FIG. 3. DHFR enzyme-inhibitor interactions at the active site. The DHFR quadruple mutant, S108N/N51I/C59R/I164L, complexed with WR99210. The flexibility of the WR99210 tail allows its binding to DHFR to be unaffected by known mutations. Reproduced with permission of the authors (Yuvaniyama et al., 2003).

From [9]

Figure 1.5 The predicted structure of *dhps*



A steric view of each domain of the *Plasmodium falciparum* PPPK–DHPS model.

From [44].

CHAPTER 2

THE EARLY HISTORY OF SOUTH AMERICAN MALARIA

Authors have differing views on when malaria was first introduced to the new world, with some arguing that it occurred prior to the voyages of Columbus [1]. The small group of Eurasians that crossed the Bering Land Bridge from 12,000 BCE -9,000 BCE was so sparse as to have been unable to support any *P. falciparum* or *P. vivax* populations. By this premise, the earliest opportunity for malaria to be introduced to the New World would have been in the 10th century by Scandinavians traveling from Greenland, though it is suggested that this too would have been a difficult introduction due to low population density [2].

It is more likely that *P. vivax* could have been spread to the New World by Europeans during the 16th century due to its long dormancy periods and prevalence in northern Europe (*P. malariae* was also present). On the other hand, *P. falciparum* was only seen in southern Europe and there seasonally. It would have been difficult for this parasite to survive the Atlantic passage. Therefore, it has been argued that *P. falciparum* was introduced, along with the trade in enslaved Africans, from West Africa, where it was highly endemic [2].

While early explorers may have successfully introduced malaria to the New World, it is likely that Hernando Cortez's invasion of Mexico from 1519-1521 was sustained enough to assure its successful introduction into the dense Aztecan population. The time he and his soldiers spent in Tenochtitlan could have also introduced *A. pseudopunctipennis* and *A. maculipennis* var. *aztecas*. By 1547, the Spaniards had conquered the Yucatan peninsula and likely spread malaria throughout the region. Francisco Pizarro y González's invasions of Peru and the Andes Mountains (1524-1532) likely spread malaria to the Incan Civilization [2].

Initially malaria would have predominated on the coastal plains, Andean foothills, and the interandean valleys, where *A. pseudopunctipennis* could survive (it is reported to support

malaria above 2,600 feet in Bolivia). However, the deserts of Chile would have blocked its spread to the south. While malaria was common in the highlands of Peru and Bolivia, it was not common north of Ecuador [2]. If it arrived prior to the Europeans, it would have had a patchy distribution; Amerindian civilizations lived along mountain plateaus and high altitude valleys, where malaria would have found it difficult to gain a foothold [1].

During the 16th century, Peru also gave the world the bark of the Cinchona tree, which is the source of quinine, an effective antimalarial[1]. There are competing histories regarding whether the indigenous population or the Spanish invaders first discovered the value of this medicinal plant for the treatment of malaria [1]. Cinchona was first was reported to have been used by Father Antonio de la Calancha in 1633 [1]. However, its more famous supposed use was between 1623-1633, when Dr. Juan de la Vega gave it to the wife of the Peruvian Viceroy (the Countess of Chinchon) for the treatment of malaria [1]. She or her doctor, in turn, introduced the drug to Spain upon their return [1]. However, more critical scholarship suggests that this story may be fabricated [1]. The other portion of this legend suggests that the Jesuits sent the bark to Spain and Rome around 1631 after its medicinal properties had been proven in Lima, Peru. Regardless it was used in Spain by 1639 [1]. In the early 1640s, "Jesuit's powder", as it was known in Europe[2], was used by the Catholic Church in Rome and then throughout the Jesuit network[2]. By 1647, it was being regularly supplied to Italy from Peru and just three years later was used at Jesuit colleges in Genoa, Lyon, Louvain, and Ratisbon [1]. During much of the rest of the century, the drug was looked on with mistrust by Protestants on political, theoretical, and religious grounds. Later, success of this antimalarial lead to the overharvesting of the tree [2].

P. falciparum was more likely introduced by Portuguese colonists to the Brazilian coast during the 1530s seeking Brazil wood and its rich red-purple dye. This industry began to incorporate a workforce made up of slaves collected during raiding trips into the interior of the

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[1] This was so important to the country that it still appears on their flag.
[2] Other names included "Powder of the Countess" (*Pulvis comitissae*) and "Powder of the Cardinal" See: *El paludismo en América* Latina and *An illustrated history of malaria* in the refererences

country. By the middle of the century, Amerindians were drawn into the sugarcane plantations in Northeast Brazil as slaves and, as they did not survive the conditions or European disease, were supplemented with African slaves. The Africans carried the Duffy mutation and were thus *P. vivax* resistant. This initial *P. falciparum* zone spread north to the coast of Guyana, and portions of the Caribbean by the end of the century [2].

North American malaria was not established until 1607 in the English colony of Jamestown. Many of these colonists came from greater London, where malaria was rampant. Malaria (*P. malariae* and *P. vivax*) became endemic in the Chesapeake Bay to the north. A substantial number of African slaves began to arrive during the 1660s and most likely established *P. falciparum* in Virginia and in South Carolina in the 1680s. As more colonies were created, with higher population densities, *P. falciparum* became established. *P. vivax* predominated all the way up to Massachusetts. As one moved inland, the number of cases decreased. As one moved south, *P. falciparum* became more common, such that it predominated in southern Virginia, South Carolina amongst the rice fields, and south all the way to the Caribbean.

This north/south division of malaria species continued across new territories as the United States began to expand west. However, malaria incidence decreased over a few generations in the north because the breeding grounds of the principal vector, *A. quadrimaculatus*, were removed by the farmer's plow. To the south, the Mississippi flood plain kept this vector from disappearing and malaria continued [2].

Much later, the Rockefeller foundation experimented with malaria control in the southern United States by "cleaning up" stagnant water, the prime breeding ground of *A. quadrimaculatus* in 1915, concluding that malaria elimination was possible in 1918. They conducted work in the Caribbean for the next few years, as well as Italy, where swamp draining, quinine, and improvements in housing and education led to a massive decrease in malaria. The Rockefeller foundation would continue to work in the United States and elsewhere in the world, including Sardinia. Its work also extended to South America, particularly Venezuela and Brazil [2].

The Brazilian sugar plantations were supplanted as an industry by the discovery of surface gold in the late 1600s, and diamonds in the early 1700s, in the interior in the towns of São Vicente and São Paulo. This led to an influx of Portuguese colonists and African slaves, which extended all the way into the province of Minas Gerais (General Mines) where they set up mining camps. This, in turn, led to the development of supporting industries including cattle ranching and slash-and-burn agriculture. Disruption of forest habitat left conditions attractive to *A. darlingi*, which would go on to be the major South American malaria vector. All of these changes acted to establish *P. falciparum* and *P. vivax* in the interior of Brazil [2].

As Argentina did not have a sugar industry, suitable vectors, or African slaves, it was the southernmost limit of *A. darlingi*'s distribution and the South American malarious zone. However, during the late 19th century Italian and Spanish immigrants settled in the northwest of the country to claim agricultural land and malaria became endemic. In 1911, the government launched an anti-malaria campaign that included education, the free distribution of quinine, swamp draining, and the use of larvicides. By the 1920s they had created their own quinine industry [2]. Figure 2.1 describes the distribution of malaria in 1970 in the new world.

In the following sections I will describe the history of malaria treatment during the 20th century in Brazil, Peru, and Venezeula. I will also describe what is known regarding the spread of CQ and SP resistance, as well as mutations in *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* that are thought to contribute to drug resistance.

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Figure 2.1 The distribution of malaria in the New World, 1970.

FIGURE 1. Status of the Malaria Eradication Program in the Americas, 31 December 1970.[2]

From [4]

CHAPTER 3

THE HISTORY OF MALARIA IN PERU

Overview

Plasmodium falciparum, P. malariae, and *P. vivax* have been reported to be present in Peru. Mosquitoes are rarely seen above 1,500 meters in altitude because of the low temperatures and humidity. Therefore the Andes Mountains break the country into two major malarious zones: the Pacific coast and the Peruvian Amazon [1].

The coast is a succession of valleys separated by desert and carved by rivers falling steeply from the Andes Mountains. When rain falls in the Andes from January to March, the rivers engorge and follow erratic courses through the valleys, creating swamps and other environments with stagnant water. This water is conducive for development of *Anopheles* mosquito larvae, especially at the end of the rainy season [1]. Malaria transmission is unstable with a peak between March and August [2]. Years with massive rainfall like 1925 or 1965 increase vector populations and create the conditions for large outbreaks of malaria. The coastal climate is also generally condusive to mosquitoes, maintaining a moderate temperature of 16˚C or above and humidity around 70% [1].

Anopheles pseudopunctipennis is the most common coastal *Anopheles* species. *A. pseudopunctipennis* deposits its eggs in shallow sunny water near algae. After the rainy season, the larval populations disappear from the lower parts of the valleys and maintain populations at higher altitudes near springs, water pools, and river backwaters. A report by PAHO/WHO claims that *A. pseudopunctipennis* is the primary malaria vector in coastal Peru [3]. However, another author argued the major main vector is *A. albimanus* [2]. The frequency of both vectors varies depending on climatic conditions and *A. pseudopunctipennis* is considered the more efficient vector (personal communication, Nancy Arrospide).

Unlike on the coast, rain in the Peruvian Amazon disrupts *Anopheles* larval habitat. In the interior, the period with the greatest rainfall is June to August. During the dry season, the rivers quiet and allow for the formation of swamps and pools where mosquitoes can breed. Rain is more persistent in the portions of the Peruvian Amazon that are below sea level, which makes it harder for *Anopheles* mosquitoes to breed. Habitat changes that lead to flooding of land generate additional anopheles breeding grounds. These changes include the cultivation of rice and gramalote (a grass used to feed cattle), deforestation, poor maintenance of canals, irrigation canals and tanks, excavations left over from constructions, roads [1] and aquaculture [4].

Prior to the reintroduction of *An. darlingi* to Peru in the 1990s, 98% of the anopheline fauna was *An. benarrochi,* where *P. falciparum* and *P. vivax* were endemic during the 1970s [5]. *An. Triannulatus* is another important vector in the eastern Peruvian Amazon. Other vectors in Loreto, a department that contains the majority of the Peruvian Amazon, include *An. oswaldoi*, *An. nuneztovari*, and *An. rangeli*. [6] (Figure 3.1). In 1995, *An. benarrochi* was found in the departments of San Martin, Huanaco, Junin, Cusco, Ucayali, and Madre de Dios and Loreto.

An. benarrochi is still the most important malaria vector in the western Amazon [6] in towns like Alianza and Yurimaguas [7] and limited eastern localities, but *A. darlingi* invaded Peru during 1990s and replaced it in the eastern and central and Peruvian Amazon [5,8]. In 1995, *An. darlingi* was reported in Cuzco, Madre de Dios, and Loreto [8]. *An. darlingi* now appears to be the most important vector around Iquitos, the largest city in the department [6]. *An. darlingi* is a highly competent and anthropophilic malaria [6] and considered the most important malaria vector in South America [4,6]. It is a riverine species that “favors large ponds, and the presence of leaf litter, algae, and emergent grasses”, but has been found in “irrigation canals, rice fields, flooded cane fields and pastures [4].

History of Peruvian Malaria

At the beginning of the 20th century, human populations on the Pacific coast and in the Peruvian Amazon were exposed to malaria as children and developed partial immunity. However, individuals from the Andes were not exposed and were thus immunologically naïve. When Andeans travelled to malarious regions they had a high risk for acquiring malaria. Migration from the mountains to the coast and jungle increased at the beginning of the 20th century because of the demand for workers during the expansion of plantations and businesses focused on sugar, rice, timber, fruit, and natural rubber. The greatest demand for agricultural workers was during the times of sowing and harvesting, which unfortunately coincided with the times of the year with the greatest density of *Anopheles* mosquitoes. As migrant labourers from Andes had little immunity to malaria they succumbed to malaria easily [1].

During the first decades of the 20th century, larval populations were controlled by the spraying Paris Green or oil (which limited the ability of larva to breathe) and the draining of water reservoirs. Mosquito nets, home fumigation, and metal roofs were also used in control efforts. Malaria was treated with quinine and later drugs including plasmoquinina (chloroquine sulfate) and atebrina (quinacrine) [1].[3]

The first official malaria control action by the federal government was in the form of a 1916 law called "Malaria Prophylaxis"[4] in response to an epidemic in Chanchamayo on the coast. Planters were required to distribute quinine for free, destroy *Anopheles* larva, and separate living quarters a minimum distance from rice paddies and sugar cane fields. In addition, quinine and metal roofing (it was assumed that mosquitoes were able to enter homes through roofs) were no longer taxed. Some planters from the northern and central coast complied, maintained a medical service, provided quinine, and cleaned canals and ditches. However, they were less likely to drain

[3] It is unclear when plasmoquine and quinacrine were first used. However, the Germans did not approve the second drug for use until the early 1930s and a report suggesting using plasmoquine and quinine together was published 1933 From: Schwartz M (1933) Treatment of Malaria By Plasmoquine And Quinine. British Medical Journal 1: 995.

[4] Ley 2364 de 1916: Profilaxis al Paludismo

wetlands which were considered irrigation reservoirs or reconstruct worker housing. In the jungle, planters formed a Philanthropic Society that managed a hospital from 1908 to 1918. After that, the state supported the hospital [1].

Despite these efforts, a major epidemic (thought to have been *P. falciparum*) occurred in the province of "La Convencion" during 1932 (in what is now the department of Cuzco in the southern inland portion of Peru). It was larger than any of the epidemics that had come before, according to the oldest residents, including those between 1898 and 1902 [1]. This epidemic, and that of 1934, killed 32% of the Peruvian population at risk of infection [9]. Workers on the outskirts of the province became infected in August, most likely from an indigenous population. Recent deforestation, road building, and heavy rains led to the puddles and pools of stagnant water that allowed mosquito breeding. The epidemic spread rapidly along the province's rivers and even reached the foothills of the Andes. The intense mortality and rapid spread of this epidemic is explained by the movement of merchants, rural workers, and those fleeing the epidemic. From November and December, a Dominican field hospital treated 2,770 cases [1].

The epidemic ran its course by the end of April 1934, as *P. falciparum* was replaced with *P. vivax*. Out of a population of 25 thousand people, 6-10 thousand people died and another 15 thousand became sick in two years. This epidemic led to the development of the Antimalaria Service of the Health Directorate of Cuzco [1].

Peru centralized its public health response in 1933 by creating the Hygiene and Prophylaxis Service of the Health Directorate, which received some support from the National Agrarian Society in the form of quinine sold at cost. Initial efforts focused on the transmission conditions and control of malaria in Carabayllo on the Northern Pacific coast. In 1937, another study was started in Cañete to the south of Lima. These groups united in the same year to form the Malaria Expert Department of the recently created Ministry of Public Health, Work, and Social Forecasting. The ministry conducted malaria campaigns in various regions, constructed malaria hospitals, and hired special personnel including engineers [1]. In 1941, the ministry also

created the National Antimalarial Service of Rural Sanitation which acted as an assistance, prevention, and statistics service for the ministry. Eight antimalaria services were created in Lima, Cuzco, Ayacucho, Cañete, Chancay, Camaná and the Moche and Tambo valleys. During the war with Ecuador in 1941, both armies had to deal with malaria [9]. In 1942, the ministry changed names and became the Ministry of Public Health and Social Assistance. With the name change, the national services gained greater autonomy, more staff, and larger budgets [1]. During the first half of the 1940s, Paris green was used as a larvicide [9]. It became expensive due to World War II and copper aresinite was manufactured locally as a replacement. Road construction and other public projects were supervised in order to impede the development of larval breeding pools. There were those that argued that, if public health was made a clear priority, the Peruvian Amazon could be made habitable and profitable [1].

At this point, Peru began to receive additional assistance from outside the country. In 1942, a Pan-American conference was held that led to the development of the Interamerican Public Health Cooperative Service (SCISP). During this period, the government also received donations from the Rockefeller foundation for disease prevention. These donations went to support the national malaria and yellow fever services, the National Hygiene Institution, an experimental sanitary service in Ica, and a test eradication of *Anopheles* in a coastal valley. Foundation support gradually decreased from 1941 to 1948 as the government began to pay for more [1].

The Rockefeller Foundation also introduced DDT to Peru [1]. DDT is a long lasting insecticide, which limited the number of sprayings required to control vectors, and cost less than other insecticides. By 1947, the National Antimalaria Service was spraying DDT in 16 coastal valleys. By 1953, DDT was being sprayed in 47 coastal valleys and 3 in the Peruvian Amazon. Malaria morbidity went from 945 per 100,000 (1941-1946) to 490 per 100,000 (1947-1958). In 1944, there were 95,349 cases of malaria, but there were only 20,000 by 1950 (~67% were on the coast and 33% were in the Peruvian Amazon) [1]. After the control efforts of the 1950s, there

were less than 1000 a year, more than 80% of which were *P. vivax* and none were *P. falciparum*, (the source is unclear regarding the cause of the remaining cases but it includes *P. malarie*)in the districts bordering with Ecuador, Colombia, and Brazil [10].

These successes, and the increased urbanization of the coast, encouraged Andean migration from the mountains to the coast and Amazon. Chimbote, for example, went from 4 thousand inhabitants in 1946 to 50,000 inhabitants by 1958. The Peruvian malaria control efforts between the 1940 and 1972 caused a massive social shift from a population that predominately lived in the rural mountains to one that lived predominately in coastal urban zones. Still, between 1946 and 1955, malaria was the first ranked cause of morbidity in Peru and the 10^{th} ranked cause of mortality [1].

By 1957, the goal shifted from the control of malaria to its eradication through the support of SCISP and the United Nations International Children's Emergency Fund (UNICEF). The country organized a National Service for the Eradication of Malaria and funding from the National Health Fund and Social Welfare. They thought that five years of aggressive application of antimalarials and insecticides would be enough to eradicate malaria. It would also have the added benefit of controlling yellow fever, dengue and murine typhus. The campaign was to have four stages: 1) preparation, defining the area of work, organizing personnel, and establishing sentinel stations, 2) attack, requiring the application of DDT every six months for four years to homes, the administration of medications to interrupt transmission, and the evaluation of blood samples from the sentinel stations, 3) consolidation, lasting 1-3 years, beginning when transmission was interrupted, during which mosquitoes and illness would be eliminated, 4) maintenance, a period during which the absence of malaria would be verified and its eradication confirmed [1].

The effort began in November, 1957 with 67,633 sprayings of DDT on the coast, with malaria disappearing from various departments that same year. Between 1959 and 1962, sprayings did not go below 600,000 per year in five different zones across the country. By 1965,

Piura, Tumbes, La Libertad, Ica, Callao, Arequipa and Huancavelica had all spent 3 years in the consolidation stage (all sites located on the coast) and only 1,500 cases were reported across the country [6]. The remaining cases were chloroquine sensitive and generally seen on the Peru/Ecuador border with a few cases on the borders of Loreto with Ecuador, Colombia, and Brazil [6]. By 1970, almost the entire coast was malaria free, as well as the interandian valleys, and in the southern portions of the Peruvian Amazon. The remaining malaria in the northern Peruvian Amazon was, in most cases, *P. vivax* or *P. malariae*. Unfortunately, 1970 was also the turning point when malaria began to increase again in Peru as funding disappeared and mosquitoes became resistant to DDT. *P. falciparum* was also found to be resistant to CQ [1].

By the 1980s there was no longer a structured program to control malaria [1]. In 1984, less than 1% of malaria cases (47 cases) were caused by *P. falciparum* and all came from the Amazon [11]. In 1988, there were no cases of *P. falciparum* reported in the country and transmission had been interrupted for many years [12], though *P. vivax* cases had been gradually increasing from a low of 1,484 cases in 1963 to 39,122 cases in 1987 [12]. In 1989, there were 65 *P. falciparum* cases in the country [12]. During the 1990s, *P. falciparum* disseminated throughout the country [1]

Between 1992 and 1997, malaria increased 4-fold in Peru and 50 fold in Loreto, a department that makes up one fourth of the land mass of (348,177 square km) [4,6]. In 1990, there were 28,882 cases of malaria in Peru, of which only 131 were *P. falciparum*, principally in the northern coastal departments of Piura and Tumbes [12]. The Peruvian Amazon basin and the northern pacific coast account for 85% of the malaria cases in Peru and 95% of *P. falciparum* cases [13].

During the 1990s, there were two major outbreaks (Figure 3.2). One was on the Pacific coast and the other in Loreto in the Peruvian interior. Interestingly, there was also an outbreak in the department of San Martin in 1993, which is in the Peruvian Amazon [14], but close to the Andes (*P. falciparum* was still in this state in the late 1990s). In response to the epidemics, the

National Malaria Control Program used insecticide spraying, active case detection, and mass drug administration [13].

Loreto and the Epidemics of the late 1990s

Loreto is a region of Amazonian lowland forest and the source of the Amazon River, where the Marañon and Ucayali Rivers join. In Loreto, humidity is usually higher than 87% and the warmest months are September to October, with a mean temperature of 28°C. Typically, Loreto has a rainy season that lasts from November to May with some rain in September during the dry season [6]. Iquitos is currently the largest city in Loreto (Figure 3.3). In 1842, Iquitos only had 200 inhabitants, but the rubber boom of the early twentieth century increased this number to 14,000 by 1903. Iquitos experienced significant population growth since then, reaching a population size of 305,514 in 1993 and 351,940 in 2006 [4] of mestizos (people of Amerindian and Spanish decent) [15]. In 1999, Loreto had 819,000 inhabitants with 474,000 spread about the Amazon tributary system and 345,000 inhabitants in the city of Iquitos [6]. This burgeoning population led to rural expansion and deforestation, with an estimated 4,257 hectares of forest cleared between 1983 and 1995 [4]. Another settlement has extended away from Iquitos by following the path of the partially built (95 km) road between Iquitos and the city of Nauta with deforestation expanding around the road [4].

In the early 1990s, malaria began to reemerge on the eastern borders of Loreto and the upper Pastaza River [6] (Figure 3.4). In 1988, there were no cases of *P. falciparum* in Loreto, but in 1991 there were 140 cases [6], with an outbreak in the Pastaza River valley [14]. The next year there were 123 cases of *P. falciparum* and 518 cases of *P. vivax*, with a malaria prevalence of 2.1/1000 [4]. In 1993, cases were reported in the northern most point of Loreto in Gueppi, to the west on the Pastaza river and in Yurimaguas, to east in Baja Putumayo and Atlántida, and to the southeast in Requena-B Lomas [16]. It was first reported in Padrecocha in 1994 [6], a riverine village of 1,400 inhabitants, 5 km from Iquitos [13]. By 1997, there were 121,268 slide confirmed

cases of malaria, of which 45% were *P. falciparum* with a malaria prevalence of 343/1000, though *P. malariae* remained rare (44 cases in 1997 in Loreto). Loreto accounted for 67.2% of all malaria cases in all of Peru that year [6].

While the majority of the cases occurred around Iquitos, there were outbreaks throughout Loreto. There were two areas of high transmission near Iquitos. One was communities along the Nanay River, downstream of Iquitos. The other was along the unfinished Iquitos-Nauta asphalt road. Elsewhere in Loreto, the communities around the Yavarí and Pastaza Rivers were areas of regional high transmission. Malaria was generally hypoendemic, with periods of mesoendemicity [6].

During this massive epidemic, *Anopheles darlingi* was reintroduced to Peru. Previous eradication efforts had eliminated *An. darlingi* from the Peruvian Amazon in the 1960s and it did not reappear until the early 1990s [17]. *An. darlingi* was not present in 1991 in Iquiotos, but was when *P. falciparum* infections rapidly increased. Once established, it made up more than 90% of the *Anopheles* mosquitoes around Iquitos during the rainy season and remained the major vector during the dry season. The rainy season was also the period during which the most cases of malaria were seen. Around Iquitos, *An. darlingi* larvae developed in "cleared land, fish hatcheries, areas of poor sanitation, swamps, and the edges of small rivers." The agricultural practices of non-nomadic farmers led to the development of habitat suitable for *An. darlingi;* namely, deforestation of the area around a village for farming and expansion farther into the forest as the land became infertile [17]. Taken together, these facts suggested that habit change by humans allowed *An. darlingi* to expand in Loreto and allowed for the rapid expansion of both *P. vivax* and *P. faliparum* [4,6].

In response to this epidemic, the National Malaria Program and Loreto Public Health Department provided both malaria diagnosis and free treatment with CQ as the first line medication, SP as the second line, and quinine with clindamycin or tetracycline as the third line. Depending on where the malaria case occurred, different drugs were used [6] (Figure 3.5).

However, drug policy changed throughout this epidemic. In 1996, SP replaced CQ as the first line of treatment in the eastern Amazon region and this was replaced by a 7 day course of quinine plus tetracycline in 1997, based on limited *in vivo* efficacy data and drug resistance monitoring by the Peruvian National Malaria Control Program [13].

CQ resistance was present on the river of Putumayo in the Gueppi, Yubineto-Angusilla and Alamo epidemics on the border with Colombia in 1979 and 1980 [16,18]. CQ resistance was reported as far back as 1986 at Aquarico-rio Alto Napo and on the Pastaza river and its tributaries on the border with Ecuador in Loreto [16]. Later, reduced CQ and SP sensitivity had been noted in a number of unpublished *in vivo* studies from 1993 to 1997 from the Peruvian Amazon [13]. However, these studies used varying methods and, as the Ministry of Health did not see them as a coordinated survey of drug resistance, they had little impact on public health policy leading up to the epidemic [13]. Yet they did cause concern about drug resistance and all patients treated for *P. falciparum* by the National Malaria Control Program were requested to come back for follow up blood smears 7 and 14 days after therapy. Self treatment in Peru was (and is) uncommon, despite the availability of antimalarial drugs available in pharmacies without prescription, because treatment at government facilities is free [13].

Insecticides were used for fogging, domiciliary spraying, and bed net impregnation. DDT use in this department ceased in 1988 and therefore pyrethroids including cyfluthrin were used. Unfortunately, the efficiency of domiciliary spraying may have been diminished because mosquitoes could enter many homes through windows or open eaves without ever touching any surfaces. Bed nets were either sprayed with cyfluthrin or deltamethrim or impregnated with permethrim or deltamethrim. The elimination of larval breeding sites was done with temephos (tetramethyl-thiodiphenylene phosphorothioate). They also experimented with biological control using *Bacillus sphaericus* and *B. thuringiensis*. El Niño's extension of the dry season in 1997 may have also assisted control efforts [6].

On the Pacific coast of Peru, there was an outbreak of *P. falciparum* in 1987 in Zarumilla, Tumbes to the north. In 1991, another outbreak occurred in Sullana, Piura also to the north [14]. Two years later, it was again reported in the coastal departments of Tumbes, Piura, Lambayeque, and Cajamarca [14]. However, the major epidemic began after heavy rains and flooding caused by the El Niño event in the late 1990s [19]. In 1997, there were 6,000 *P. falciparum* cases and the next year there were 51,000 [13]. During this epidemic, 10-20% of malaria cases were *P. falciparum* [2].

Peruvian Public Health Response to Epidemics

In response to these epidemics across Peru, the Peruvian Ministry of Health became concerned about drug resistance. In the central Amazon, drug policy shifted to SP as the first line drug in 1996 based on limited *in vivo* efficacy data and drug resistance monitoring by the Peruvian National Malaria Control Program [13]. Early studies into the drug resistance in the Peruvian Amazon suggested there multiple *P. falciparum* strains in the area. One author argued that there were three *P. falciparum* strains that converged in Loreto and Iquitos based on *in vivo* drug resistance: a Brazilian strain that was completely resistant to SP and CQ, Loretana, which had variable resistance to SP and CQ, and Pastazan/coastal, which was CQ susceptible. This author also argued that they were introduced through routine travel or narcotrafficking [6]. Another author argued that there were two lineages of *P. falciparum* meeting in the Peruvian Amazon based on *pfcrt* genotypes (SVMNT and CVMNT) [20].

From 1997-1998, the Ministry, with the support of international partners conducted, a 14-day *in vivo* study to compare CQ and SP efficacy for uncomplicated *P. falciparum* cases, based on WHO recommendations around Iquitos. The study took place from March to May, 1998 in two health facilities in the area. In the study, more than 60% of patients had moderate to high level CQ and SP resistance. Markers that indicate drug resistance in both *dhfr* and *dhps* suggested a similar conclusion: 46% of the samples were triple mutant for both genes (*dhfr*, 62/108/164 and

dhps, 437/540/581), another 11% were double mutant for *dhfr* (51/108) and triple mutant for *dhps* (437/540/581). The remainder were either *dhfr* single 108 mutant combined with the wild type *dhps* (41%) or a *dhfr* double mutant 108/164 combined with a wildtype *dhps* (3%) [13], which suggest low levels of resistance [21].

Two samples from the 1998 study with triple *dhfr* and *dhps* triple mutations were later examined for *pfcrt* genotypes. One carried S_{agt}VMNT and the other S_{tct}VMNT [22]. Two additional samples, apparently not reported in the earlier paper, were *dhfr* 51/108 and *dhps* single mutant (437) along with *pfcrt* genotype CVMNT [22]. In another study from 1999, 44% of samples examined had the *dhfr* triple mutant (51/108/164) and 56% only had only the 108 mutation [15]. For *dhps*, 34% were triple mutant (437, 540, 613), 6% were double mutant (437, 581) and the remaining 56% were wild type [15]. For *pfcrt*, 53% of samples carried CVMNT and 47% carried S_{tct}VMNT. The situation for *pfmdr1* was more complicated, but triple mutants (144, 184, 1042 and 184, 1034, 1042) as well as quadruple mutants (184, 1034, 1042, 1246) were circulating [15].

In September 1998, it was decided to use SP with Q, plus tetracycline as the first line treatment in three districts around Iquitos, but this transition was not completed until early 2000 [13]. The drug policy changes did not appear to have an immediate impact on CQ resistance as a study conducted from 2001 to 2002 showed that 19/31 samples carried the S_{tct}VMNT genotype and the 12/31 carried the CVMNT genotype.

SP resistance in Iquitos was similar to that found in eastern Loreto. Thirty kilometers from the borders of Brazil and Colombia, there is a town called Caballococha with 3,300 inhabitants. SP resistance (RII/RIII) appeared to be similar (56%, [23]) to that found in Iquitos in 1998. This is in agreement with another study from 1999 where 65% of samples carried the *dhfr* triple mutant (51, 108, 164) and 35% carried only the 108 single mutant. For *dhps*, 18% carried the triple mutant (437, 540, 581), 48% carried the double mutant (437, 613) and 35% carried the wild type [15]. In addition, both *pfcrt* CVMNT (33%) and S_{tct}VMNT (77%) were seen. For

pfmdr1, double mutants were reported (184, 1042) as well as a triple mutant (184, 1034, 1042) and a quadruple mutant (184, 1034, 1042, 1246) [15].

In contrast to *P. falciparum* found in the eastern and central Peruvian Amazon, there was a different pattern of drug resistance in the northwestern Peruvian Amazon. There, malaria transmission peaked between March and August and 30% of cases were *P. falciparum,* with the remainder being *P. vivax*. The main vector was *Anopheles benarrochi*, which is considered to be a less efficient vector. SP use in the western Peruvian Amazon towns of Alianza, Pampa Hermosa, and Ullpayacu had been minimal in years prior to the epidemics of the late 1990s and CQ had been the first line treatment until late 2002. In January, 2003, this portion of the Peruvian Amazon switched to MQ plus AS. While roads from the coast lead directly to the western Peruvian Amazon, it is isolated from the remainder of the Peruvian Amazon as it is only accessible by river [7].

In Pampa Hermosa, there had been a *P. malariae* outbreak during 1986 and 1987, and there was circulating *P. vivax,* but the first *P. falciparum* cases occurred in 1992. In 1993, Pampa Hermosa had 1,680 inhabitants and an economy based on coca, plantains, yucca, rice, and beans.There were 436 *P. falciparum* cases, 205 *P. vivax* cases, 3 *P. malariae* cases, and 3 mixed infections. The authors recommended that SP be used instead of CQ as the first line of treatment for *P. falciparum* [16].

A study of *P. falciparum* drug resistance was undertaken in Alianza and Pampa Hermosa in 2000 and in Ullpayacu, a town to the north that is located on the Pastaza River and has 900 inhabitants, in 2002. In 2000, Pampa Hermosa and Alianza had 4,000 inhabitants [7]. Taking Pampa Hermosa and Alianza results together, only 14.3% of the 35 patients in the CQ arm of the trial had adequate clinical and parasitological response. RII/RII levels at both sites were 22.9 and 34.3 %. Among the 65 patients that received SP, 92.3% had adequate clinical and parasitological response. There were no cases of treatment failure in Alianza or Pampa Hermosa, though there were five in Ullpayacu. In Ullpayacu, all 29 analyzed samples had only one mutation in *dhfr*

(108). At Alianza/Pampa Hermosa 3 of the 26 samples analyzed had the S108 mutation, but the other codons (51, 59, 164) in all of the samples were wild type. All of these samples were wildtype for *dhps*. These samples included the five patients that failed SP treatment and therefore are likely to reflect the entire range of *dhfr*/*dhps* genotypes at these sites [7]. In summary, it appeared that the Peruvian Amazon was CQ resistant, but SP sensitive.

Coastal CQ resistance may go as far back as the 1970s because many of these cases appeared close to the border with Ecuador and may, therefore have spread from that country. In Ecuador, CQ resistance may go back as far as 1976 (Clyde 1987), but certainly at least as far back as 1987 [19] and the SP sensitivity was also noted there in 1987 as well [19]. In 1999, three coastal health centers were investigated for drug resistance including Zarumilla in Tumbes and La Arena and Bellavista in Piura [19]. CQ and SP sensitivity were evaluated at all sites, but MQ sensitivity was only tested in Zarumilla because it had been used in nearby Ecuador. Parasites had RII and RIII resistance to CQ and it was 53%, 65% and 58% in Bellavista, La Arena, and Zarumilla respectively. In contrast, RII and RIII resistance to SP was 10%, 3%, 0% in Bellavista, La Arena, and Zarumilla, respectively. In June 1999, SP replaced CQ as the first line treatment [19], though it was also used during the 1997/1998 epidemic in several districts [13]. No resistance to MQ was noted in Zarumilla [19].

At a national malaria treatment policy meeting in Lima in August 1999, it was decided that the existing first line treatments for *P. falciparum* malaria needed to be changed and artesunate (AS) based combination therapy (ACT) was introduced [13]. On the northern pacific coast, the first line *P falciparum* treatment was shifted to AS plus SP, as SP was still efficacious, while in the Amazon basin AS plus MQ was used. *P. vivax* treatment policy continued to be CQ, as *in vivo* studies showed there was no resistance on the coast and little in the Amazon [13].

On the coast, the efficacy of SP and SP plus AS were 97% and 99% for the treatment of *P. falciparum* [2]. In Iquitos, both MQ and MQ plus AS had 100% efficacy for *P. falciparum* [13]. A study that examined mutations in *pfmdr1* (a gene indicated in MQ resistance) to establish

a baseline of prevalent genotypes took place in June through September of 2000. Both a triple mutant genotype (S1034C, N1042D, D1246Y, 48.5 % of samples) and a double mutant (S1034, N1042D, 16.5%) were present. The remaining genotypes of the population are ambiguous in the literature [24,25].

Another national meeting was held in 2000, and it was agreed the new *P. falciparum* treatments should be implemented—the coast would use SP (25 mg/kg) plus AS (4 mg/kg/day for three days) [26] and the Amazon would use MQ plus AS [13]. New first, second, and third line drug treatments were approved in August, 2001 and pregnant women continued to be treated with quinine plus clindamycin [13]. MQ plus AS therapy was implemented in Iquitos and its surrounds in 2001. In the northwestern Amazon, CQ was still being used for the first line treatment for *P. falciparum* in 2002. By January 2003, MQ plus AS was used in this region as well [7].

In Iquitos, these drug policies appear to have had a positive effect on the levels of SP resistance. As opposed to an earlier study [27], triple mutant *dhfr* genotypes had dropped from 46.7% of the population to only 16.9% in 2005/2006 [28]. Triple mutant *dhps* genotypes had undergone an even greater change, going from 46.7% in 1997 to 0% in 2005/2006 [28]. These results are echoed from another study that examined samples from Iquitos from 2006/2007. For d*hfr*, 79% were 108 single mutants and the triple mutant was still maintaining 16% of the population. However, a new triple mutant (50, 51, 108) had appeared and made up the remaining 5% of the population [15]. For *dhps*, 6% of the population carried the triple mutant (437, 540, 613), 82% had the double mutant (437, 613) and 8% were wild type. Together, these two studies showed for the first time that the removal of SP drug pressure resulted in the decline of highly resistant triple mutant *dhfr* and *dhps* genotypes, which were apparently less fit in the absence of drug pressure. It also appeared the S_{tct}VMNT alleles were taking over as 66% of the population carried this genotype and 44% carried CVMNT [15]. For *pfmdr1*, the genotypes are more complicated but there were double mutants (184, 1042), triple mutants (144, 184, 1042 and 184 1042, 1246) and quadruple mutants (184, 1034, 1042, 1246) in the same 1999 study [15].

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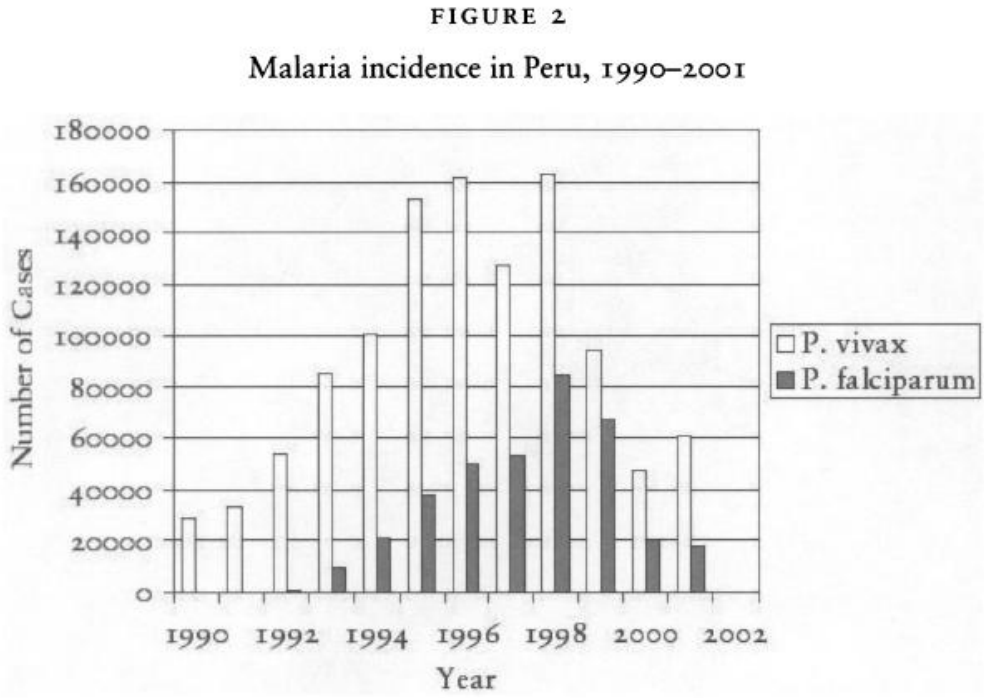
Figure 3.1 The mosquito species of Peru, 1995

ESPECIES DE ANOPHELINOS (a)	TUMBES	PIURA	LAMBAYEQUE	LA LIBERTAD	CAJAMARCA	ANCASH	LIMA	ICA	AREQUIPA	MOQUEGUA	TACNA	AMAZONAS	SAN MARTIN	HUANUCO	PASCO	JUNIN	HUANCAVELICA	AYACUCHO	APURIMAC	CUSCO	PUNO	LORETO	UCAYALI	MADRE DE DIOS
1 An.(Ano.)eiseni	X	X	X		X								X		X	X		X	X	X	X	X		X
2 An.(Ano.)mattogrossensis																						X	X	X
3 An.(Ano.)peryassui													X							X		X	X	X
4 An.(Ano.)pseudopunctipennis	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				X
5 An.(Ano.)tibiamaculatus																X		X		X				
6 An.(Ano.)calderoni (1) (&)	X	X	X	X		X	X	X																
7 An.(Ano.)fluminedius (1)					X							X	X			X		X		X		X	X	X
8 An.(Ano.)intermedius (1)					X								X			X						X		
9 An.(Ano.)mediopunctatus (1)													X	X	X					X		X	X	X
10 An.(Ano.)neomaculipalpus (1)														X								X	X	
11 An.(Ano.)pseudomaculipes (1)																						X		
12. (An.(Ano.)shannoni (1)																						X		X
13. An.(Lop.)squamifemur (&)																						X	X	
14. An.(Nys.)albimanus	X	X	X	X	X	X																		
15. An.(Nys.)albitarsis	X				X							X	X									X		X
16. An.(Nys.)argyritarsis	X				X								X							X	X	X	X	X
17. An.(Nys.)benarrochi													X	X		X				X		X	X	X
18. An.(Nys.)braziliensis													X											
19. An.(Nys.)darlingi																				X		X		X
20. An.(Nys.)dunhami (=trinkae) (&)																X								X
21. An.(Nys.)evansae (=noroestensis)(&)																						X	X	X
22. An.(Nys.)galvaoi (&)																								X
23. An.(Nys.)nuneztovari															X	X						X	X	X
24. An.(Nys.)oswaldoi					X								X	X		X		X				X	X	X
25. An.(Nys.)rangeli					X							X	X	X	X	X	X	X		X	X	X	X	X
26. An.(Nys.)strodei																						X		
27. An.(Nys.)triannulatus	X				X								X		X	X		X		X		X	X	X
28. An.(Nys.)hutzi (2)																					X			
29. An.(Nys.)parvus (2)																X		X						
30. An.(Ker.)boliviensis																		X						
31. An.(Ker.)bambusicolus																		X				X		
32. An.(Ker.)cruzii																		X				X		
33. An.(Ker.)homunculus																				X				
34. An.(Ker.)lanearus																				X				
35. An.(Ker.)lepidotus (&)																X								
36. An.(Ker.)neivai																						X		
37. An.(Ste.)acanthotorynus																						X		
38. An.(Ste.)kompi																						X		X
39. An.(Ste.)nimbus																						X		
40. An.(Ste.)thomasi																						X		
41. Chagasia bathana	X												X	X						X		X		X
42. Ch. Bonneae														X		X						X		
43. Ch. fajardoi (&)																X								

(1) *Anopheles (Anopheles)* serie Arribalzagia (2) *An (Nyssorhynchus)* grupo Myzorhynchella (&) Nuevos records
(a) No se incluye An.(Ano) apicimacula porque esta especie se distribuye sólo del norte de Colombia hasta México.

Mosquito species are listing in the column to the left. Peruvian locations of sample are listed in the following columns. Entries with ampersands are new records. An. apicimacula was not included because this species's distribution goes from Mexico to Northern Colombia. From [8].

Figure 3.2 Malaria incidence in Peru, 1990-2001



From [13].

Figure 3.3 Loreto and the city of Iquitos

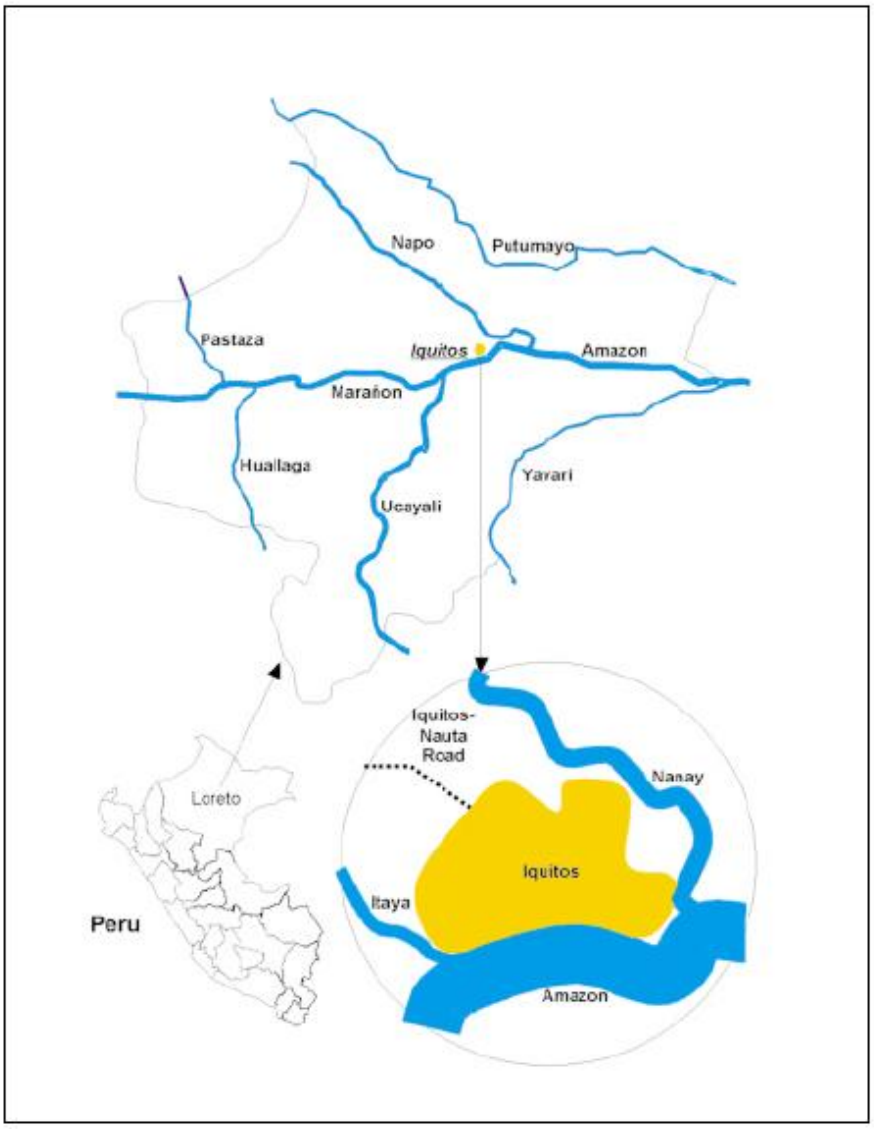
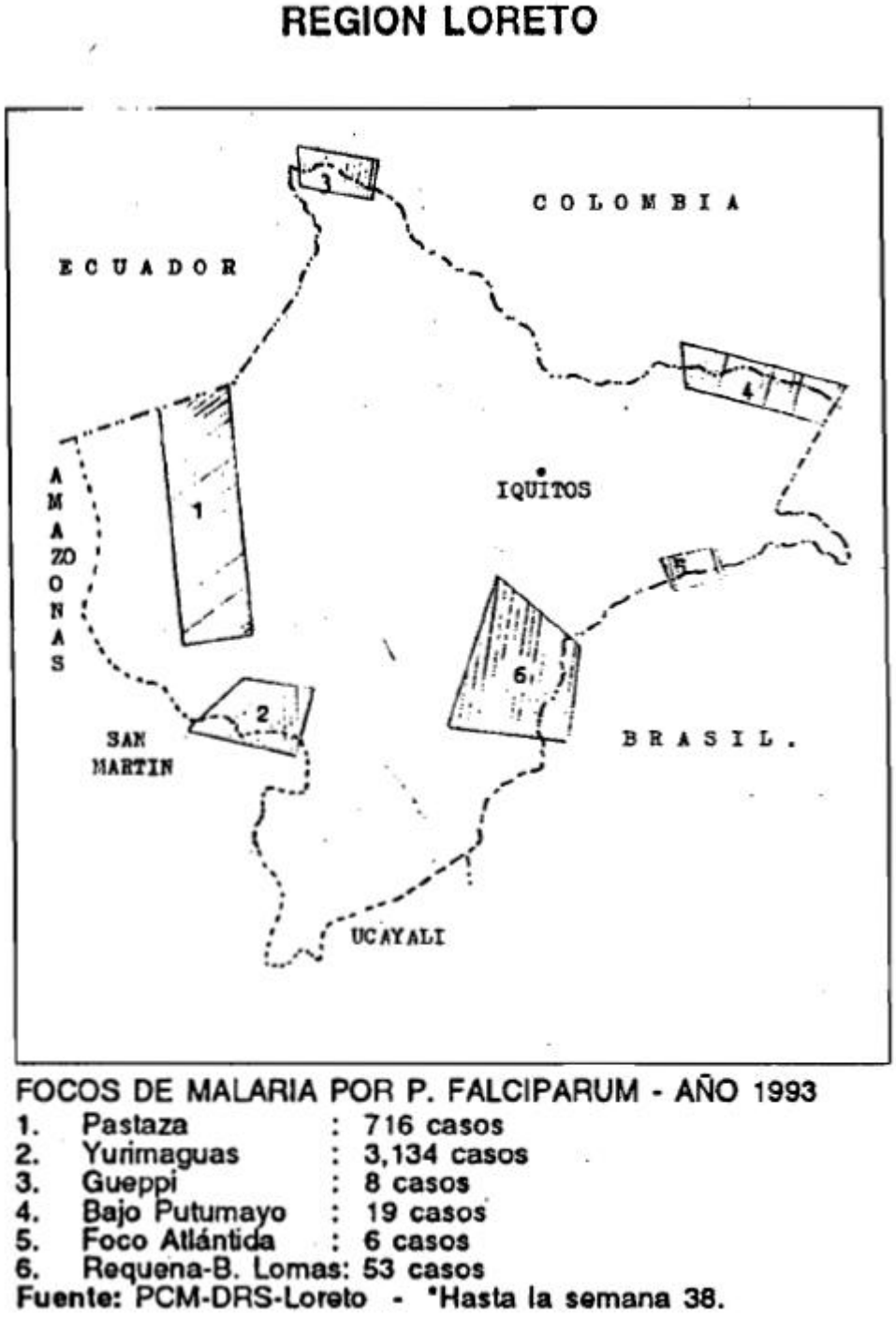


Figure 1: The department of Loreto and the city of Iquitos in Peru.

From [6].

Figure 3.4 Outbreaks of *P. falciparum* malaria in the Loreto region in 1993.



From [16]

Figure 3.5 Initial *P. falciparum* malaria treatment schemesin Loreto by district, 1998.

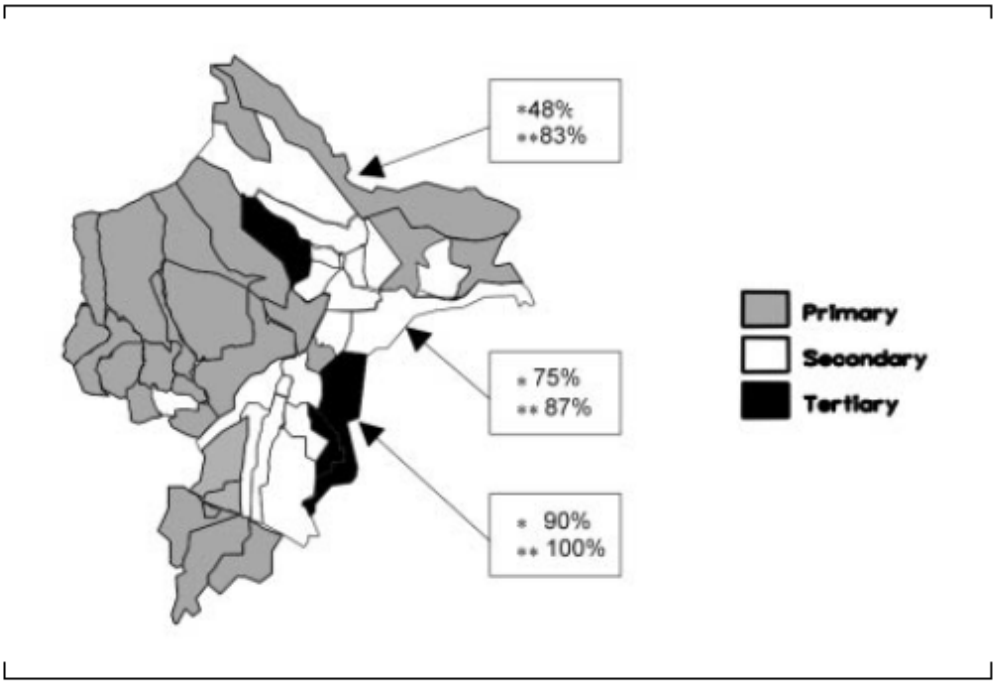


Figure 7: Initial *Plasmodium falciparum* malaria treatment schemes in Loreto by district, 1998. Treatment efficiency* (cases cured/cohort number x 100) and efficacy** (cases cured/[cases cured + resistant cases] x 100) shown for each treatment scheme region. Treatment schemes: Primary-chloroquine; secondary-pyrimethamine-sulfadoxine; tertiary-quinine.

From [6].

CHAPTER 4

THE HISTORY OF MALARIA IN VENEZUELA

Overview

In Venezuela, the temperature is consistently over 18 ºC and the mean annual temperature varies between 23 ºC and 28 ºC. Humidity is high and rarely goes below 60 ºC, even during the dry season. Malaria transmission is determined by rainfall [1,2]. Prior to the use of DDT in the 1940s, epidemics appeared to follow a five year cycle, along with repeated invasions of *A. darlingi* onto the coast. Later epidemics seemed to have followed a similar pattern and this was attributed to the influence of the El Niño Southern Oscillation [3].

Venezuela can be broken into three regions which vary geographically, economically, and demographically: the Costa-Cordillera, the Llanos, and Guayana. The Costa-Cordillera (which could be translated as the Coast-Mountain Range) lies to the north and is a coastal plain that leads to an inland mountain range. The Costa-Cordillera is only 18% of the country's area. In the 1940s, this region contained 70% of the country's population. Malaria rarely occurred at altitudes above 500 m. and would be transmitted by *A. pseduopunctipennis* below 1,000 m. The brackish marshes of the coast precluded *A. darlingi*, leaving *A. albimanus* as the major vector [1]. The highest endemicity was found where *A. darlingi* predominated, though rates were high where *A. albitarsis* was found. Regions with moderate endemicity tending to have *A. albimanus*, which contributed to local epidemics due to heavy rainfall or rice cultivation [2].

For the purposes of early control efforts, the Costa-Cordillera was broken into three sectors (west, central, and east). To the west, there were Lake Maracaibo's valleys and the Andean cordillera. In the north of this sector, *A. albimanus* was dominant, with *A. darlingi* in areas with greater rainfall. Several epidemics were caused by *A. albimanus* after heavy rain. *A. darlingi* predominated in the central portion and this was therefore where the most malaria cases

occurred. In the southern foothills, *A. nuneztovari* and *A. pseudopuntipennis* were present. In the central portion of the Costa-Cordillera, there were valleys to the west and east, and the mountains to the south. The distribution of vectors was similar, though the mountains also had *A. darlingi* at Lake Valencia and a southern valley had *A. albimanus*. In the western part of the Costa-Cordillera (made up of Nueva Esparta and Sucre), the mountains were free of mosquitoes. Nueva Esparta had reported little malaria in the past. Sucre had *A. darlingi* and *A. albimanus* in the west and *A. aquasalis* to the east [1].

The Llanos (which could be translated as the Plains) are in the middle of the country and behind the mountains. The Llanos are grass-covered plains intersected by rivers abutting jungle that periodically floods. In the grasslands between these rivers there are pools, ponds, and lagoons where vectors can breed. This region is 36% of Venezuela landmass. In the early 20th century, 20% of the population lived there and it had the highest malaria prevalence in the country. However, there were no large epidemics. Malaria cases typically occurred between 500 and 1,000 meters on the Gran Sabana (Grand Savannah) plateau, where *A. darlingi* predominated. To the southwest, near the Apure River, there was a region mostly free of malaria due to the absence of *A. darlingi*. In the northern portion, malaria was hyperendemic with some spleen indexes (the proportion of a sampled population with palpable enlargement of the spleen) above 100. To the south, spleen indexes were below 50 [1].

Finally, farther south is Guayana, made up of the states of Amazonas and Bolivar. Guayana borders Brazil, Colombia and Guyana. It is covered with a thick tropical forest with small patches of open country to the north and a rolling plateau covered by savannah to the south. Though Guayana made up 46% of country, it had only 3% of the population early in the 20th century. While the density of people was low (0.2 per square kilometer), it was distributed in pockets of higher density. Such urban congregation may explain why Guayana had less malaria than the Llanos. Spleen indexes were usually below 50, but 85 occurred in one locality. In the northeast, *A. darlingi* was absent and spleen indexes were ~5. The southwest was free of malaria

as the Atabapo and Guaynia Rivers were ‘black’ rivers, filled with tannic acid that kept villages on their banks free of *A. darlingi* [1].

Much of the malaria found in Venezuela in the current day is relegated to Bolívar state in the Guayana region. Bolivar ranges between 327 to 1700 meters above sea level (Figures 4.1, 4.2). Its borders are the Venezuelan states of Anzoátegui, Apure, Guarico, Monagas, Amazonas, Delta Amacuro, and the countries of Guyana and Brazil. It covers 238,000 km^2, which is 26% of Venezuela’s territories. The climate varies between tropical savannah and forest. There is a short dry season, annual temperatures vary between 24 and 27ºC, and humidity is 80 to 82%.

As of 1996, Bolívar had 900,310 inhabitants that lived along the Orinoco River and its tributaries. Livelihoods include cattle ranching and gold and gem mining had been part of the state since it was originally colonized. Indigenous populations were dispersed throughout the forest and the high plains. [4]. Rudimentary housing was and is improvised in small encampments within the forest. Both the miners and indigenous populations are migratory. Self-medication is common among miners, but often inadequate and using whatever antimalarial is available. The predominant vector is *An. Darlingi*, which is present in all municipalities below 800 meters. Parasites have been multidrug resistant at least since 1996. Miners in search of new claims extended drug resistance to the rest of the state [4]. Malaria outbreaks tend to occur at the beginning and ending of the rainy season [5].

Amazonas State has the second most malaria cases. *A. darlingi* is the predominate vector and 63.8% of cases are *P. falciparum*. There is much less mining activity in this state. There is, however, a sizeable Indian community, including the Yanomami that live on the upper Orinoco River. Malaria is extremely common among the Yanomami, with an Annual Parasite Index (API) of 1,279 per 1,000 inhabitants – which means some people are being infected multiple times each year.

The Yanomami are estimated to have a population of 22,786 people and live along the Brazilian-Venezuelan border [6]. The Yanomami frequently migrate and the villages have not

been sprayed with insecticides since 1993. While transmission occurs throughout the year, it peaks in September and June when the Orinoco River floods during the rainy season, creating breeding grounds for *A. darlingi*, which crests as well. It appears that the majority of cases occur in children under the age of 10 with infection occurring in the home [7].

History of Venezuelan Malaria

In the 1820 and 1830s, malaria epidemics were a problem for the armies involved in the Venezuelan War of Independence in the Llanos. Epidemics were again a problem in the 1890s [8]. The malaria parasite was first identified in samples from Venezuelan patients by Dr. Santos Aníbal Dominici in 1894 at the Vargas Hospital, three years after it was founded [9,10]. Venezuelan public health began on March 17, 1909 with the creation of the Public Health Commission. The National Health Office was opened in November, 1911 along with an Institute of Hygiene and Chemistry, Bacteriology, and Parasitology Laboratories [10]. During the 1920s, quinine was distributed for free in some regions [8]. In 1926, the National Health Office began to study malaria in collaboration with the Rockefeller Foundation around Lake Valencia in Maracay, Aragua. They recommended spraying Paris Green, creating drainage, and cultivating the fields around Maracay. Both Caracas and Guaira were reported to be malaria free [8]. During May to December, 1930, many malaria cases occurred in Maracay. In one district alone every one of the 500 inhabitants contracted *Plasmodium falciparum*. The outbreaks were attributed to the recent introduction of *A. darlingi* [11].

It has been claimed that Venezuela was the most malarious Latin American republic prior to 1936 and the affected region covered 600,000 km. Death rates and reduced birth rates in affected regions led to negative “vital indices” and a reduction in population in a region of 319,000 km2 from 1891 to 1920. [12]. No disease, not even the 1918 influenza epidemic, caused a higher mortality in Venezuela than malaria during 1905 to 1945 [13]. Early epidemics had death rates between 60 and 70 per 1,000 and malaria death rates were as high as 531 and 1,125 per

100,000 in the states of Carabobo and Cajedes as late as 1941. Some municipalities had endemicity ratios that were between 10 and 15 and death rates between 30 and 50 per 1,000. However, most municipalities[5] had endemicities below 8 and death rates of 20 to 25 [2]. The Ministry of Health and Social Assistance (MSAS) and its internal Malaria Division began the fight against malaria in 1936 [14,15]. In that year, malaria was disseminated throughout the entire country [16].

The success of DDT spraying was startling; in areas where *A. darlingi* and *A. albimanus* predominated malaria disappeared after only 3-5 years, without any additional measures except the occasional use of quinacrine. Perhaps assisting this eradication, the populace in this region did not move very far, which limited the number of introduced cases. Eradication was slower to the east, where *A. emilanus* was present, and to the west, where *A. nuneztovari* was present [12]. Malaria transmission was interrupted and reduced its range by 500,000 km, relegating it to southern (Bolívar) and western regions [4]. *P. falciparum* was the most common parasite, though *P. vivax* predominated in children below five years of age [2]. Holoendemic malaria was unseen, though hyperendemic malaria was seen in several municipalities [2].

The country had also developed an impressive public health network to eradicate malaria by the 1950s [2], so much so that the WHO used it as a model for other such eradication programs [15]. The first 8 years of DDT spraying succeeded in eradicating *A. darlingi*, and thus endemic and epidemic malaria, from the center of the Costa-Cordillera, where ~50% of Venezuela's population lived. In fact, *A. darlingi* was still absent from central Venezuela in 1983. After these initial successes, 28% of the remaining malaria cases occurred in a tiny coastal portion (0.1% of the malarious zone) of the Coasta-Cordillera, where *A. aquasalis* acted as the vector from at least 1950-1952 [12]. *A. aquasalis* was able to survive spraying because it lived and bit outdoors and thus continued to maintain malaria [17]. Another 50.6% of malaria cases

[5] In Venezuela, the first political subdivision is estado: state, followed by municipio: municipality

occurred on the western border of Costa-Cordillera with Colombia (3.3% of the malarious zone), where *A. darlingi*, *A. nunez-tovari*, and *A. pseudopunctipennis* were the vectors [12]. Control efforts were generally successful, but not for inhabitants that lived too close to the forest or banana plantations [17]. Like *A. aquasalis*, *A. nunez-tovari* was also able to resist DDT due to its exophilic habits (this was still a problem in the 1990s in Apure, Barinas, and Tachira) [18]. Just to the north, still in Costa-Cordillera and bordering Colombia (5% of the malarious zone), 5.6 percent of malaria cases occurred and the vectors were *A. albimanus* and *A. darlingi*. [12]

The remainder of malaria cases occurred to the south in the Llanos and Guayana regions. There 56.6% of the malarious zone contained only 14.7% of malaria cases in 1952 and the main vectors were *A. albimanus*, *A. albitarsis*, and *A. darlingi* [2]. These eradication methods were not used within Coasta-Cordillera, along the Colombian border and to the north in a small coastal area, in the states of Apure and Delta Amacuro in Llanos, or in the states of Bolivar and Amazonas in Guayana. This is because most cases occurred due to out-of-doors transmission. In addition, outside of the Coasta-Cordillera, distances were too large for such control efforts to be economically feasible [2].

The malaria free zone was ~180,000 square km in size in October, 1954 [2]. To the west and south, where malaria was more intractable, control measures were limited to only the localities with the highest levels of endemicity based on spleen indices (which reached 90-100% in some). DDT spraying was usually conducted every four months. They also used weekly mass drug administration (chloroquine and primaquine) for periods less that 3 months in length in villages with monthly parasite incidences above 50/1000 [12]. The number of malaria cases in the country was at its nadir in 1959, when only 911 cases were reported [16]. In that year, Venezuela reported to PAHO that the territory now free of malaria had expanded to 407,945 km2 [12]. In June 1961, the WHO declared that malaria had been eradicated from two thirds of Venezuela [19]. The DDT campaign lasted until 1965 without fully eradicating malaria from Venezuela [2,4].

Before DDT spraying, *A. albimanus* and *A. darlingi* had been the primary vectors, followed by *A. albitarsis*, *A. aquasalis, A nuñez-tovari,* and *A. pseudopunctipennis*. Afterwards DDT spraying, *A. albitarsis, A. aquasalis, A. albimanus,* and *A. pseudopunctipennis* populations remained [2]. In the Western Amazon, *A nuñez-tovari* survived DDT fumigation and was present in the late 1980s, even after the National Control Program changed to fenitrothion in 1984 [20]. The most prevalent species of malaria in Venezuela prior to the use of DDT was *P. falciparum*, followed by *P. vivax*, followed by *P. malariae*. After, *P. vivax* predominated with a few cases of *P. falciparum*, and no cases of *P. malariae* [12].

In 1971 the region of Venezuela that was free of malaria had grown to 460, 054 km^2, though cases continued to be introduced by agricultural laborers coming from regions not treated by insecticides. These lead to more than 100 new foci per year, often close to areas with endemic malaria, usually in naïve populations and therefore easy to spot by the vigilance services [12]. Unfortunately, malaria cases began to increase again during the 1970s [16] and spread to regions previously malaria free due to changes in vector habitats, insecticide resistance, inadequate rural housing, difficulty reaching the last bastions of malaria (especially Amerindians [15], the development of drug resistant parasites, as well as politics, economics, and mining activity) [4,21]. In 1972, malaria was being transmitted persistently in western Venezuela and the primary vector was *A. nuñeztovari*. However, the majority of malaria cases occurred in Bolívar. In both regions, DDT was being sprayed every three months, along with the application of focal chemotherapy [22].

In Bolivar, malaria still had persistent transmission in the southwest portion in 1970, where populations were nomadic and lived in primitive structures built in sylvan habitats. Malaria was found in the municipalities of La Urbana Caicara, Santa Rosalía, Santa Elena, and La Paragua. These localities were only accessible by river or air. It was also found in some of the municipalities of the Territorio Federal Amazonas and others in Apure. In these affected regions, *A. darlingi* was the principal vector. The district of Sucre was given as an example of the spread

of malaria within Bolivar. In 1961, Sucre had been considered malaria free for 10 years. In 1970, 17 *P. vivax* cases occurred in Moitaco, Bolívar. A year later, there were 113 cases (100 *P. vivax* and 13 *P. falciparum*), in 1972, 117 cases (53 *P. vivax* and 64 *P. falciparum*) and in 1973 148 cases (16 *P. vivax* and 132 *P. falciparum*) [21]. One author suggested that the rapid spread and resurgence of malaria in Bolívar state was due to the movement of miners [4].

In 1984, it was said that malaria was confined to the south and east of the country among only 6% of the population formerly living in malarious regions [23]. To the north, the state of Sucre had been free of *P. vivax* for 15 years when, in May 1985, cases first occurred and began to spread across the state – in 1990, there were 6,831 cases, though still no *P. falciparum*. Stake holders thought that local control would be more advantageous, along with more research into vector biology (particularly *A. aquasalis*). However, they thought the first would be difficult to wrest from the Ministry of Health, and the second difficult to encourage within the Ministry at that time [24]. Meanwhile, the state of Amazonas had an Annual Parasite Index (API), which was 25 times that of the average for the country in 1987 [16]. In 1988, the API in Amazonas was 17 in 1988 and 33.4% of cases were *P. falciparum* [25]). There was a notable increase in cases between 1988 and 1992 [16]. In addition, *A. aquasalis* in this region was DDT refractory [18].

In 1990, 56.7% of malaria cases occurred to the South in Bolivar. Of these, 85% occurred in forested areas where mining was being conducted. The prevalence of parasitemia was 5.9% among Amerindians and 2.9% among miners and 70% of the population had been infected in the last 2 years. One third of those sick with malaria were treated by the national control service and the remainder dealt with pharmacists, local doctors, or traditional healers [18]. In 1994, Bolivar had 5,917 cases, Amazonas, 4,435 cases, Apure, 1,786, and the Delta Amacuro 1,020, with Táchira, Sucre, and other states contributing a few cases [26]. In Bolívar, 76% of cases were introduced (imported from elsewhere), 20% indigenous, and 2% were from other regions of the country in 1993. In 1994, 74% of cases were *P. vivax* and 26% were *P. falciparum*. [4].

By the late 1990s, Bolívar state contributed 60% of Venezuelan malaria cases, of which 20% were *P. falciparum*. The average infection reached 2 cases per 1,000 people. Malaria was endemic in this region of the country. In 1997, there were 10,000 malaria cases in Bolívar. Of those, there were 1,634 cases of *Plasmodium falciparum* in Domingo Sifontes. Furthermore, 35-40% of malaria cases occurred in the municipality of Domingo Sifontes, Bolívar in a population of 10,000 to 20,000 due to 15 years of uncontrolled mining and the introduction of naïve hosts [27]. From 1997 to at least 2002, there was a notable increase in the number of malaria cases [16].

In 2000, many of the problems that had plagued Venezuela control efforts in past decades continued unabated to the south. Experts were sent to deal with serious outbreaks, but the costs associated with covering such a large areas covered in Amazonian jungle, mountains, and swamps were prohibitive [28]. A year later, there were 22,714 cases of malaria in Venezuela [29] and 60% occurred in Bolívar, where 3.4 infections per 1,000 inhabitants were reported. Of those, 20% of cases were *P. falciparum* [5].

By 2002, the distribution of malaria was similar to that noted in 1936. The greatest number of cases was observed in Bolívar, Sucre, and Amazonas where malaria seemed to be endemic. The national API in 2002 was 3.87 per 1,000 inhabitants in 2002[16]. The state with the most cases was still Bolívar. In Amazonas, the API was 42.16 and cases were patchy, non-existent in some localities and hyperendemic in others (specifically, Manapiare and Alto Orinoco). In Sucre, it was 19.96. In Bolívar, it was 4.99 [16].

In 2004, there were 46,244 cases of malaria in Venezuela. The API for Amazonas and Bolívar had worsened (102.2 and 19.2, respectively, while it improved in Sucre to 5.5). Delta Amacuro had an API of 12.5 per 1,000. The number of malaria cases was similar in 2005 (45,328) and 2007 (41,570) [25,30]. Of these cases, 20% were *P. falciparum* and 80% were *P. vivax* [25]. In 2007, Amazonas contributed 22% of Venezuelan cases, had an API of 68.4 cases per 1,000 inhabitants, and 15.4% of cases were *P. falciparum* [25].

Venezuelan Drug Policy and Resistance

Venezuela utilized relatively few antimalarials in public health response over the 20th century. Unfortunately, resistance developed rapidly to all of these drugs. During the 1950s, Gabaldon started administering weekly or monthly dosing regimens of chloroquine [2] and for much of the 20th century the official treatment for *P. falciparum* in Venezuela was three days of chloroquine and primaquine. CQ resistance was first noted in Venezuela among parasites that were also pyrimethamine resistant in July, 1959 [31,32,33], near the border with Colombia. A few months later, a Colombia strain from the Magdalena valley that was CQ resistant was used to infect neurosyphilitic patients in South Carolina. Patients showed CQ resistance, but pyrimethamine sensitivivity [34]. CQ resistance rapidly spread throughout eastern Colombia, aided by the presence of *A. nunez-tovari* which is exophilic and therefore not susceptible to DDT [32].

In Venezuela, CQ resistance was first reported on the border with Colombia in 1959 in Táchira [4] and in two samples collected by Silvio Maberti in 1960 in the municipalities of Milla, Zerpa, and Alberto Ariani in the state of Mérida [33] to the northwest of the country. These CQ resistant parasites were fought with a treatment campaign with 40 mg of CQ per kg between 1960 and 1962 [32].

In 1956, Gabaldon and others experimented with treating malaria with weekly suppressive treatments of pyrimethamine on a tiny portion of the eastern coast (covering 16,416 inhabitants) and along the border with Colombia (covering 95,579 inhabitants). In total, they treated 111,995 inhabitants. Houses were generally made with mud walls and thatched roofs [17]. The health visitors made sure all members of each household took pyrimethamine in their presence every week. Their rounds took them by bicycle, horseback, or motor boats. Patients were encouraged to finish the 24 week treatment by lottery tickets for prizes like sewing

machines and bicycles and sugar candy for the children. Pyrimethamine was withheld from areas where it appeared that administration would be irregular [17].

In the west, this study was conducted by Maberti in Mérida and appears to have led to pyrimethamine resistance in the late 1950s. The study population had been treated with CQ every month in the presence of Rural Visitors starting in 1955, with additional CQ held in reserve for fevers and weekly patient self treatment. A massive campaign of pyrimethamine treatment was also conducted there from July 1957 to December 1957 [33]. This treatment was applied every week in 50 mg. doses for adults. Flattering results were noted within 3 months of use [33]. An intensive vigilance program was then conducted until January 1958, wherein only patients suspected to have been infected or febrile were treated with drugs. As imported, and then autochthonous, cases began to increase, pyrimethamine was again used until September 1958, on a biweekly basis until May, and then weekly from June until August 1959. At this point, pyrimethamine use ceased due to the development of resistance [33].

To the south, CQ resistance was first reported in Bolívar state in 1973 (RI) around the district of Sucre [21], though it has been argued that local immunity may have masked earlier resistance [4]. Indeed, a study of volunteers infected with a parasite collected from a patient in Amazonas suggested that there may have been moderate resistance to pyrimethamine and RI resistance to CQ around 1968 [35]. During the 1970s pyrimethamine and sulfamethoxipyridazine[6] were used, but resistance was reported in Bolívar State in 1977 [36].

During the 1980s, SP resistance had developed [5]. In Amazonas, small *in vitro* studies showed CQ, amodiaquine, and mefloquine resistance during the early 1990s [37,38]. In 1990, *P. vivax* was treated with CQ and primaquine and *P. falciparum* was treated with amodiaquine and primaquine. Resistant *P. falciparum* cases were treated with SP or quinine [18]. During 1999 and 2000, patients from Bolívar were treated with CQ or SP at three sites. All sites showed a significant amount of clinical failures (greater than 25%), though only two out of the three sites

[6] This is a sulfa drug, but not the same as sulfadoxine.

showed significant CQ resistance. None of the sites showed quinine resistance [39]. Another study reported moderate resistance to SP was reported in Domingo Sifontes, Bolívar clinically (RI, 17.1%; RII, 2.9%) and *in vitro* in 1999 (11%). CQ resistance was also reported clinically (RI, 28.5, RII, 14.2%, RIII, 5.7%) and *in vitro* (21%). Resistance to both drugs varied across the municipality. No resistance was seen to mefloquine or quinine [27]

Drug policy also changed. In Bolívar, CQ was used by the Bolívar State Malaria program before 1983 and half of 1984. CQ and primaquine were distributed weekly but reached less that 70% of the population [27]. It appears that Midekel and Fansidar were also used during these decades, though the timing is unclear. From 1984 to 1994, patients were treated with a single dose of sulfa (1,500 mg) plus pyrimethamine (75 mg), followed by two doses of primaquine (90 mg) (from 1988 to 1996) [4]. Policy shifted back to three days of CQ and primaquine in March 1995 [4,27]. In Bolívar, miners also self-medicated with medicinal plants, CQ and other drugs [27]. SP was banned from use in 1998. As of 2002, CQ was still occasionally used for uncomplicated *P. falciparum* cases [5]. By mid 2004, Venezuela had adopted artesunate combination therapy (artesunate plus mefloquine) for the primary treatment of malaria [40].

For *P. vivax* treatment shifted from CQ for three days (1,500 mg), plus 14 days of primaquine doses (15 mg), to six years of amodiaquine and primaquine using the same treatment scheme (from 1986 to 1992) and then returned to CQ/primaquine [4]. Mefloquine was used to treat uncomplicated malaria and in combination with parenteral quinine for treating complicated disease [5]. It has been argued that these changes in Venezuelan public health policy were made without enough drug sensitivity studies [4].

Venezuelan Molecular Findings Related to *P. falciparum* Drug Resistance

A number of studies have been conducted in Venezuelan since the 1990s that have examined genes known to confer CQ and SP resistance. An examination of 54 *P. falciparum* samples collected in Bolívar showed that 96% had a *dhfr* mutation to I at codon 51 and a

mutation to N at codon 108 in 1995. The remaining 4% of samples were wildtype for both codons. In addition, 64% of samples at a mutation to arg at codon 50 [41].

Another study examined *P. falciparum* samples collected between 1998 and 2000 from Bolívar State health clinics and the VEN line, which was established in 1987 [42]. The VEN sample had a 108 T *dhfr* mutant, a wild type *dhps*, a CVIET pfcrt mutant, and *pfmdr*1 was wild type at codon 1246 (D). Samples generally carried the 50R/51I/108N *dhfr* mutant, the 437G/540E/581G *dhps* mutant, the S_{tct}VMNT or the S_{agt}VMNT pfcrt alleles, and a tyr mutation at codon 1246 in *pfmdr*1. One sample carried a 51ile/108 asn *dhfr*, a 437 gly *dhps*, CVMET, and a mutation to tyr at codon 1246 in *pfmdr*1. Another carried a 51ile/108 asn *dhfr*, a 437G/581G *dhps* mutant, CVMET, and a mutation to Y at codon 1246 in *pfmdr*1. [42].

A different study conducted collected samples in 6 out of 10 municipalities in Bolívar during 1998 and 2000 (Cedeño, Sucre, Piar, Raul Leoni, Sifontes, and Gran Sabana). The authors concluded that "*pfcrt* and *dhfr/dhps* allelotypes were not exclusive to a particular region of the state." For *pfcrt*, 167 out of 168 samples showed the K76T mutation. For a subset of the samples (91), *pfcrt*, *dhfr*, and *dhps* genotypes were more closely examined. Of these, 64% carried the *S_{tct}VMNT* allele, 21% carried *S_{agt}VMNT*, 5% carried *CVMET*, 4% carried CVIET, and 1% carried CVMNK (this sample was wild type). The *S_{tct}VMNT* and *S_{agt}VMNT* alleles were associated with most of the SP resistant alleles. A 50R/51I/108N *dhfr* mutant, combined with a 437G/540glu/581G *dhps* mutant, was seen in 34% of samples. Another 26% of parasites carried 51I/108N *dhfr* and a 437G/540E/581G *dhps* mutant. Much of the remaining parasites (25%) carried 51I/108N *dhfr* and a 437G/581G *dhps*. The *CVMET* and *CVIET* alleles were associated with the 13% of samples carrying 51I/108N *dhfr* mutant and a 437 gly *dhps* mutant. Two more samples had different combinations of these alleles. Despite the authors' assurances that there were no regional patterns, the parasites carrying CVMET and CVIET were not reported in eastern Bolívar [5].

A study of *P. falciparum* samples collected in 2003-2004 from Domingo Sifontes showed that all samples either had a combination of SP resistant *dhfr* 50R/51I/108N and *dhps* 437G/540E/581G (90.7% of the samples) or *dhfr* 51I/108N and *dhps* 437G/581GI (9.3% of the samples). There was a larger region of depressed variation around *dhps* (99 kb), due to hitchhiking than around *dhfr*. This suggests that the resistant *dhfr* allele originated first with sufficient time to allow recombination to increase surrounding variation. They concluded that SP resistant alleles of *dhfr* and *dhps* originated once in South America and were locally fixed [40].

Venezuelan Molecular Findings Related to *P. falciparum* Population structure

Previous studies established that Venezuelan *P. falciparum* had limited genetic diversity. A study using 75 samples collected in 1995 genotyped *msp-1*, merozoite surface protein 2 (*msp-2*), the ring-infected erythrocyte surface antigen (*resa*), and the circumsporozoite surface protein (*csp*). They found significant linkage disequilibrium for these genes and suggested that clonal propagation was important to Venezuelan population structure. Furthermore, they argued that this clonal propagation could be due to low transmission rates leading to high rates of self fertilization [43]. Another study of *P. falciparum* in Venezuelan Amazon examined the glutamate-rich protein (*glurp*), *msp1* and *msp2*, and a polymorphic microsatellite marker (*pfrrm*) using samples collected from 1995-1997 [44]. The profiles of 32 out of 40 parasites for these four molecular markers were described by three multiallelic clonets (respectively making up 62.5%, 12.5% and 5% of the sample set). In addition, three parasites selected from each of these clonets shared few *var* gene sequences [45]. It was again proposed that *P. falciparum* followed an epidemic or clonal population structure in the Venezuelan Amazon [44].

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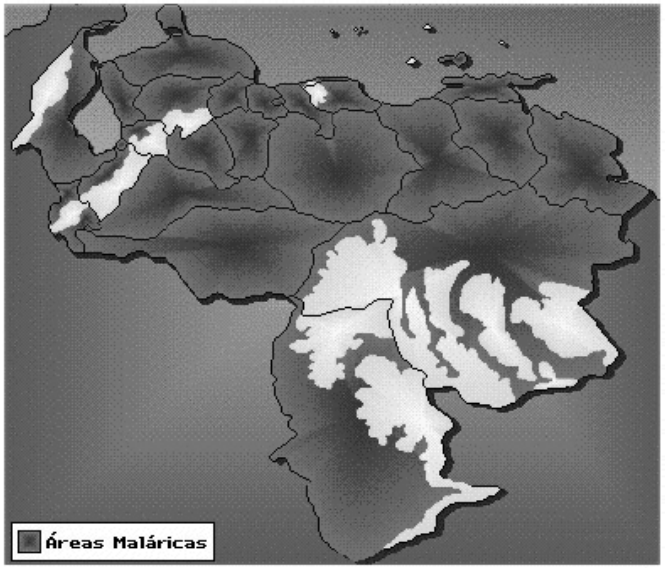
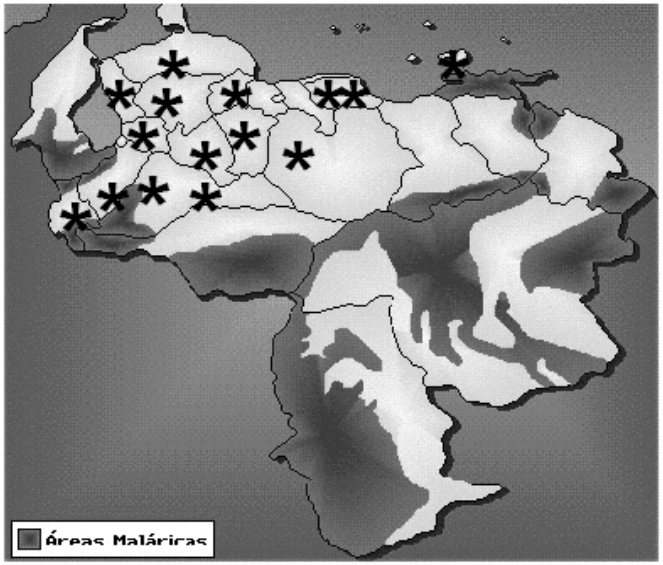
Figure 4.1 The historical and modern distribution of malaria in Venezuela.



(a) **1936**



(b) **2002**

From [16]

Figure 4.2 The distribution of malaria in Venezuela, 1979-1981

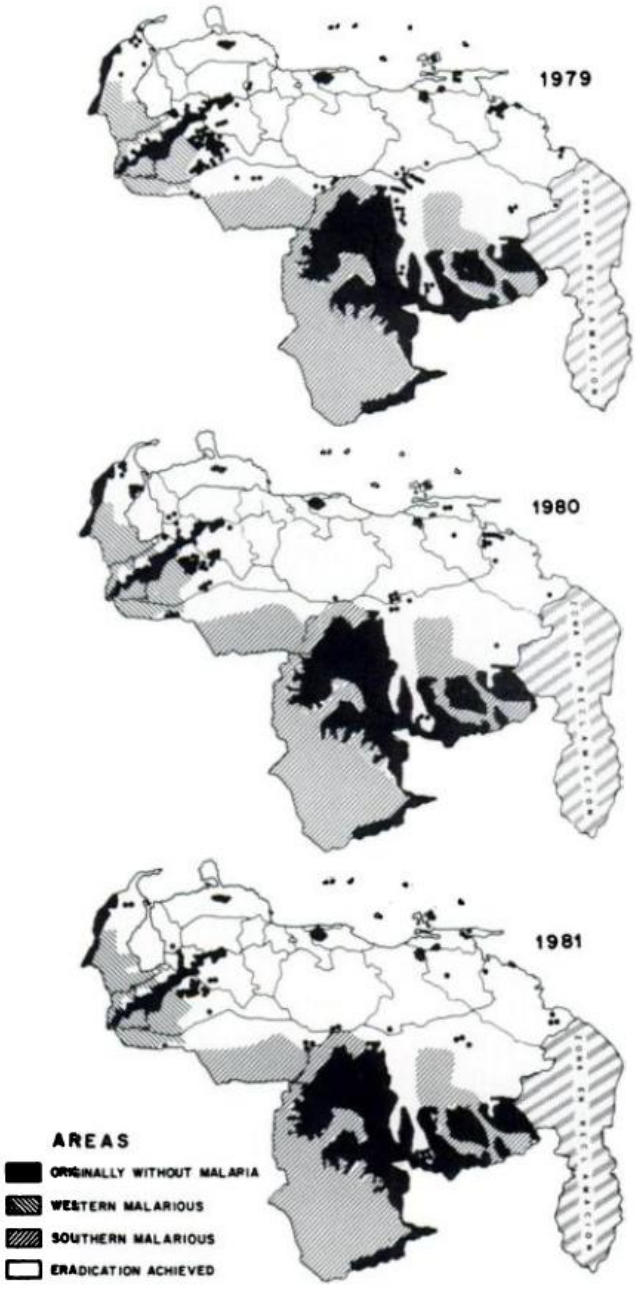


FIGURE 1. Maps of Venezuela showing localities with indigenous and introduced malaria cases, 1979–1981. Black dots show the reinfected localities with introduced and indigenous cases in the area where malaria has been eradicated during the past 20 years. One dot may correspond to several neighboring localities.

Figure from [12].

CHAPTER 5

THE HISTORY OF MALARIA IN BRAZIL

Overview

Brazil has three macro regions: Amazonia, the arid/semiarid northeast, and the southern region. While *A. darlingi* is the most important vector on the interior, the most important vector on the coast is *A. aquasalis* [1]. Amazonia had 99% of malaria cases in 1990 and has nine states: Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima and Tocantins. This region is at or near sea level and covered in a humid tropical forest with high rainfall, and elevated temperatures [2]. As of 1990, the principle vector was *A. darlingi* [2] and the states with the most malaria were Rondônia (33.4 percent of all Brazilian cases), Pará (20.1 percent) and Mato Grosso (25.0%), where miners are active. Mining destroys the forest and diverts local streams, leading to the development of puddles in which vectors can breed. In addition, agricultural practices in Acre and Rondônia also lead to malaria cases [2].

Northeastern Brazil is made of up the states of Bahia, Ceará, Paraíba, Pernambuco, Piauí, Rio Grande do Norte, and Sergipe. It also has high temperatures with periodic droughts [2]. Northeastern Brazil can also be broken into three regions: the Zona da Mata, the Agreste, and the Sertão. The Zona da Mata is a southeastern coastal plain which had traditionally supported plantation crops like cotton and sugercane. Inland, and to the west, the Agreste makes up the majority of northeast Brazil. Agreste is a transitional zone between the dry interior and the humid coastal plain and supports industries including coffee and cattle ranching. Historically, land in both regions has been owned by a few stakeholders. To the north, and east of the Zona and Agreste iss the Sertão. The Sertão is arid and contains the states of Ceará and Rio Grande do Norte [3,4].

The South of Brazil is the most developed portion of the country and is made up of the states of Espírito Santo, Goiás, Mato Grosso do Sul, Minas Gerais, Paraná, Rio de Janeiro, Rio Grande do Sul, Santa Catarina, São Paulo, and the Distrito Federal. It has a temperate climate. There are sometimes outbreaks of malaria in the region, as occurred in 1989 in Foz do Iguaçu, Paraná, which was attributed to growth of the *A. darling* population and the immigration of people from Amazon region [2].

History of Brazilian Malaria

Malaria was first reported in 1587 by Gabriel Soares de Souza in "Noticia do Brazil" as "tertian and quarten fevers" affecting the Tupinambá Indians. Yet large scale epidemics were not reported during the colonial period [5,6]. However, the "Baixada Fluminense", a lowland area where four river basins meet in the state of Rio de Janeiro, was filled with malarious swamps during this period [7]).

By the 1870s, malaria was attacking migrants leaving the northeast due to drought for the Amazon interior and the rubber industry [5,6]. In 1888, the Brazilian slaves were freed and soon after the coastal plains of Rio de Janeiro State, the Baixada Fluminense, and other similar sites in São Paulo State were highly malarious for decades [6]. This was due to breakdown in agricultural practice and the depopulation of the Baixada Fluminense. Commissions were started in 1891 to combat malaria with poor results [7]. From 1892-1906, 26.6 people out of every 10,000 died of malaria in northeast Brazil, with about half coming from outside the region [4]. Malaria was present in all of Brazil at the end of the 19th century and it is estimated there were 6 million cases each year, which was 50% of the country's population [5]

In the 19th century, and going into the 20th century, natural rubber was important to Brazil's economy. From 1890 to 1900, ~500, 000 Brazilians came into the Amazon in a "chaotic flood," with many becoming rubber tappers. The industry was not based on rubber plantations, but rather the gathering of rubber from wild plants. From 1900 to 1910, natural rubber from

Hevea brasiliensis was Brazil's second export crop. In just 1900, 40,000 people from Ceará went to the Amazon [8]. In that year, Dr. Oswaldo Gonçalez Cruz was made the director of the Instituto Federal de Soroterapia in Manguinhos, Rio de Janeiro (later renamed the Instituto Oswaldo Cruz) [9]. From 1905 to the 1920s, researchers from the Institute went to northeast Brazil, Amazonia, Mato Groso, Paraná, Minas Gerais, and São Paulo [8]. The first person to make the connection between anopholine mosquitoes and malaria in Brazil was Adolfo Lutz in 1903, when he identified *A. cruzi* as the vector spreading malaria along the São Paulo Santos railway [1].

In the same year, Carlos Chagas, later known for his discover of Chagas disease, received his medical degree with a thesis entitled "Hemological Studies of Malaria." He went on to publish a number of papers on malaria prophylaxis which culminated in the novel proposal that malaria was an infection of the domicile. In 1905, Chagas was fighting a malaria outbreak in the port city of Santos, São Paulo by burning sulfur indoors to kill endophilic mosquitoes [9]. Meanwhile, Cruz was conducting a government survey of sanitary conditions in major Brazilian ports (Belém, Santarém, Óbidos, and Manaus).

In 1907, Chagas and Dr. Arthur Neiva initiated an antimalarial campaign at the Instituto Federal de Soroterapia. They planned to give quinine in the most compromised parts of the state of Rio de Janeiro to all workers. These workers were involved in development of infrastructure including water works for the city and the development of a central Brazilian railroad [10]. Neiva was in charge of malaria prophylaxis among 3,500 workers damming the Xerém River [8]. Work on the railroad in Xerém and Mantiquira halted in January and February due to epidemics and 96% of workers had malaria. Interestingly, he noted that the dosage required to treat malaria increased over 20 months from 50 centigrams every 3 days to the same dose daily and concluded that parasites had developed quinine resistance in 1910 [10].

Meanwhile, there was also a massive epidemic in Rondônia [9,10]. Rondônia had only been inhabited by Amerindians until the middle of the 1800s, when a few immigrants came to

grow cocoa along the rivers. Later immigrants came for the rubber industry until 1912. Attempts to build a railroad started in 1878, but the Madeira-Mamoré Railroad was finally built between 1907 and 1912. This was constructed to connect the rubber production of Bolivia to what is now Porto Velho, Rondônia. Thousands of workers died and Dr. Cruz was called in for advice [8,11]. Cruz was based at the Candelaria hospital outside of Porto Velho and Cruz examined the conditions of the 113 km of completed railroad and nearby villages along the Madeira River. Cruz stated that 90% of the thousands of workers had been infected with malaria and 75% of those had infected with *P. falciparum* [8]. Quinine resistance was reported as well [9,10], which was a considerable problem because Cruz relied upon mandatory quinization as a proplylatic [8]. In 1912, Cruz returned to the Amazon to create a plan for sanitizing the entire Amazon basin at the behest of the Committee for the Defense of Rubber, created by the Ministry of Agriculture, Industry, and Commerce. His team, led by Chagas traveled along the rivers of Brazil from the coastal city of Belém, to the interior city of Manaus, and most sites of major rubber extraction in the Amazon. Malaria control was made more difficult because, as the major cities filled puddles and water channels, new construction would generate additional vector habitats. In addition, there was not enough quinine, Brazilians disliked taking it, and it was too expensive and adulterated. The survey team visited the Amazon during the time of year when most rubber tappers were unreachable in the forest. Still, they came to the conclusion that the state of Acre deserved its nickname as the "champion of death." The splenectomies of children suggested they had been infected with malaria multiple times. Malaria killed 400 out of the 800 residents of the town of São Felipe in just six months in 1911. Chagas wrote "never had I encountered such high lethality for an endemic disease and never had I seen more widespread morbidity. [8]. In 1917, there was a large malaria epidemic in the Paraná region which affected soldiers in the Barra del Norte fortress and civilians in the surrounding area [10].

The Brazilian government created the National Department of Public Health (DNSP) in 1920. Its division of Public Health focused on Chagas' disease, hookworm, and malaria. Run by

Barros Barreto, the initial goal was to establish a hookworm service across multiple states with attached malaria services where needed [7]. In the Baixada Fluminense there was a severe epidemic that eventually spread throughout the state of Rio de Janeiro, but the public health response was more successful. The mayor of Nova Iguaçu, who later became the Brazilian Minister of Health, led the first health unit of the Rural Prophylactic Service in Baixada Fluminense [7].

From February 17th 1922 to June, 1925, Mark Boyd and the Rockefeller Foundation studied the epidemiology of malaria in the plains surrounding Guanabara Bay, Rio de Janeiro in the Baixada Fluminense. Their goal was to see if the malaria control methods used in the United States could be used in the tropics and create guidelines for tropical malaria control; from a foundation progress report to "ascertain a simple, economical and effective method of malaria control adapted for a tropical area[7], which will offer prospects of permanent relief with a minimum of maintenance." Their plan was to spend the first year in observation, followed by a two-year campaign of control and maintenance. Mechanistically, this consisted of drainage projects combined with the use of Paris green and biological control. After a 6 month survey, Francis Root was the first to describe *A. darlingi*. At the end of the project, no malaria epidemics occurred in the study areas. Their work would influence future Brazilian malaria control and contributed to the control of malaria locally [7,9].

In 1926 and 1927, control efforts at the sites covered by Boyd broke down. This is because the Rockfeller Foundation generally expected local government to contribute funding to public health projects. In Brazil, the Rockfeller Foundation sought funding from municipal government at the same time that finances were consolidating at higher levels. In 1927, another contract was signed with the state of Rio de Janeiro where 50% of survey costs and 100% of maintenance costs would be covered by the state government [7]. Work continued until 1929

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[7] The secondary source I reference actually says "adopted for a topical area." I assume it was incorrectly entered.

[10]. However, the maintenance phase of control generally broken down due to a lack of support at the municipal level. According to Barros Barreto, "the attempts from 1891 to 1933 to re-establish the former prosperity of Baixada were fruitless [7]. In 1931, *A. darlingi* was first reported to be infected with sporozoites in Belém, Pará [1].

Much of the history of malaria during this period focuses on the introduction of *A. gambiae* to Brazil. *A. gambiae* (or possibly *A. arabiensis* according to recent molecular work [12]) was introduced to Natal in the state of Rio Grande del Norte, Brazil from Dakar, Senegal in March, 1930 or earlier [3]. It was most likely introduced by the "Avisos" ships (service started in 1928) [3]. In 1928, Dr. Adolph Lutz at the Instituto Oswaldo Cruz had voiced concerned that African mosquitoes could be introduced by these ships. In that year, there were 28 cases in Natal [5,6,10]. Afterwards a Rockefeller Foundation entomologist named Raymond Shannon, who was working on the Yellow Fever Program, reported 2000 *A. gambiae* larvae 1 km from the port city of Natal in March, 1930 [13]. In 1930, there were 139 cases in Natal [10]. Dr. Frederick Soper intervened. He also sent a telegram to the Brazilian Department of Health which read "Poor Brazil" [5]. By the following year there were 344 deaths in Natal. Theses epidemics occurred in the Alecrim neighborhood, near where the Avisos docked, which "had no precedence in Brazil for the number of illnesses or deaths" [10].

Soper had been the director of the international health division in South America of the Rockefeller Foundation [14] since 1927 and had been working on yellow fever elimination. The yellow fever service assisted the Brazilian government in the eradication of *A. gambiae* in Natal using Paris Green, with work ceasing in 1932 (http://profiles.nlm.nih.gov/VV/Views/Exhibit/narrative/campaign.html; July 21, 2010). Soper was unable to interest the state government or the federal health authorities in a larger scale *A. gambiae* elimination program and the Rockefeller Foundation was disinterested as well [4,10].

Greater Northeastern Brazil also had malaria epidemics during the 1930s. Epidemics in northeastern Brazil were often caused by people from Sertão returning from work in the interior

or coast. After a three year drought that started in 1932, migrants returning to Sertão introduced malaria and this led to an outbreak starting 1934 and ending in 1937 [3,4].

A. gambiae quietly spread west, to the less arid valleys of Assu, Apodí, and Jaguaribe rivers by 1938 [4,9,13]. Another drought had occurred in 1936 in Sertão and forced more migrants into Zona and Agreste. This meant malaria cases occurred during 1938-1939 [3], but this time in the presence of *A. gambiae*. For example, after epidemic malaria was reported Ceará in April 1938, *A. gambiae* was found to be present in October (http://profiles.nlm.nih.gov/VV/Views/Exhibit/narrative/campaign.html; July 21, 2010). The presence of *A. gambiae* led to a much greater epidemic throughout northeast Brazil in 1938-1939, which put the problem of malaria on the national stage, though it has also been argued that much of this epidemic could have been due to migration patterns previously described. Many of the victims were from the Sertão because, unlike Brazilians from the coast or the interior, they had little acquired immunity [3,4]. The epidemic started on the coast and spread up the river valleys into the interior, leading to the deaths of more than 5,000 people [3], reaching 14,000 in eight months [9].

During the summer of 1938, a "pandemic" was declared in the states of Rio Grande del Norte and Ceará, in zones where *A. gambiae* was present. In certain Brazilian states, the invasion of *A. gambiae* was followed in a few weeks by epidemic malaria [14]. Around 40,000 people were infected with more than 20,000 deaths [10,13]. By the end of the epidemic there were 600,000 cases [6].

On August 5th, 1938 President Getulio Vargas created a new emergency antimalaria service [13]. The Brazilian Government ,with the support of the Rockefeller Foundation, created the Northeast Malaria Service to eradicate *A. gambiae* [15]. This effort led to elimination of *A. gambiae* by 1940 [10,13]. Control efforts were aided by the northeast dry season, during which adult mosquitoes found it more difficult to find breeding grounds [13,14]. During the 1940s, there were 4-6 million malaria cases per year in Brazil out of a population of 55 million [3,5,6]. Greater

than 50% of cases occurred outside of the Amazon [6]. A portion of these cases was due to the government enlisting 50,000 people from Northeastern Brazil to be "rubber soldiers" in the Amazon. This was in support of the Allies during 1942 and 1943 after the Japanese cut off supplies [5,6,11]. Outside of the Amazon, valleys with large rivers also supported malaria including Sao Francisco, Paraná, and the Baixada Fluminense [6]. From the 1940s and the 1950s, *A. aquasalis* was the main malaria vector along the coast, and *A. darlingi* elsewhere. *A. (Kerteszia) bellator* and *A. (Kerteszia) cruzii* were also important in the south [16,17]. Prior to the beginning of the malaria eradication campaign, there were 6 million cases per year of malaria in Brazil in the early 1940s (which was then 1/7 of the country's population) [9].

In 1941, the National Malaria Service (SNM) was created. The SNM was run by Mario Pinotti starting in 1942 [9]. There were actually three organizations involved in eradication effort: the Special Public Health Service in the Amazon (SESP), the São Paulo State Antimalaria Service in that state, and the National Malaria Service, which covered the remainder of the country and took over in the Amazon in 1950 [9,18]. SESP was founded in 1942 in cooperation with the U.S. Institute of Inter-American Affairs to increase rubber production during World War II [8]. During the War, 17.7 million tablets of Ateprine were distributed by SESP with little impact on malaria incidence. There were 2-2.5 million malaria cases each year and half occurred in the Amazon [8]. During the 1940s, there were about 4-5 million cases each year in a population of 45 million, with more than half coming from outside the Amazon [9]. In Rondônia, malaria consisted of atabrine prophylaxis and occasional engineering projects [11]

DDT was first used in an organized way in 1945 in Breves, Pará by the SESP. Houses were sprayed every two months and later every four months, with a drastic drop in malaria. DDT used began to expand throughout the country. CQ tablets were also given out to doctors, malaria inspectors, and local influential people to give to malaria patients [9] and from1946-1947 in hospitals in Belém and Santarém with total doses of 1500 mg [19]. DDT was systematically used in Amapá, Amazonas (including neighborhoods in Manaus), Rondônia (Porto Velho in 1946,

extending to other villages in 1947, and reaching the railroad in 1948 [11]), and Pará (including the Bolonha and Agua Preta dams) to reduce the impact of *A. darlingi* by 1948 [20]. By 1950, the National Malaria Service had moved into the Amazon and was in charge of control everywhere except the state of São Paulo. Malaria had been wiped out in the northeast, dropped in the coastal plain, and was reduced in the Amazon. By 1954, DDT spraying covered regions occupied by 3 million Brazilians [9].

Mario Pinotti was made director of the National Department for Rural Endemic Diseases in 1956. He changed the National Malaria Service into the Malaria Eradication Campaign (CEM) in 1957 [9]. One of Pinotti's more interesting ideas was to put CQ in table salt, "Pinotti's method", particularly in locations where insecticides would be difficult to use. After trials in the early 1950s, chloroquinized salt was given in Pará from 1952-1953, Paraná from 1952-1954, Maranhão from 1953-1954, Minais Gerais starting in 1956, Santa Caterian State starting in 1956, Amapá starting in 1957, and along the Amazon River starting 1959 [21]. CQ salt was successfully used by the ICOMI mining company in Amapá [9,15]. As Pinotti's political career began to fade in 1960, so did the use of chloroquinized salt due to concerns that the targeted population was unevenly protected with regards to its intake (for example, due to its taste) [22]. Still other countries used it as well including French Guiana (1967-1971), Guyana (1961-1965), and Suriname (1966-1972) [21]. It has been argued that this use of CQ may have encouraged the development of early resistance [21].

The malaria eradication campaign reached the state of São Paulo in 1959. The eradication effort focused on the entire area with malaria (198 municipalities) in São Paulo State, which had 2.7 million inhabitants. The attack phase of control went from 1960 to 1963 and consisted of spraying houses with DDT every six months. Inhabitants with fever were actively sought out and 10% of the population was tested for malaria every year. In addition, 5,000 posts were created for passive surveillance and malaria treatment. By 1968, 68% of the population was living in a

malaria free zone, with much of the rest of the population in the consolidation phase (31%). It took four years to completely interrupt malaria transmission [23].

In Amapá, the Industria e Comercio SA mining company began providing its miners in Serra do Novio, and all other inhabitants with chloroquinized salt in 1963 and apparently continued to do so for the next 30 years. Their malaria control program, which included biannual DDT spraying, locally eradicated *A. darlingi* for the next forty years [16].

During the 1960s, malaria cases were confined to the Amazon region. The remaining transmission was attributed to the dispersed Amazon population, which made it hard to apply control, housing which facilitated vector contact but hindered DDT spraying, and CQ resistant *P. falciparum* [24]. Different habitats in the Amazon had different malaria profiles. During this decade, the government began to secure Brazil's borders by way of colonization and the construction of roads linking the north with other regions [3,25]. The cities of Manaus and Porto Velho had epidemics due to the influx of immigrants from the rest of the Brazilian Amazon and the creation of slums where mosquitoes could breed [24].

On September 6, 1965, the Brazilian government passed law 4,709 and adopted a Malaria Eradication Campaign (CEM) model recommended by the WHO. CEM was created along the lines of eradication described by the WHO, based around DDT spraying and the use of antimalarials where malaria was present. CEM eliminated malaria from the Northeast, the Southeast, the Center West, and the South. As the number of malaria tests increased to 1.7 million per year by 1969, the number of positive tests diminished. *A. darlingi* predominated with lesser vectors including *A. albitarsis*, *A. aquasalis*, *A. cruzii* and *A. bellator*. However, these control efforts could not eradicate transmission outside of the home and *P. falciparum* began to show signs of CQ resistance. Additional factors that led to the failure of CEM included the absence of social and health infrastructure and an at risk population of miners, agricultural colonists, and loggers that were often in contact with vectors [5,26]. Prior to the 1970s, there had been little deforestation in the Brazilian Amazon [27].

In 1970, the malaria eradication effort was suspended due to the drop in the number of cases, criticism of the public health administrative model, and concerns over DDT brought on by the book *Silent Spring* [22]. There were only 28,557 cases of *P. falciparum* in Brazil [24] out of a population of 92.3 million people in 1970 [2]. In all, 73% of the remaining cases occurred in the Amazon due to its climate and wall-less dwellings [9] and malaria had been reduced to 1% of its occurrence in 1950 [8]. Rondônia had grown to 113,659 thousand inhabitants [11]. Most of São Paulo State was deforested and mechanized agriculture had increased. Seasonal migration of agricultural workers from other states had also increased, but the number of rural residents decreased. There were very few autochthonous malaria cases (less than 100 per year).

In 1974, all malaria regions in Brazil had already been through the attack phase and there were only 50,000 cases in the entire country [23]. Of these, 24,000 cases were registered in Acre, Amazonas, Pará, and Maranhão and more than 12,000 occurred along the transamazon highway and its surroundings[7]. The Superintendent of the Public Campaigns of Brazil (SUCAM) integrated the malaria eradication programs with other public health programs and the resources for malaria control were reduced [26]. DDT resistance was reported by 1975 in Brazil and Colombia [10]. Starting around this time, malaria transmission began to increase, including all of the areas where malaria transmission had been interrupted, due to more Brazilians moving to the Amazon for colonization, and the construction of hydroelectric projects and roads [23,25]. As late as 1975, only 0.6% of the Amazon had been cleared (3,000,0000 hectares). By 1976, malaria was under control in the South, Southeast, Northeast, and part of the Center-West. Malaria was only in the prevalent in theAmazon and Northeast [5].

Historically, Brazilians had moved from the north/northeast to the south, but between 1970 and 1980 movement inverted [7]. In total, 1 million Brazilians moved into the Amazon, with the majority going to Rondônia [3] and other gold mining areas [3,28]. Most of the immigrants were immunologically naïve with regards to malaria [29]. The construction of roads, hydroelectric plants, livestock and agricultural projects, and innumerable mines lead to an

increase in malaria transmission. The number of malaria cases between 1970 and 1980 tripled – from 52,469 to 169,871 cases [24,25]. In Rondônia there were 116,000 inhabitants and the API was 50/1,000 in 1970, by 1990 there were 1,130,000 inhabitants and the API was 216.7/1,000 [30]. Malaria cases went from 5,848 in 1970 to 278,408 in 1988 [29]. During the 1940s, *A. darlingi* had made up 26% of the anopholine species composition, but it was 77.7% in the 1980s, and greater than 90% in the 1990s. *A. darlingi* behavior seems to have become less endophilic when those caught outdoors outnumbered those caught indoors by 5-10 times in the 1990s [31].

In 1980, 97.5% of malaria was still confined to the Amazon (34.8% occurred in Rondônia, 22.4% in Pará, 11.3% in Maranhão, 9% in Mato Grosso, and 8% in Roraima) [24]. Outside of the Amazon, the most cases occurred in Goiás, followed by Paraná, São Paulo, and Mato Grosso do Sul. Cases in these states came from the Amazon states and constantly presented the risk of the reintroduction of autochthonous malaria [24]. Acre had many imported cases arriving from Rondônia, with a few coming from Amazonas. Amazonas had cases that came from gold miners arriving from Rondônia and others arriving from Roraima. Amazonas also spread malaria by way of miners going to Itaituba, Pará [7]. Other miners have entered Pará from north of Mato Grosso, particularly from the mines around Peixoto de Azevedo river. Maranhão received numerous cases from Pará and Mato Grosso. Cases from Rondônia spread to Mato Grosso as well. Goiás received cases from Pará, Mato Grosso and Maranhão. Roraima had cases from multiple Amazon states including Rondônia, Amazonas, and Maranhão [7].

During the 1980s, SUCAM accepted that eradication efforts needed to be abandoned in favor of more nuanced local projects with epidemiological stratification and ‘microzoning’[24]. They divided the Amazon region into priority areas I and II based on various risk factors that could impact epidemiology [5,32]. Priority I regions had “frank” transmission and should be examined by SUCAM district boards for classification of cases and identification of provenance. Priority II regions either had low intensity, stable transmission, or did not have transmission at all [25] and the largest number of cases possible for classification and identification of possible local

transmission [25]. The new approach was first used in Pará and Rondônia [26]. New techniques were also used including outdoor ultra low volume nebulization, mass treatment, impregnated curtains, and new insecticides. In other areas, including areas of colonization in Rondônia, control efforts remained unchanged despite their lack of success [24]. Traditional control methods were ineffective in states like Rondônia that had Amazonian riverine communities. In such communities, screening homes made no sense because walls were porous for air flow, sylvatic vectors were behaviorally resistant to DDT spraying of homes, and inhabitants would have to stay under bed nets from 5 PM to 6 AM. This left patient care as the only viable control method and this might prove ineffective due to asymptomatic cases [29].

Studies suggested that there were multiple migratory channels in Brazil including: southern Pará and northern Mato Grosso to Maranhão; northern Mato Grosso to São Paulo, Paraná, Goiás, and Mato Grosso do Sul; and Rondônia to São Paulo, Pará, Acre, and Amazonas [24]. The largest migration was into Rondônia from everywhere but the north, followed by northeasterners moving into Pará. Most settled along the Transamazon highway or at mining locations [33].

In 1988, deforestation had declined because of a recession and hyperinflation [27]. In following year, the Brazilian government went to the World Bank to fund the Amazon Basin Malaria Control Project (PCMAM) due to increasing problem of malaria and regional political pressure. The World Bank provided Brazil with $99 million and this was matched by a government counterpart for another $99 million dollars. The funds were to be used over the next five years (1989-1993). The goals of PCMAM were to 1) reduce the occurrence of malaria; 2) develop SUCAM and the state secretariats of health; 3) focus attention on the health of indigenous communities. PCMAM developed local public health services with regards to diagnostics and treatment [26]. Given the difficulties implementing PCMAM during this period, it was extended for another three years, though the funding for the program was reduced by $40 million. The Integrated Malaria Control Program (PCIM) was also created during this period,

which was basically PCMAM adjusted to match strategies of the WHO at a recent meeting in Amsterdam [5]. With PCMAM back on track, the Brazilians increased the diagnostic laboratory and treatment network and the number of doctors and support staff [26]. They also made malaria treatment widely available through shops in mining areas and all fevers were presumptively treated. Though CQ was the first line treatment, mefloquine was the second line drug [34]. From 1989 to 1996, 81% of malaria cases were *P. vivax* and 17.1% were *P. falciparum* [35] (*P. falciparum* made up 59% of cases in 1988, 34% in 1992, and 25% in 1996 [30]). The incidence of malaria began to diminish in 1989 due to control strategy changes and a shift in colonization zones to occupation rather than deforestation [24]. They claimed to have reduced malaria from 577,787 cases in 1989 to 221,600 cases in 1996 [3] and the rate of mortality went from 7/1,000 in 1988 to 1.8/1,000 in 1995 [26].

Unfortunately, as the funding for PCMAM ran out, there was a resurgence of malaria. FUNASA created a new plan to intensify malaria control at the end of 1996, which focused on 100 municipalities with API's of greater than 50/1,000 or in some municipalities and state capitals where malaria was a serious problem despite lower APIs. Between 1998 and 1999 there was a 26% [26] or 34% [24] increase in malaria cases. In 1999, there were 631,000 malaria cases, of which 99.7% occurred in the Brazilian Amazon [3,36] (Figure 5.1). During the 1990s, malaria was in all the Amazon states (Maranhão: 7.2/1,000, Annual Parasite Index; Amazonas: 16.9/1,000; Pará: 22.6/1,000; Mato Grosso: 28.8/1,000; Acre: 38.5/1,000; Amapá: 43.2/1,000; Rondônia: 128.3/1,000; and Roraima: 146.5/1000). In Amazonas, the highest API occurred around Manaus among migrants in areas of poor sanitation where vectors could breed. There were 21, 234 malaria cases in Manaus in 1997 and 83% were *P. vivax* [35]. In Pará, the highest APIs were where mining and colonization projects were taking place [16]. In Amapá, cases occurred in important mining areas [24] and *A. marajoara* was reported as a new emerging vector [16]. In Acre, cases were localized around agricultural and rubber plantations around Abunã River [24]. In Rondônia, the high APIs occurred where mining and colonization projects were

taking place. By 2000, deforestation of this state had reached the northern city of Porto Velho and it was argued that this process had encouraged *A. darlingi* populations to increase, which also fed on the cattle that replaced farm crops [28].

The Brazilian Government announced that they would reduce malaria cases by 2001 to half the number seen in 1999 and half the mortality by 2002, at the first international meeting of the Roll Back Malaria program of the Pan American Health Organization in Lima, Peru in October, 1999. They would do this through the Program for the Intensification of Malaria Control in the nine-state Legal Amazon (PIACM),which was started in 2000 and cost $50.2 million [26,28,37].

PIACM had new elements: 1) political involvement at all levels of government, 2) regional development; 3) an assessment of the social development cost of malaria; 4) integration of related government offices including the Ministries of Health and Agrarian Reform;4) a structured service strategy; 5) periodic assessments of progress; 6) a guarantee of consistent funding from all levels of government [26]. Vector control was expanded with more equipment, vehicles, and personnel. This allowed for more indoor insecticide spraying and the spatial treatment of outbreaks. Drainage projects in urban centers including Manaus and Porto Velho were also undertaken. In addition, it was decided that all new settlements would have to be extensively evaluated for malaria prevention [28].

By 2001 there was a drastic reduction in the incidence of malaria (1999: 630,985 cases; 2001: 383,654 cases). *P. falciparum* cases were reduced by 35% and *P. vivax* cases by 41%. Such a reduction had not occurred in the last 41 years and was attributed to the implementation of PIACM in 2000 in most states, its implementation in 2001 in Amapá, and a similar Amazonas plan that had been implemented in 1999 [26]. Success varied by state. Amazon and Acre had more than a 60% reduction, while Amapá reduced cases by 15% and Rondônia , 9%[38].

In 2002 and 2003 there was another increase in malaria transmission, including Manaus in Amazonas [39]. In 2004, there were 350,000 cases of malaria in the country [37]. By 2005,

malaria control had passed to the National Program for Malaria Prevention and Control (PNCM). Its objectives were to reduce mortality, severe malaria, and overall malaria incidence [5].

Brazil *P. falciparum* Population Structure and Surface Proteins

The studies which have examined Brazil *P. falciparum* population structure have sometimes provided conflicting results, but suggest that population structure might be different than that reported in Peru and Venezuela. There was significant linkage disequilibrium among 10 neutral microsatellite loci that were examined in196 samples from Marabá, Pará; Tailândia, Pará; Porto Velho, Rondônia; Rio Branco, Acre; and Serra do Navio, Amapá from the 1990s. This was most true in locations were there were no mixed *Plasmodium falciparum* infections. Tailândia and Rio Branco showed strong linkage disequilibrium, Serra do Navio showed moderate linkage disequilibrium, and Porto Velho and Marabá showed the least LD. The populations with the most LD had the least multiple infections and vice versa. Regarding the sites with strong LD, they argued that an additional analysis (where the common haplotypes were collapsed into single data points) implied that there had not been recent epidemic expansions. Another analysis suggested there was no evidence for epidemic expansion of clones [40].

Though the populations appeared different, isolation by distance was also rejected. Furthermore, none of the populations showed evidence of recent bottlenecks, in a comparison of the observed heterozygosity (H_e) with that theoretically expected based on the number of alleles reported using an infinite allele model. Marabá, however, did show evidence of a recent population expansion if a subset of markers were used and a stepwise mutation model assumed. The authors argued that Brazilian populations had distinct population structure with little gene flow based on Fixation indices (F_{st}) values that varied between 0.08 and 0.30. Rio Branco appeared to be the most different and had a number of neutral haplotypes that grouped using eBurst. The authors suggested that Brazil did not have the extremely low genetic diversity and linkage disequilibrium seen elsewhere in Central and South America [40].

In contrast to the study just described, another found that there was relatively free gene flow across the Amazon basin and limited genetic diversity, based on at samples collected in the Pacific Coast of Colombia; Iquitos, Peru; Manaus and Tabatinga, Amazonas; Porto Velho, Rondônia, Peixoto de Azevedo and Apiacás, Mato Grosso. They found minimal evidence for population substructure with most diversity found within populations. Like the previous study, the authors used multiple microsatellite markers from the same chromosomes. Again, the geographic distance between sites did not explain genetic variation. Their eBurst analysis included a single haplotype group which connected 58 isolates collected in Manaus, Amazonas; Mato Grosso; Tabatinga, Rondônia in Brazil; and Iquitos in Peru. Some of these findings should be viewed with caution as fewer chromosomes were examined in this study than the previous study; the 15 microsatellite makers came from just 4 chromosomes (five of which coming from chromosome 7, which carried *pfcrt*) [41].

In another study, it was shown that isolates collected from Porto Velho, Rondônia were highly differentiated from samples collected in Colombia. Their results also suggested that there was a loss of rare alleles in Brazil, which suggesting that they were not at mutation drift equilibrium and that there had been a recent bottlenecks [42]. The differences between Colombia and Brazil were supported by another study which suggested that they carried highly different *pfmdr1* alleles. This same study also said that South American parasites had lower genetic diversity than either Africa or Asia based on *pfcrt* and *pfmdr1* alleles [43].

Surface proteins, ostensibly under pressure to be highly diverse in order to avoid the immune systems of hosts and vectors, also showed reduced diversity. A study of the *P. falciparum* merozoite surface protein 1 (*msp*-1) gene showed limited diversity (12 of 24 possible types) across the Amazon basin (Rondônia, Pará, Mato Grosso, Amapá, Tocantins, Amazonas) and between 1985 and 1997 [44]. In Rondônia, it was shown that *msp*-1 had 10 out of the 24 possible genotypes in 1995 based on variable blocks 2, 4a, 4b, and 10 (n=54). Only a single *msp*-1 type was found in 76% of samples, with 21% had two, and the remainder had three or more.

This finding was not significantly different than the null hypothesis of random allele association [45]. The *var* gene family repertoire, which encodes the *P. falciparum* erythrocyte membrane protein 1, was limited independent of location or time in the Brazilian Amazon based on samples from Rondônia in 1985 and 2001-2006, Amazonas in 2002, samples collected between 1999 and 2008 from Acre and Amazonas, samples from Mato Grosso, as well as 61 sequences from Venezuela [46]. Another study showed that *Pfs*48/45, a surface protein, had only a single allele in Amapá (n=40) and Rondônia (n=55) based on five SNPs [47].

Taken together, these studies suggest that Brazilian *P. falciparum* has limited diversity and may or may not have the extensive clonality seen in other South American countries. When combined with the history of Brazilian malaria described earlier, it appears that *Brazilian P. falciparum* may have gone through bottlenecks, multiple reintroductions due to human migration, and potentially clonal expansions.

Summary of Drug Use and First Reports of Resistance

In Brazil, quinine was already the drug of choice by the beginning of the 20th century. However, as previously described, resistance was noted as early as 1910 after quinine had been distributed as a prophylactic on a large scale to railway works in the Amazon [8]. CQ was tested in Pará during the late 1940s, first tested in the field during the early 1950s with success, and distributed during the mid 1950s as chloroquinized salt to the public [21], leading quickly to reports of the resistance [48]. Resistance to amodiaquine by *P. falciparum* was also reported in the 1960s.

SP was first used in trials at the beginning of the 1960s in response to these reports of resistance, with its use in public health by the 1970s. In 1968, 18 strains collected from Brazil, Colombia, and Brazil were used to inoculate patients in São Paulo, Brazil. Some of the strains were shown to be either mildly resistant (collected in Belém, Pará and Boa Vista, Roraima) or moderately resistant to pyrimethamine (Machado River, Rondônia; El Pescado, Colombia;

Igarapé Mirim, Pará; Barcarena, Pará; Puerto Ayacucho, Venezuela; and Cripori River close to Pará), with one strain reported to have RII resistance (collected in Goiânia, Goiás). However, all all appeared to be sensitive to pyrimethamine when used in combination with various sulphonamides. In the same study 3 out 15 'attacks' treated with quinine were not cured [49]. By 1972, SP resistance was reported in Goiás, Brazil and later reported again in Maranhão in 1978 [48,50]. In another study of patients infected in the Brazilian Amazon between 1986 and 1990, all had CQ and amodiaquine resistance at the RI or RII levels, while one patient showed quinine resistance at the RIII level. *In vivo* tests showed that all parasites were CQ and amodiaquine resistant, while 11% were quinine resistant [51].

By the end of the 1980s, 90% of parasites were SP resistant and 100 percent were CQ resistant. Brazil shifted to quinine-tetracycline as the standard treatment for uncomplicated *P. falciparum*, followed by quinine and doxycycline, and mefloquine plus primaquine as a secondary-line drug [52,53]. However, a report from 2001 showed that 23.8% of uncomplicated malaria cases in Acre were resistant to quinine plus doxycycline [54]. Parasites collected from Mato Grosso showed reduced quinine sensitivity, though only a few showing high levels of quinine resistance in the late 1990s. The sample parasites were also susceptible to mefloquine and halofantrine [55]. In 2007 and 2008, the Ministry of Health changed to artemisinin plus lumefantrine and also artesunate plus mefloquine for the treatment of *P. falciparum*. In rural locations in the Amazon, quinine plus antibiotics was still wildly used to treat *P. falciparum*. However, large hospitals in the Amazon used artemisinin plus lumefantrine. [56].

CQ resistance

CQ was first used from 1946-1947 in Belém and Santarém hospitals in Pará at a dosage of 1500 mg. *P. falciparum* recrudescence was reported, leading one author to suggest that there were already CQ resistant parasites circulating that existed prior to CQ treatment [19]. Chloroquinized salt was first distributed to the public in Pará from 1952-1953, Paraná from 1952-

1954, Maranhão from 1953-1954, Minais Gerais starting in 1956, Santa Caterian State starting in 1956, Amapá starting in 1957, and along the Amazon River starting 1959 [21]. This most likely led to its use at less than adequate dosages by the general population in these regions and facilitated the development of resistance.

In 1954, two doctors named Brito and Pinheiro reported that there was CQ resistance in Rondônia. After having reported their findings to Heath Secretary of the Federal Territory of Guaporé, they then reported their results at a National Health meeting [48]. After CQ resistance was reported in Colombia in 1961, CQ resistance was reported in Brazil, in Porto Velho, Rondônia; Belém, Pará; and along the 300 km Belém-Brasilia road [19,48,57]. Malaria recrudescence after CQ treatment was reported in Amapá, Amazonas, and Roraima in the same year[19]. In response to this resistance, an investigative center was created by OPAS/OMS at the Santa Teresa psychiatric hospital in Ribeirão Preto, São Paulo in the same year. By 1965 CQ resistance was again reported in Belém, Pará, as well as Manaus, Amazonas. Over the next two years it was again reported in Pará and Roraima, as well as Espírito Santo and Minas Gerais [19]. CQ resistance was reported in Mato Grosso by 1969. CQ resistance was first reported in the state of Acre in 1980 [19]. Overall, drug susceptibility studies suggested that 56% of cases were chloroquine resistant during the 1960s [58].

By the end of the following decade CQ resistance had increased substantially [58]. By the 1980s CQ resistance was reported throughout Brazil [19]. In Belém, Pará, patients treated with CQ, amodiaquine, and SP had a cure rate of 10% and less than 20% in Goiania, Goiás in 1987 [59]. In Acre, 73% of samples were resistant to amodiaquine and 84% were resistance to CQ in the same year [60]. Another study conducted in 1997 with 10 patients showed that 100% of the samples were resistant to CQ and amodiaquine and 11% were resistant to quinine (four from Rondônia, four from Mato Grosso and two from Pará) [51]. In 1998 in Pará, only 4% of parasites were CQ sensitive in Macapa and Serra do Navio, Amapá where miners around the Serra do

Navio had been using chloroquinized salt for prophylaxis. However, they were all susceptible to mefloquine, amodiaquine, and quinine [61].

A study suggested that *P. falciparum* was CQ resistant in Peixoto de Azevedo, Mato Grosso, but still susceptible to MQ and halofantrine in the late 1990s. This study also examined *pfmdr1* genoypes. The samples had N86/184F/C1042/1246Y [55]. In 1998 in Amapá, almost all samples (50/51) had the 1042 asp mutation in *pfmdr1*. All isolates had the tyr mutation at 1246 codon. There was not a tyr mutation at codon 86 [61]. A different study found that there were four *pfcrt* genotypes circulating in Brazil (S_{tct}VMNT, S_{agt}VMNT, CVMNT, CVMET and CVIET) using samples collected in the pacific coast of Colombia; Iquitos, Peru; Manaus and Tabatinga, Amazonas; Porto Velho, Rondônia; Peixoto de Azevedo and Apiacás, Mato Grosso between 1992 and 2002. CVMNT was only reported on the border with Peru and was argued to be due to Brazilians returning from that country. CVIET occurred in only a few isolates and was proposed to have originated from Asia or Africa within the last 20 years. The authors were unsure of the relationship between S_{tct}VMNT and S_{agt}VMNT, but argued that CVMNT had developed from SVMNT and that S_{agt}VMNT may have originated in Mato Grosso [41].

Samples collected between 1996 and 2000 from Peixoto de Azevedo, Mato Grosso; Porto Velho, Rondônia; and Manaus, Amazonas all carried the K76T mutation in *pfcrt*, which is considered key to CQ resistance [62]. Another study also supported this conclusion [63]. As did another study utilizing samples from Porto Velho, Rondônia, which showed that 93% of samples carried *pfcrt* S_{tct}VMNT and 7% S_{agt}VMNT. The same study also reported that all samples the 184F/1034C/1042D/1246Y *pfmdr1* allele [43].

In 2005, isolates collected in Tucuruí, Pará showed that all parasites had *pfcrt* mutations 75N and 76T. For *pfmdr1*, they were wildtype at codon 86 (N), had a 1042D mutation in 86% of the samples, and had a fixed mutation 1246Y [64]. Another study, published in 2009 possibly using samples from the same period, showed that most samples from Paragominas, Pará and

Porto Velho, Rondônia carried the *pfcrt* SVMNT allele. One sample from Paragominas carried the wildtype CVMNK profile and will be described further in the next section [52].

SP Resistance

Pyrimethamine was used in neighboring Venezuela in 1956 [65] with early reports of drug resistance by 1959 [66]. However, in Brazil, SP was first used in trials at the beginning of the 1960s with its use in public health by the 1970s [48]. Between 1965-1967, a study conducted in four Brazilian localities showed that 102 out of 104 infections were cured when treated with SP [58]. SP resistance was first reported in 1972 in Brazil and later in Colombia [48]. A retrospective study of treatment between 1974 and 1979 showed that, in Goiás, five patients did not clear parasites from their blood with the application of SP and another showed that 164 patients showed RII level resistance to SP in Amazonia. SP resistance was also reported in Maranhão in 1978 [67]

In the early 1980s, SP treatment failed 16-63% of the time in the country [68]. In the western Amazon, only 30% of cases treated with SP were cured, though the cure rate was 75% in the eastern Amazon [48]. During 1980 to 1981, RI SP resistance was seen 25% of parasites in Paragominas, Brazil [69]. In 1981, 16% of parasites were resistant to SP in Maranhão and 25% were resistant to CQ [70]. By 1982, RI resistance to SP was reported in patients from the Rio Tapajós, Pará; Maués, Amazonas; Ariquemes, Rondônia and Mato Grosso. RII resistance was reported in Maués and Humaita, Amazonas. Perhaps more troubling, RIII resistance was reported along BR-319, a road that connects Manaus, Amazonas to Porto Velho, Rondônia [50]. By 1984, it was stated that 30-50% of infections were no longer cured by SP in Brazil [58], though it was as high as 60% in the eastern Amazon [48]. In 1985, 52 patients infected with Amazon strains and treated with SP showed RI in 32.7%, RII in 42.3% RII, and RIII in 7.7% [67]. SP resistance in eastern Amazon increased to 90% by 1987 [48]. In the same year 92% of *P. falciparum* cases were SP resistant in Acre [60].

A study of parasites from Rondônia, Pará, Mato Grosso, and Amazonas showed the *dhfr* 108 mutation was present 90% of samples collected between 1987-1990 (with the exception of a few samples collected in Rondônia and Pará) [68]. A study of samples from the Brazilian Amazon collected in 1997 suggested that SP resistant *dhfr* and *dhps* alleles were common [63]. Another study examined samples from 1996 collected in Peixoto de Azevedo (n=17) and Apiacás (n=10), Mato Grosso and samples collected in 1998 in Porto Velho (n=15), Rondônia. Samples collected in Peixoto de Azevedo all had the 50R/51I/108N *dhfr* genotype. Apiacás had the 50R/51I/108N allele in 60% of the samples and 40% of the samples carried 51I/108N/164L [8]. Isolates from Porto Velho, Rondônia were reported to have various genotypes, which should be treated with suspicion because of earlier errors in the paper and the presence of alleles that have not been reported elsewhere in South America (including a mutation to T at 108, and a 50R/108N allele). Nonetheless, only a few samples with only the 108Asn mutation, as well others carrying the 50R/51I/108N genotype. All samples from Mato Grosso and Rondônia had the 437G/540E/581G genotype, but Rondônia also had an additional mutation, 436A, in 47% of the samples. The greater variation seen in Rondônia was attributed to more communities being sampled as well as migration from neighboring Bolivia and Peru [71].

Later some of these same samples, presumably those from Mato Grosso as their point of origin was referred to as "the southern Amazon", were sequenced for *pfcrt*, *pfmdr1* codon 1246, and *dhps*. These samples carried SVMNT (generally with S coded for by tct) as well as a mutation at *pfmdr1* codon 1246 to Y, and the 437G/540E/581G dhps *allele*. Using these samples as well as a limited number of laboratory strains, as well as isolates from Colombia, Ecuador, Haiti, Honduras, Peru, and Venezuela, they concluded that parasites with multiple mutations in *dhfr* and *dhps* had a single origin. This included the two major triple mutant profiles: 50R/51I/108N and 51I/108N/164L. They also argued that multidrug resistant *P. falciparum* had

[8] The paper and tables had conflicting reported alleles and have been corrected based on general knowledge of circulating alleles. The results for Porto Velho should be taken with additional caution.

moved in a north-northwest direction across the continent starting from the lower Amazon. In particular, they pointed out that the RIII SP resistance came from the Brazil-Bolivia Border [63].

Another study, published in 2009 and possibly using samples from the same period, showed that most samples from Paragominas, Pará and Porto Velho, Rondônia had mutant *dhfr* and *dhps*. The major multiallele profiles for these samples were *dhfr* 50R/51I/108N with *dhps* 437G/540E/581G, and *pfcrt* SVMNT (42.5%); *dhfr* 51I/108N/164L with *dhps* 437G/540E/581G, and *pfcrt* SVMNT (27.6%); *dhfr* 50R/51I/108N with *dhps* 437G/540E, and *pfcrt* SVMNT (10.6%); *dhfr* 51I/108N/164L with *dhps* 437G/540E, and *pfcrt* SVMNT (6.3%); *dhfr* 51I/108N with *dhps* 437G/540E, and *pfcrt* SVMNT (4.2%). There were three samples that had unique multidrug profiles: *dhfr* 108N with *dhps* 437G/540E/581G, and *pfcrt* SVMNT (Porto Velho); wildtype *dhfr* with *dhps* 437G/540E, and *pfcrt* wildtype (reported in Paragominas); and *dhfr* 59R/108N with *dhps* 437G, and *pfcrt* SVMNT (Porto Velho and another sample with 51I/59R/108N was reported) [52].

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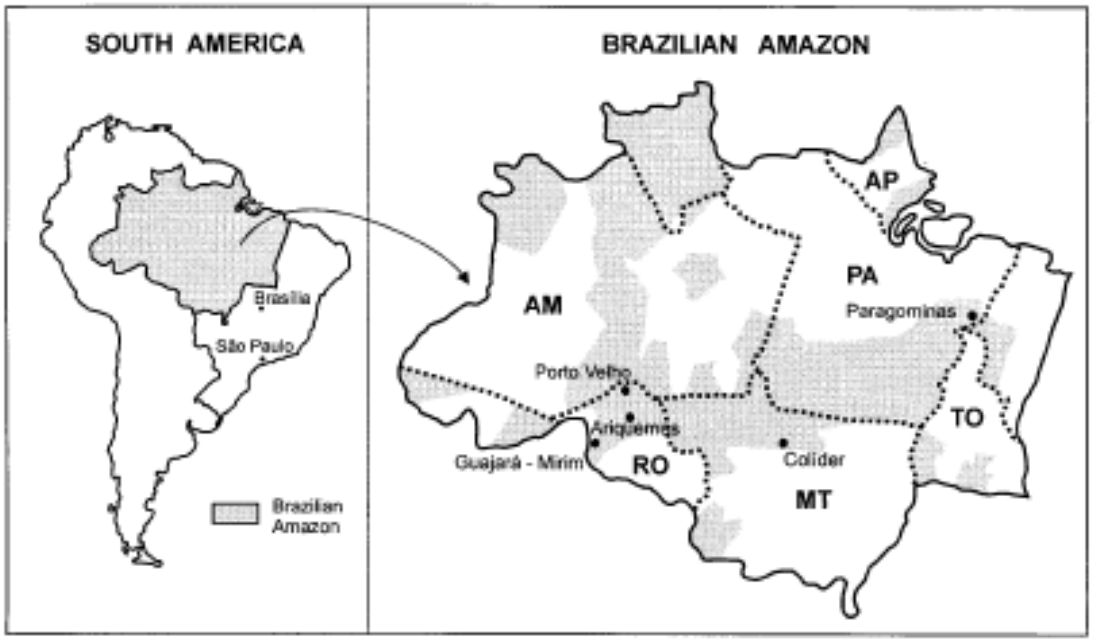
Figure 5.1 Portions of Brazil with the highest malaria transmission in the early 1990s



"(Left Panel) Map of South America showing the Brazilian Amazon region. (Right Panel) Collection sites of the Amazonian *P. falciparum* isolates analyzed in this study (Table 1). The shaded portions represent the areas with highest malaria transmission in the early 1990s…States are abbreviated as follows: AM, Amazonas; RO, Rondônia, Mato Grosso; PA, Pará; TO, Tocantins; and AP, Amapá."

From [44].

CHAPTER 6

PFMDR1 AMPLIFICATION AND FIXATION OF CHLOROQUINE RESISTANT *PFCRT* ALLELES IN VENEZUELA

Sean Griffing[1, 2], Luke Syphard[2,3], Sankar Sridaran[2, 3], Andrea M. McCollum[2], Tonya Mixson-Hayden[2], Sumiti Vinayak[2], Leopoldo Villegas[4], John W. Barnwell[2,] Ananias A. Escalante[5], Venkatachalam Udhayakumar[2*]

[1]Emory University, Atlanta, GA, United States of America, [2]Malaria Branch, Centers for Disease Control and Prevention, Atlanta, GA, United States of America, [3]Association of Public Health Laboratories, Silver Spring, MD, United States of America, [4]Asociación Civil Impacto Social, Tumeremo, Venezuela, [5]Arizona State University, Tempe, AZ, United States of America

Address of Corresponding Author and Institution Where Work was Performed:

Centers for Disease Control and Prevention

4770 Buford Highway, NE

Bldg. 22, Rm. 2

Atlanta, GA 30341

Phone 770-488-4047

Fax 770-488-4454

Email:vxu0@cdc.gov

ABSTRACT

Molecular tools are valuable for determining evolutionary history and the prevalence of drug-resistant malaria parasites. These tools have helped to predict decreased sensitivity to antimalarials and fixation of multidrug resistant genotypes in some regions. In order to assess how historical drug policies impacted Venezuelan *Plasmodium falciparum*, we examined molecular changes to genes associated with drug resistance. We examined *pfmdr1* and *pfcrt* in samples from Sifontes, Venezuela and integrated our findings with earlier work describing *dhfr* and *dhps* in these samples. We characterized *pfmdr*1 genotypes and copy number variation, *pfcrt* genotypes, and proximal microsatellites in 93 samples originating from 2003-2004 surveillance. Multi-copy *pfmdr1* was found in 12% of samples. Two *pfmdr1* alleles, Y184**F**/N1042**D**/D1246**Y** (37%) and Y184**F**/S1034**C**/N1042**D**/D1246**Y** (63%), were found. These alleles share ancestry and no evidence of strong selective pressure on mutations was found. Chloroquine resistant *pfcrt* alleles are fixed with two alleles: $\mathbf{S_{tct}}$VMN**T** (91%) and $\mathbf{S_{agt}}$VMN**T** (9%). These alleles are associated with strong selection. There was also an association between *pfcrt*, *pfmdr*1, *dhfr*, and *dhps* genotypes/haplotypes. Duplication of *pfmdr1* suggests a potential shift in mefloquine sensitivity in this region, which warrants further study. A bottleneck occurred in *P. falciparum* in Sifontes and multidrug resistant genotypes are present. This population could be targeted for malaria elimination programs to prevent the possible spread of multi drug resistant parasites.

INTRODUCTION

Amplification of the *P. falciparum* multi drug resistance (*pfmdr*1) gene has been implicated in mefloquine (MQ) resistance in Thailand and Cambodia [1,2,3,4,5,6] but not elsewhere. It is not known if amplification has occurred in Venezuela, where MQ monotherapy was used between 2001-2004 and artesunate (AS)+MQ thereafter. *Pfmdr*1 amplification is also implicated in resistance to lumefantrine, halofantrine, quinine, and AS [7] and may decrease resistance to chloroquine (CQ) [8]. Also, single nucleotide mutations in *pfmdr1* such as N86**Y**, D142**G**, Y184**F**, S1034**C**, N1042**D**, and D1246**Y** are postulated to modulate drug response. While these mutations may or may not contribute to CQ resistance [9], mutations at codons 1034, 1042 and 1246 make parasites more MQ sensitive [9] . Studies suggest at least two lineages of mutant *pfmdr*1 genotypes have evolved in South America [10,11].

In South America, CQ and sulphadoxine-pyrimethamine (SP) were used to treat *P. falciparum* prior to the use of artemisinin-based combination therapy (ACT). Resistance to CQ and SP evolved independently in South America [12,13]. Point mutations in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene were correlated with CQ resistance [14]. The *pfcrt* point mutation K76T is critical, but C72**S**, M74**I**, N75**E**, and N75**K** are also associated with resistance [15]. There are at least four different origins of CQ resistant *pfcrt* alleles: one in Papua New Guinea (**S**VMN**T)**, where the genotype represents amino acids at codons 72-76 and **S** and **T** are mutant, one in Southeast Asia (CV**IET**) that spread to Africa, and two in South America (**S**VMN**T**/CVMN**T** in Brazil/Peru and CVM**ET**/CVMN**T** in Ecuador/Colombia) [16].

Molecular surveillance showed that, after drug removal, CQ resistant genotypes in Malawi and China [12,17] and SP resistant genotypes in the Peruvian Amazon [18] declined. Thus, the reduction in the frequency of resistant parasites likely occurred because resistant parasite populations are at fitness disadvantage in the absence of drug pressure. In Bolívar State, Venezuela, mutant *pfcrt* alleles remained after removal of CQ in 1986 [19] and mutant dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes remained fixed after SP

removal [20]. Whether the recent use of MQ and AS+MQ led to the evolution of *pfmdr*1 genotypes associated with AS and MQ resistance is unknown.

This study in Bolívar assessed: 1) whether *pfmdr*1 duplication has occurred, 2) the frequency of *pfmdr*1 and *pfcrt* mutations 3) whether MQ and CQ drug pressure has affected variation surrounding these genes, and 4) linkage disequilibrium between *dhfr, dhps, pfcrt* and *pfmdr*1 alleles.

MATERIALS AND METHODS

Study site and subjects. Sifontes municipality, located in Bolívar, is an epicenter of multi-drug-resistant *P. falciparum*, contributing 35-40% of Venezuelan malaria cases in 1999 [21,22]. We tested 93 blood samples taken from a Sifontes surveillance study during 2003-2004. Patients were adults with confirmed *P. falciparum* parasitemia and generally uncomplicated malaria. These samples were previously characterized for neutral microsatellites and those surrounding *dhfr* and *dhps* [20]. Previous analysis of these samples did not reveal any multiple infections; each sample possessed a single genotype at all loci. Informed consent was obtained from patients and the study protocol was approved by the bioethics commission of the Instituto de Altos Estudios in Venezuela.

DNA isolation, amplification, and genotyping methods DNA was isolated from whole blood using the QIAamp DNA mini kit (QIAGEN, Valencia, CA). Genomic DNA was used for sequencing and real time PCR. Amplified DNA (Qiagen's REPLI-g Whole Genome Amplification Kit, Valencia, CA) was used for microsatellite characterization.

*Pfmdr*1 copy number was determined by TaqMan real-time PCR (Stratagene MX3005P; Agilent Technologies, LaJolla, CA) with published primers and probes [5] labeled with 3' black hole quencher (BHQ) and 5' FAM (*pfmdr*1*)* or 5' HEX (*B-tubulin*) (Table 6.1). Amplification reactions were multiplexed. Samples were run in triplicate, with clone 3D7 as a normalizer. Two

reference DNAs were included, DD2 has 3-4 copies of *pfmdr*1, [23] and W2-mef has 3 copies [24]. Assays were repeated if Ct values were > 32 or if the 95% confidence around the estimation was > 0.4 [5]. Copy number was calculated with the comparative ΔΔCt method [5]. Copy number estimates were rounded to the nearest integer, and parasites with greater than 1.5 copies were considered multicopy [5]. Following the convention established by Price et al., the copy numbers reported are based on mean values after rounding, even if the final confidence intervals calculated contained more than one integer after rounding. Two tailed 95% confidence intervals were calculated from the individual replicate ΔΔCt calculations [25].

We examined *pfmdr*1 for mutations in codons 86, 144, 184, 1034, 1042 and 1246. Mutations in codons 86 and 184 of *pfmdr*1 were detected using a Stratagene MX3005P Real-Time PCR system [26] (Table 6.1). Wild type probes were labeled with FAM and minor groove binder-nonfluorescent quencher, while mutant probes were labeled with VIC (Applied Biosystems, Foster City, CA). Direct sequencing was used to analyze polymorphisms in codons 1034, 1042 and 1246 (Table 6.1).

A 264 bp region of *pfcrt* containing codons 72-76 was amplified in 91 samples (Table 6.1). Residual dye terminators were removed by ethanol precipitation followed by a 70% ethanol wash. Pellets were resuspended in 10 μL HiDi Formamide (Applied Biosystems, California) and sequenced using an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, California).

Microsatellite analysis Samples were assayed for 12 microsatellite loci spanning 382 kb around *pfcrt* on chromosome 7 and 15 microsatellite loci, spanning 538 kb around *pfmdr*1 on chromosome 5. PCR primer sequences are provided in Supplementary Table 6.3 following the cycling conditions detailed in [27] and [28]. PCR products were separated on an Applied Biosystems 3130xl sequencer and scored using GeneMapper software v.3.7 (Applied Biosystems, Foster City, CA). Multiple alleles were not detected (Supplementary Table 6.4), supporting earlier results [20] that suggested the samples were all monoclonal. Two samples were removed

due to contamination. Haplotypes were classified as different if they contained ≥ 2 different alleles across all loci. eBurst [29] was used to examine the microsatellite haplotypes of both *pfcrt* (-4.8 kb to 7 kb) and *pfmdr*1 (-4.2 to 3.7 kb). Missing data was reported but not considered when defining haplotypes. Previously published data for microsatellite loci and genotype for *dhfr* and *dhps* were also incorporated [20].

Statistical analysis

Expected H_e was calculated for each locus as $[n/(n-1)][1-\Sigma p_i^2]$, where n is the number of isolates sampled and p_i is the frequency of the ith allele [30]. The sampling variance for H_e was calculated as $[2(n-1)/n^3][2(n-2)][\Sigma pi^3-(\Sigma pi^2)^2]$ [30]. The Excel Microsatellite tool kit calculated the number of alleles per locus and allele frequencies [31]. An α of 0.05 was our threshold of statistical significance. Significant associations between microsatellite pairs were determined using an exact test of linkage disequilibrium [32] with 10,000 Monte Carlo steps in Arlequin version 3.1 [33]. We also noted whether there was any linkage between *pfcrt*, *pfmdr*1, *dhfr*, or *dhps* genotypes and *pfcrt*+*pfmdr*1 versus *dhfr*+*dhps* using the same conditions. In a panmictic population, the null hypothesis is linkage equilibrium between loci located on different chromosomes. P-values for microsatellites were examined after a Bonferonni-Holms correction [34].

RESULTS

***Pfmdr*1 Copy Number Variation and Genotypes:** *Pfmdr*1 copy number analysis was successful for 90 samples: 79 (88%) had 1 copy, 7 (8%) had 2 copies, 1 (1%) had 3 copies, and 3 (3%) had 4 copies (Figure 6.1). We found only two *pfmdr*1 mutant alleles, Y184**F**/N1042**D**/D1246**Y** (triple mutant) and Y184**F**/S1034**C**/N1042**D**/D1246**Y** (quadruple mutant) at frequencies of 37% and 63%, respectively (Table 6.2). Codon 144 of *pfmdr*1 was always wild type. Duplication of *pfmdr*1 was found on both triple and quadruple mutant *pfmdr*1 lineages.

***Pfcrt* Genotypes:** No wild type CVMNK *pfcrt* genotypes were present. We found two alleles $\mathbf{S}_{tct}$VMN**T** and $\mathbf{S}_{agt}$VMN**T** at a frequency of 91% and 9%, respectively (Table 6.2).

Microsatellite characterization: Supplementary Table 6.2 details the microsatellite haplotypes around *pfcrt* and *pfmdr1*. Quadruple *pfmdr*1 mutant parasites carrying the *pfcrt* allele S_{agt}VMNT had only one haplotype. The mean H_e of loci surrounding *pfmdr*1 was 0.25 (Table 6.2). H_e was reduced in microsatellite loci closest to *pfmdr*1 (Figure 6.2). There was no marked difference in the H_e curves of single and multicopy *pfmdr*1 parasites (data not shown).

The mean estimated H_e for *pfcrt* was 0.07 (Table 6.2). Little variation was found immediately surrounding *pfcrt* with the exception of markers at -5 kb (Table 6.2). The majority of the variation around *pfcrt* was attributable to the different haplotypes of $\mathbf{S}_{tct}$VMN**T** or $\mathbf{S}_{agt}$VMN**T** lineages. For $\mathbf{S}_{tct}$VMN**T,** an increase in variation is seen ~45 kb 5', but no increase is seen within ~60 kb 3' of *pfcrt* (Figure 6.2). The lack of variation in the loci around $\mathbf{S}_{agt}$VMN**T** is striking; however the small number of parasites with this genotype warrants caution. There are only 1-2 alleles at each of the microsatellite loci around *pfcrt* (Table 6.2).

Visual inspection (Supplementary Table 6.4) and eBurst analysis (eBurst analyses not shown) suggests the *pfcrt* genotypes $\mathbf{S}_{tct}$VMN**T** and $\mathbf{S}_{agt}$VMN**T** are closely related with the only evidence

for differentiation or mutation found at the -5 kb marker. The triple and quadruple *pfmdr*1 genotypes are related and clustered around a single haplotype (203, 126, 196, 206, 221, 191, 168).

Linkage disequilibrium between genotypes and haplotypes

Previously, *dhfr* and *dhps* were shown to be in linkage disequilibrium (LD) in this population [20]. LD existed between each pair of genes: *pfcrt* vs. *pfmdr*1 (p = 0.02), *dhfr* vs. *dhps*, (p = 0.00), *pfcrt* vs. *dhfr* (p = 0.00), *pfcrt* vs. *dhps* (p = 0.00), *pfmdr*1 vs. *dhfr* (p = 0.03), *pfmdr*1 vs. *dhps* (p = 0.02). LD was also significant for a comparison of combined *pfcrt/pfmdr*1 genotypes vs. *dhfr*/*dhps* genotypes (p = 0.00). Each gene occurs on a separate chromosome and, here, had two alleles. A maximum of 16 possible combinations of *dhfr*, *dhps*, *pfcrt*, *pfmdr*1 alleles would be expected, assuming independent assortment. We only saw three: $\mathbf{S}_{agt}$VMN**T** *pfcrt*/quadruple mutant *pfmdr*1/double mutant *dhfr* /double mutant *dhps;* $\mathbf{S}_{tct}$VMN**T** *pfcrt* /triple mutant *pfmdr*1/triple mutant *dhfr*/triple mutant *dhps*; $\mathbf{S}_{tct}$VMN**T** *pfcrt*/quadruple mutant *pfmdr*1/triple mutant *dhfr*/triple mutant *dhps*. In addition, only the two S_{tct}VMT 'types' had multiple copies of *pfmdr*1.

There was extensive linkage disequilibrium among microsatellites around all four genes and neutral markers (Figure 6.3). We compared 1275 pairs of loci on chromosomes 2, 3, 4, 5, 7, and 8. We expected 63.75 pairs (0.05*1275 pairs) to be statistically significant in a panmictic population; here, 325 pairs, or 26%, showed significant disequilibrium.

$\mathbf{S}_{agt}$VMN**T** allele had only a single microsatellite haplotype and was found with one quadruple mutant *pfmdr*1 haplotype and none of the parasites with multicopy *pfmdr*1. This *pfcrt*/*pfmdr*1 haplotype appeared with only a single double mutant *dhps* (minor variation at -297 kb) and one *dhfr* (minor variation noted at -17, 5.87, and 350 kb) microsatellite haplotype. This variation continued to the neutral markers, where 7/8 of the $\mathbf{S}_{agt}$VMN**T** samples shared a haplotype (the eighth differed at 2/7 markers), suggesting clonal expansion. Conversely, the $\mathbf{S}_{tct}$VMN**T** *pfcrt* genotype occurs with both *pfmdr*1 genotypes, multiple related haplotypes of the

triple *dhfr* and triple *dhps* alleles (n=82), and with multiple neutral marker profiles. **S**$_{tct}$VMN**T** had a single haplotype, with the exception of variation of -257, -200 kb, and 245 kb.

DISCUSSION

Our findings raise concerns about the potential development of *de novo* MQ resistance in South America. Twelve percent of the samples tested from Sifontes carried multiple copies of *pfmdr*1. This had previously only been reported in Southeast Asia, where it was linked to MQ failure and decreased ACT efficacy [1,2,3,4,5,6]. The retrospective nature of our study prevented testing whether *pfmdr*1 amplification was induced by MQ monotherapy or its implications for MQ treatment. However, a few studies have shown reduced MQ sensitivity *in vitro* or prophylaxis failure in South America [35]. Our data highlights the importance of testing more recently collected samples for shifts in *pfmdr*1 copy number prevalence and potential MQ resistance. In contrast to Venezuela, there is no evidence of multi-copy *pfmdr*1 in isolates from the Peruvian Amazon, where AS+MQ therapy has been the first line treatment since 2001 [10].

We found only two alleles for *pfmdr*1: Y184**F**/N1042**D**/D1246**Y** and Y184**F**/S1034**C**/N1042**D**/D1246**Y** and gene duplication occurred with both alleles. Parasites with the Y184F mutation and higher copy number are reported to have higher IC 50s *in vitro* to MQ and other drugs [36]. Previously reported multicopy *pfmdr*1 occasionally carried a mutation at codon 86, but not mutations at 1034, 1042, or 1246 [5,36,37]. There are at least two explanations: 1) the 1042 mutation imposes a severe fitness cost imposed on parasites with multiple copies of *pfmdr*1 [36] or, 2) there is underreporting of mutations due to the limited number of studies [38]. Our results support the latter hypothesis because all parasites carrying multi-copy *pfmdr*1 had the 1042 mutation and 2-3 other mutations. However, if a fitness cost is associated with the 1042 mutation, then the additional *pfmdr*1 mutations seen in this population may be compensatory.

In contrast to the multiple origins of *pfmdr*1 amplification, point mutations associated with *pfmdr*1 resistance have a common founder lineage in our study. There is a shared haplotype

for both the triple mutant and quadruple mutants between -4.2 and 3.7 kb and an additional quadruple mutant haplotype. The latter, while differing at both -3.4 and 0.56 kb, appears to be due to slippage (Supplementary Table 6.2). This data suggests the triple mutant is ancestral to the quadruple mutant, or vice versa. While the overall H_e around *pfmdr*1 is lower than in Southeast Asia, we see a similar relative reduction in variation close to *pfmdr*1 [39]. There is a smaller region of reduced He around *pfmdr*1 than *pfcrt* (Figure 6.2). This suggests *pfmdr*1 may have 1) experienced little to no selection or 2) the selective event(s) for *pfmdr*1 occurred earlier than for *pfcrt* allowing recombination to break down LD. The latter possibility appears unlikely given history of antimalarial policy. Additionally, point mutations in *pfmdr*1 may be under selection by multiple drugs, which could complicate the signal of selection [9]. The two most recent influences on *pfmdr*1 in Sifontes are MQ and CQ, which may have differing directions of selection for mutations at 1042, 1034, or 1246. Our data could be interpreted as evidence of selection for multiple alleles or soft selective sweeps, as shown at the Thailand-Myanmar border [39].

Recent drug policy in Sifontes may have influenced preexisting *pfmdr1* alleles since nothing in our data indicates mutations occurred locally. For example, South American isolates collected in 1984 carried the same quadruple mutant *pfmdr1* genotype found in our samples, though we could not compare microsatellite haplotypes [40]. The quadruple mutant *pfmdr1* allele has been seen in Peru, Guyana, and Brazil [11,41], and the triple mutant allele has been seen in Peru [10] and Colombia [11]. Whether all of these alleles share microsatellite haplotypes is unknown. However, *pfmdr*1 haplotypes in Guyana and Brazil are more closely related to each other than those found in Colombia [11]. Our data indicate that Venezuelan *pfmdr*1 haplotypes are closely related to one of the two major haplotypes (MDR-A1 and MDR-A8) found in the Peruvian Amazon [10]. If we assume that these *pfmdr1* alleles existed prior to the gene duplication event(s), then the amplification evolved multiple times in South America, as seen in Southeast Asia [39].

To clarify whether reduced H_e around *pfcrt* is due to a sweep or a bottleneck, we looked for a U-shaped depression in H_e surrounding the gene. For $\mathbf{S}_{tct}$VMN**T**, a selective sweep is suggested by the reduced H_e in a long surrounding region and the observation that distant markers are approaching neutral heterozygosity (Figure 6.2). The lack of variation surrounding $\mathbf{S}_{agt}$VMN**T** may be due to low sample size or a bottleneck followed by clonal expansion. The second possibility appears more likely given the lack of variation associated with *dhfr*, *dhps*, and *pfmdr*1 genotypes/haplotypes. Additional data is required to test whether a selective sweep influenced H_e. around $\mathbf{S}_{agt}$VMN**T**. Nonetheless, the depressed H_e around the $\mathbf{S}_{agt}$VMN**T** allele, compared to that around $\mathbf{S}_{tct}$VMN**T**, suggests it is a recent introduction with a smaller number of founders.

The evolutionary relationship between $\mathbf{S}_{agt}$VMN**T** and $\mathbf{S}_{tct}$VMN**T** in South America is unclear in the literature. Proximal microsatellite alleles are shared between the two genotypes suggesting that the two alleles are closely related. Some of the remaining variation in $\mathbf{S}_{tct}$VMN**T** haplotypes could be explained by recombination with $\mathbf{S}_{agt}$VMN**T** haplotype. While the limited variation around *pfcrt* does not define which arose first, our results suggest they originated from the same lineage [11,42]. Our haplotype data also suggests $\mathbf{S}_{agt}$VMN**T** was introduced to Sifontes along with a related $\mathbf{S}_{tct}$VMN**T** haplotype (Supplementary Table 6.4). It had been hypothesized that $\mathbf{S}_{agt}$VMN**T** originated in Mato Grosso, Brazil [42], but $\mathbf{S}_{agt}$VMN**T** is also found in our study and Guyana, Peru, Suriname, and Venezuela [11,19,43,44], which makes its point of origin obscure.

There are at least three possible explanations for the fixation of CQ resistant *pfcrt* SVMNT alleles in Sifontes. First, the at-risk population may continue to expose *P. falciparum* indirectly due to CQ-based *P. vivax* treatment. Second, **S**VMN**T** may have little or no fitness disadvantage in the absence of drug pressure. In Africa, CQ resistant parasites with CV**IET** declined after CQ was withdrawn, but CV**IET** is more likely to revert to CQ sensitivity in the presence of verapamil than **S**VMN**T**, suggesting the alleles differ in biological fitness [45]. Third, there are no wildtype parasites present to replace the less fit CQ resistant genotype. This is

supported by our results and earlier work which found only **S**$_{agt}$VMN**T** and **S**$_{tct}$VMN**T** in Sifontes and Gran Sabana in 1998-2000 [19].

Fixation of CQ resistance in Sifontes is likely to continue because of its isolation and the fixation of CQ resistance in neighboring populations. According to one study the K76T mutation was fixed across Bolívar [19]. Sifontes is isolated from Orinoco river basin flow, which influences travel through Bolívar [21,22] and, to the west, it is separated from Bolívar by a region of higher elevation and a large reservoir. To the south, Sifontes is separated from most of Gran Sabana and Brazil by a mountain range, though a road does connect them. Even if migration occurs from Brazil, the K76**T** mutation was fixed in Manaus in 2000-2002 (n=38) [42]. To the east, there are few geographic barriers with Guyana, where **S**$_{agt}$VMN**T** is at high frequency and two studies indicate the K76**T** mutation was fixed or nearly fixed [11,46].

The association between alleles of *pfcrt*, *pfmdr*1, *dhfr* and *dhps* alleles amongst our samples indicates inbreeding, a bottleneck, and/or that each subsequent resistant gene was established from a population already fixed for other resistant genes. Our results for the **S**$_{agt}$VMN**T** *pfcrt* lineage support clonal propagation. These eight samples carried a single quadruple mutant *pfmdr*1 haplotype, and always exhibited the double *dhfr* mutation (N51**I**/S108**N**) and double *dhps* mutation (A437**G**/A581**G**), as well as an exclusive neutral marker haplotype. Our results for **S**$_{tct}$VMN**T** also support this hypothesis, albeit with a larger starting population. Only a small portion of the *pfcrt* alleles found in another study of Bolívar (CV**IET**, CVM**ET**, CVMN**T,** CVMNK) [19] were seen in Sifontes. This lack of allelic diversity, in comparison to the rest of the state, extends to *dhfr* and *dhps* genotypes [19,20].

Clonal propagation is argued to play a significant role in the population structure of *P. falciparum* in Venezuela [47]. Low transmission leads to high rates of self fertilization, and thus de facto clonal propagation. For example, with 1% recombination, markers 5 cM apart could maintain linkage disequilibrium for longer than 400 years [48]. Our results suggest that the level

of transmission, genetic diversity, and migration should be considered when predicting whether drug resistant alleles will decline after new drugs are introduced.

Demographic history may also explain the strong linkage disequilibrium across multiple chromosomes. *P. falciparum* populations in Sifontes likely originated from a recent population expansion after a bottleneck. In 1970, Bolívar had a malarious zone to west and another in the middle of the state, yet in Sifontes, malaria had been eradicated [49]. By 1983, *P. falciparum* reemerged in El Dorado, the capital of Sifontes, and presumably acted as a founding population [22]. Since CQ and SP resistance were already present in the 1970s in Venezuela [50,51], it is unlikely that the drug resistant alleles originated in Sifontes; resistance was noted elsewhere before and during the time Sifontes was malaria free. It has been postulated that the SP resistant alleles in Bolívar came from Brazil [19]. Therefore, the limited diversity and linkage we see across all markers and genes in this population may be due to rapid expansion from a small parasite population over 20 years, resulting in a semi-clonal population of multi-drug resistant parasites.

Our results suggest how multi-drug resistant *P. falciparum* can develop in isolated populations with low genetic diversity. If resistance to an antimalarial (CQ) reaches fixation, then a mutant allele is at no fitness disadvantage until a more fit allele with fewer mutations appears through back mutation or migration. Successful back mutation is unlikely due to the low probability of facilitory mutations and genetic drift. Successful migration is unlikely given this region's isolation and the lack of nearby wild type source populations. Given these restrictions, if resistance is fixed for a drug (CQ) and a second drug (SP) is introduced, then resistance to the second will occur on a background of prior resistance. Such multi-drug resistant strains will remain stable and increase in the population as inbreeding renders chromosomal reassortment ineffective. The generation of MQ resistant multicopy *pfmdr*-1 in CQ and SP resistant parasites may give resistance to additional drugs like halofantrin, quinine, and AS [5,9,36,37] and challenge the effectiveness of ACT.

Whatever the mechanism, potentially MQ-resistant P. *falciparum* are evolving on a background of CQ and SP resistance in Sifontes. Therefore this is a region of special concern for malaria treatment and elimination because migrants could spread multidrug resistance to other countries. It remains to be seen whether *pfmdr*1 amplification in Sifontes has resulted in increased levels of MQ resistance and less AS+MQ sensitivity. Future molecular surveillance will be critical for determining whether the prevalence of pfmdr1 duplication has increased since the time of our study and whether it is associated with ACT resistance.

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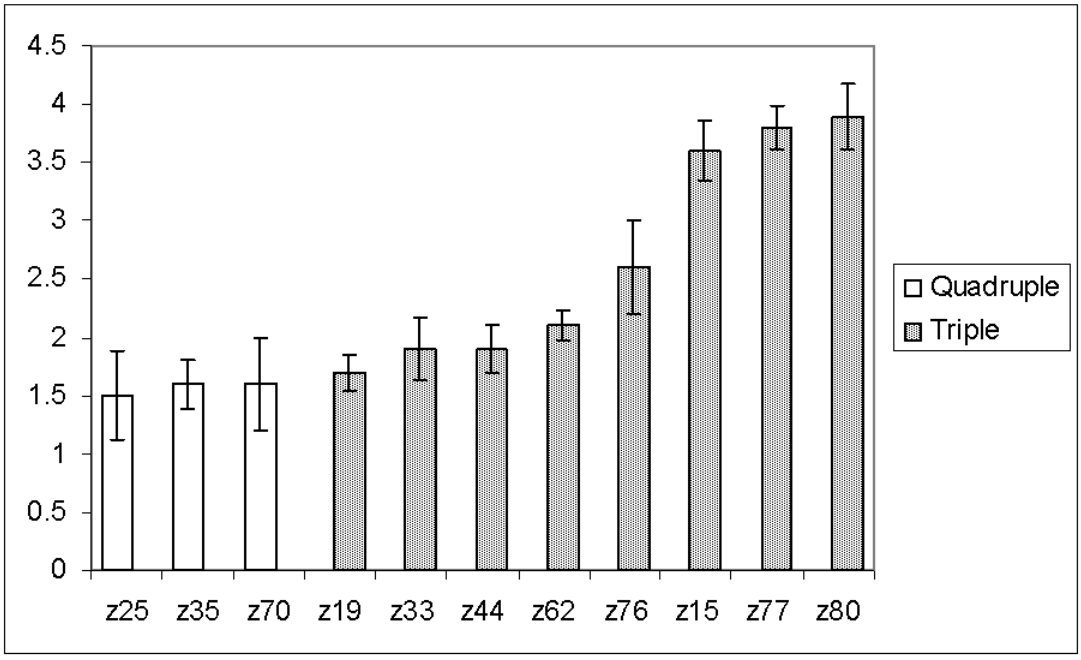
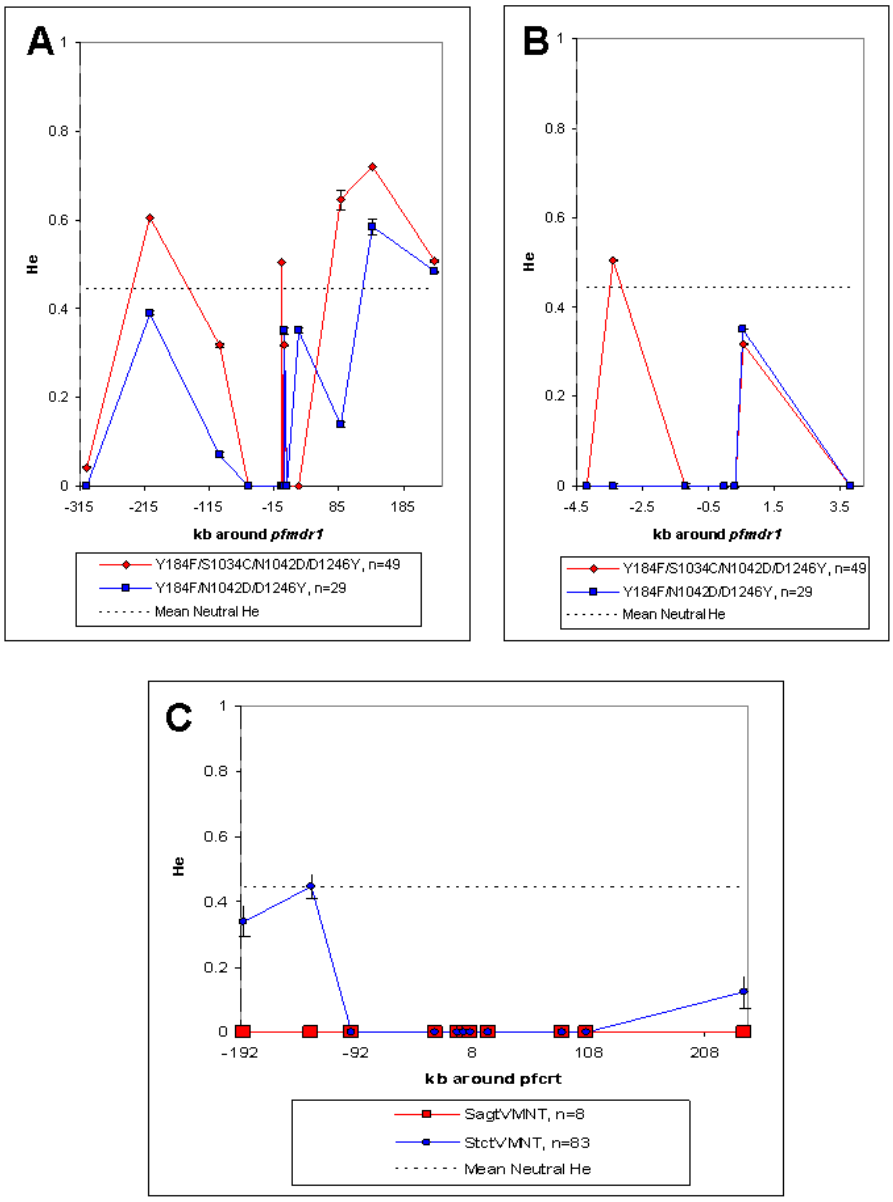
Figure 6.1 The copy number of *pfmdr1* in Sifonties, Venezuela



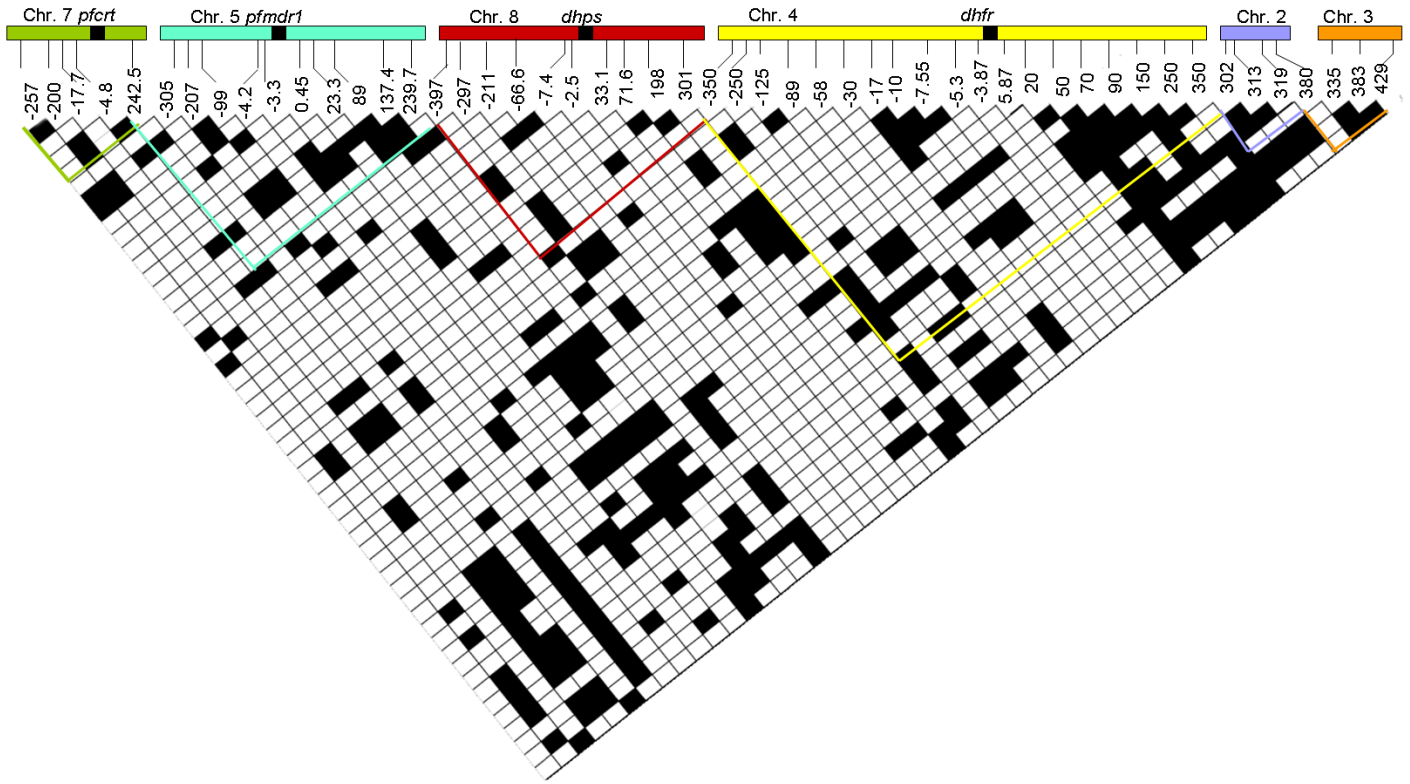
Estimates of *pfmdr1* copy number with confidence intervals from samples with *pfmdr1* amplification. Open bars represent quadruple mutant *pfmdr*1 parasites, while shaded bars represent those with triple mutant *pfmdr1* parasites.

Figure 6.2 Variations in H_e around *pfmdr1* and *pfcrt*.



Graphical displays of $H_e \pm 1$ standard deviation around *pfmdr1* and *pfcrt*. The dashed lines in each graph is the mean neutral H_e calculated from loci on chromosomes 2 and 3 [20]. On the *x* axis, negative numbers are positions 5' to the gene and positive numbers are positions 3' to the gene. (A) The entire region surrounding *pfmdr1* characterized by microsatellite markers on chromosome 5. (B) A close-up of the pfmdr1 region with low H_e (C) The entire region surrounding *pfcrt* characterized by microsatellite markers on chromosome 7. For S_{agt}VMNT, the error bars for the microsatellite markers are all 0 due to the lack of variation.

Figure 6.3 Pairwise linkage disequilibrium between microsatellite loci on different chromosomes.



Pairwise linkage disequilibrium between microsatellite loci on different chromosomes. Each box represents one comparison between polymorphic pairs of loci; non-polymorphic pairwise comparisons are not included. Bonferroni correction for multiple comparisons was conducted for each comparison. Black cells represent significance at 0.01 and white cells were not significant. The location of each microsatellite locus is given on the x and y axis (loci are named according to their position relative to *pfcrt*, *pfmdr*1, *dhfr*, *dhps*, or position along chromosome 2 or 3 according to the 3D7 genome sequence available from NCBI)

TABLE 6.1. *pfmdr1* and *pfcrt* methods

	*pfmdr*1 TAQMAN	*pfmdr1* *CODONS* 86 & 184 STRATAGENE	*pfmdr1* CODONS 1034, 1042, & 1246 SEQUENCING	*pfcrt* PCR AMPLIFICATION	*pfcrt* CYCLE SEQUENCING
Conditions	1 cycle at 95°C, 10 min.; 50 cycles at 95°C for 20 sec.. & 60°C, 1 min..	96°C for 10 min.; 50 cycles of 96°C for 15 sec. and 59°C for 1 min. (codon 86) or 61°C for 1 min. (codon 184).	95°C for 3 min.; 40 cycles of 93°C for 30 sec., 58°C for 40 sec., 72°C for 45 sec.; final extension at 72°C for 2 min..	1 cycle at 94°C for 10 min.; 38 cycles of 94°C for 30 sec., 56°C for 30 sec., 72°C for 45 sec.; 72°C for 10 min..	1 cycle at 96°C for 60 sec.; 25 cycles of 96°C for 10 sec., 50°C for 5 sec., 60°C for 4 min..
Forward Primer	[5]	[26]	5’-GCATTAGTTCAGATGATGAAATG-3’	5' TTTTTCCCTTGTCGACCTTAAC 3'	NA
Reverse Primer	[5]	[26]	5’-CCATATGGTCCAACATTTGTATC-3’	5' AGGAATAAACAATAAAGAACATAATCATAC 3'.	5' AGGAATAAACAATAAAGAACATAATCATAC 3'.
Secondary Reaction Conditions	NA	NA	94°C for 10 min.; 35 cycles of 94°C for 1 min., 59°C for 1 min., 72°C for 1 min.; and a final extension at 72°C for 10 min..	NA	3.0µL of PCR product, 2.0 µL of Dye Termin.ator 5x Sequencing Buffer, 0.8 µL of the reverse primer at 10 uM, 0.3 µL of Big Dye Termin.ator (Applied Biosystems, California), and 3.9 µL of water in a final volume of 10.0 µL.
Nested Forward Primer for Secondary Reaction	NA	NA	5’-TA TGCATACTGTTATTAATTATGG-3’ (used either primer for sequencing)	NA	5' AGGAATAAACAATAAAGAACATAATCATAC 3'.

TABLE 6.2. Frequency of *pfcrt* and *pfmdr*1 genotypes and number of alleles (A) and expected heterozygosity (H_e) per microsatellite locus

pfmdr1	NUMBER (FREQUENCY)	*pfcrt*	NUMBER (FREQUENCY)
Y184F/N1042D/D1246Y	29 (0.37)*	S_{tct}VMNT*	83 (0.91)*
Y184F/S1034C/N1042D/D1246Y	49 (0.63)*	S_{agt}VMNT*	8 (0.09)*

LOCI ON CHROMOSOME 5 AROUND *pfmdr1*			LOCI ON CHROMOSOME 7 AROUND *pfcrt*		
Distance from *pfmdr*1	*A*	H_e ±SD	Distance from *pfcrt*	*A*	H_e ±SD
-305 kb	2	0.0227±0.0006	-257 kb	2	0.3209±0.0026
-207 kb	3	0.5424±0.0014	-200 kb	2	0.4796±0.0004
-99 kb	2	0.2360±0.0029	-45 kb	1	0.0000±0.0000
-54 kb	1	0.0000±0.0000	-17.7 kb	1	0.0000±0.0000
-4.2 kb	1	0.0000±0.0006	-4.8 kb	2	0.1655±0.0024
-3.4 kb	2	0.4086±0.0007	-4.5 kb	1	0.0000±0.0000
-1.4 kb	1	0.0000±0.0000	4.6 kb	1	0.0000±0.0000
Within gene	1	0.0000±0.0000	7 kb	1	0.0000±0.0000
0.2 kb	1	0.0000±0.0000	22 kb	1	0.0000±0.0000
0.5 kb	2	0.3524±0.0025	48 kb	1	0.0000±0.0000
3.7 kb	1	0.0000±0.0000	60 kb	1	0.0000±0.0000
23 kb	2	0.3905±0.0020	245 kb	3	0.2630±0.0058
89 kb	4	0.5405±0.0029			
137 kb	4	0.7247±0.0049			
240 kb	2	0.5058±0.0007			
mean	1.93	0.2487	Mean	1.18	0.0728

NOTE. – *Only samples with complete genotypes reported

SUPPLEMENTARY TABLE 6.3. List of PCR primers used for microsatellite amplification around *pfcrt* and *pfmdr1*

***pfcrt* MICROSATELLITES**			***pfmdr1* MICROSATELLITES**		
Locus	Sequence (5'→3') - direction	Original Source	Locus	Sequence (5'→3') - direction	Original Source
-257 (-189.041)	TTTATAAGGCACACATGAAT *(h) GTACATCTTATGGAAGAAGC	[30]	-305	GGGAAAAGTTATAGTTCACA (f) AATATATTTCCCCAGCTTT	[39]
-200 (-131.339)	AATTGTCCAAACAAATAAAA TGATAGGATAAGTTTTTGAA *(f) CTCTTAAAATTGTCCAAACA	[30]	-207 (-208)	ATGGAAATAAGATAGCATCA (f) TATTTCACATAATCAGCAAA	[39]
-45 (-96)	(f) TGTAATGAATGATTCTAATACCAC TTGGACCATGCTTCACAG	[16]	-99	ATGCACATGTCATATTCTTA (f) AAAATTACATTTCCATTGAG	[39]
-17.7 (-24)	(h) AAGGTAGCATTATGTAAGTA ATTAAGGAAACAAAATGAAAG	[16]	-54	CAATGCTGATATGCTAAATA (f) ATTTCAACCTTGTATTTTTG	[39]
-4.8 (-5)	(f) TCCAGAGGAATAAAAAATAATA AAACACACACATGAACACA	[16]	-4.2	ATCGGATGTTAGTTTTTATG (f) TCGATGTTATCATTTTATTGT	[39]
-4.5 (-4.382)	GGTGTCAATTTTATTTTGTT ATACAATTTGGGGTGAAA *(h) GTCAATTTTATTTTGTTTCT	[30]	-3.3	AAAATTAATGTCTTCCTCAA (f) TTGGCTTTTATTTTATTTTC	[39]
1.5 (1)	(h) ATATATTCCAGTATGTTCGC AATGATACAATGGGATTTAC	[16]	-1.4 (-1.2)	AAAATGCGCTGACTTTAT (f) AGGTGCAAAATGTAATATAGA	[39]
3.9 (6)	(f) TCTGATACAAAAGGGGTGTC AAAAGGGGGTATAAGACACA	[16]	Within gene	TTGAAAGGAAATGAAAATAG (f) CATGTGTACCTTGTTCAATA	[39]
18.8 (22)	(h) ATCTTTAAGTTCAATCTGGA CGAGAACGCAAAGGTGCC	[16]	0.2 (0.3)	ACTCTTGTCCGTTATATTGA (h) AAAAAGGAAGAAGGAAAAA	[39]
45.3 (86)	(f) AAATAATGAAATGATGAG TCAACATAAGATTCTTTG	[16]	0.45 (0.56)	AGTTTACCAATTGTGTGATT (f) CAAATGTTTGCAAATAAATAC	[39]

57.1 (106)	(h) CTGTGGATAATGATATTC GTCCATTGAAAAGATAGG	[16]	3.7 (3.8)	TTTAAAATTAAAACCGTTAG (h) CAAAACTTAAAATTTCTTCAC	[39]
242.5 (241)	TTATGTTTTCATCGTTTTCT TGTCATATTTGTGAAAGTCA *(h) TGTGTATTATGTTTTGATCG	[30]	23.3	GTGTGAGGTGATGTAAGAAT (f) CTTTCCTGTTGTTTTGTAAT	[39]
			89	AATCATTAGGAGTTTCCTTT TTTAGCTTTCTTTTGCTTAG	[39]
			137.4 (137)	TGTTTATGTGCTAATTGAAA GCACAATATTAGCTACAAAAG	[39]
			239.7 (233)	AAAGGAACATAAATAGCAAA TCTTCATGTTTTTCCATATC	[39]

The locus positions of upstream loci are measured with respect to the start codon of the gene and the downstream loci are measured with respect to stop codon. Forward primers are listed first and reverse primers are listed second for each locus. Primers with fluorescent tags are denoted by either (f) or (h) for HEX and FAM, respectively. Values in parentheses in the locus column represent previous estimates of kb distance from *pfcrt* or *pfmdr1* as described in the original papers referenced above. Values not in parentheses are updated kb distance stimates by our lab group.

*Denotes that a semi-nested primer for the secondary reaction, (-189 is modified; the 1st reaction was not used due to generation of noisy results).

SUPPLEMENTARY TABLE 6.4. Microsatellite haplotypes for *pfmdr1* and *pfcrt*

		pfmdr1 haplotypesS														
Number of Samples	Genotype (184/1034/1042/1246**)**	-305 kb	-207 kb	-99 kb	-54 kb	-4.2 kb	-3.3 kb	-1.4 kb	0 kb	0.2 kb	0.45 kb	3.7 kb	23.3 kb	89 kb	137.4 kb	239.7 kb
10	FCDY[a]	246	193	224	137	203	130	196	206	221	193	168	126	124	190	165
1	FCD?	246	193	224	137	203	130	196	206	221	193	168	126	124	190	165
8	FCDY	246	189	231	137	203	130	196	206	221	191	168	126	139	198	152
1	FCDY	244	193	224	137	203	130	196	206	221	191	168	126	124	198	152
1	FCDY	246	193	224	137	203	130	196	206	221	191	168	126	124	190	152
1	FCDY	246	193	231	137	203	130	196	206	221	191	168	126	126	184	165
9	FCDY	246	193	224	137	203	126	196	206	221	193	168	126	124	190	165
1	FCD?	246	193	224	137	203	126	196	206	221	193	168	126	124	190	165
8	FCDY	246	134	224	137	203	126	196	206	221	191	168	126	124	184	165
1	FCDY	246	134	224	137	205	126	196	206	221	191	168	126	124	190	152
7	FCDY	246	189	224	137	203	126	196	206	221	191	168	126	122	194	165
1	FCD?	246	189	224	137	203	126	196	206	221	191	168	126	122	194	165
1	FCDY	246	193	224	137	203	126	196	206	221	191	168	126	139	198	152
1	FCDY	246	193	224	137	203	126	196	206	221	191	168	126	124	190	152
1	FCDY	246	193	224	137	203	126	196	206	221	191	168	126	124	184	165
1	FCD?	246	189	231	137	203	130	196	206	221	191	168	126	139	198	167
14	FDY[b]	246	193	224	137	203	126	196	206	221	191	168	128	124	184	152
1	FDY	246	193	224	137	203	126	196	206	221	191	168	128	139	194	152
1	FDY	246	193	231	137	203	126	196	206	221	191	168	126	139	198	152
6	FDY	246	189	224	137	203	126	196	206	221	193	168	126	124	190	165
1	FDY	246	189	231	137	203	126	196	206	221	191	168	128	124	190	152
1	FDY	246	189	224	137	203	126	196	206	221	191	168	128	124	190	152
4	FDY	246	193	224	137	203	126	196	206	221	191	168	128	124	190	165
1	FDY	?	193	224	137	203	126	196	206	221	191	168	128	124	190	?

Supplementary Table 6.5 *pfcrt* haplotypes

		pfcrt haplotypes											
Number of Samples	Genotypes Codons 72-76	-257 kb	-200 kb	-45 kb	-17.7 kb	-4.8 kb	-4.5 kb	4.6 kb	7 kb	22 kb	48 kb	60 kb	245 kb
49	S_{TCT}VMNT	186	182	119	155	183	232	160	305	186	113	131	192
1	S_{TCT}VMNT	186	?	119	155	183	232	160	305	186	113	131	192
1	S_{TCT}VMNT	?	182	119	155	183	232	160	305	186	113	131	192
1	S_{TCT}VMNT	?	182	119	155	183	232	160	305	?	113	?	192
1	S_{TCT}VMNT	186	182	119	155	183	232	160	?	186	113	?	192
2	S_{TCT}VMNT	?	?	?	?	?	?	?	?	?	?	?	?
2	?	186	182	119	155	183	232	160	305	186	113	131	192
1	S_{TCT}VMNT	179	182	119	155	183	232	160	305	186	113	131	192
15	S_{TCT}VMNT	179	174	119	155	183	232	160	305	186	113	131	192
1	?	179	174	119	155	183	232	160	305	186	113	131	192
9	S_{TCT}VMNT	186	174	119	155	183	232	160	305	186	113	131	190
1	S_{TCT}VMNT	186	174	?	155	183	232	160	305	186	113	131	190
8	S_{AGT}VMNT	186	174	119	155	187	232	160	305	186	113	131	182

Loci are named relative to their position to *pfcrt* or *pfmdr*1. The numbers under each microsatellite maker represent the fragment length in base pairs. Incomplete genotype or haplotype data with missing data is denoted by "?" and attached to its most likely genotype/haplotype. [a]**FCDY** denotes Y184**F**/S1034**C**/N1042**D**/D1246**Y**. [b]**FDY** denotes Y184**F**/N1042**D**/D1246**Y.** We were not able to amplify microsatellites for three samples with the **S_{TCT}**VMN**T** allele and this missing data is not presented here.

CHAPTER 7

SOUTH AMERICAN *PLASMODIUM FALCIPARUM*: SUCCESSFUL SURVIVORS AND RECENT INTRODUCTIONS

Authors

Sean M Griffing[1, 2, 3], Tonya Mixson-Hayden[2, 3], Sankar Sridaran[2, 4], Md Tauqeer Alam[2], Andrea M. McCollum[2, 3], César Cabezas[5], Wilmer Marquiño Quezada[5], John W. Barnwell[2], Alexandre Macedo De Oliveira[2], Carmen Lucas[7], Nancy Arrospide[5], Ananias A. Escalante[8], David Bacon[7], Venkatachalam Udhayakumar[2*]

Affiliations

Emory University, Atlanta, GA, United States of America[1], Malaria Branch, Centers for Disease Control and Prevention, Atlanta, GA, United States of America[2], Atlanta Research and Education Foundation, Atlanta, Georgia[3], Association of Public Health Laboratories, Silver Spring, MD, United States of America[4], Instituto Nacional de Salud Lima, Peru[5], Parasitology Program, Naval Medical Research Center Detachment, Lima, Peru[7], Arizona State University, Tempe, AZ, United States of America[8].

***Address of Corresponding Author:**

Centers for Disease Control and Prevention

Division of Parasitic Diseases, Malaria Branch

1600 Clifton Road NE, Mail Stop D-67; Atlanta, GA 30329 USA

Phone: 404-718-4418

Fax: (404) 718-4440

Email:vxu0@cdc.gov

Abstract

Population genetics approaches have explained the impact of natural selection on the spread, maintenance, and decline of drug-resistant alleles and global population structure of *Plasmodium falciparum*. South American *P. falciparum* populations are the least diverse in the world, yet highly differentiated. Other authors have not explained this differentiation, beyond pointing to genetic drift or, rarely, admixture. In Peru, malaria control reduced malaria incidence after the 1950s, but multiple epidemics of malaria occurred in the 1990s. We tested the hypothesis that Peruvian *P. falciparum* populations expanded from locally bottlenecked populations or neighboring founding migrants. We investigated the genetic relatedness of *P. falciparum* parasites (n=220) by comparing samples from the western, central, and eastern Peruvian Amazon, and the Peruvian Pacific Coast collected during the peak epidemics in 1999-2000. We sequenced drug-resistant genes *dhfr*, *dhps*, *pfcrt*, and *pfmdr1*; 54 proximal microsatellite markers; and 12 neutral markers. Parasite lineages demonstrated limited genetic diversity. There were at least five clonal lineages (designated as clonets A, B, C, D and E). The coast only had the E clonet. In the Amazon, the west had clonets C, D, and E; the center had all lineages except E; and the east had A, B, and D. Clonets A, B, and C may have come from the Amazon after sulfadoxine - pyrimethamine resistance (A and B) or chloroquine resistance (C) developed. Clonets D and E may have undergone bottlenecks and come from the Pacific Coast after CQ resistance emerged but before SP resistance. We demonstrate how recent admixture of different clonets, due to human/vector migration, can lead to cryptic parasite population substructure. Understanding the population substructure of *P. falciparum* in South America has implications for epidemiologic studies, including monitoring malaria during and after the elimination phase.

Author Summary

During the mid-20th century, there was a malaria eradication campaign in many parts of the world. In South America, eradication dramatically decreased malaria incidence. For example, there were less than six *Plasmodium falciparum* cases reported each year during the 1970s in Peru. Later, South American malaria populations expanded. In Peru, this resurgence peaked in the late 1990s. We examined Peruvian *Plasmodium falciparum* isolates from 1999-2000 to examine the influence of eradication, intense antimalarial use, and parasite expansion on the dynamics of such populations. We examined genes associated with drug resistance and neutral molecular markers that gave insight into parasite population structure. We found that five clonal lineages described most parasite diversity at collections sites across Peru. Furthermore, we hypothesized their approximate periods of introduction and migration routes by comparing drug resistant markers with first reports of drug resistance and drug policy changes. We illustrated how human/vector migration can lead to cryptic parasite population substructure. To our knowledge, previous studies of South American malaria have not interpreted *P. falciparum* population dynamics to this degree. Understanding the population substructure of *P. falciparum* in South America has implications for vaccine, drug, and epidemiologic studies, including monitoring malaria during and after the elimination phase.

Introduction

Knowledge of malaria parasite population structure can contribute to epidemiologic investigations, interventions and malaria control, including elimination programs, by providing tools and concepts that support surveillance of drug- and vaccine-resistant parasites in terms of frequency and migration. Population genetics approaches have been used to explain the impact of natural selection on the spread, maintenance, and decline of drug-resistant alleles [1,2,3,4] in *Plasmodium falciparum*. The greatest *P. falciparum* genetic diversity is in Africa, with intermediate genetic diversity in Southeast Asia and the Pacific, and low diversity in South America. In Africa, most diversity is captured within site and there is therefore little differentiation between parasite populations that are 2,000 km apart. On the other hand, in Bolivia, Brazil, and Colombia, parasites are highly differentiated. In addition, neither Colombian nor Bolivian populations appeared to be at mutation drift equilibrium (MDE). This indicates their effective population sizes have not been maintained over time and may have dramatically shrunk in what are referred to as bottlenecks [5].

In general, South American parasites have limited genetic variation at microsatellite loci and genes including antigen-coding loci and the *var* gene family [1,5,6,7,8,9,10,11,12]. It was predicted that reduced diversity would lead to multilocus genotypes being maintained over multiple generations in regions with low recombination [5,13]. This appears to be the case in Sifontes, Venezuela, where there were drug-resistant lineages in multilocus linkage disequilibrium for sulfadoxine-pyrimethamine-(SP-) resistance conferring dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*), the *P. falciparum* chloroquine-resistance transporter (*pfcrt*), and *P. falciparum* multidrug- resistant (*pfmdr1*) alleles [1]. Indeed, the selective pressure applied by such drugs could also reduce population diversity. Yet disequilibrium also existed in Amazonas, Venezuela for GLURP, MSP1, MSP2, PFRRM, and potentially *var*, as well as MSP-

1, MSP-2, RESA, and CSP in Bolívar, Venezuela [8,14,15]. It may also be the case elsewhere in South America [12,16].

There could be multiple causes for the low population diversity, high differentiation, and lack of MDE seen in South American populations. First, the transmission of South American malaria is low (<1%), leading to inbreeding parasites and reduced effective population sizes [5]. Second, malaria control during the 20th century led to population fragmentation and bottlenecks. Third, low effective population size left *P. falciparum* populations susceptible to allele loss due to random changes in allele frequencies (genetic drift). If these differentiated parasite populations subsequently migrated between sites, admixture might have occurred where previously separated populations begin interbreeding. It has been suggested that this occurred in Bolivia [5]. Populations founded by migrants would have limited diversity as they represent a fraction of their source population's diversity (founder effect). Determining the exact causes of low genetic diversity in South America would be difficult because these factors could be intertwined [5,17]. Yet in one South American region we have the unique opportunity to explain *P. falciparum* population structure by explaining new molecular findings using existing knowledge of epidemic history, eradication efforts, and drug resistance.

Peruvian *P. falciparum* populations can be subdivided into three regions based on geographic barriers, vector differences, and drug resistance profiles: the northern Pacific coast, the western Amazon, and the central/eastern Amazon. The Andes Mountains divide the Pacific coast from the Peruvian Amazon, thereby separating *P. falciparum* populations because mosquitoes are rarely reported above 1,500 meters [18]. During this study, the predominant vectors were *Anopheles pseudopunctipennis* and *A. albimanus* on the coast [18,19], *A. benarrochi* in the Western Peruvian Amazon, and *A. darlingi* in the central and eastern Peruvian Amazon [20,21]. The coast and the western Amazon were chloroquine (CQ) resistant, but SP sensitive, while the remainder of the Peruvian Amazon was CQ and SP resistant [22,23].

Peruvian malaria control dramatically reduced the incidence of malaria during the 1950s [18]. Between 1966 and 1989, only two years had more than 68 *P. falciparum* cases [24]. This lull ended when Peru suffered multiple epidemics of malaria during the 1990s [25]. Heavy El Niño precipitation caused flooding and contributed to malaria cases increase on the northern Pacific Coast from 6,000 in 1997 to 51,000 in 1998 and 10-20% were caused by *P. falciparum* [19,23]. Yet most *P. falciparum* cases were in the Peruvian Amazon, the majority of which is within the department of Loreto (in 1997, 121,268 cases). Loreto has ~819,000 inhabitants, of which 345,000 live in the city of Iquitos [26]. Factors contributing to this Amazonian epidemic included habitat changes, human population expansion, and the reinvasion of *A. darlingi* during the 1990s [20,21,27,28]. In 1993, multiple *P. falciparum* foci were reported along the western, northern, and eastern borders of Loreto [29], and there ~75,000 malaria cases in 1998 [23]. One study suggested there were two parasite lineages in this region based on *pfcrt* alleles (SVMNT and CVMNT) [30]. Another suggested there were three based on clinical-resistance (a Brazilian, a Loreto, and a Western Amazon/Pacific type) [26].

We investigated genetic relatedness of *P. falciparum* parasites from the western, central and eastern Peruvian Amazon, as well as the Peruvian Coast, using parasites collected during the peak of malaria transmission (1999 and 2000) after the malaria eradication era of the 1950s-1960s (Figure 7.1). We sequenced *dhfr*, *dhps*, *pfcrt*, and *pfmdr1*, as well as characterized microsatellite loci around each gene and at numerous neutral markers. We hypothesized that *P. falciparum* populations from the late 20th century would represent locally bottlenecked Peruvian parasite populations or migrants from neighboring Ecuador or the greater Amazon. Additionally, we tested the hypothesis that the Andes Mountains could serve as a geographical barrier to genetic exchange within Peru and greater South America.

Results

Pairwise F_{ST} and Mantel test: We analyzed putatively neutral microsatellite markers in *P. falciparum* isolates from multiple collection sites across Peru to understand underlying population structure. Statistically significant pairwise F_{ST} ranged from 0.25 (Padrecocha and Pampa Hermosa; Padrecocha to Caballococha) to 0.9 (Zarumilla and Ullpayacu), which suggests the sites are differentiated (Table 7.1). Padrecocha is most similar to Caballococha and Pampa Hermosa, and more differentiated from La Arena, Ullpayacu, and Zarumilla. The significant differentiation between Zarumilla and La Arena (F_{ST} =0.58) may be due to the limited sampling of La Arena (n=11), as they had the same circulating neutral alleles. Ullpayacu is highly differentiated from all the other sites. Pampa Hermosa is most similar to La Arena and Padrecocha. No isolation by distance was found in this population based on the Mantel test (R^2=0.01, p = 0.447).

There were five clonal haplotypes in multilocus linkage disequilibrium (LD) based on the seven neutral satellite markers. Hereafter, we shall refer to these haplotypes as clonets A, B, C, D, and E. Clonets are defined as being genetically identical for a set of markers, but possibly genetically different with additional markers [31]. They could have a common ancestor that is a few weeks or hundreds of years old [32]. Statistically significant pairwise F_{st} values between the clonets exceeded 0.70, suggesting high differentiation between clonal lineages (Table 7.2). To investigate the contribution of the A, B, C, D, and E clonets to neutral population substructure, we subdivided the seven neutral markers from each collection site by these clonets.

AMOVA: When all sites were treated as a single population, 55% of variation among the seven selected neutral markers was explained (AMOVA, Table 7.2). Partitioning the sites between coastal sites vs. interior sites explained 27% of the variation. When we organized the data by clonet, more variation was explained. All clonets grouped as a single population explained 82% of the variation (Table 7.2). When we disregarded geographic subdivision and grouped the data

by clonet alone (i.e. all samples in the A clonet compared to all samples in the B clonet, etc.), 68% of the variation was explained.

If clonets actually exist in Peru, we would expect an AMOVA of all 66 markers organized by clonet to explain the majority of variation seen in our samples. We therefore expanded the AMOVA to all markers used in this study. This AMOVA result may have been influenced by markers hitchhiking with alleles under selection. When we treated all of the clonets as a single population, 90% of the variation was explained. If each of the clonets is considered a separate population, 76% of the variation is still explained (Table 7.2). Furthermore, partitioning the data by collection site only explained 7.03% of the variation suggesting that clonets may better explain the population structure seen in Peru.

Network diagrams: A median joining network based on neutral markers is shown in Figure 7.2 and reflects both clonets and study sites. The Pacific coast sites (Bellavista, La Arena, and Zarumilla) only had the E clonet. Ullpayacu, a western Amazon site, had only the D clonet, with the exception of one sample from the C clonet. Despite limited data from Pampa Hermosa, only the C and E clonets were found. In Padre Cocha, clonets A, B, C, and D were found, while in the Caballococha only the A, B, and D clonets were found. The geographic distribution of these clonets suggests that the highest amount of admixture can be found in Padre Cocha, the site closest to Iquitos. We also created network diagrams for the drug resistance genes based on proximal microsatellite markers (Figures 7.4-7.7)

Bottleneck analysis: We evaluated whether the clonets had experienced recent bottlenecks. Organizing the data by clonets controlled for migration and population substructure, which would have violated Bottleneck's assumptions. For clonet E, three of the markers on chromosomes associated with drug resistance appeared monomorphic, and we extrapolated limited gaps to be monomorphic as well. MDE was not rejected for any of the lineages, though it was nearly

significant for clonet B (p=0.07) and also clonet E if the markers on the four chromosomes associated with drug resistance were excluded (p=0.09). Only clonet B showed a significant H_e deficit (p=0.03, but p=0.13 when the same four markers were excluded). Clonet E had a significant H_e excess (p=0.037, but p=0.06 when four markers were excluded).

Pairwise linkage disequilibrium: If the haplotypes based on the seven neutral markers represent clonets, then we would expect each clonet to have few polymorphic markers even after inclusion of all 66 markers. We would also expect high levels of pairwise LD for the remaining polymorphic markers (Table 7.4). However, LD could also be caused by markers hitchhiking along with beneficial alleles. Two markers that failed to amplify for a number of samples in lineage D were removed from the analysis (*dhfr*: 0.52 kb and *dhps*: 9.0 kb) and appeared to be monomorphic within each lineage, though polymorphic between lineages. Samples with missing data across any of the 66 markers were removed, leaving 29 samples in A, 23 samples in B, 33 samples in C, 39 samples in D, and 84 samples in E. All lineages had many monomorphic markers (A: 70%; B: 58%, C: 40% and D: 44%, and E: 83%). The remaining polymorphic markers had more pairwise LD (A, 11% B, 12%, C, 30%, D, 31%, and E, 21%) than expected by chance (5%).

Resistance associated alleles: The multilocus LD between the 66 microsatellite markers was also apparent between *pfcrt*, *pfmdr1*, *dhfr*, and *dhps* alleles (Table 7.5). However, the multilocus lineages have partially broken down, particularly in Padre Cocha, which suggests some reassortment and recombination. In addition, genotype data highlighted regional differences (Tables S1-S7). Highly resistant *dhfr* and *dhps* lineages were restricted to central and eastern Amazon region. On the coast, the majority of samples carried wild type *dhfr* and *dhps*. In the western Amazon, both Ullpayacu and Pampa Hermosa only had *dhfr* alleles with a single mutation (108N). Pampa Hermosa had *pfcrt* CVMNT-A and CVMNT-B, but Ullpayacu only had

CVMNT-B. We were not able to successfully sequence *pfmdr1* in Pampa Hermosa, but only the ND**FCD**D was seen in Ullpayacu. Pampa Hermosa had only the silent *dhps* mutant, but Ullpayacu also had the true wild type. A silent mutation in *dhps* at codon 540 (AAA to AAG) was found in Pampa Hermosa, Ullpayacu, Padre Cocha, and Caballococha, but not on the coast.

Regional variation was noted for *pfcrt* and *pfmdr1* as well. The *pfcrt* SVMNT-A haplotype was seen only in the eastern and central Peruvian Amazon. The CVMNT-A haplotype (closely related to SVMNT-A) was seen only in the Padre Cocha and in Pampa Hermosa. On the other hand, the CVMNT-B allele was seen at all sites. Two major *pfmdr1* lineages (α and β) were defined based on microsatellite fragment sizes at -1.40 and 0.45 kb. The α lineage contained samples with a 197 bp fragment at -1.20 kb and a 191 bp fragment at 0.45 kb, whereas the β lineage contained samples with a 203 bp and a 178 bp fragment at -1.40 and 0.45 kb, respectively. The α lineage was predominately seen in the A, B, and C clonets, with a few samples from the D clonets. The β *pfmdr1* lineage was only seen on the C, D, E clonets. No mutations in *pfmdr1* other than 184F and 1042D were noted at the coastal sites. In the interior, there were a number of different alleles and a novel 144G mutation (misidentified as 142G in [33]). Close haplotypes, and H_e tables for *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* are reported in Tables 7.5-7.7.

Discussion

Low transmission rates have led to inbreeding in *P. falciparum* populations in South America [15]. Low transmission also left parasite populations particularly responsive to the malaria eradication efforts during the 20th century. In South America and elsewhere, historical efforts to eradicate malaria left a patchy network of 'islands' where parasites survived [34,35]. The structure of such populations might initially have followed Sewell Wright's isolation by distance model, which assumes a uniform population with mating limited to surrounding areas [41]. However, it is unlikely populations followed this model on a regional scale, as the patchy distribution would violate the model's assumption of a uniform population. It is also unlikely populations would follow this model over time because the dispersal of infected humans and hitchhiking vectors is not limited to nearby areas, which would lead to admixture. This scenario would explain our findings in Peru, where the model of isolation by distance was rejected.

In areas of low *P. falciparum* transmission like South America, Sewall Wright's simple island model of population structure may be more appropriate. In this model, the population is considered to be subdivided into subgroups, each breeding within itself, except for migrants [36]. Wright suggested that such subdivision could be due to geography, ecology, or time [37]. As malaria began to expand and spread in South America, there would be two kinds of 'islands':, the allopatric 'island' refuges left after control efforts, which would eventually lead to unique clonets due to genetic drift and inbreeding, and 'islands' of sympatric inbreed clonets after migration, which allowed for admixture [31].

Our study demonstrates what happens when clonets begin to expand and migrate across regions that had recently been malaria free. We show that multiple clonets can be maintained at different locations as long as large epidemics do not allow outcrossing. Our findings suggest that the *P. falciparum* population structure in areas such as those studied in Peru is the result of the dynamics of clonets. In Peru, five distinct lineages were identified. Clonal substructure was found at all of the collection sites in the Amazon interior (clonets A, B, C, D, and E) and a single clonet

expansion was found on the northern Pacific coast (clonet E). While the Andes appeared to act as a semi-permeable geographic barrier, there were no obvious barriers to gene flow in the Peruvian interior. Future studies should examine other areas where multiallelic LD is found, including Bolivia, Brazil, Colombia, the Thai-Burmese border, Papua New Guinea, and Zimbabwe [5], for similar cryptic population substructure. For example, a similar scenario may have occurred in Bolivia, where admixture has already been suggested [5], and Brazil, where populations were independent yet isolation by distance was rejected [38].

Though the coastal (clonet E) and western Amazon sites (C and E vs. D and C) do not share many clonets, Caballococha (A, B, C lineages) and Padre Cocha (A, B, C, D lineages) share three. The presence of clonet E in Pampa Hermosa suggests it has recently been introduced to the Western Amazon by roads that extend over the Andes Mountains and terminate in Yurimaguas (near Pampa Hermosa) due to the movement of infected human hosts or mosquitoes. Therefore, at least some of 1993 epidemic in Yurimaguas [29] may have been due to parasites from this clonet. The diversity seen in Padre Cocha is not surprising given its close proximity to Iquitos, where ~42% of Loreto's population lives [26]. Iquitos has a large enough population to support multiple lineages, is a hub of human movement throughout the state, and many of the *P. falciparum* cases reported from the peak of the epidemic occurred there. Iquitos is therefore the most likely location in Peru to have patients infected with multiple clonal lineages of *P. falciparum.* This may explain why the majority of samples that appear to have undergone reassortment and recombination of the clonets came from Padre Cocha. Padre Cocha is connected by rivers to Caballococha and thereafter the rest of the Amazon basin, perhaps explaining their shared diversity.

Clinical reports, and also molecular studies, of drug resistance suggest the earliest dates that the clonets could have been introduced to Peru. All of the Peruvian clonets are at least CQ resistant. Yet neither CQ nor SP resistance had been postulated to have developed in Peru. Furthermore, the Peruvian Amazon had little or no malaria during the period that CQ and SP

resistance were first reported, which would have limited any potential sexual reproduction with existing susceptible Peruvian parasites. This implies that all of the clonets may have been introduced to Peru during the 20th century. In South America, it has been argued that CQ resistance first developed on the border of Colombia and Venezuela in 1959 and to the south in Rondônia in southeastern Brazil in 1960. It was first reported in coastal and interior Ecuador in 1976 [26,39,40]. Parasites in southern Peru were CQ-sensitive until at least 1965, with the first reports of resistance in the eastern Peruvian Amazon in 1979-1980 [29,41]. Together, these facts suggest that CQ resistance spread from the north, into Ecuador, over the Andes, and into Peru [39]. Molecular data suggested at least two independent origins for CQ resistance in South America [42], which are identified as the *pfcrt* CVMNT-B allele and the CVMNT-A/SVMNT-A alleles in this study. Although the precise locations where resistance developed are unknown, the CVMNT-A/SVMNT-A alleles are generally in the Amazon region and the CVMNT-B allele on the Pacific coast [42]. On the other hand, SP resistance was proposed to have developed in the Southern Amazon and spread north [16].

In our study, clonets A and B always had the S_{tct}VMNT *pfcrt* allele associated with highly resistant *dhfr* and *dhps* alleles, which was also true in an earlier study [16] and the *pfmdr1* α lineage. SVMNT may have swept through the Amazon basin by hitchhiking with SP resistance when countries shifted to that treatment, as it may have done as such resistance spread in Peru during the 1990s. This would indicate that clonets A and B swept into Peru recently from Brazil. The absence of the A and B clonets in the western Peruvian Amazon and the coast may be due to their recent introduction to Peru, limited internal migration, the lack of widespread SP use, control efforts, differences in vector populations, and/or the Andes Mountains.

Clonet C carried the CVMNT-A, which was only found in Padre Cocha and Pampa Hermosa, and lacked the highly resistant SP- resistant genotypes. We speculate this clonet represents a remnant of the CQ-resistant lineage hypothesized to have developed in southern Rondônia. This would imply it entered eastern Peru sometime after the development of CQ

resistance in Rondônia in 1960, but prior to the development of SP resistance. The CVMNT-A allele has only been reported in Padre Cocha (based on a sample labeled "PC 17" and appearing on a map in that area) [42]. However, a CVMNT allele that grouped with SVMNT was reported among 12 samples collected in Iquitos and two samples from Tabatinga, Brazil. Tabatinga neighbors Caballococha and is where patients infected at the border go for treatment [43]. Our data also suggests that clonet C may represent an ancestral lineage for clonets A and B for three reasons. First, CVMNT shares a haplotype with SVMNT and is closer to the wildtype CVMNK, though an earlier study argued that S_{tct}VMNT might be the oldest CQ resistant *pfcrt* haplotype in South America [43]. Second, clonet C's wildtype *dhps*-A is also shared with the wildtype allele seen in clonet A, as well as the double 437/581 and 437/540/581 mutants seen in clonet A and B. Third, clonet C's 108-B *dhfr* haplotype shares 6/13 markers with the 51/108/164-A *dhfr* haplotype seen in both clonet A and B.

Clonets D and E were associated with the CVMNT-B allele, the *pfmdr1* β lineage, and neither carried highly resistant SP genotypes. Clonet D carried a unique 108-C *dhfr* haplotype and a unique synonymous 540 *dhps* mutation not reported elsewhere in South America (potentially indicating genetic drift). We speculate that clonet D represents the CQ-resistant coastal lineage, which was argued to have spread from the coast of Ecuador into the interior and down into northeastern Peru between 1976 and 1980. Therefore, it would be logical to expect to find it in Padre Cocha and Caballococha. It may have spread from these sites into Ullpayacu prior to the 1993 epidemic in the Pastaza River valley or from a bordering Ecuadorian site [29], which would explain why clonet D was monomorphic at 79% of markers in Ullpayacu (considerable more homogenous than for this lineage overall in Peru, 43%). Clonets D and E are highly divergent. This suggests that, while they sharing ancestry, they may not have migrated from the same immediate source. Clonet E, which carried the same CVMNT-B but a different neutral background, may have reached Peru from a more direct coastal migration sometime after 1976.

Based on the drug resistance patterns, we suggest a possible way that clonets A, B, C, D, and E spread throughout Peru in Figure 7.3.

If our lineages were truly clones, we would expect them to be highly monomorphic and potentially show evidence of recent bottlenecks or expansions. However, testing for evidence of bottlenecks relies upon polymorphic markers, which is inherently difficult when working with clonets. Overall, clonets A, B, and E had more monomorphic markers (70%, 58%, and 83% respectively), than clonets C and D (39% and 43%). Neither clonet A nor B had a significant H_e excess, which suggests they have not undergone recent bottlenecks. Yet clonet B had a significant H_e deficit (0.03), indicative of a rapid expansion. It is unlikely that sufficient time has passed for the signature of recent expansion to disappear from clonet A, therefore the H_e deficit in clonet B suggests clonet A had greater diversity or has outbred with other clonets (B and/or C based on shared neutral markers). The relative abundance of polymorphic markers in clonet C and D implies that they had sufficient time to develop additional microsatellite alleles, more diverse founding populations (i.e., locally bottlenecked rather than introduced), or recombined with more recent introductions. All three hypotheses suggest that clonets C and D have been in Peruvian Amazon longer than A, B, and E.

Furthermore, in clonet C and D, MDE was not rejected and there was neither a H_e excess or deficit. This suggests that these two clonets may have reached a new MDE after their introduction. Clonet E has a significant H_e excess, which indicates a recent bottleneck and the least microsatellite variation of any of our clonets. Therefore, it appears that E is a locally bottlenecked coastal population. However, this hypothesis relies on the assumption that there were Peruvian *P. falciparum* cases below the threshold of coastal epidemiological surveillance and discounts that most cases occurred close to the border with Ecuador. Alternatively, Clonet E may have been recently invaded the western Peruvian Amazon and rapidly expanded. This invasion could have occurred as early as 1976. If this were true we would have expected a significant H_e deficit, which was rejected ($p=1.00$). It is possible that more than one clonet E-like

lineage invaded and masked this deficit, but this would be difficult to prove without access to the unknown source population.

Our findings regarding the clonets suggest that the two *pfmdr1* lineages, α and β, reflect the isolation of the South American coast from the interior. The α haplotype was associated with clonets from the Amazon interior (A, B, C, and a few from D; Table 7.3), while the β *pfmdr1* haplotype was seen in the coastal clonets (D and E) and one of the interior clonet (C). The break down in this pattern in clonet C and D is presumably due to outcrossing. Therefore, it appears that the α lineage originated in the Amazon and the β lineage originated on the coast. This is supported by a study of *pfmdr1* from Colombia, Brazil, and Guyana, which found that *pfmdr1* haplotypes from Colombia and Guyana were quite distinct [44].

It would be difficult to perform association studies that aim to link mutations with drug resistance, or other traits, in such inbred populations given the strong LD that even extends to neutral markers. In areas of low transmission sexual recombination is limited. It has been suggested that the low H_e reported in South America might be due to low effective population sizes, which would heighten the influence of genetic drift, leading to a similar number of alleles in Africa and South America. In our study, the overall neutral H_e calculated from coastal and interior samples combined was 0.64, which is equal to the intermediate H_e reported from Southeast Asia and the Pacific (0.51-0.65), but not Africa (0-76-0.8) [5].

Our study is unique for South America and other areas of low transmission in the world, due to the quantity of samples and markers examined, the breadth of geographic sampling, and our ability to explore how resistant alleles may have influenced expansion of multiresistant lineages. Previous studies have suggested that low transmission could maintain linkage disequilibrium in *P. falciparum* [1,5,10,38,44,45,46,47]. They have not explained the mechanisms underlying population differentiation in low transmission areas, beyond pointing to genetic drift [5,16,48] or, rarely, admixture [5]. Our study demonstrates the utility of the clonet concept (identical for a set of markers, but possibly genetically different [31]) as a tool for

understanding underlying population structure of *P. falciparum* in areas of low transmission. The admixture of clonets in Peru has lead to cryptic parasite population substructure after the rapid expansion of parasite populations during the 1990s. This admixture was most likely the product of human movement along Andean roads and the major waterways of the Amazon basin in response to regional economic shifts. We demonstrate that demography (inbreeding) in low transmission areas alone can maintain and facilitate the spread of stable multidrug-resistant genotypes over long distances and multiple years. Clonets may remain stable provided transmission rates do not increase sufficiently for outcrossing to occur and drug policies do not change the selective environment. Our previous work in Venezuela [1,10], and now in Peru, suggest that clonets may be an important aspect of South American *P. falciparum* population structure. Therefore, reductions in drug use may not lead to an increase in susceptibility in populations where only highly resistant clonets are present. Understanding the population substructure of *P. falciparum* in areas of low transmission will have implications for epidemiologic studies including monitoring malaria during and after the elimination phase.

Materials and Methods

Ethics Statement: The protocols that collected the samples used in this study were approved by Ethical Review Committees of the Instituto Nacional de Salud (for northern Pacific Coast), US Naval Medical Research Center Institutional Review Board and the National Institutes of Health of Peru (for Peruvian Amazon); and Institutional Review Boards of the U.S. Army, the U.S. Navy, and the Universidad Cayetano Heredia (for central and northeastern Amazon) [7, 15, 13, 16, 17, 18], as well as the Centers for Disease control and Prevention. Written informed consent was provided by study participants and/or their legal guardians.

Study sites and *P. falciparum* clinical isolates: We examined 220 Peruvian *P. falciparum* clinical isolates collected during 1999-2000. The samples from the northern Pacific Coast (Bellavista, n=2; La Arena, n=11 and Zarumilla, n=67) and the western Peruvian Amazon (Pampa Hermosa, n=10; Ullpayacu, n=25) [22] were collected during drug efficacy trials in patients > 2 or 5 years of age [19,22,49]. The Bellavista and La Arena are located around the city of Sullana, which had ~150,000 inhabitants at the time of sample collection. Zarumilla had ~18,000 inhabitants and is located near Ecuador. Pampa Hermosa has less than 4,000 people and is located on a highway connecting Yurimaguas and Tarapoto. Ullpayacu is a village of 900 people on the Pastaza River. Samples from the central Peruvian Amazon (Padre Cocha, n=65) and the eastern Peruvian Amazon (Caballococha, n=40) were collected during drug efficacy trials and surveillance studies with all ages included [33,50,51]. Padrecocha is a village of 1,400 people on the Nanay River, 5 km from Iquitos and quickly accessible by boat. Caballococha is a village of 3,300 people located in northwest Peru (~30 miles from Brazil and Colombia) and is only reachable by river.

Study site malaria history: DDT application reduced malaria in Peru from 95,349 cases in 1944 to 20,000 by 1950 (~67% were on the coast and 33% were in Amazon) [18]. After the 1950s,

there were fewer than 1,000 cases a year confined to the borders with Brazil, Colombia, and Ecuador [26,52]. By 1970, almost the entire coast was malaria free, as well as the interandian valleys, and the southern Peruvian Amazon [18]. Less than six *P. falciparum* cases were reported in nine out of the following ten years. On the Pacific coast of Peru, there was an outbreak of *P. falciparum* in 1987 in Zarumilla, Tumbes. In 1991, another outbreak occurred in Sullana, Piura [53]. Two years later, *P. falciparum* was reported in the coastal departments of Tumbes, Piura, Lambayeque, and Cajamarca [53]. However, the major coastal epidemic began after heavy rains and flooding caused by the El Niño event in the late 1990s [49]. In the western Amazon there was an outbreak in the Pastaza River valley in 1991, which includes Ullpayacu [53]. During 1992 and 1993, an epidemic was reported along a road connecting Alianza (San Martin) and Pampa Hermosa (Loreto) [29]. In the eastern Amazon, *P. falciparum* was reported in eastern and southeastern Loreto in 1993 [29]. In the central Peruvian Amazon, *P. falciparum* was first reported in Padre Cocha in 1994 [26]. By 1997, Loreto accounted for 67.2% of all malaria cases in Peru [26] with the majority occurring around Iquitos. In 1998, Peruvian *P. falciparum* reached its peak with more than 80,000 cases [23].

DNA isolation, PCR amplification and genotyping of *dhfr, dhps, pfcrt,* and *pfmdr1*: DNA was isolated from filter paper blood spots [22,33,49] or whole blood [33] using the QIAamp DNA blood mini kit (QIAGEN, Valencia, CA). Samples from Padre Cocha and Caballococha were previously sequenced for point mutations in *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* [33]. Limited samples from these sites were resequenced for *dhps* to test for a novel synonymous *dhps* mutation at codon 540 (AAG). Sequencing of *dhfr* and *dhps* in Pampa Hermosa and Ullpayacu samples were previously reported [22] and were resequenced for confirmation. Samples from Bellavista, La Arena, Pampa Hermosa, Ullpayacu, and Zarumilla were sequenced for *pfcrt*, *pfmdr1*, and *dhps* using protocols described previously [1,54]. For *dhfr*, we used a nested PCR amplification protocol. For the first reaction, we used 5'-TCCTTTTTATGATGGAACAAG-3' (forward) and

5’-AGTATATACATCGCTAACAGA-3’ (reverse) primers and, for the secondary reaction, we used 5’-TTTATGATGGAACAAGTCTGC (forward) and 5’-ACTCATTTTCATTTATTTCTGG-3’ (reverse) primers. The cycling conditions for the first reaction were 94˚C/5 min; (35 cycles of 95/30 sec; 50/30 sec; 68/1 min); 68˚C /5 min and for the second reaction conditions 94˚C /5 min; (30 cycles of 95˚C /30 sec; 52˚C /30 sec; 68˚C /1 min); 68˚C/5 min.

Microsatellite typing: Whole genome amplified DNA (Qiagen’s REPLI-g Whole Genome Amplification Kit, Valencia, CA) was used for microsatellite characterization. Samples were assayed for 12 microsatellite loci spanning 499.5 kb around *pfcrt* on chromosome 7; 15 microsatellite loci spanning 544.7 kb around *pfmdr*1 on chromosome 5; 13 microsatellite loci spanning 700 kb around *dhfr* on chromosome 4; and 16 microsatellite loci spanning 406.3 kb around *dhps* on chromosome 8 [42,55,56]. Primer sequences and their PCR parameters were described earlier [1,10]. We previously reported microsatellite data close to *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* for Caballococha and Padre Cocha [33]. In addition, we examined 12 putatively neutral microsatellite loci. Five were selected from neutral markers previously described (TA1, chromosome 6; poly α, ch. 4; PfPK2, Ch. 12; TA109, ch. 6; and 2490, ch. 10) [5,57]. The remaining seven markers were C2M33, C2M34, C2M29, C2M27 on ch. 2; and C3M40, C3M69, and C3M39 on ch. 3 [10].

Statistical analysis: We used seven microsatellites on different chromosomes (TA1, poly α, PfPK2, TA109, 2490, C2M34, and C3M69) to examine Peruvian *P. falciparum* population structure. A locus by locus hierarchical analysis of molecular variance (AMOVA) was used to partition variation among and between all populations, as well as between coastal and interior sites using Arlequin version 3.1. Significance of the fixation indices was determined using a non-parametric approach. F_{ST} was calculated among all populations, with the exception of Bellavista,

and between all pairs of populations. The significance of F statistics and genetic variance components were tested using 1,000 permutations [58]. We excluded Bellavista because it was represented by only two samples. F_{ST} is a measure of population differentiation, expressed as the proportion of genetic diversity that is due to differences between populations; it ranges between zero, which would indicate populations are one population, and one, which would indicate there is no gene flow between the populations.

Isolation by distance was tested by regressing pairwise F_{ST} on pairwise geographic distances among populations [59] and significance determined with Mantel's tests (1,000 permutations) using Arlequin [58]. We tested whether grouping our samples by apparent ancestral populations explained more genetic variation than grouping them by collection sites using analysis of molecular variance (AMOVA, locus by locus), which estimates population differentiation at different hierarchical levels.

We initially examined the seven neutral markers and then expanded to all microsatellite markers. We also examined population differentiation by comparing fixation indexes between the different sites or clonal lineages (F_{ST}). A median joining network of neutral loci was created using Network v. 4.516 (fluxus-engineering.com) [60] that reflects study sites and lineages.

Expected H_e was calculated for each locus as $[n/(n-1)][1-\Sigma p_i^2]$, where n is the number of isolates sampled and p_i is the frequency of the ith allele [56] using the Excel Microsatellite Tool Kit [61]. The H_e sampling variance was calculated as $[2(n-1)/n^3][2(n-2)][\Sigma pi^3-(\Sigma pi^2)^2]$ [56]. Mean neutral H_e was based on the seven markers on Ch. 2 and 3. H_e reported for microsatellites surrounding each gene were calculated based on alleles regardless of study site, as well as after having been subdivided by clonal lineage. Significant associations between microsatellites within clonets were determined using an exact test of linkage disequilibrium [62] and 10,000 Monte Carlo steps in Arlequin version 3.1 [58] and a Bonferronni-Holms correction [63].

We tested for bottlenecks using the Bottleneck application available at www.ensam.inra.fr. Bottleneck assumes that populations lack substructure, migration, and

hybrids; and that markers used are neutral and not in linkage disequilibrium. When a population is at MDE, each microsatellite should have an equal probability of having an observed H_e deficit or excess in comparison to the expected H_e based on the number of alleles. After a reduction in the effective population size (bottleneck), there will be a reduction in the number of alleles and H_e at polymorphic loci. However, allelic diversity decreases at a faster rate than H_e during a bottleneck. Therefore, a bottleneck is indicated if a significant number of loci have a H_e excess compared to that expected if the population was in mutation-drift equilibrium. Conversely, if there is an H_e deficit, the population will also no longer be in MDE and a rapid population expansion is indicated [64]. To test whether our populations were in MDE, we used a sign test. The sign test has low statistical power, according to Bottleneck's documentation; therefore failing to reject the null hypothesis of MDE should be treated with caution. To test for H_e deficits and excesses, we used a Wilcoxon sign-rank test, which can be used with as few as four polymorphic markers. We used a two-phased model of mutation for all tests [64] and included the seven neutral markers from different chromosomes, as well as four markers from each chromosome carrying one of the genes (Ch. 4, 347.1 kb; Ch. 5, -305 kb; Ch. 7, -257 kb; and Ch. 8, -196.6). We selected each marker to be as far from the gene as possible and note when our results would be statistically insignificant when excluded.

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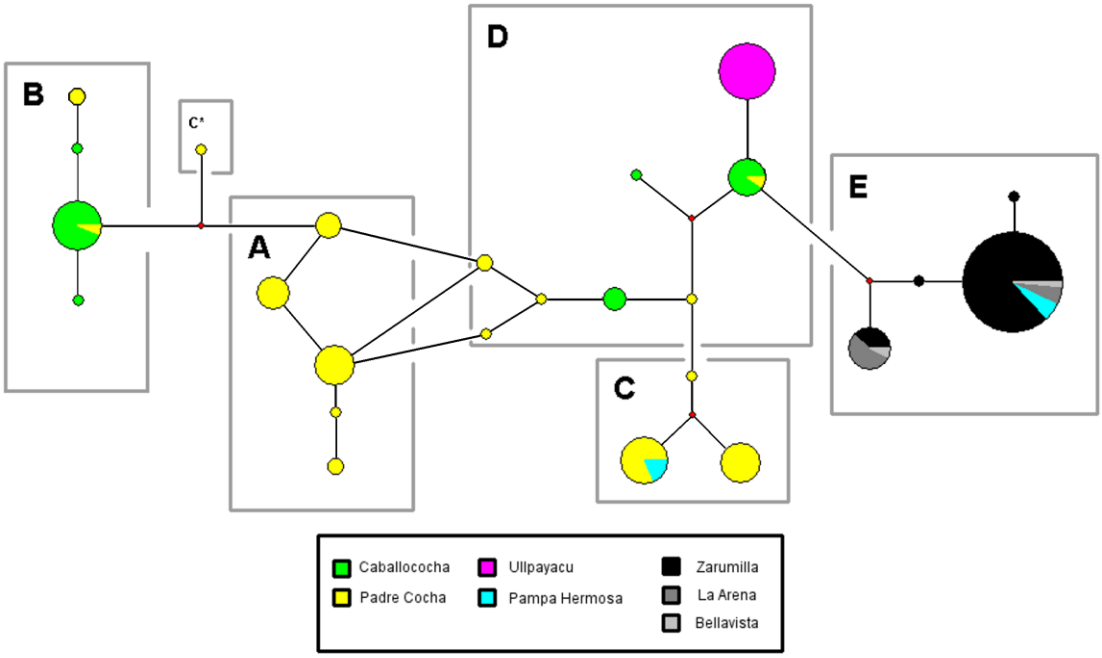
Figure 7.1. Collection Sites.

A map of Peru that shows the collection sites, as well as the Andes Mountains (dark gray), roads of interest (black lines over Andes), and rivers of interest in the Amazon (light gray).

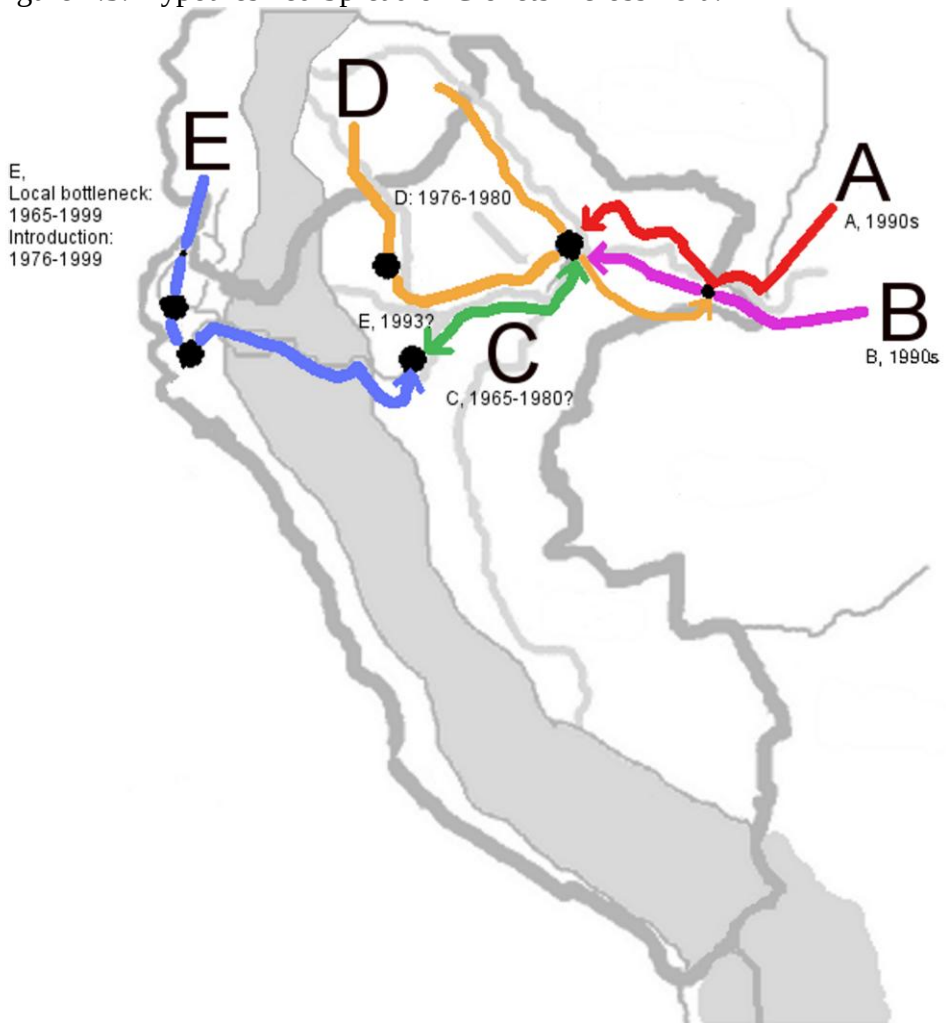
Figure 7.2. Clonets and Collection Sites.



This Network diagram shows the genetic relationships among the A, B, C, D, and E clonets and collection sites using the seven neutral microsatellite markers described in the text. Small red circles represent hypothetical parasites that link haplotypes seen in our samples.

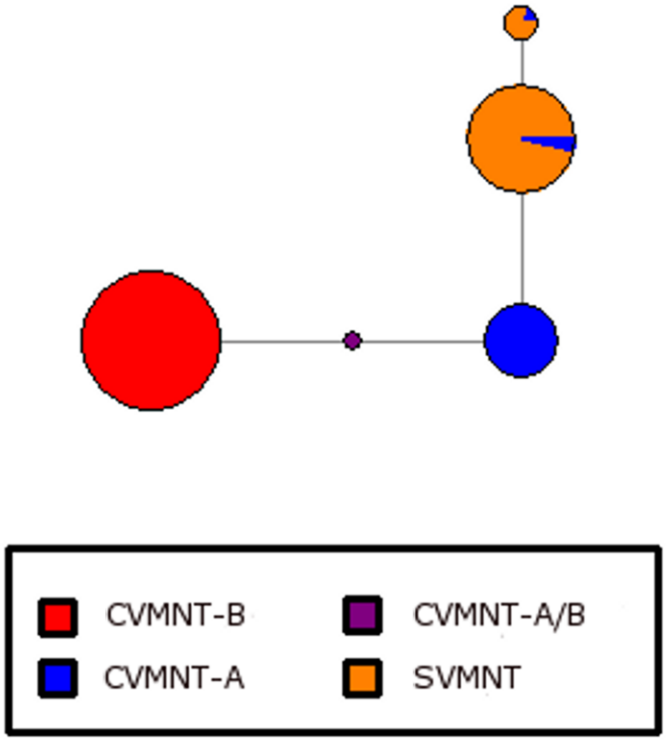
*This Padre Cocha sample shared two of the neutral markers with the C group and otherwise showed the multilocus LD of the C clonet.

Figure 7.3. Hypothesized Spread of Clonets Across Peru.



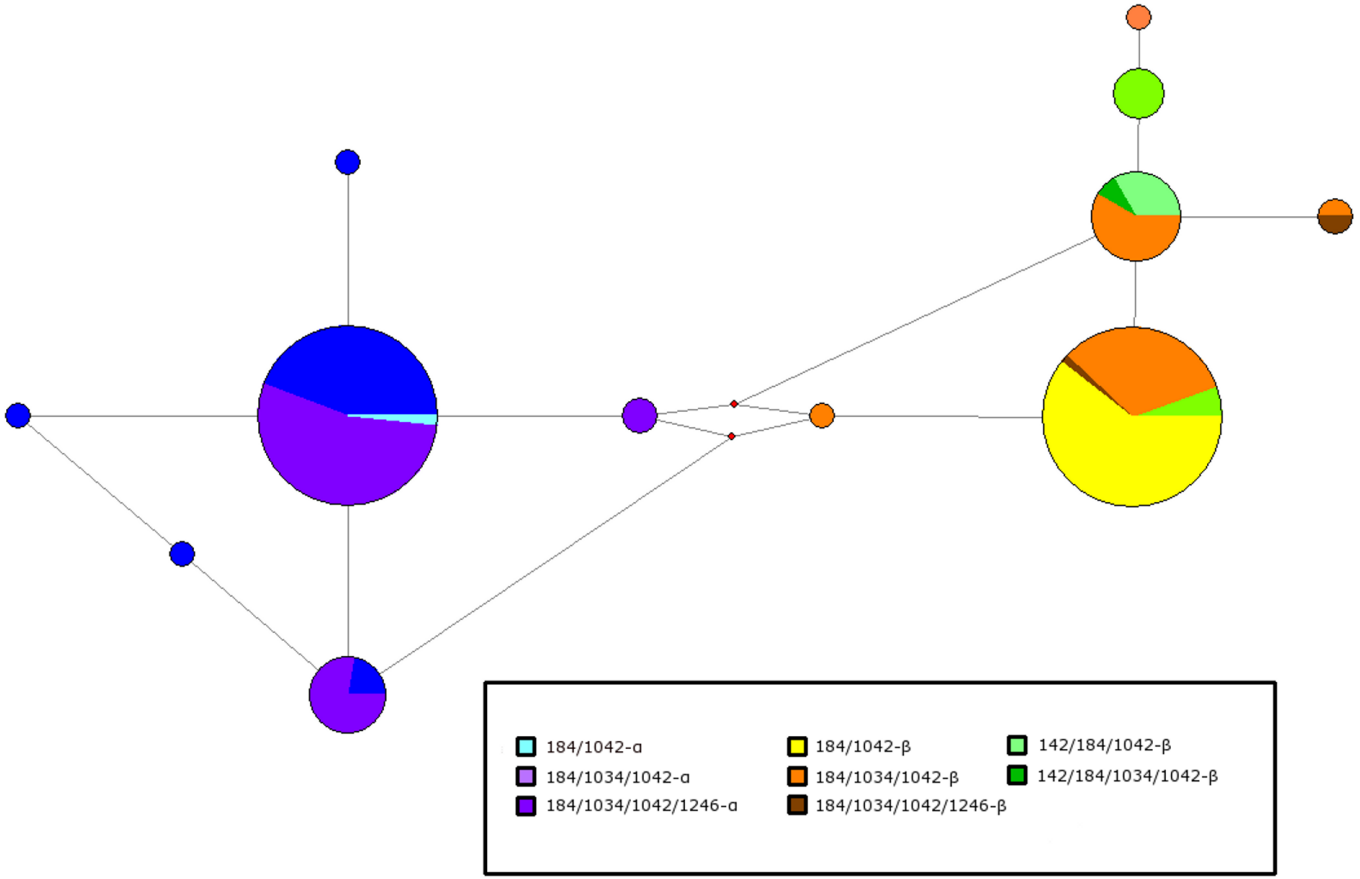
Clonet A: red, B: purple, C: green, D: orange, E: blue.)

Figure 7.4. Network diagram for *pfcrt* close microsatellite markers



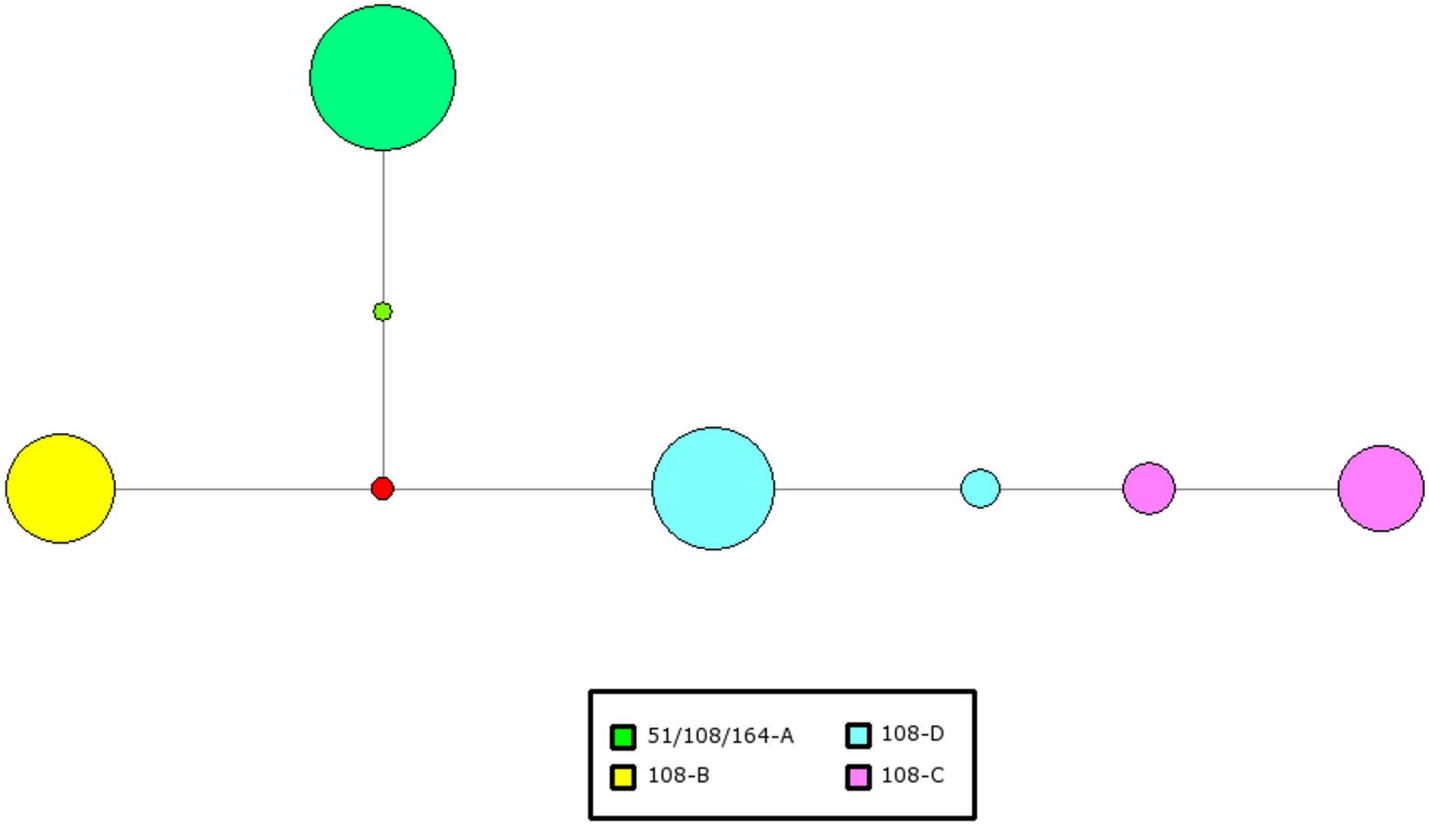
This network diagram shows the genetic relationships among the p*fcrt* alleles using proximal microsatellite makers. The purple group links the two CVMNT alleles and may be due to recombination.

Figure 7.5. Network diagram for *pfmdr1* close microsatellite markers



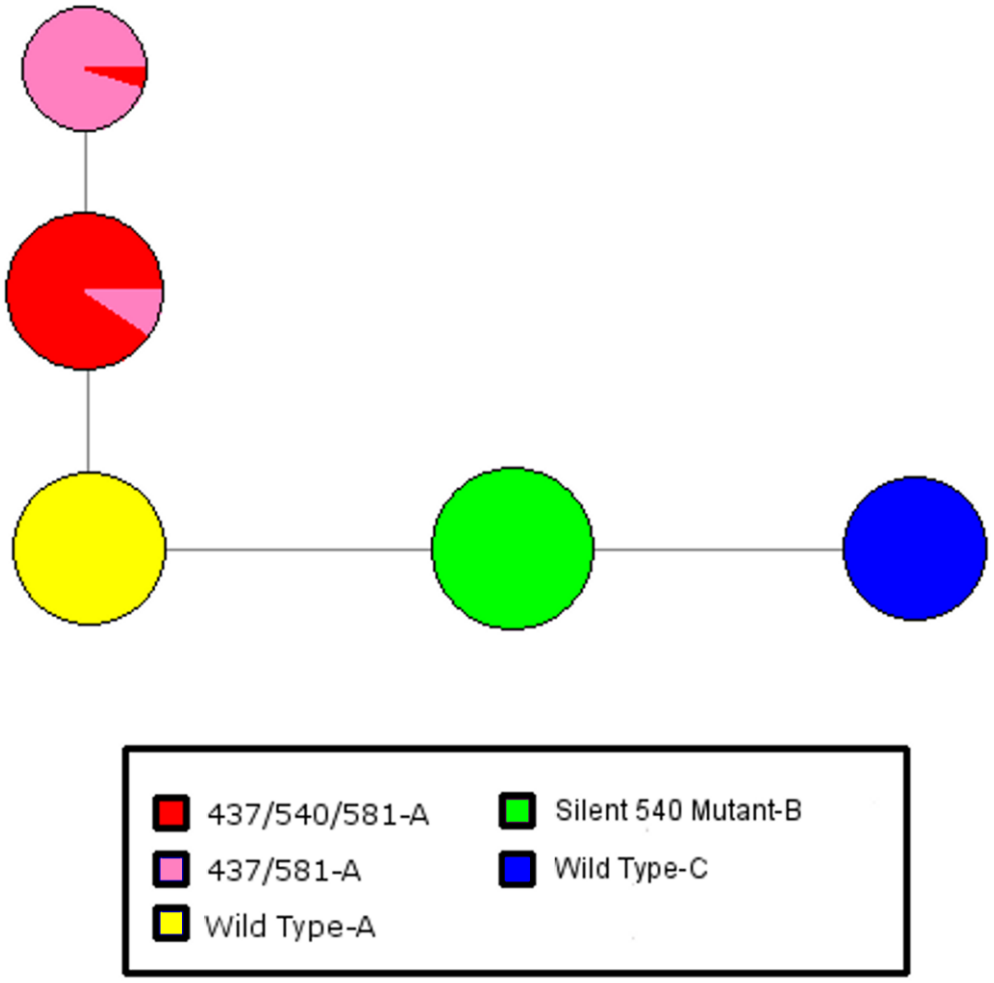
This network diagram shows the genetic relationships among the *pfmdr1* alleles using proximal microsatellite makers. Notice that the α and β lineages described in the text are clearly separate in the figure. Small red circles represent hypothetical parasites that link haplotypes seen in among our samples.

Figure 7.6. Network diagram for *dhfr* close microsatellite markers



This network diagram shows the genetic relationships among the *dhfr* alleles using proximal microsatellite makers. Small red circles represent hypothetical parasites that link haplotypes seen in our among our samples.

Figure 7.7. Network diagram for *dhps* close microsatellite markers.



This network diagram shows the genetic relationships among the *dhps* alleles using proximal microsatellite makers. Notice that the A group clusters across different numbers of mutations.

Table 7.1. Pairwise F_{ST} by Collection Site.

Study Sites	**Zarumilla**	**La Arena**	**Padrecocha**	**Caballococha**	**Pampa Hermosa**
Zarumilla (n*=66)	-	-	-	-	-
La Arena (n=11)	0.58	-	-	-	-
Padrecocha (n=58)	0.61	0.40	-	-	-
Caballococha (n=38)	0.62	0.39	0.22	-	-
Pampa Hermosa (n=7)	0.48	0.28	0.22	0.31	-
Ullpayacu (n=24)	0.90	0.85	0.52	0.55	0.77

This table shows the pairwise F_{ST} values calculated when comparing different collection sites using the 7 neutral markers described in the text. All values are significantly different from zero ($p \leq 0.05$).

*n denotes sample size

Table 7.2. AMOVA Results.

Divisions	Source of Variation	Variation (%)	Fixation Indices	p-value
Neutral data by study site:				
1 group: Padre Cocha (n=*58), Caballococha (n=38), Zarumilla (n=66), La Arena (n=11), Bellavista (n=2), Ullpayacu (n=24), Pampa Hermosa (n=7)	Between populations	54.89%	$F_{ST,}$ 0.548	0.00
	Within populations	45.11%		
Neutral data by study site:				
Coastal Peru: Zarumilla, La Arena, Bellevista) ***vs*** Interior Peru: Padre Cocha, Caballococha, Ullpayacu, Pampa Hermosa)	Between Regions	26.78 %	F_{ST} 0.268	0.001
	Populations/regions	32.79 %	F_{IS} 0.448	0.000
	Within populations	40.44 %	F_{IT} 0.596	0.000
Neutral data by clonal lineages (neutral loci)				
One group: A, B, C, D, E	Between populations	82.22%	$F_{ST,}$ 0.822	0.00
	Within populations	17.78%		
Neutral data by clonal lineages (neutral loci):				
A: Padre Cocha (n=22), Caballococha (n=7) *vs* B: Padre Cocha (n=3), Caballococha (n=17) *vs* C: Padre Cocha (n=29), Pampa Hermosa (n=3), Ullpayacu (n=1) *vs* D: Padrecocha (n=4), Caballococha (n=14), Ullpayacu (n=23) *vs* E: Zarumilla (n=66), La Arena (n=11), Bellavista (n=2), Pampa Hermosa (n=4)	Between Regions	68.16%	$F_{ST,}$ 0.682	0.00
	Populations/ regions	15.40%	$F_{IS,}$ 0.484	0.00
	Within populations	16.44%	$F_{IT,}$ 0.836	0.00
All 66 loci by clonal lineages:				
A, B, C, D, E	Between populations	89.60%	$F_{ST,}$ 0.896	0.00
	Within populations	10.40%		
All 66 loci by clonal lineages:				
A: Padre Cocha (n=23), Caballococha (n=7) *vs* B: Padre Cocha (n=19), Caballococha (n=3) *vs* C: Padre Cocha (n=28), Pampa Hermosa (n=4), Ullpayacu (n=1) *vs* D: Padrecocha (n=5), Caballococha (n=14), Ullpayacu (n=23) *vs* E: Zarumilla (n=64), La Arena (n=7), Bellavista (n=2), Pampa Hermosa (n=4)	Between Regions	75.59%	$F_{ST,}$ 0.756	0.00
	Populations/ regions	14.66%	$F_{IS,}$ 0.601	0.00
	Within populations	9.74%	$F_{IT,}$ 0.903	0.00

***** The number of samples in each group are designated by "n=."

Table 7.3. Pairwise F_{ST} by Clonet.

Clonal lineage	**A**	**B**	**C**	**D**	**E**
A (n*=30)	-	-	-	-	-
B (n=22)	0.80	-	-	-	-
C (n=33)	0.80	0.83	-	-	-
D (n=42)	0.83	0.85	0.70	-	-
E (n=81)	0.86	0.84	0.89	0.78	-

We used seven neutral markers described in the text for this figure. All values were significantly different from zero (p=0.00).
*The number of samples in each group are designated by "n=."

Table 7.4. Pairwise Linkage Disequilibrium in Clonets.

Lineage	Polymorphic Neutral MS	Polymorphic Neutral MS + Four from Ch. with genes	All Polymorphic MS	Pairwise Comparisons of All Polymorphic Markers	Expected Pairwise LD of All Polymorphic Markers	Observed Pairwise LD of All Polymorphic Markers
All samples	7/7	11/11	58/64	1658	83	1632
A	4/7	7/11	19/64	176	9	20
B	2/7	4/11	27/64	359	18	42
C	4/7	11/11	39/64	741	37	108
D	4/7	7/11	36/64	630	32	196
E	3/7	4/11	11/64	62	3.1	13

This table shows the number of makers that were in significant pairwise linkage disequilibrium after a Bonferonni correction using various groups of markers (p-value=0.05).

Table 7.5. Multilocus Linkage Disequlibrium and Clonets.

Clonet	CC	PC	UL	PH	ZA	Haplotype group	*dhfr* haplotype	*pfcrt* haplotype	*dhps* haplotype	*pfmdr1* haplotype	Neutral haplotype
	7	8				AAAα	51/108/164-A	SVMNT-A	437/540/581-A	184/1034/1042/1246-α	A
A		1				AAAα	51/108/164-A	SVMNT-A	WT-A	184/1034/1042/1246- α	A
		13				AAAα	51/108/164-A	SVMNT-A	437/540/581-A	184/1034/1042-α	A1
		1				AAAα	51/108/164-A	SVMNT-A	437/581-A	184/1034/1042-α	A1
B	18	3				AAAα	51/108/164-A	SVMNT-A	437/581-A	184/1034/1042/1246-α	B
	1					AAAα	51/108/164-A	SVMNT-A	437/581-A	184/1042-α	B
C		18				BAAα*	108-B	CVMNT-A	WT-A	184/1034/1042-α	C
		10				BAAα	108-B	CVMNT-A	WT-A	142/184/1042- β	C1
D	12	2	15			CBBβ	108-C	CVMNT-B	540-Silent-B	184/1034/1042-β	D, D1 & D2
	2					CBBβ	108-C	CVMNT-B	540-Silent-B	184/1034/1042/1246-β	D1
					33	DBCβ	WT-D	CVMNT-B	WT-C	184/1246- β	E
					1	DBCβ	WT-D	CVMNT-B	WT-C	184/1042/1246- β	E
E					4	DBCβ	108-D	CVMNT-B	WT-C	184/1246- β	E
				2		DBCβ?	108-D	CVMNT-B	WT-C	β?	E
				2		?BCβ?	?	CVMNT-B	WT-C	β?	E
	Parasites with multi-locus profiles that suggest reassortment or recombination										
C		1				AAAα	51/108/164-A	CVMNT-A	437/581-A	184/1034/1042/1246- α	C_A
		1				AAAα	51/108/164-A	CVMNT-A	437/540/581-A	184/1034/1042/1246- α	C_A
		1				BBAβ	108-B	CVMNT-B	WT-A	184/1034/1042- α	C?
C		1				BBAβ	108-B	CVMNT-B	WT-A	142/184/1034/1042-β	C1
		2				BBAβ	108-B	CVMNT-B	WT-A	142/184/1042-β	C1
		3				CABα	108-C	SVMNT-A	540-Silent-B	184/1034/1042/1246-α	D_B
D			1			CBBα	108-C	CVMNT-B	540-Silent-B	184/1034/1042-α	D
				3		?AAα?	?	CVMNT-A	540-Silent-A	α?	C
C				1		?AAα?	?	A?	540-Silent-A	α?	C?
C			1			?BCβ?	?	CVMNT-B	?	β?	C

This table illustrates the multiallelic disequilibrium seen across multiple genes and microsatellite markers. The final column suggests the subvariants seen within each clonet amongst the seven neutral markers. In cases were reassortment or recombination appears to have occurred, we have suggested the secondary clonet with a subscript (e.g. C_A was most likely a cross between clonet A and C.)

Table 7.6. Bottleneck results for 11 markers.

Clonet	Sign test	H_e deficit	H_e excess
A	p=0.53	p=0.65	p=0.41
B	p=0.07	p=0.03	p=1.0
C	p=0.14	p=0.18	P=0.84
D	p=0.47	p=0.59	p=0.47
E	p=0.20	p=1.0	p=0.03

This table gives the p-values for our Bottleneck analysis

Table 7.7. Common *dhfr* genotypes and microsatellite markers from study isolates.

dhfr allele[a]	Number of samples							Microsatellite haplotype[b]					Haplotype group
	CC (n=40)	PC (n=64)	PH (n=2)	UL (n=15)	BE (n=2)	LA (n=10)	ZA (n=52)	-5	-3.77	-0.06	0.45	5.78	
C**I**N**L**	25	28						223	216	101	97	122	DHFR-A1
C**I**N**L**	1							223	220	101	97	122	DHFR-A2
CN**N**I		31						223	195	125	97	108	DHFR-B1
CN**N**I	14	5						202	209	97	103	108	DHFR-C1
CN**N**I				4				202	209	97	-	108	DHFR-C1?
CN**N**I				7				202	202	97	103	108	DHFR-C2
CN**N**I				1				202	202	-	103	108	DHFR-C?
CN**N**I				1				202	202	97	-	108	DHFR-C?
CN**N**I				1				202	202	-	-	108	DHFR-C?
CN**N**I				1				202	-	97	-	108	DHFR-C?
CN**N**I			1			2	4	227	213	101	103	108	DHFR-D1
CN**N**I			1					227	213	101	103	108	DHFR-D1?
CN**N**I					1	2	1	230	213	101	103	108	DHFR-D2
CN**N**I						1	1	-	-	101	103	108	DHFR-D?
CNSI					1	1	31	227	213	101	103	108	DHFR-D1
CNSI						1	7	227	-	101	103	108	DHFR-D1?
CNSI						1	1	227	213	101	-	108	DHFR-D1?
CNSI							6	-	213	101	103	108	DHFR-D?
CNSI						1	1	-	-	101	103	108	DHFR-D?
CNSI						1		-	-	-	103	108	DHFR-D?

This table shows the *dhfr* allele distribution across the different collection sites (CC, Caballacocha; PC, Padrecocha; PH, Pampa Hermosa; UL, Ullpiyacu; BE, Bellavista; LA, La Arena; ZA, Zarumilla).

[a]Indicates amino acids at dhfr codons 50, 51, 108 and 164 [b]Microsatellite loci (in Kb) are named according to their position from the gene; negative positions are 5' and positive positions are 3' to the gene

Table 7.8. Common *dhps* genotypes and microsatellite markers from study isolates.

***dhps* allele[a]**	**Number of samples**							**Microsatellite haplotype[b]**									**Haplotype group**
	CC[a]	**PC[b]**	**PH[c]**	**UL[d]**	**BE[e]**	**LA[f]**	**ZA[g]**	**-11.1**	**-7.5**	**-2.8**	**-1.5**	**-0.1**	**0.03**	**0.50**	**1.4**	**6.4**	
S**GEG**A	7	24						232	180	197	171	134	124	140	266	310	DHPS-A1
S**G**K**G**A		1						232	180	197	171	134	124	140	266	310	DHPS-A1
SAKAA		31						232	180	197	171	134	124	140	266	310	DHPS-A1
S**G**K**G**A	19	3						232	178	197	171	134	124	140	266	310	DHPS-A2
SA**K***AA			1					232	180	197	171	134	124	140	249	-	DHPS-A?
SA**K***AA			1					232	180	197	171	134	124	140	-	308	DHPS-A?
??**K***??			1					-	178	-	171	134	124	140	-	-	DHPS-A?
??**K***??			1					-	-	-	-	134	124	140	-	-	DHPS-A?
SA**K***AA	14	5		15				220	180	189	190	134	130	140	247	285	DHPS-B1
SA**K***AA				2				-	180	189	190	134	130	140	247	285	DHPS-B1?
SA**K***AA				2				220	180	189	190	134	130	140	247	-	DHPS-B1?
SA**K***AA				1				220	180	189	190	134	-	140	247	285	DHPS-B1?
SA**K***AA				1				220	180	189	190	134	130	140	247	-	DHPS-B1?
SA**K***AA				1				220	180	-	190	134	130	140	247	285	DHPS-B1?
SA**K***AA				1				-	180	-	190	134	130	140	247	285	DHPS-B1?
SAKAA				2	1	1	27	220	169	203	163	136	136	143	255	305	DHPS-C1
SAKAA				1			1	220	169	-	163	136	136	143	255	305	DHPS-C1?
SAKAA				1			1	220	169	-	163	-	136	143	255	305	DHPS-C1?
SAKAA						1	5	220	169	203	163	136	-	143	255	305	DHPS-C1?
SAKAA							6	220	169	203	163	136	136	143	255	-	DHPS-C1?
SAKAA						1		-	169	203	163	136	136	143	255	305	DHPS-C1?
SAKAA							1	220	169	203	163	136	136	143	-	305	DHPS-C1?
SAKAA							1	220	169	-	163	136	136	-	255	305	DHPS-C1?
SAKAA							1	220	169	-	-	136	136	143	255	305	DHPS-C1?
SAKAA							1	220	-	-	163	136	136	143	255	305	DHPS-C1?
SAKAA							1	220	169	203	163	136	136	143	-	-	DHPS-C1?
SAKAA							1	220	-	203	163	-	136	143	255	-	DHPS-C1?
SAKAA							1	-	169	-	163	-	136	143	255	305	DHPS-C1?
SAKAA							1	220	169	203	163	-	136	-	-	305	DHPS-C1?
SAKAA					1			-	-	203	-	136	136	143	255	305	DHPS-C1?
SAKAA						1		220	169	-	163	-	136	143	-	-	DHPS-C1?
SAKAA							1	220	169	-	-	-	-	143	-	-	DHPS-C1?

This table shows the *dhps* allele distribution across the different collection sites. Microsatellite locations were updated from previous references for accuracy. The following are the previous locations followed by their corrected locations: H_e data, *dhfr* -7.4 is 6.4, -2.47 is 1.4, -1.64 is 0.50, -0.8 is 0.03, 0.06 is -0.1, 0.144 is -1.5, 1.591 is -2.8, 6.19 is -7.5, and 9.79 is -11.1.

[a]Indicates amino acids at dhps codons 436, 437, 540, 581 and 613; [b]Microsatellite loci (in Kb) are named according to their position from the gene; negative positions are 5' and positive positions are 3' to the gene. CC, Caballacocha; PC, Padrecocha; PH, Pampa Hermosa; UL, Ullpiyacu; BE, Bellavista; LA, La Arena; ZA, Zarumilla; *11/14 samples from Caballocha and 2/5 samples from Padrecocha in this group had a synonymous mutation at this codon (AAG instead of AAA) that was not reported in our previous paper. We were not able to verify the remaining samples. All samples from Ullipayacu had this mutation.

Table 7.9. Common *pfcrt* genotypes and microsatellite markers from study isolates.

pfcrt allele[a]	**Number of samples**							Microsatellite haplotype[b]				Haplotype group
	CC	PC	PH	UL	BE	LA	ZA	-4.8	-4.4	1.5	3.9	
CVMN**T**		26	1					186	233	160	305	CRT-A1
CVMN**T**			1					-	233	160	-	CRT-A1
CVMN**T**			1					-	-	160	305	CRT-A1
CVMN**T**			1					-	-	160	305	CRT-A1
CVMN**T**		2						183	233	160	305	CRT-A2
CVMN**T**		1						152	233	160	305	CRT-A3
SVMN**T**	24	26						183	233	160	305	CRT-A2
SVMN**T**		2						152	233	160	305	CRT-A3
SVMN**T**	1							183	231	160	305	CRT-A4
SVMN**T**	1							152	233	160	294	CRT-A5
SVMN**T**		1						135	233	160	305	CRT-A6
CVMN**T**	1							186	231	155	289	CRT-B1
CVMN**T**	12	5	3	21	1	1	46	186	231	155	305	CRT-B2
CVMN**T**			1	2			7	-	231	155	305	CRT-B2?
CVMN**T**				1			1	186	-	155	305	CRT-B2?
CVMN**T**						1	1	186	231	155	-	CRT-B2?
CVMN**T**							1	-	-	155	305	CRT-B2?
CVMN**T**							2	-	231	155	-	CRT-B2?
CVMN**T**							1	-	231	-	305	CRT-B2?
CVMN**T**						1	1	-	231	155	-	CRT-B2?
CVMN**T**							1	186	-	155	-	CRT-B2?
CVMN**T**							1	-	231	155	-	CRT-B2?
CVMN**T**		1						152	231	155	305	CRT-B3
SVMN**T**	1							186	231	155	305	CRT-B2

This table shows the *pfcrt* allele distribution across the different collection sites.

[a]Indicates amino acids at pfcrt codons 72, 73, 74, 75 and 76; [b]Microsatellite loci (in Kb) are named according to their position from the gene; negative positions are 5' and positive positions are 3' to the gene. CC, Caballacocha; PC, Padrecocha; PH, Pampa Hermosa; UL, Ullpiyacu; BE, Bellavista; LA, La Arena; ZA, Zarumilla

Table 7.10. Common *pfmdr1* genotypes and microsatellite markers from study isolates.

***pfmdr1* alleles**[a]	**Number of samples**							**Microsatellite haplotype**[b]						Haplotype group
	CC	**PC**	**PH**	**UL**	**BE**	**LA**	**ZA**	**-4.2**	**-3.4**	**-1.4**	**0**	**0.45**	**3.7**	
ND**F**S**D**D	1							204	127	197	206	192	169	MDR-A1
N**GF**S**D**D		4						196	133	203	206	178	189	MDR-B1
ND**F**S**D**D						2	41	196	133	203	206	178	189	MDR-B1
?D**F**S**D**D						1		-	133	-	206	178	189	MDR-B1?
???S**D**D						1		196	133	-	206	178	189	MDR-B1
ND**F**???						3	7	196	133	203	206	178	189	MDR-B1
ND**F**???					1			-	133	203	206	178	189	MDR-B1?
ND**F**???							1	-	133	203	-	178	-	MDR-B1?
ND**F**???						1		195	133	-	-	178	-	MDR-B1?
ND**F**???						1		-	133	202	-	-	189	MDR-B1?
ND**F**S**D**D							1	196	-	203	206	178	189	MDR-B1?
ND**F**S**D**D							4	196	133	203	206	178	-	MDR-B1?
ND**F**S**D**D							2	196	133	-	206	178	189	MDR-B1?
ND**F**S**D**D							1	196	133	203	-	178	189	MDR-B1?
ND**F**S**D**D							1	196	133	203	-	178	-	MDR-B1?
ND**F**S**DY**							1	196	133	-	-	178	189	MDR-B1?
???S**D**Y							1	196	133	-	206	178	189	MDR-B1?
ND**F**???							6	196	133	203	206	178	189	MDR-B1
ND**F**???							1	196	133	203	-	178	189	MDR-B1?
ND**F**???							1	-	133	-	206	178	189	MDR-B1?
?D**F**???							1	196	133	-	206	178	-	MDR-B1?
???S**DY**							1	-	133	-	-	-	189	MDR-B1?
???S**DY**							1	-	-	-	-	-	189	MDR-B1?
ND**F**???					1			196	133	-	206	178	189	MDR-B1?
ND**FSDY**						1		-	133	-	-	178	-	MDR-B1?
ND**F**S**DY**						1		196	-	203	-	178	-	MDR-B1?
???S**DY**						1		196	133	203	-	178	189	MDR-B1
ND**F**S??							1	-	-	-	-	178	189	MDR-B1
???S**DY**							1	-	-	-	206	-	189	MDR-B1
???S**DY**							1	-	133	-	-	-	189	MDR-B1
ND**F**???							1	-	-	-	206	178	189	MDR-B1
?D**F**???							1	-	-	-	206	178	189	MDR-B1

ND**FS**D**Y**							1	196	133	203	206	178	189	MDR-B1
N**GF**S**D**D		4						196	135	203	206	178	189	MDR-B2
N**GF**S**D**D		4						196	127	203	206	178	189	MDR-B3
ND**FCD**D		26		1				204	127	197	206	192	169	MDR-A1
ND**FCD**D		1						204	127	200	206	192	169	MDR-A2
ND**FCD**D		2						204	133	197	206	192	169	MDR-A3
ND**FCD**D		1						204	133	200	206	192	169	MDR-A4
ND**FCD**D		1						204	119	197	206	192	169	MDR-A5
ND**FCD**D		1						204	121	197	206	192	169	MDR-A6
ND**FCD**D	4			17				196	133	203	206	178	189	MDR-B1
ND**FCD**D				1				196	133	-	206	178	-	MDR-B1?
ND**FCD**D				1				196	133	203	206	178	170	MDR-B1
ND**FCD**D		1						196	135	203	206	178	189	MDR-B2
ND**FCD**D	7							196	127	203	206	178	189	MDR-B3
ND**FCD**D		1						196	133	197	206	178	189	MDR-B4
ND**FCD**D	1							196	127	203	206	178	169	MDR-B5
ND**FCDY**	19	14						204	127	197	206	192	169	MDR-A1
ND**FCDY**	4	3						204	133	197	206	192	169	MDR-A3
ND**FCDY**	2							204	127	197	206	192	189	MDR-A7
ND**FCDY**	1							196	133	203	206	178	189	MDR-B1
ND**FCDY**	1							196	127	203	206	178	169	MDR-B5
N**GFCD**D		1						196	127	203	206	178	189	MDR-B3

This table shows the *pfmdr1* allele distribution across the different collection sites. The A and B haplotype identifiers are approximately equivalent to α and β lineages and were used in this table to maintain continuity with an earlier work by our group [31]. No *pfmdr1* alleles were successfullly amplified in Pampa Hermosa.

[a]Indicates amino acids at *pfcrt* codons 86, 144, 184, 1034, 1042 and 1246; [b]Microsatellite loci (in Kb) are named according to their position from the gene; negative positions are 5' and positive positions are 3' to the gene. CC, Caballacocha; PC, Padrecocha; PH, Pampa Hermosa; UL, Ullpiyacu; BE, Bellavista; LA, La Arena; ZA, Zarumilla,

Table 7.11. Microsatellite Heterozygosity around *dhfr*.

Lineage	Neutral He	Allele	n =	-349 kb	-249 kb	-88.9 kb	-29.6 kb	-5 kb	-3.77 kb	-0.06 kb	0.45 kb	5.78 kb	48.7 kb	90.5 kb	249 kb	347.1 kb
All	0.64, N=207	51/108/164-A	54						0.04 ±0.03				0.509 ±0.00	0.509 ±0.00	0.602 ±0.03	0.509 ±0.03
		108-B	31	0.52 ±0.01												
		108-C	39	0.15 ±0.07	0.11 ±0.07	0.16 ±0.07			0.49 ±0.03				0.25 ±0.08	0.24 ±0.08	0.26 ±0.08	0.23 ±0.08
		108-D	14	0.53 ±0.03				0.56 ±0.09								
		WT	55	0.09 ±0.06												
A	0.09, n= 30	51/108/164-A	30										0.37 ±0.08	0.37 ±0.08	0.37 ±0.08	0.37 ±0.08
B	0.00, n= 22	51/108/164-A	22						0.09 ±0.08				0.25 ±0.10	0.25 ±0.10	0.25 ±0.10	0.25 ±0.10
C	0.14, n= 38	108-B	31	0.52 ±0.02												
		51/108/164-A	2													
D	0.01, n= 42	108-C	39	0.15 ±0.07	0.11 ±0.07	0.16 ±0.08			0.49 ±0.03				0.25 ±0.08	0.23 ±0.08	0.26 ±0.08	0.23 ±0.08
E	0.08, n= 84	Wildtype-D	55	0.09 ±0.06												
		108-D	14	0.53 ±0.03				0.49 ±0.08								

All empty cells represent a H_e of zero. Microsatellite locations were updated from previous references for accuracy. The following are the previous locations followed by their corrected locations: He data, *dhfr* -350 is -349, -250 is -249, -89 is -88.9, -30 is -29.6, -5.3 is -5, -3.8 is -3.77, -0.3 is -0.06, 0.52 is 0.45, 5.87 is 5.78 , 50 is 48.7, 90 is 90.5, 250 is 249, and 350 is 347.1

Table 7.12. Microsatellite Heterozygosity around *dhps.*

Lineage	Neutral He	Allele	n =	-196.6 kb	-72.8 kb	-34.6 kb	-11.1 kb	-7.5 kb	-2.8 kb	-1.5 kb	-0.1 kb	0.03 kb	0.50 kb	1.4 kb	6.4 kb	9.0 kb	36.1 kb	66.1 kb	210 kb
All	0.64, n=207	437/540/581	29																
		437/581	25	0.23 ±0.09				0.22 ±0.09									0.08 ±0.07	0.08 ±0.07	
		Silent 540-AAG	47	0.50 ±0.02	0.17 ±0.07		0.10 ±0.06		0.09 ±0.06	0.13 ±0.06		0.16 ±0.07		0.05 ±0.04	0.05 ±0.04			0.10 ±0.06	0.61 ±0.04
		Wildtype A	92	0.48 0.03	0.48 0.02		0.46 0.03	0.46 0.03	0.48 0.03	0.52 0.03	0.49 0.02	0.46 0.03	0.46 0.03	0.46	0.48 0.03	0.50 0.02	0.49 0.03	0.50 0.03	0.47 0.03
A	0.09, n= 30	437/540/581-A	28																
		437/581-A	1	Not enough data for calculation, but extended haplotype is the same as the first entry (437/540/581-A)															
		Wildtype-A	1	Not enough data for calculation, but extended haplotype is the same as the first entry (437/540/581-A)															
B	0.00, n= 22	437/581-A	22																
C	0.14, n= 38	Wildtype-A	31																
		Silent 540-AAG	4		?									?	?	?	?		
		437/581-A	2	1 ±0													1 ±0	1 ±0	
		437/540/581-A	1	Not enough data for calculation, but extended haplotype is the same as the first entry (437/581-A)															
D	0.01, n= 42	Silent 540-AAG	43															0.05 ±0.04	0.55 ±0.03
E	0.08, n= 84	Wildtype-C	61																

All empty cells represent a H_e of zero.

Microsatellite locations were updated from previous references for accuracy. The following are the previous locations followed by their corrected locations: -211 is 209.7, -66.6 is 66.1, -36.8 is 36.1, -10.1 is 9.0, -7.4 is 6.4, -2.5 is 1.4, -1.64 is 0.50, -0.8 is 0.03, 0.1 is -0.1, 0.144 is -1.5, 1.591 is -2.8, 6.19 is -7.5, 9.8 is -11.1, 33.1 is -34.6, 71.6 is -72.8, 198 is -196.6.

Table 7.13. Microsatellite Heterozygosity around *pfcrt.*

Lineage	Neutral He	Allele	n =	-257 kb	-200 kb	-45 kb	-17.7 kb	-4.8 kb	-4.4 kb	1.5 kb	3.9 kb	18.8 kb	45.3 kb	57.1 kb
All	0.64, n=207	SVMNT-A	55	0.57 ±0.04	0.50 ±0.02			0.14 ±0.06						
		CVMNT-A	30	0.07 ±0.06				0.20 ±0.09		0.07 ±0.06				
		CVMNT-B	116		0.48 ±0.02	0.31 ±0.05				0.07 ±0.03				0.10 ±0.04
A	0.09, n= 30	SVMNT-A	30	0.13 ±0.08	0.13 ±0.07			0.19 ±0.08						
B	0.00, n= 22	SVMNT-A	22	0.26 ±0.10				0.10 ±0.08						
C	0.1354, n= 38	CVMNT-A	32	0.13 ±0.08				0.19 ±0.09		0.18 ±0.08				
		CVMNT-B	4											
D	0.0134, n= 42	CVMNT-B	39			0.50 ±0.02				0.05 ±0.05				0.11 ±0.07
		SVMNT-A	3											
E	0.08, n= 84	CVMNT-B	72		0.11 ±0.05					0.08 ±0.04				

All empty cells represent a H_e of zero.

Microsatellite locations were updated from previous references for accuracy. The following are the previous locations followed by their corrected locations: -189 is -257, -131 is -200, -96 is -45, -24 is -17.7, -5 is -4.8, -4.4 is -4.5, 1 is 1.5, 6 is 3.9, 22 is 18.8, 86 is 45.3, and 106 is 57.1.

Table 7.14. Heterozygosity around *pfmdr1*.

Lineage	Neutral He	Allele	n =	-305 kb	-207 kb	-99 kb	-54 kb	-4.2 kb	-3.4 kb	-1.4 kb	0 kb	0.45 kb	3.7 kb	23 kb	89 kb	137.4 kb	239.8 kb
All	0.64, n=207	184/1034/1042/1246-α	42	0.22 ±0.08	0.43 ±0.07	0.05 ±0.05			0.28 ±0.07				0.09 ±0.06	0.51 ±0.00	0.51 ±0.00		
		184/1034/1042-α	33	0.06 ±0.06	0.12 ±0.07				0.23 ±0.09	0.12 ±0.07							
		184/1034/1042- β	33	0.63 ±0.04	0.51 ±0.02				0.42 ±0.08	0.06 ±0.07			0.06 ±0.07				0.06 ±0.07
		142/184/1042- β	14				0.26 0.12		0.70 0.03								
		184/1042-β	54	0.20 ±0.07	0.33 ±0.08												0.17 ±0.06
		184/1042/1246-β	2	?	?					?	?						1 ±0
		184/1034/1042/1246-β	2		1 ±0				1 ±0							?	
		142/184/1034/1042- β	1														
		184/1042- α	1														
A	0.09, n= 30	184/1034/1042/1246-α	16	0.46 ±0.08	0.59 ±0.05				0.33 ±0.11				0.23 ±0.11				
		184/1034/1042-α	14		0.14 ±0.11				0.38 ±0.13	0.14 ±0.11							
B	0.00, n= 22	184/1034/1042/1246-α	21			0.1 ±0.08			0.32 ±0.10								
		184/1042- α	1	Not enough data for calculation, but extended haplotype as majority 184/1034/1042/1246-β haplotype													
C	0.14, n= 38	184/1034/1042/1246-α	2														
		184/1034/1042-α	19	0.11 ±0.08	0.11 ±0.08				0.11 ±0.09	0.11 ±0.08							
		142/184/1042- β	12				0.17 ±0.12		0.73 ±0.0								
		142/184/1034/1042- β	1	Not enough data for calculation, but extended haplotype similar to 142/184/1042- α													
D	0.01, n= 42	184/1034/1042/1246-α	3														
		184/1034/1042/1246-β	2		1 ±0				1 ±0							?	

		184/1034/1042- β	33	0.63 ±0.04	0.51 ±0.02				0.42 ±0.08	0.06 ±0.05							
E	0.08, n= 84	184/1042/1246- β	2	?	?					?	?						1 ±0
		1034/1042- β	54	0.20 ±0.07	0.33 ±0.08												0.17 ±0.06

All empty cells represent a H_e of zero. Microsatellite locations were updated from previous references for accuracy. The following are the previous locations followed by their corrected locations: -208 is -207, -1.2 is -1.4, 0.56 is 0.45, 3.8 is 3.7, 137 is 137.4, and 233 is 239.8.

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CHAPTER 8

EVIDENCE FOR RECOMBINATION BETWEEN *PLASMODIUM FALCIPARUM* CLONETS IN THE PERUVIAN AMAZON AFTER A MULTIYEAR EPIDEMIC

Authors

Sean M Griffing[1, 2, 3], Tonya Mixson-Hayden[2, 3], Sankar Sridaran[2, 4], Md Tauqeer Alam[2,3], Andrea M. McCollum[2, 3], Alexandre Macedo De Oliveira[2], Carmen Lucas[7], John W. Barnwell[2], Ananias A. Escalante[8], [*], David Bacon[7] Venkatachalam Udhayakumar[2]

Emory University, Atlanta, GA, United States of America[1], Malaria Branch, Centers for Disease Control and Prevention, Atlanta, GA, United States of America[2], Atlanta Research and Education Foundation, Atlanta, Georgia[3], Association of Public Health Laboratories, Silver Spring, MD, United States of America[4], Instituto Nacional de Salud Lima, Peru[5], Pan American Health Organization[6], Parasitology Program, Naval Medical Research Center Detachment, Lima, Peru[7], Arizona State University, Tempe, AZ, United States of America[8].

***Address of Corresponding Author:**

Centers for Disease Control and Prevention

Division of Parasitic Diseases, Malaria Branch

1600 Clifton Road NE, Mail Stop D-67; Atlanta, GA 30329 USA

Atlanta, GA 30333

Phone 404-718-4418

Fax 404-718-4440

Email:vxu0@cdc.gov

Abstract

During the 1990s, the Peruvian Amazon suffered large scale malaria epidemics. By 1998, Peruvian *Plasmodium falciparum* cases reached a peak of more than 80,000 cases. We previously examined parasites collected at sites across the country during the peak of epidemic expansion in 1999-2000. We found that parasites fell within five clonets (A, B, C, D, and E) and we proposed that they had rapidly expanded during the epidemics. The breakdown of such clonets by the mating of such clonets, or outcrossing, is likely limited by the lack of local diversity and low transmission. Yet within the Peruvian *P. falciparum* clonets, there were significant polymorphic microsatellite markers at site closest to Iquitos, the nexus of the epidemics which occurred within the Peruvian Amazon. We therefore hypothesized that outcrossing could have occurred in Iquitos because the most cases occurred there and that locality had the greatest number of clonets. We investigated the genetic relatedness of *P. falciparum* parasites from Iquitos collected in 2006 and 2007 to see whether the clonets had broken down after the epidemic peak and the removal of SP in favor of ACT in 2001. The four *P. falciparum* clonets previously reported in the vicinity of Iquitos region in 1999 outcrossed. Most of the parasites found in Iquitos clustersed with clonet B, while a few clustered with clonet A and C, and D. Clonets B and C had recombined and clonets C and D outcrossed based on a qualitative analysis. The remaining minority of samples suggest there may have been recombination events between clonets A, B, and C or B, C, and D leading to hybrids possibly better suited to the changed drug policy.

Introduction

In South America, low transmission, inbreeding, population crashes (bottlenecks), and/or epidemic expansions have led to *Plasmodium falciparum* populations with limited genetic diversity [1,2,3,4,5,6,7,8,9,10,11,12,13,14] [Dissertation Chapter 7]. Recent studies in Venezuela [5,6,12,14], Peru [15,16] [Dissertation Chapter 7], and Colombia [17] suggest that there is linkage between alleles on different chromosomes, indicating that clonal structure may be common in South America. Recently, we have proposed that these clonal groups can also be referred to as clonets [Dissertation Chapter 7]. By definition, clonets are genetically identical for a particular set of markers, but potentially variable at other markers [18] and estimates of the time to their most recent common ancestor can range from a few weeks to hundreds of years [19].

In Peru, *P. falciparum* parasites were found to fall within five clonets (A, B, C, D, and E) using isolates collected at sites across the country during the peak of epidemic expansion in 1999-2000. Clonets A and B were found in the eastern and central Peruvian Amazon.These two clonets carried CQ and SP resistant genotypes and it appeared they may have been expanded rapidly during the epidemic in late 1990s due to the selective pressure caused by the use of SP use in this region. Clonet C may have represented a remnant of an earlier Amazonian lineage as most of the parasites of this lineage carried CQ resistant genotype found in the Amazon region, but did not have SP resistant triple mutant genotypes. This clonet was mostly found in the village of Padrecocha in the Central Amazon, which is 5 km outside of Iquitos the largest city in the Loreto region. Clonet D was present at almost all Peruvian Amazon sites, though it only predominated at one in the Western Amazon. It carried a CQ resistant genotype related to the coastal lineage, which was different from that carried by clonets A,B, and C, and mostly SP sensitive genotypes (with the exception of a *dhfr* codon 108 mutation) and a unique silent mutation in *dhps* codon 540 and have come from the Pacific Coast originally. Clonet E represented a coastal lineage (CQ resistant SP sensitive) that had recently invaded the Peruvian interior. [Dissertation Chapter 7]. The breakdown of such clonets by the mating of such clonets, hereafter referred to as outcrossing,

is likely limited by the lack of local diversity and low transmission in South America [1,2,3,4,5,6,7]. Yet within the Peruvian *P. falciparum* clonets, there were still polymorphic microsatellite markers in 1999-2000 [Dissertation Chapter 7]. These polymorphic markers could be remnant diversity from the founding population, additional alleles generated over time, or chromosomal reassortment and recombination by outcrossing clonets [Dissertation Chapter 7]. More recently, 14 isolates were sequenced for their entire genomes in Iquitos during 2006. The isolates were essentially clonal with at most four parental haplotypes. It was posited that the remaining high frequencies of mutations in subtelomeric highly variable genes and internal var genes indicated that mutations had developed during self-mating or mitotic replication [20].

During the 1990s, the Peruvian Amazon suffered large scale malaria epidemics, which started on the periphery of Loreto [21]. *P. falciparum* was first reported in Padre Cocha in 1994 [22], and by 1997 the majority of malaria cases in Loreto, which accounted for 67.2% of all malaria cases in Peru, occurred around Iquitos [22]. In 1998, Peruvian *P. falciparum* reached its peak with more than 80,000 cases [23]. Public health policy also changed in Iquitos during 2001 from sulfadoxine-pyrimethamine (SP) to artemisinin-based combination therapy (ACT), consisting of artesunate plus mefloquine [23]. Two studies noted the prevalence of SP resistant alleles of dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) decreased between 1997 and 2007 [15,16]. It was argued that this decrease in frequency of SP resistant alleles was due to the higher fitness of SP sensitive alleles in the absence of SP. Notably, a shift from chloroquine (CQ) to SP in 1995 did not trigger a similar change in the prevalence of CQ resistant alleles [24,25] because the essential mutation (K76T) required to engender CQ resistance in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) [26] was fixed in the population in 1999-2000 [Bacon, et al. and Griffing not published]. The shift to combination therapy may also be responsible for changes in *P. falciparum* multidrug resistance 1 (*pfmdr1*) [15].We proposed that during these epidemics clonet A, B, C, D, and E rapidly expanded.

Here, we propose that the limited diversity seen at most sites would have limited the amount of outcrossing that could have occurred in Peru. However, we hypothesize that outcrossing could have occurred in and around Iquitos during the late 1990s and subsequent years during which control efforts began to decrease malaria cases. This region differed from the other sites for two reasons. First, this is where the most malaria cases occurred in Peru and, second, it was where it appeared the greatest number of clonets were present [Dissertation Chapter 7].We investigated the genetic relatedness of *P. falciparum* parasites from Iquitos collected in 2006 and 2007 to see if the previously reported clonets [Dissertation Chapter 7] had broken down after the epidemic peak and the change in drug policy. We tested the hypotheses that the four *P. falciparum* clonets previously reported in the vicinity of Iquitos region in 1999 (A, B, C, and D) may have undergone recombination and resulted in the generation of new hybrids by 2006-2007 as malaria transmission continued in this region. Furthermore, we tested the hypothesis that the frequency of clonets A and B, which carried highly SP resistant genotypes, may have decreased to low levels in response to the removal of the SP drug pressure due to change in public health policy.

Methods

Study sites and *P. falciparum* clinical isolates: We examined 62 Peruvian *P. falciparum* clinical isolates collected during 2006-2007 in Iquitos, Peru. The samples were collected during an ongoing febrile surveillance study approved by the US Naval Medical Research Center Institutional Review Board (approval no. NMRCD.2000.0006).

Samples from Iquitos were compared to previously reported data from the Pacific Coast of Peru (Bellavista, La Arena, and Zarumilla), the Western Peruvian Amazon (Pampa Hermosa and Ullpayacu), the central Peruvian Amazon (Padre Cocha) and the eastern Peruvian Amazon (Caballococha). Specifically, we utilized data regarding 11 microsatellites markers on different chromosomes (described below) and *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* proximal haplotypes and alleles. We used Padre Cocha as a historical comparative time point for Iquitos, due to its extreme geographic proximity [Dissertation Chapter 7].

DNA isolation, PCR amplification and genotyping of *dhfr, dhps, pfcrt,* and *pfmdr1*:
DNA was isolated from whole blood using the QIAamp DNA mini kit (QIAGEN, Valencia, CA). Samples were previously sequenced for point mutations in *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* [15]. Two samples from these sites were resequenced for *dhps* to test for a novel synonymous mutation at codon 540 (AAG) reported in 1999 [Dissertation Chapter 7], using a previously described protocol [5,27].

Microsatellite typing: Whole genome amplified DNA (Qiagen's REPLI-g Whole Genome Amplification Kit, Valencia, CA) was used for microsatellite characterization. Samples were assayed for 12 microsatellite loci spanning 499.5 kb around *pfcrt* on chromosome 7; 15 microsatellite loci spanning 544.7 kb around *pfmdr*1 on chromosome 5; 13 microsatellite loci spanning 700 kb around *dhfr* on chromosome 4; and 16 microsatellite loci spanning 406.3 kb around *dhps* on chromosome 8 [28,29,30]. Primer sequences and their PCR parameters were

described earlier [5,6]. We previously reported microsatellite data close to *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* for Iquitos [15]. In addition, we examined 12 putatively neutral microsatellite loci. Five were selected from neutral markers previously described (TA1, chromosome 6; poly α, ch. 4; PfPK2, Ch. 12; TA109, ch. 6; and 2490, ch. 10) [1,31]. The remaining 7 markers were C2M33, C2M34, C2M29, C2M27 on ch. 2 and C3M40, C3M69, and C3M39 on ch. 3 [6].

Network Analysis: Two median joining networks were created to illustrate the genetic relationships between isolates collected in Iquitos and clonets reported in our earlier study [Dissertation Chapter 7] using Network v. 4.516 (fluxus-engineering.com) [32]. We initially used seven neutral microsatellites on different chromosomes (TA1, poly α, PfPK2, TA109, 2490, C2M34, and C3M69) to examine Peruvian *P. falciparum* genetic relationships over time, as well as four microsatellite markers on chromosomes that carried drug resistance alleles (Ch. 4, 347.1 kb; Ch. 5, -305 kb; Ch. 7, -257 kb; and Ch. 8, -196.6). We selected each of these last four markers to be as far from the gene as possible. In the second network, we controlled for potential recombination along the chromosomes that contained drug-resistance alleles (Ch. 4, Ch. 5, Ch. 7, and Ch. 8) by creating pseudo alleles, for the purposes of Network, based on the genotype of each gene and their surrounding microsatellite haplotypes. For example, for *pfcrt*, CVMNT-A was labeled as allele 1, SVMNT-A was labeled as allele 2, and CVMNT-B was labeled as allele 3. For *pfmdr1*, there were too many genotypes for this to be useful and we therefore created only 2 alleles based on the α and β lineages previously described. In 1999, p*fmdr1* showed more variation in haplotypes than the other genes. To control for recombination, we used the two microsatellite loci closest to the gene (-1.40 and 0.45 kb) to define α and β and lineages. We previously concluded that α was an Amazonian lineage and β was a Pacific coast lineage [Dissertation Chapter 7].

Statistical Analysis: Expected heterozygozity (H_e) was calculated for each locus as $[n/(n-1)][1-\Sigma p_i^2]$, where n is the number of isolates sampled and p_i is the frequency of the ith allele [29] using the Excel Microsatellite Tool Kit [33]. The H_e sampling variance was calculated as $[2(n-1)/n^3][2(n-2)][\Sigma pi^3-(\Sigma pi^2)^2]$ [29]. Mean neutral H_e was based on the seven markers on Ch. 2 and 3. Significant associations between microsatellites used in this study were determined using an exact test of linkage disequilibrium [34], 10,000 Monte Carlo steps in Arlequin version 3.1 [35], and a Bonferronni-Holms correction [36].

To illustrate the impact of population substructure previously seen in Padre Cocha, and the population structure of Iquitos a few years later, we used allele frequency distributions generated by the Bottleneck application (www.ensam.inra.fr). In a population at mutation drift equilibrium (MDE), the distribution of allele frequencies is expected to be L-shaped; most alleles in the population should occur at frequencies between 0-10%, with a rapid decline in the percentage of alleles that occur more often (i.e. 10-20%, 20-30%, etc.). Allele frequency distributions indicate recent bottlenecks if they have mode-shifts; that is, if most allele frequencies occur in an intermediate range (most often 10-20%). This test assumes the population is mating randomly, has no substructure, loci are neutral, and that the samples are representative [37]. For this analysis, we used the same 11 neutral microsatellite markers described in the Network analysis section.

Results

Network diagrams: Figures 8.1 and 8.2 show the median joining networks generated based on 1) the 11 neutral markers and 2) seven neutral markers plus the four pseudo markers, respectively. Most of the parasites found in Iquitos cluster with clonet B, while a few cluster with clonet A and C, and D (Figure 8.1). After controlling for recombination by utilizing pseudohaplotypes, most of the Iquitos parasites are more similar to clonet C, a minority of parasites fall within clonets B and C, and a few clustered with A and D (Figure 8.2). A qualitative analysis of the raw data used to make Figure 8.2 indicates that clonets B and C have recombined. It also indicates that clonets C and D have recombined. The remaining minority of samples indicate there may have been additional recombination between clonets A, B, and C or B, C, and D.

Bottleneck allele frequency distributions:

We illustrate the distribution of allele frequencies in Padre Cocha in two different ways in order to show how cryptic substructure can alter allele frequency distributions. First, we show the allele distributions for clonet A (n=17) and C (n=24) (Figure 8.3). These clonets represented 89% of the samples in 1999. Both clonets appear to have undergone bottlenecks as the greatest number of alleles do not fall within the 0-10% allele frequency class and they lack the L-shaped distribution expected in a nonbottlenecked population (rather, clonet A is bimodal and clonet C is trimodal).

We also combined all data from Padre Cocha to illustrate how ignoring substructure would lead to the misleading conclusion that a bottleneck had not taken place (Figure 8.4).

The largest percentage of alleles (38%) fall within the 0-0.1 frequency class and the allele distribution approximates the L shaped distribution expected in populations at MDE (Figure 8.4). Note the lack of alleles in frequency classes above 0.6-0.7; this is most likely due to having two different clonets in approximately equal frequency.

We also illustrate the allele frequency distribution of Iquitos in 2006-2007, where the 0-0.1 frequency class again has the greatest percentage of alleles (48%), indicating that no bottleneck has taken place (Figure 8.4). In Iquitos, there were very few samples that appeared to have maintained the clonets seen in 1999 (clonet B, n=5, clonet C, n=2).

Multilocus haplotypes and diversity at microsatellites:

The prevalence of mutations in *dhfr*, *dhps*, *pfcrt*, and *pfmdr*, their haplotypes, and multilocus linkage in Iquitos are given in Table 8.1. Special note is made of the few samples which represent clonets found in 1999 in Padre Cocha or simple combinations of them. It is evident that there is substantial change in the proportion of different clonal lineages between 1999 to 2006-2007. Most of the samples either appear to be either combinations of more than two lineages or occur so rarely that conclusions regarding their origins would be imprudent. While there was haplotype variation in the parasites carrying the 50/51/108 *dhfr* alleles, it appeared that they were closely related to clonet A.

Overall, there was extremely reduced genetic diversity at microsatellite loci flanking *dhfr* (mean H_e ranged from 0 to 0.11), *dhps* (mean H_e ranged from 0 to 0.16), and *pfcrt* (mean H_e ranged from 0.03 to 0.12) (Table 8.2, 8.3, 8.4). *Pfmdr1* (mean H_e ranged from 0.06 to 0.16) appeared to have more markers with H_e greater than zero (Table 8.5). The neutral H_e was 0.43±0.05.

Pairwise linkage disequilibrium:

For this analysis, only the 32 samples with complete data for all 66 markers were used. Given the apparent connectivity of isolates within Iquitos, the data set was not subdivided. Only 8 of the 66 markers were monomorphic. Of the remaining 58 markers, 28% of comparisons were in significant linkage, which was greater than the 5% expected by chance.

Discussion

Clonets in Iquitos have outcrossed after multiple years of increased transmission and changes in drug policy. There is evidence for some parasite migration from the greater Amazon basin as the *dhfr* 50/51/108 allele reported in Iquitos had not been previously reported in Peru [8]. Yet much of the diversity in this population is most likely due to the parasites that were previously present in Iquitos or the Peruvian Amazon. As few of the samples from Iquitos appear to represent the previously reported clonets B and C (Table 8.1), the parasites have likely outcrossed multiple times over the preceding 7-8 years. In particular, the data suggested that clonets B and C, as well as C and D, have undergone sexual chromosomal reassortment. A few samples appeared to be the product of reassortment between clonets B, C, and D or A, B, and C. These results are in line with another recent study that suggested that parasites collected around Iquitos in 2006 were clonal with up to four parental haplotypes. While that study argued that the remaining variation seen was most likely due to self-mating or mitotic replication, we suggest that the remaining variation was more likely due to the reassortment of the clonets we previously described [20]

In contrast, the amount of recombination around *dhfr*, *dhps*, and *pfcrt*, and *pfmdr1* appears to be minimal (Tables 8.2, 8.3, 8.44, and 8.5). Given the high frequency of clonet A in 1999, we were surprised that few samples were associated with this clonet. There are three reasons that could explain the lack of parasites similar to clonet A in Iquitos: 1) loss due to random genetic drift; 2) after the removal of SP, the selection pressure to maintain clonet A was removed, and other clonets, including B, increased in frequency due to a higher relative fitness; 3) after the removal of SP, the selection pressure to maintain clonet A was removed, and clonet B, along with other clonets, increased in frequency due to a higher relative fitness; 3) there could have been migration of clonets from other regions which overtook clonet A; For example, clonet B was much more common in Caballococha in 1999 and could have been migrating to Iquitos between our two time points.

Allele frequency distributions have been impacted by population substructure and time. Without controlling for population structure (Fig. 3), Padre Cocha would appear to be at MDE (Fig. 4). This result underscores the importance of making sure that theBottleneck's model assumption of no population substructure is met. Between 1999 and 2006-7, the number of samples with the previously defined clonets was considerably reduced and the network interconnectivity of parasites in Iquitos had increased (Fig. 1 and 2). Therefore, controlling for substructure would be ill advised due to apparent interbreeding. This interbreeding has also led to the reversion to MDE because the allele frequency distribution is now L-shaped (Fig. 4). The lack of diversity on Ch. 4, Ch. 5, Ch. 7, and Ch. 8, after controlling for drug resistance alleles, suggests that much of this reversion is due to the reassortment and change in frequencies of entire chromosomes, rather than recombination.

In South America, *P. falciparum* clonets are likely to be maintained over the short to mid term unless three criteria are met: 1) there is more than one clonet present in a locality; and 2) there is sufficient transmission for outcrossing to occur, 3) offspring from such unions have resistance 'profiles' which are advantageous in the current drug use environment. Over the long term, if South American parasite populations remain small and fragmented, then clonets may change due to genetic drift. Iquitos meets the first two criteria for the breakdown in existing clonets because multiple clonets converged there in the middle of a large epidemic. Iquitos also meets the third criteria, based on the isolates collected between 2006-2007.

During this time, the plurality of samples (17/60 samples) in Iquitos carried a clonet that appeared to be the product of a union of clonet B and C. These parasites carried *dhfr* 108-B, *dhps* wild type-A, *pfcrt* SVMNT-A, and *pfmdr1* 184/1034/1042- α. It is possible that this profile may have been best suited to respond to the change in treatment policy (indeed, another 9 samples carry the same combination of *dhfr* 108-B, *dhps* wild type-A, and *pfcrt* SVMNT-A). As previously noted, the change in drug policy from SP use to ACT in 2001 was associated with a decrease in highly-resistant alleles of *dhfr* and *dhps* in Iquitos [15,16]. These 17 parasites carry

the more advantageous *dhfr* 108 single mutant and *dhps* wildtype alleles. They also carried the SVMNT *pfcrt* allele, suggesting this allele may have some fitness advantage over the CVMNT genotype (however, the shift from CVMNT and SVMNT was previously shown to not be statistically significant). If Peruvian epidemics are now under control, and drug policy remains constant, this new hybrid clonet may become dominant.

Therefore, the third criteria has been met in Iquitos in 2006-2007, as the offspring of clonets collected in 1999-2000 appear to have resistance 'profiles' which were advantageous in the changed drug use environment. In contrast, the survival of such a SP sensitive lineage would have been unlikely prior to the movement away from SP as the drug of choice. In 1999, prior to the removal of SP, there were only a few parasites in the Iquitos region that appeared to be the product of outcrossing and none had gained SP sensitivity (though three had gained the SVMNT allele) [Dissertation Chapter 7]. At the time, these outcrossed parasites also met the third criteria for survival because SP was still in use. However, the change in drug policy appears to have made them less fit in the absence of SP pressure and potentially led to their rapid decline by 2006-2007. Indeed, this is consistent with earlier demonstrations that SP resistant genotypes declined after the removal of SP in the Iquitos region [15,16].

Given the large scale migrations within the Amazon basin, and periodic epidemics [38], there may have been other opportunities for outcrossing. However, there may be few locations in the Amazon where such different clonets could have outcrossed. For example, it appears that SP resistance may be fixed in the remaining Amazon basin [8]. Therefore, multiresistant clonets are likely to persist. Yet there may be a potentially positive implication from this study. The SP sensitive parasites reported in Iquitos could disperse into the greater Amazon as the potential for movement throughout the region is increasing due to the development of the Interoceanic Highway. As countries in the region have moved away from SP use, these SP sensitive parasites may out compete the already established resistant clonets. Indeed, this may have already occurred, based on the presence of SP sensitive *dhfr* and *dhps* alleles in the Colombian Amazon

[17]. In summary, this study has provided evidence that already established *P. falciparum* clonets in the Peruvian Amazon have broken down due to opportunity for sexual recombination caused by the recent local epidemics. In addition, changes in drug pressure have contributed to rapid decline of some nascent hybrid clonets with low fitness in the new environment, as well as potentially contributed to the recent success of others.

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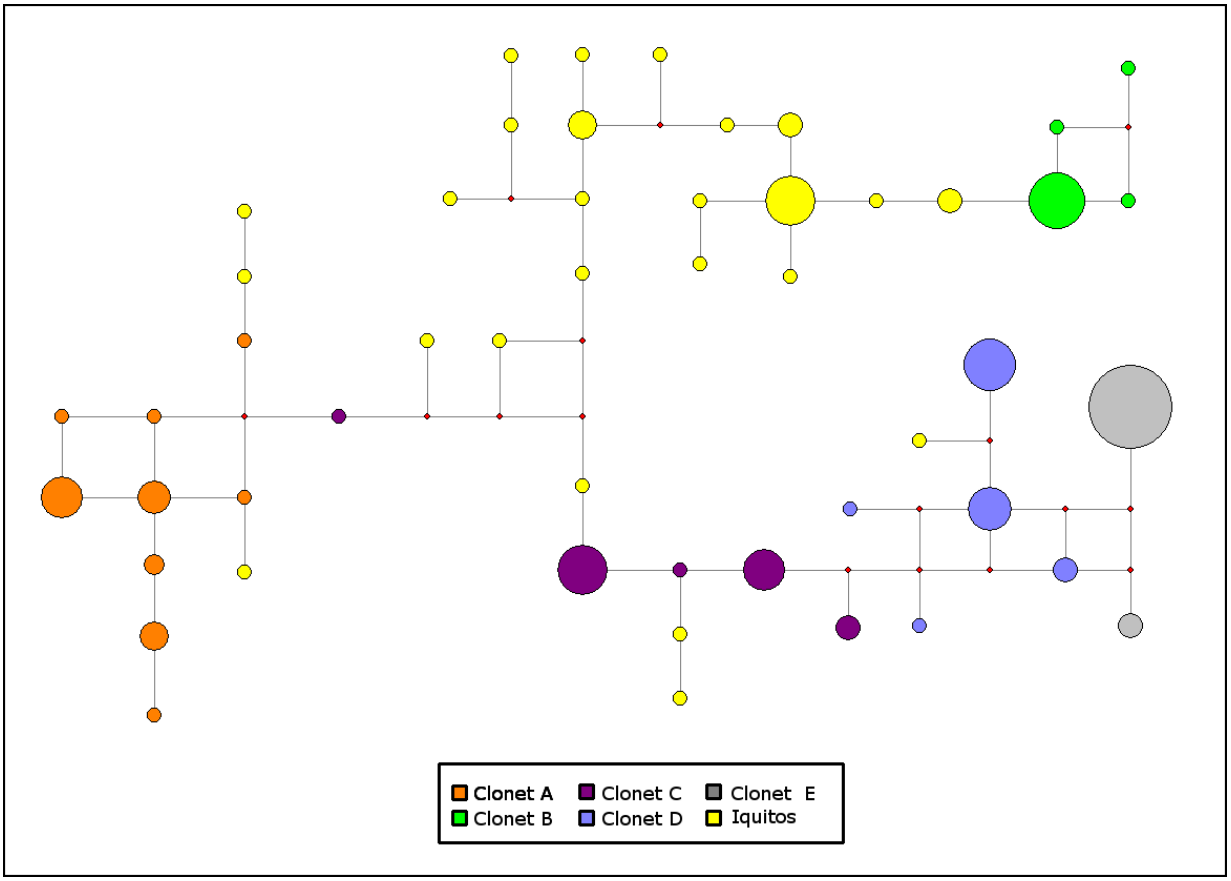
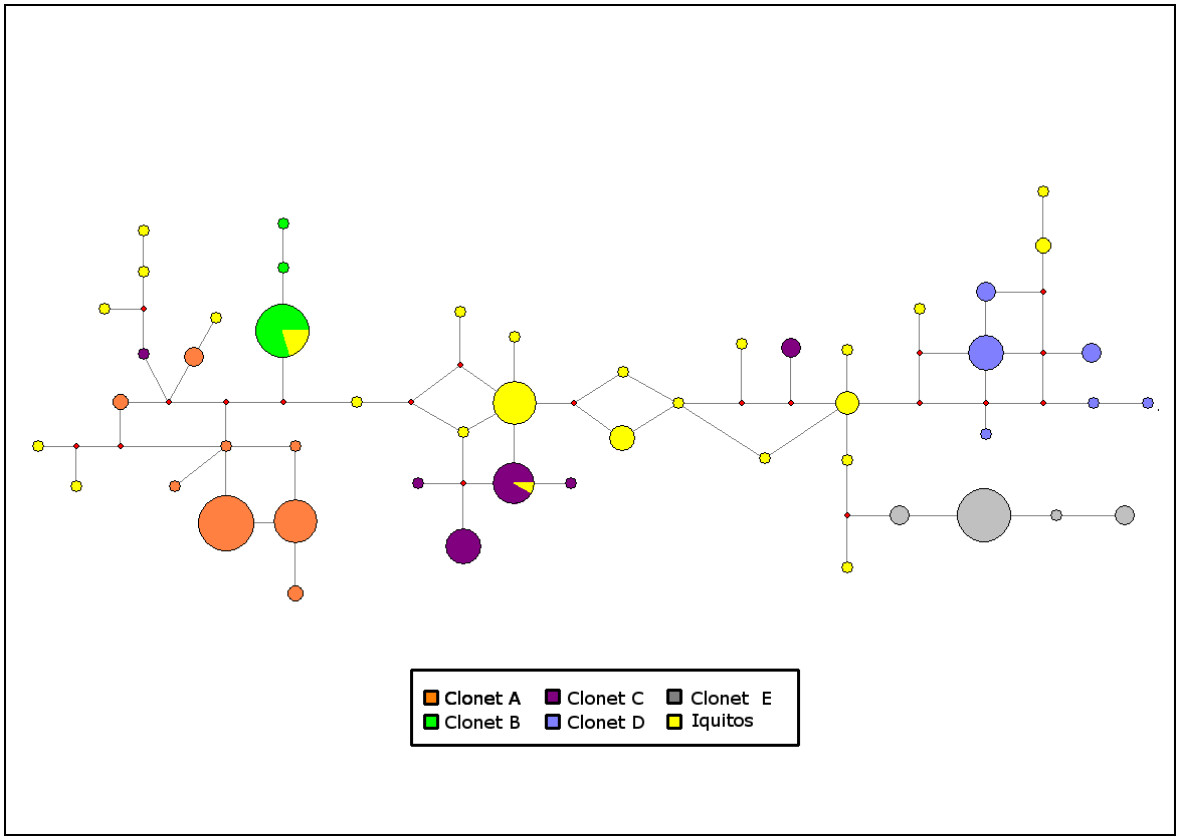
Figure 8.1: A network diagram of Iquitos in comparison to earlier clonets from neutral markers



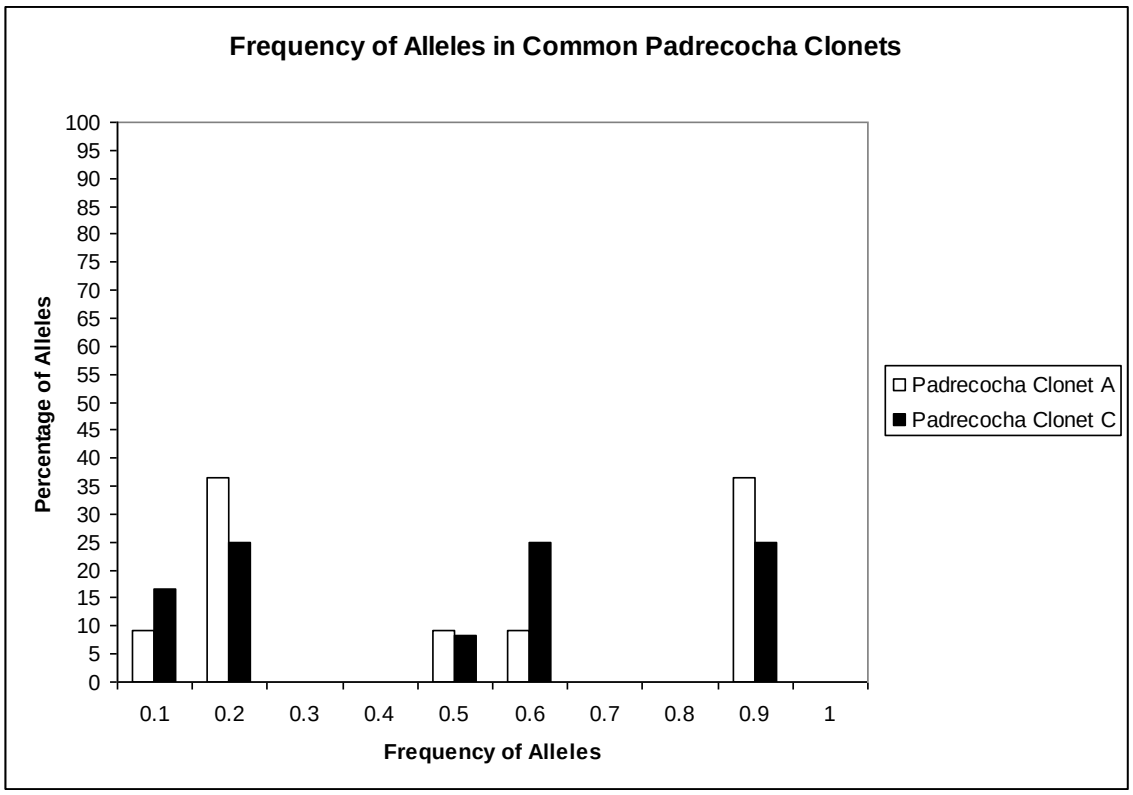
This network diagram shows the genetic relationships between Iquitos and the previously reported clonets A, B, C, D, and E using the eleven neutral microsatellite markers described in the text. Small red circles represent hypothetical nodes that link haplotypes seen among our samples.

Figure 8.2: A network diagram of Iquitos in comparison to earlier clonets from neutral markers and pseudo markers



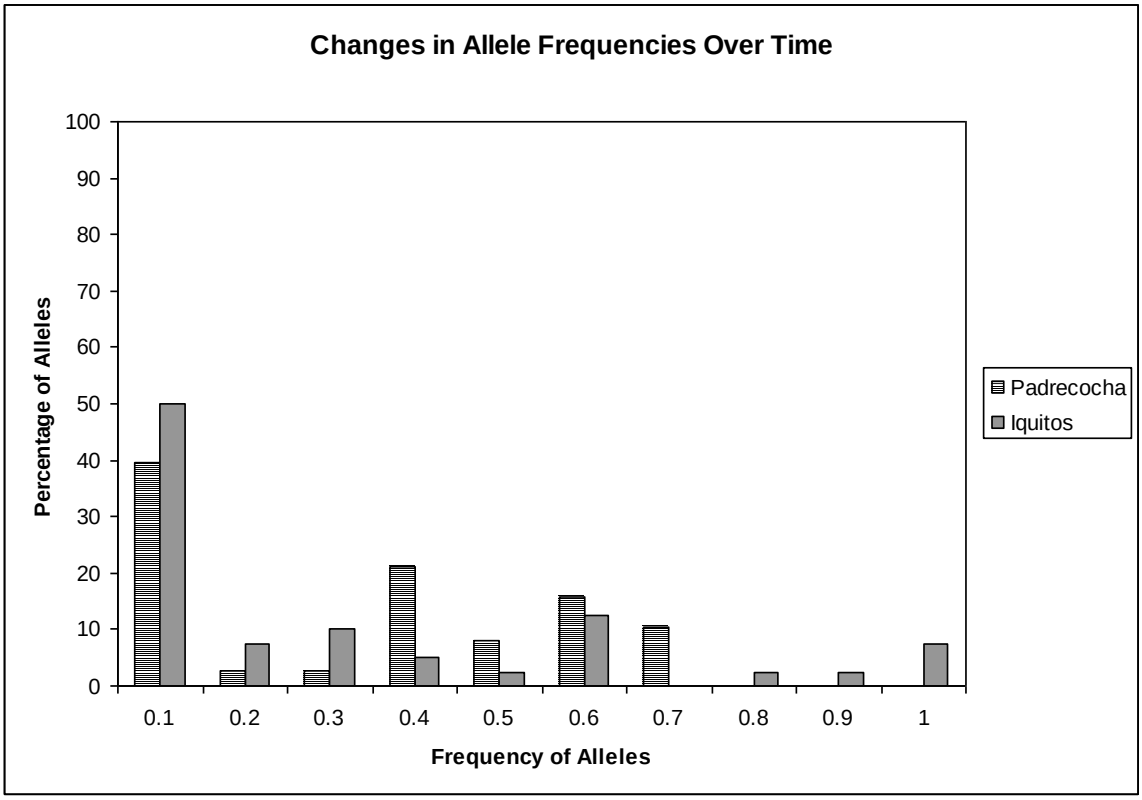
This network diagram shows the genetic relationships between Iquitos and the previously reported clonets A, B, C, D, and E using seven neutral microsatellite markers and four additional pseudo markers described in the text. Small red circles represent hypothetical nodes that link haplotypes seen among our samples.

Figure 8.3: Microsatellite allele frequency distributions for clonets A and C in Padre Cocha



This figure illustrates the percentage of alleles in different frequency classes for the most common clonets (A: n=17; C: n=24) seen in Padre Cocha in 1999.

Figure 8.4: Allele frequency distributions for Padre Cocha and Iquitos



This figure illustrates the percentage of alleles in different frequency classes for Padre Cocha (n=46) in 1999 and from Iquitos (n=42) in 2006-2007.

Table 8.1: Drug resistance allele haplotypes seen in Iquitos in 2006-2007

n	Dhfr	dhps	pfcrt	pfmdr1
1	51/108/164-A	437/540/581-A	SVMNT-A	α-184/1034/1042
6[i]	51/108/164-A	437/581-A	SVMNT-A	α-184/1034/1042/1246
1	51/108/164-A	437/581-A	SVMNT-A	β-142/184/1042
3	50/51/108-D	437/540/581-A	SVMNT-A	α-184/1034/1042/1246
1	51/108/164-A	WT-A	SVMNT-A	β-142/184/1042
1	51/108/164-A	WT-A	CVMNT-B	β-142/184/1042
17[ii]	108-B	WT-A	SVMNT-A	α-184/1034/1042
5	108-B	WT-A	SVMNT-A	α-184/1034/1042
1	108-B	WT-A	SVMNT-A	β-142/184/1034/1042
2	108-B	WT-A	SVMNT-A	β-142/184/1042
1	108-B	WT-A	SVMNT-A	β-184/1042
3[iii]	108-B	WT-A	CVMNT-A	α-184/1034/1057
12[iv]	108-B	WT-A	CVMNT-B	β-142/184/1048
1	108-B	WT-A	CVMNT-B	β-142/184/1034/1042
1	?	WT-A	CVMNT-B	β-142/184/1049
2	108-C	WT-C	CVMNT-B	β-184/1034/1042
2[v]	108-C	WT-AAG-B	CVMNT-B	β-184/1034/1042

This table shows the multiallelic linkage disequilibrium between drug-resistance alleles. It does not include neutral haplotypes because of the extent of chromosomal reassortment. [i] 5/6 of these samples represented clonet B. [ii]16/17 of these samples appear to represent the same combination of clonet B and C. [iii] 2/3 of these samples represented clonet C. [iv] 7/12 of these samples represented the same combination of clonet C and D. [v]These samples represented a combination of clonet C and D.

Table 8.2: H_e around *dhfr*

Locus	108-B n=44	50/51/108-D n=3	51/108/164-A n=10	108-C n=4
-350	0.10±0.06	0	0	0
-250	0	0	0	0
-89	0	0	0	0
-30	0	0	0	0
-5.3	0	0	0.2±0.13	0
-3.87	0	0	0	0
-0.3	0	0	0	0
0.52	0	0	0	0
5.87	0	0	0.2±0.13	0
50	0	0	0.2±0.13	0
90	0	0	0.36±0.13	0
250	0	0	0.51±0.13	0.67±0
350	0	0	0	0.67±0

Table 8.3: H_e around *dhps*

Locus	WT-A n=47	437/581-A n=7	437/540/581-A n=4	WT-C n=2	WT-AAG n=2
-211	0	0	0.67±0	0	0
-66.6	0	0	0	0	0
-36.8	0	0	0	0	1±0
-10.1	0	0	0.67±0	0	0
-7.4	0	0	0	0	0
-2.47	0	0	0	0	0
-1.64	0	0	0	0	0
-0.8	0	0	0	0	0
0.06	0	0	0	0	0
0.144	0	0	0.5±0.13	0	0
1.591	0	0	0	0	0
6.19	0	0	0.5±0.13	0	0
9.79	0	0	0	0	0
33.1	0	0	0	0	0
71.6	0.45±0.05	0	0	0	0
198	0.56±0.04	0.29±0.15	0	0	0

Table 8.4: H_e around *pfcrt*

Locus	CVMNT-B n=19	CVMNT-A n=3	SVMNT-A n=40
-189	0.11±0.09	0.67±0.09	0.37±0.09
-131	0.11±0.09	0	0.05±0.05
-96	0	0	0.06±0.05
-24	0	0	0
-5	0	0.67±0.09	0
-4.3	0.11±0.09	0	0
1	0	0	0
6	0	0	0
22	0	0	0.06±0.05
86	0	0	0
106	0	0	0.06±0.05

Table 8.5: H_e around *pfmdr1*

Locus	α-184/1034/1042 n=28	α-184/1034/1042/1246 n=9	β-142/184/1042 n=18	β-184/1034/1042 N=4	β-1142/184/1034/1042 N=2
-305	0.07±0.06	0	0.12±0.16	0	
-208	0.15±0.08	0	0.24±0.12	0	
-99	0	0.22±0.14	0.12±0.16	0	
-54	0	0	0	0	
-4.2	0	0	0.21±0.11	0	0
-3.4	0	0	0.11±0.09	0.5±0.13	1±0
-1.2	0	0	0.29±0.11	0	1±0
0	0	0	0	0	0
0.56	0	0	0	0	0
3.8	0	0	0	0	1±0
23	0	0.61±0.12	0	0	
89	0.15±0.08	0.43±0.12	0	0.67±0	
137	0.08±0.09	0.48±0.11	0	0	
233	0.34±0.10	0.43±0.12	0.23±0.13	0.67±0	

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CHAPTER 9

HISTORICAL SHIFTS IN BRAZILIAN *P. FALCIPARUM* POPULATION STRUCTURE AND DRUG RESISTANCE MARKERS.

Introduction

Malaria has been reported in Brazil since at least the 1500s. At that time, *Plasmodium falciparum* was spread along the coast along with the sugarcane industry. As Brazilian industry shifted to gold panning and diamond mining, migrating colonists and slaves spread the parasite inland into Minas Gerais (General Mines). This colonization led to supporting industries, including slash and burn agriculture and cattle ranching, which further supported the spread of malaria along with the principal vector of the region, *Anopheles darlingi* [1]. Similar colonization of the interior by susceptible and infected hosts due to the natural rubber industry in the late 1880s helped to propagate additional malaria epidemics, [2,3] as did later colonizations for other economic reasons. Usually, population movement went from the coast into the interior and waves of colonization would continue to support malaria in Brazil.

The dawn of the 'modern' era of public health control of malaria in Brazil began in 1900s when quinine was given on a very large scale to railroad workers in the state of Rio de Janeiro and Rondônia [4,5], and to workers building a dam of Xerém River [6]. These early studies reported that some infections were becoming refractory to quinine [4,5,6]. By the 1930s, malaria control had begun to rely heavily on vector control thanks to Dr. Soper's early efforts to eradicate the recently introduced *A. gambiae* mosquito species [7]. There were 6 million cases per year of malaria in Brazil in the early 1940s (which was then 1/7 of the country's population) [5]. After the application of DDT [5] and the mass use of chloroquine (sometimes in table salt) [8],there were only 28,557 *P. faliparum* cases in 1970 [9] out of a population of 92.3 million people in 1970 [5]. The incidence of malaria in Brazil was 1% of that reported in 1950 [6] and 73% of cases were reported in the Amazon region [10].

Unfortunately the removal of DDT, changes in public policy, Amazonian infrastructure projects, increasing colonization and deforestation of the Amazon, and drug resistance led to a gradual increase in the malaria [10,11,12]. Malaria eradication programs were integrated into other public health programs and the resources were reduced [13]. The construction of roads, hydroelectric plants, livestock and agricultural projects, and innumerable mines lead to an increase in malaria transmission. The number of malaria cases between 1970 and 1980 tripled – from 52,469 to 169,871 cases [12,14], as did the number of *P. falciparum* cases [9].

Brazilians had historically moved from the poorer north/northeast to the more wealthy south [15]. However, during the 1970s the direction of migration flipped, with the majority of colonists going to the gold mining areas, especially Rondônia [16,17]. Many of these colonists were immunologically naïve to malaria [18]. Migration between the different malarious regions was multifaceted with malaria being imported from multiple states in different directions [15]. By 1990, 99% of malaria in Brazil occurred in states around the Amazon basin: Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima and Tocantins [9].

For chloroquine (CQ) and sulfadoxine pyrimethamine (SP), drug resistance developed relatively quickly. In 1954, physicians in Rondônia reported CQ resistance [19].After CQ resistance was reported in Colombia in 1961, resistance was reported in Brazil: in Porto Velho, Rondônia; Belém, Pará; and along the 300 km Belém-Brasilia road [19,20,21]. A later study using samples from 1992 to 2002 in Manaus and Tabatinga, Amazonas; Porto Velho, Rondônia; Peixoto de Azevedo and Apiacás, Mato Grosso found that all parasites carried CQ resistant *pfcrt* alleles. Almost all isolates carried S_{tct}VMNT and S_{agt}VMNT, with a few on the border of Peru carrying CVMNT or a CVIET of apparent African origin. The authors argued that SVMNT was the ancestral resistant allele, though they were unsure of the relationship of the two variants [22]. Various other studies agreed that CQ resistance was fixed in Brazil [23,24,25]. Similarly, studies found mutant alleles of the *Plasmodium falciparum* multidrug resistant gene (*pfmdr1*) were fixed at various sites in Brazil. In Rondônia, only the 184F/1034C/1042D/1246Y allele [24] was

reported, but another study in Mato Grosso reported that all that isolates carried N86/184F/C1042/1246Y [26].

SP was first used in Brazil during the early 1960s. In a study conducted in 1968, it was found that *P. falciparum* strains were resistant to pyrimethamine in Goiás (RII resistance), Pará (mild to moderate resistance) and Roraima (mild resistance), Rondônia (moderate resistance), though parasites appeared to be still susceptible to sulphonamides [27]. Resistance to pyrimethamine and sulfadoxine combination (SP) resistance was first reported in Goiás and six years later in Maranhão [19,28]. In 1982, RIII SP resistance was reported along the road between Manaus, Amazonas, and Porto Velho, Rondônia [28]. By the end of the 1980s, 90% of parasites were SP resistant and 100 percent were CQ resistant.

In the southern state of Mato Grosso, *dhfr* 50R/51I/108N and 51I/108N/164L alleles were reported. Rondônia was reported to have the 50Arg/51Ile/108Asn *dfhr* allele among other less resistant alleles, but the paper appeared unreliable in its reporting [29]. In both states, parasites had *dhps* mutants with the profile 437G/540E/581G [29]. A study which looked at *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* concluded that both triple mutant *dhfr* had originated from a single origin in the Southern Amazon and spread in a north-northwest direction across the continent [23].

While *P. falciparum* in Brazil may have linkage disequilibrium and limited genetic diversity, it may not be to the extent described earlier in this dissertation for Peru. An examination of 10 microsatellites in isolates from Marabá, Pará; Tailândia, Pará; Porto Velho, Rondônia; Rio Branco, Acre; and Serra do Navio, Amapá from the 1990s showed that varying levels of linkage disequilbrium, with Tailândia and Rio Branco showing the most linkage disequilbirum and Porto Velho and Marabá showing the least. According to their analysis, even the sites with strong LD did not have evidence of recent bottlenecks or epidemic expansions of clones and isolation by distance was rejected. They also argued that there was little gene flow between sites based on F_{ST} values that varied between 0.08 and 0.30. The authors suggested that

Brazil did not have the extremely low genetic diversity and linkage disequilibrium seen elsewhere in Central and South America [30].

However, a different study found that there was indeed gene flow across the Amazon basin and limited diversity based on samples which included isolates from Manaus and Tabatinga, Amazonas; Porto Velho, Rondônia, Peixoto de Azevedo and Apiacás, Mato Grosso. Most diversity was found within populations and isolation by distance was again rejected. In addition, they showed that some parasites collected from Amazonas, Mato Grosso, Rondônia, and Peru clustered together [22]. Both of these studies used multiple microsatellites from the same chromosomes and may have therefore influenced their analysis of population structure. In particular, the second study only examined four chromosomes, one of which carried *pfcrt*.

Still another study argued that there had been a loss of rare alleles in Brazil based on samples collected in Porto Velho, Rondônia, which suggested a potential bottleneck [31]. They also argued that samples collected were highly differentiated from samples collected in Colombia, which was supported by others as well [24]. Additional studies suggested that the *P. falciparum* merozoite surface protein 1 (*msp*-1), *var*, and *Pfs*48/45 gene had limited diversity across the Amazon basin [32,33,34,35]. Taken together, these studies suggest that Brazilian *P. falciparum* has limited diversity and may or may not have the extensive clonality seen in other South American countries. When combined with the history of Brazilian malaria described earlier, it appears that Brazilian *P. falciparum* may have gone through bottlenecks, multiple reintroductions due to human migration, and potentially clonal expansions.

In this study we have examined samples from across Brazil temporally and geographically from one site in Amapá, two sites in Rondônia, and multiple sites in Pará. By examining many more microsatellites markers on more chromosomes than previous studies, we hoped to define the extent of low diversity, linkage disequilibrium, and parasite migration within Brazil. By examining samples from the 1980s and the 1990s, we hoped to illuminate how

multiresistant lineages, as well as individual alleles of *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* spread across Brazil. In addition, we planned to test these sites for isolation by distance as well as bottlenecks.

Methods

Study sites and *P. falciparum* clinical isolates: We examined 190 Brazilian samples collected during the 1980s and 1990s in Brazil in Amapá, Pará, and Rondônia (Table 9.1). Amapá is a coastal state in the northeast known for its gold mining. Pará is a coastal state just to the south which includes the terminus of the Amazon river, a major port city (Belém), and various hydroelectric projects. Rondônia is a Brazilian state in the very west of the country. It has been economically important for rubber tapping as well, as gold mining, and has also been colonized by subsistence farmers during the 20^{th} century.

Sequencing and microsatellite fragment analysis: The methods for these procedures have remained the same as in previous studies. However, one microsatellite locus 'distant' from the genes under selection have been removed from analysis based on its continued poor performance during this dissertation (-208 kb, *pfmdr1*). Another marker (50 kb, *dfhr*) was removed, despite its previous acceptable performance, because it did not amplify in these samples.

Analysis tools: The analysis tools used for this chapter are the same as used throughout the rest of the dissertation. The same 11 microsatellites used in Chapter 8 were used in this chapter again for Network diagrams and F_{ST} analysis.

Heterozygozity curves for microsatellites around each gene and its alleles were calculated based on all sites at two time points: the mid-1980s and the late 1990s. A decision was made not to divide the data by site based on the lack of diversity and the relatively low F_{ST} results.

Results

dhfr, *dhps*, *pfcrt*, and *pfmdr1* genotypes: Parasites collected at all time points and locations in Brazil carried one of three *dhfr* alleles (51/108, 50/51/108 or 51/108/164). The temporal and geographic breakdown of these alleles is given in Table 9.2. Changes in H_e between the 1980s and 1990s for each one of the genes are given in Figures 9.1-9.4. Interestingly, only the *dhfr* 51/108/164 allele showed a large increase in surrouding H_e.

Parasites collected at all time points and locations in Brazil carried the *dhps* 437G/540E/581G (156/166 of the samples amplified) with the following exceptions. A sample collected during 1983 in Itaituba, Pará carried 437G/581G, as did another sample in 1999. One sample from 1983 and two samples from 1984 carried this genotype in Tucuruí, Pará. This genotype was also found in three samples collected in 1983, 1984, and 1985 in Paragominas, Pará. Interestingly, Paragominas had the only single mutant 437G genotype reported in our sample set during 1983. Another sample collected in Amapá in 1983 also carried this genotype. All samples collected in Brazil carried the same close microsatellite haplotype (A1).

Most parasites collected carried the *pfcrt* S_{TCT}VMNT (164/182 of the samples amplifed) at all time points and locations with all of the remaining samples carrying S_{AGT}VMNT (18/182 of the samples amplifed). The samples carrying S_{AGT}VMNT consisted of two samples collected in 1983 and 1984 in Tucuruí, Pará. There was only one other sample carrying this allele in 1983 in Paragominas, Pará, though another isolate with this genotype was collected in 1985 in Amapá. The remaining samples were collected in 1996 (1 isolate, Porto Velho, Rondônia), 1998 (Porto Velho, Rondônia) 1999 (1 isolate in Itaituba and Marabá, Pará, as well as Porto Velho, Pará). All parasites carried the same close microsatellite profile.

Parasites collected at all time points and locations in Brazil carried the *pfmdr1* N86/184F/1034C/1246Y (137/144 of the samples amplifed) with the following exceptions. In Itaituba in 1999, there was one sample carrying 1034C/1042D/1246Y and another carrying 184F/1042D/1246Y. In Porto Velho, Rondônia, five samples collected in 1998 carried

1034C/1042D/1246Y. While most parasites (133/144) carried the α close marker haplotype, a few carried a slightly different marker at -1.4 kb (200 versus 197 in most samples). This type, α2, was only seen with N86/184F/1034C/1246Y and then generally during the 1980s. In particular, It was reported in seven samples in Amapá (1983: 1; 1985: 6). It was also reported in Pará (Marabá: 1, 1984, 1, 1986, 1, 1998; Itaituba: 1, 1999).

Due to the lack of diversity seen among most genes examined in this study, we divided the multidrug resistant profiles by *dhfr* alleles (Table 9.3-9.5).

Network Diagrams: I have created three network diagrams for Brazil. The network diagram in Figure 9.6 only includes data collected prior to 1996 and is meant to represent historical population structure. It illustrates that none of the sites in Amapá, Pará, or Rondônia has a single group of parasite neutral haplotypes (though Tucuri, Pará haplotypes almost cluster). The network diagram in Figure 9.7 includes only data collected in 1996 or later and is meant to represent later population structure. Generally speaking, there does not appear to be clustering of neutral haplotypes in any particular site. However, there is one cluster at the top of the figure which includes all four sites and seems to be somewhat different from the general population. The network diagram in Figure 9.8 includes all of the data collected in the study regardless of date or location. It illustrates that isolates from all time points and locations share overall connectivity, which implies that there is internal migration between sites and that parasite populations were conserved over the period we examined.

F_{ST} Analysis: This analysis was confined to only contemporaneous samples collected between 1996 and 1999 in order to control for shifts in parasite populations over time. The results of the F_{ST} analysis were generally significant with the exception of one comparison (Table 9.6). Values were generally low in comparison to earlier values reported in our other studies. The sites that appeared most similar were Rondônia/Marabá, Pará (F_{ST} =0.07) and Rondônia/Itatiuba, Pará (F_{ST}

= 0.09). Interestingly, Tailândia, Pará did not share a similar low F_{ST} with Rondônia or the other sites in Pará.

Bottleneck Analysis: There were only a few sites and times with sufficient samples to conduct bottleneck analysis. Rondônia appeared to be in mutation drift equilibrium (though the results suggest that with a larger sample set we might argue that it was bottlenecked as well), but Pará gave indications of recent bottlenecks (Table 9.7).

Discussion

According to our study, it appears that Brazilian *P. falciparum* malaria has reduced genetic diversity and gene flow across the Amazon basin. It appears that local populations did not follow the isolation by distance model because F_{st} comparisons between Rondônia and Pará were lower than comparisons within Pará state. Rather, the population structure of Brazil appears to be based on the previous admixture and continuing reassortment of parasite lineages that may have undergone genetic drift in partial isolation during the late 1960s and early 1970s. In short, we did not see the sort of population structure we reported earlier for Peru though similar processes may occurred there in earlier decades. While the data suggested there was significant migration between sites in the Brazilian Amazon Basin, there were no obvious structure to that migration (Figures 9.6, 9.7, 9.8). In support of this, our pairwise F_{ST} calculations suggested that Brazilian populations generally had low values (Table 9.6). Interestingly, the two sites in Pará had lower F_{ST} values in comparison to Rondônia than to each other. The low reported F_{ST} for the comparison of Porto Velho, Rondônia and Marabá, Pará is also supported by the findings of a previous paper [30]. It appeared that parasites in Pará had gone through statistically significant bottlenecks, but those in Rondônia had not. This suggests one of two scenarios. Either Rondônia acted as a source population multiple times for different sites in Pará or multiple different parasites populations from Pará have populated Rondônia.

Our study agrees with earlier findings [31] which found that most parasite diversity was found within sites in the Brazilian Amazon. Our limited F_{ST} comparisons also imply, as in earlier work, that isolation by distance is rejected. We found that some isolates from all of our sites in Brazil had haplotypes which grouped together. We found evidence for bottlenecks in Brazil in Pará, though our data from Rondônia did not appear bottlenecked, which is in conflict with an earlier study which reported a potential bottleneck in Rondônia [31]. We suspect that their study may be the accurate finding as Rondônia had a skewed allele frequency distribution (indicative of a bottlenecked population) and almost significant H_e excess in our dataset. A qualitative

examination of the isolates used in this study do not suggest that there is varying levels of linkage disequilibrium at different Brazilian sites, which is in conflict with the conclusions of an earlier study. This study also concluded that there was limited gene flow in the Brazilian Amazon and that the low genetic diversity seen elsewhere in South America was not found in Brazil [30]. Our study disagreed with all of these results, perhaps because they used fewer markers on fewer chromosomes.

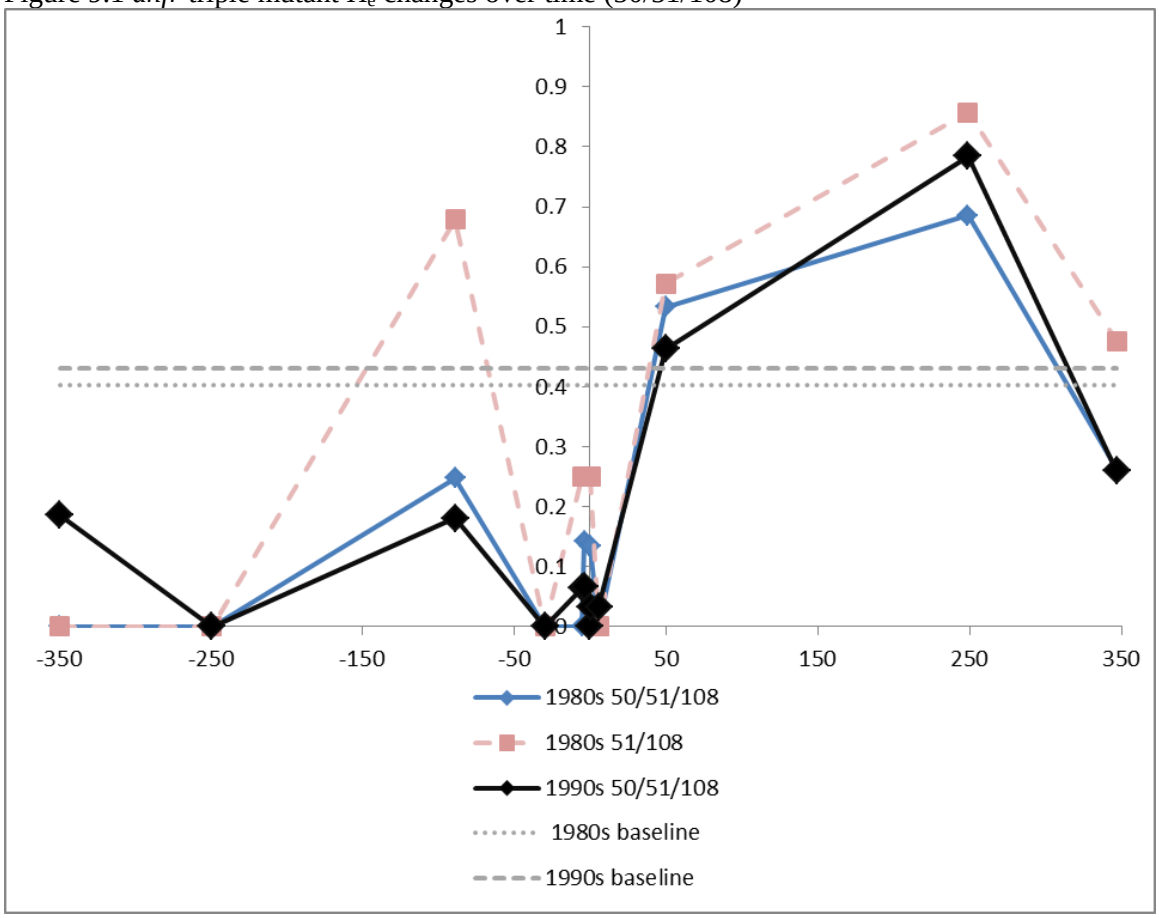
It has been previously argued that highly resistant alleles of *dhfr* and *dhps* spread from the southern Brazilian Amazon [15] based on their understanding that high levels of SP resistance had first developed on the border of Bolivia and Brazil. However, RII pyrimethamine resistance was first reported in Goiás in 1968 [27], which is in the center-south of the country, and resistance to the sulfadoxine-pyrimethamine drug combination was first reported in Brazil in that locality in 1972 [19,28]. The later RIII cases were reported between Manaus and Porto Velho (which is located in the west of the country, but not on the border with Bolivia) in 1982 [28]. While we do not have parasites from 1982, samples from approximately same period in Amapá (1984) and Itaituba, Pará (1985) carry *dhfr* 51/108/164 *dhfr* and also 50/51/108 (Itaituba, 1983; Rondônia, 1984). In addition, a parasite carrying the *dhfr* 50/51/108 allele and a 437/540/581 allele was collected in 1984 in Paragominas, Pará. It would be impressive if the highly resistant *dhfr*/*dhps* alleles were able to spread across Brazil in ~ two years, which implies that high levels of SP resistance may have been present in the country prior to the first report of RIII in 1982. This lack of certainty regarding the origins of highly resistant *dhfr*/*dhps* alleles within Brazil weakens the hypothesis that such highly resistant alleles developed in the south of the country and spread north [23].

. On the other hand, parasites carrying less SP resistant alleles of *dhfr* (51/108) and *dhps* (437/581) generally disappeared around 1985 among our samples (the one sample collected in Rondônia in 1999 with this genotype could potentially have migrated from Peru). This suggests that such resistance did not develop initially in northeastern Brazil. Interestingly, the isolates

carrying these double mutants were all associated with the 184/1034/1042/1246 *pfmdr1* allele, which suggests that, whatever the value of this *pfmdr1* allele, its fixation occurred earlier than the mid-1980s. Finally, a higher percentage of these samples carried the *pfcrt* S_{AGT}VMNT allele than for the other two multidrug resistant profiles. While we have not yet tested the statistical significance of this finding, it suggests that the either the S_{AGT}VMNT may predate S_{TCT}VMNT or argue that this allele was originally an eastern allele that later spread west (which might explain why it was not reported in Peru).

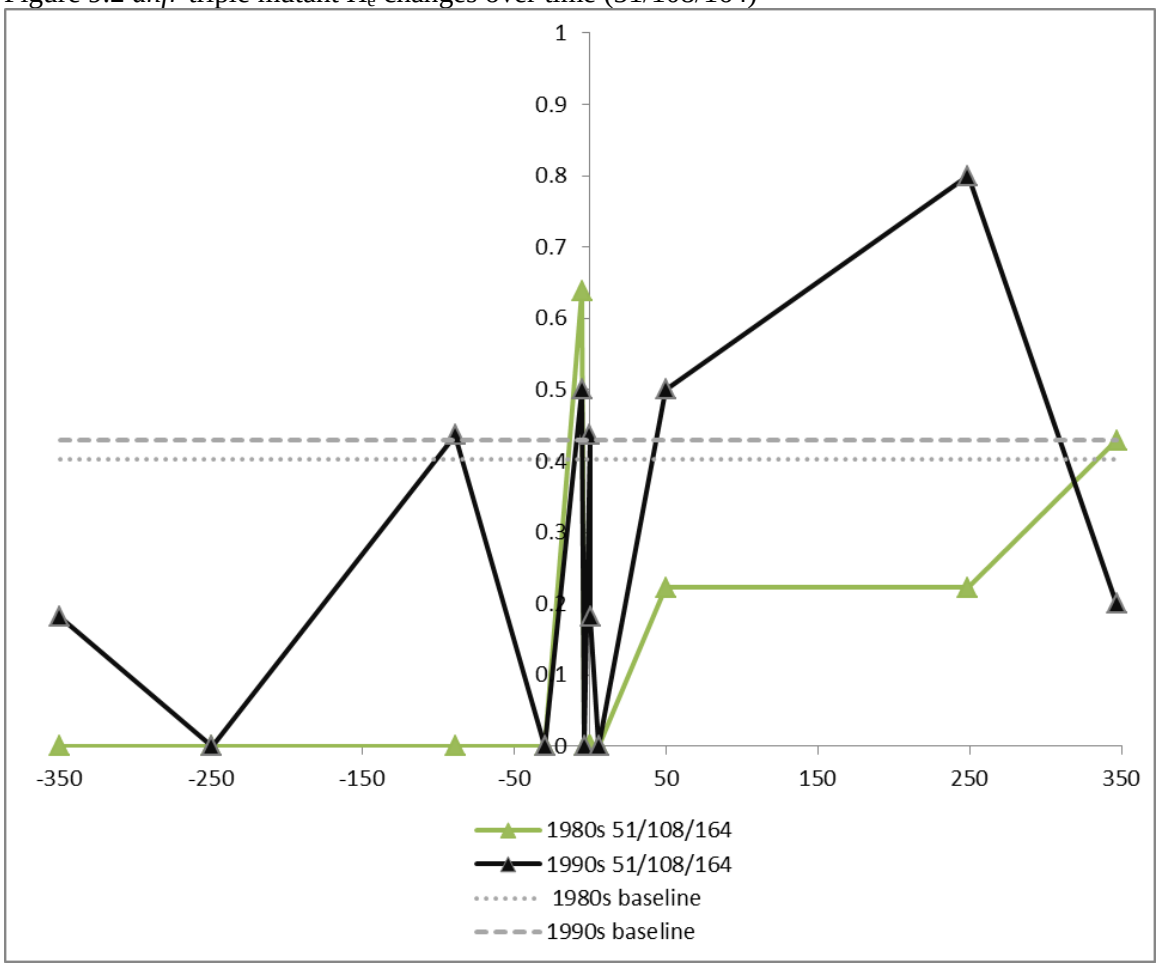
Though our sampling of Brazil begins in the early 1980s, it appears that it may have been too late for us to understand how SP resistance spread across the basin or to fully describe the complex internal migration of Brazilian parasites after the colonization efforts of the past few decades. It may be that the kind of clonet dynamics we reported in Peru occurred during the late 1970s in Brazil and have been masked over time due to extensive admixture and reassortment. On the other hand, even at its best Brazilian malaria control efforts never completely suppressed the incidence of malaria and therefore populations may have maintained high enough effective population sizes to avoid clonal expansions. Indeed, our bottleneck tests did reject this as a possibility though they suggest that at least coastal populations in Pará went through bottlenecks. From the standpoint of public health policy, our results suggest that the Brazilian Amazon basin has sufficient internal migration that drug resistance reported in any one particular region will most likely rapidly spread to other sites in the basin with similar drug pressure.

Figure 9.1 *dhfr* triple mutant H_e changes over time (50/51/108)



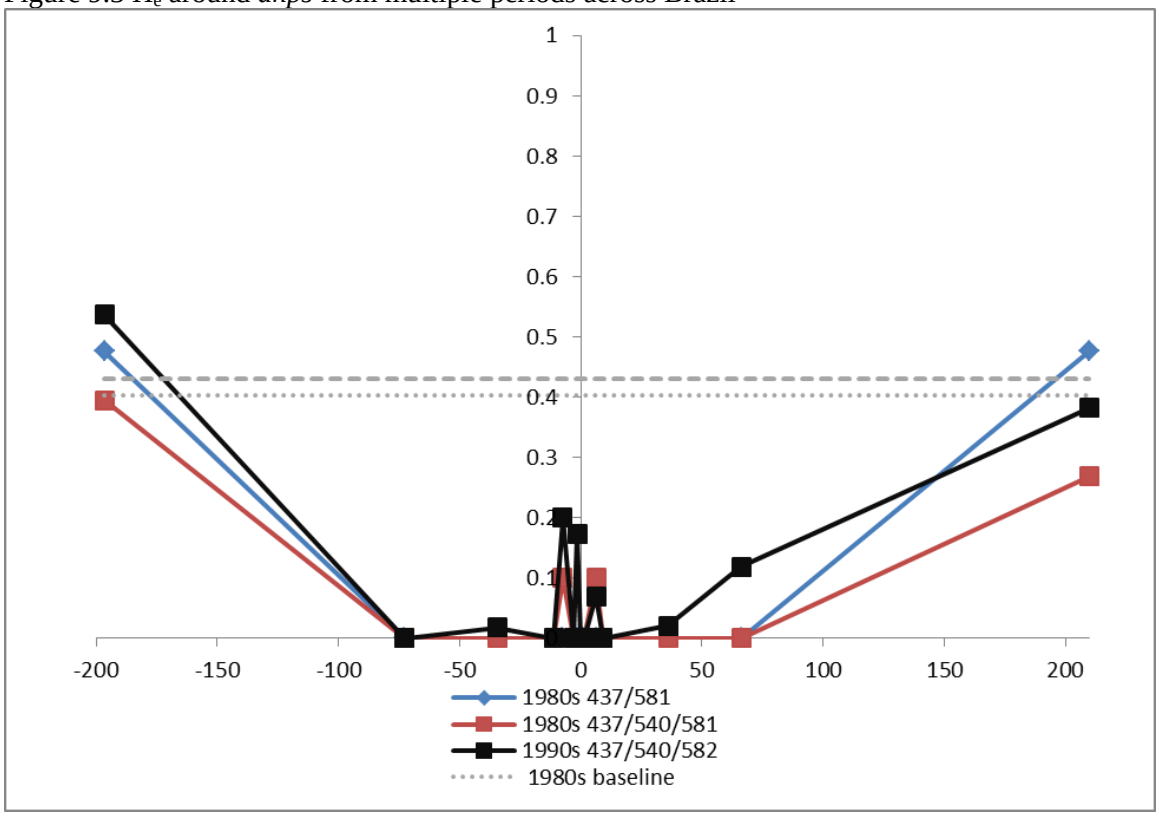
The figure shows variation in H_e surrounding *dhfr* for parasites carrying a 51/108 double mutant during the 1980s (n=8). It also shows variation in H_e surrounding *dhfr* for parasites carrying a 50/51/108 mutants during the 1980s (n=15) and 1990s (n=63). Note that H_e around the triple mutants seems similar between the two decades. The higher variation noted close to the gene in the 1980s may be due to a small number of samples being used to estimate these values. The two flat, dashed lines represent two different estimates of neutral H_e in Brazil.

Figure 9.2 *dhfr* triple mutant H_e changes over time (51/108/164)



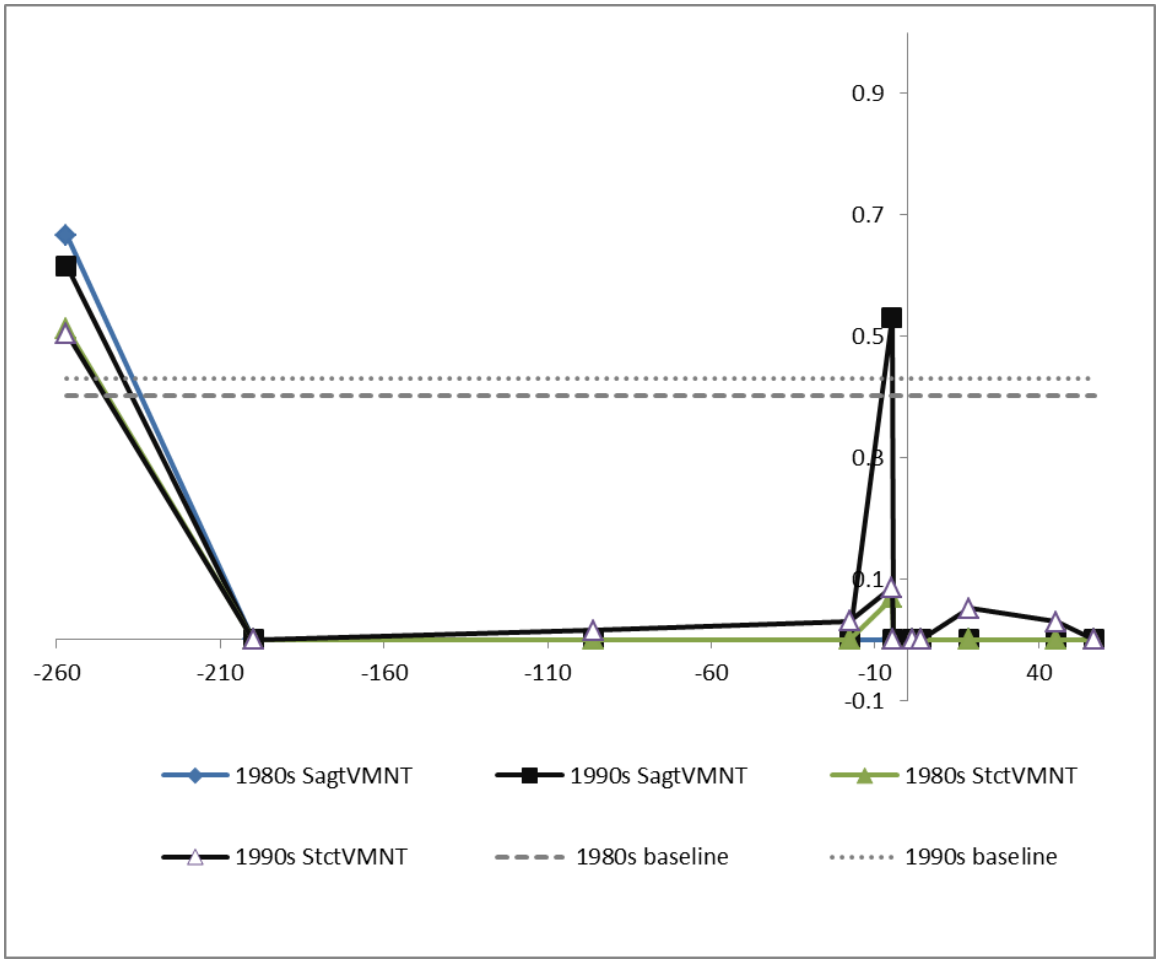
The figure shows variation in H_e surrounding *dhfr* for parasites carrying the 51/108/164 mutant during the 1980s (n=9) and 1990s (n=11). It does appear that H_e surrounding this gene increased between the two periods. The two flat, dashed lines represent two different estimates of neutral H_e in Brazil.

Figure 9.3 H_e around *dhps* from multiple periods across Brazil



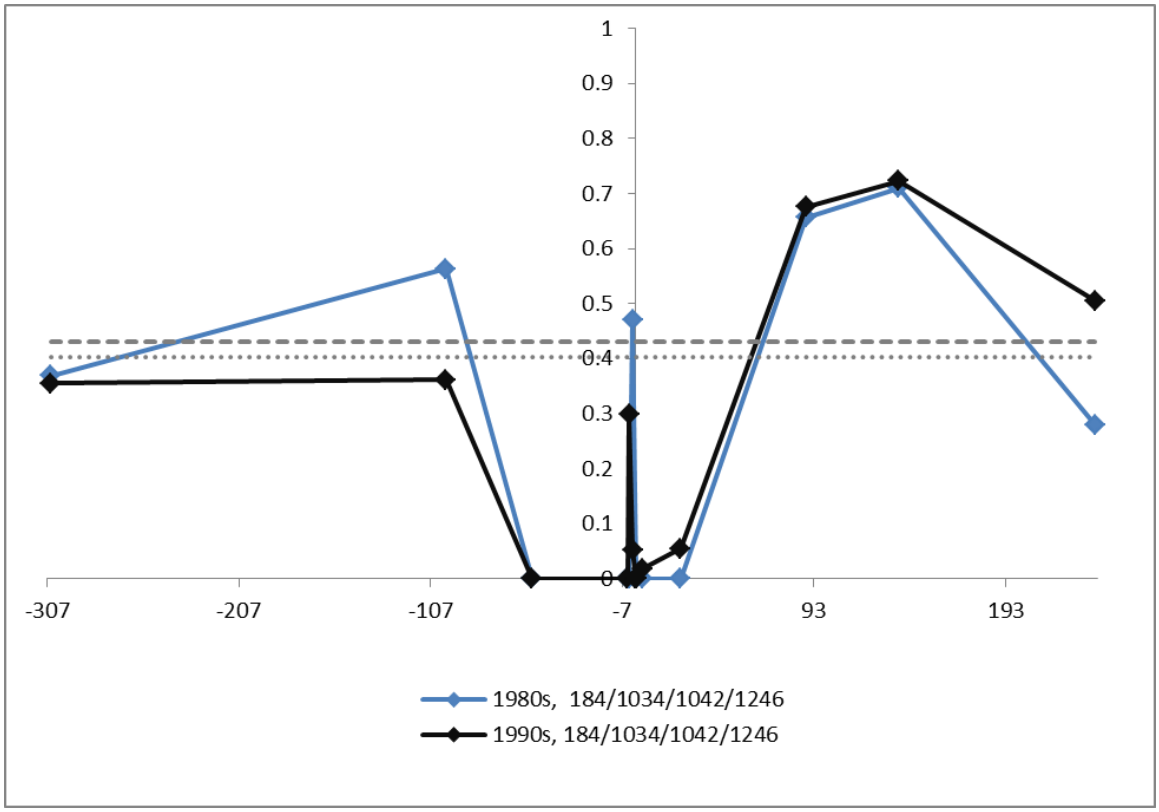
The figure shows variation in H_e surrounding *dhps* during the 1980s and 1990s. It appears that H_e has increased between the 1980s (n=21) and 1990s (n=126) for the triple mutant. The double mutant seems to have a similar shape as the triple mutants (n=8). The two flat, dashed lines represent two different estimates of neutral H_e in Brazil.

Figure 9.4 H_e around *pfcrt* from multiple periods across Brazil



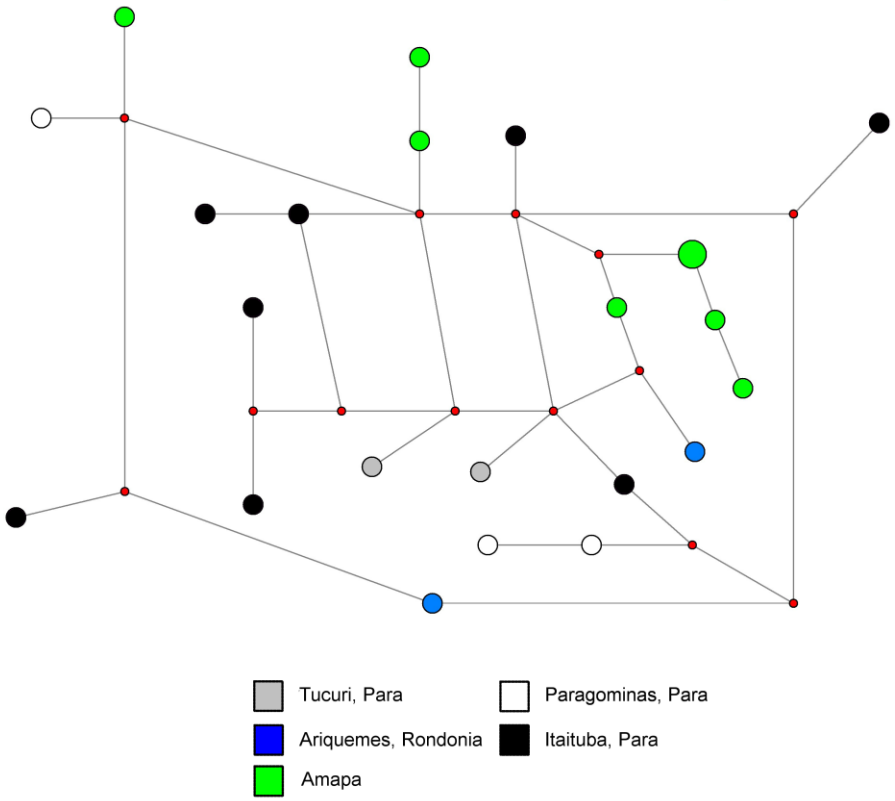
The figure shows variation in H_e surrounding *pfcrt* during the 1980s and 1990s. Given the small number of samples with the S_{AGT}VMNTin the 1980s (n=4) and the 1990s (n=14), their H_e estimates should be taken with caution. There have been only slight increases in the H_e around *pfcrt* for S_{TCT}VMNT from the 1980s (n=30) to the 1990s (n=133). The two flat, dashed lines represent two different estimates of neutral H_e in Brazil.

Figure 9.4 H_e around *pfmdr1* from multiple periods across Brazil l



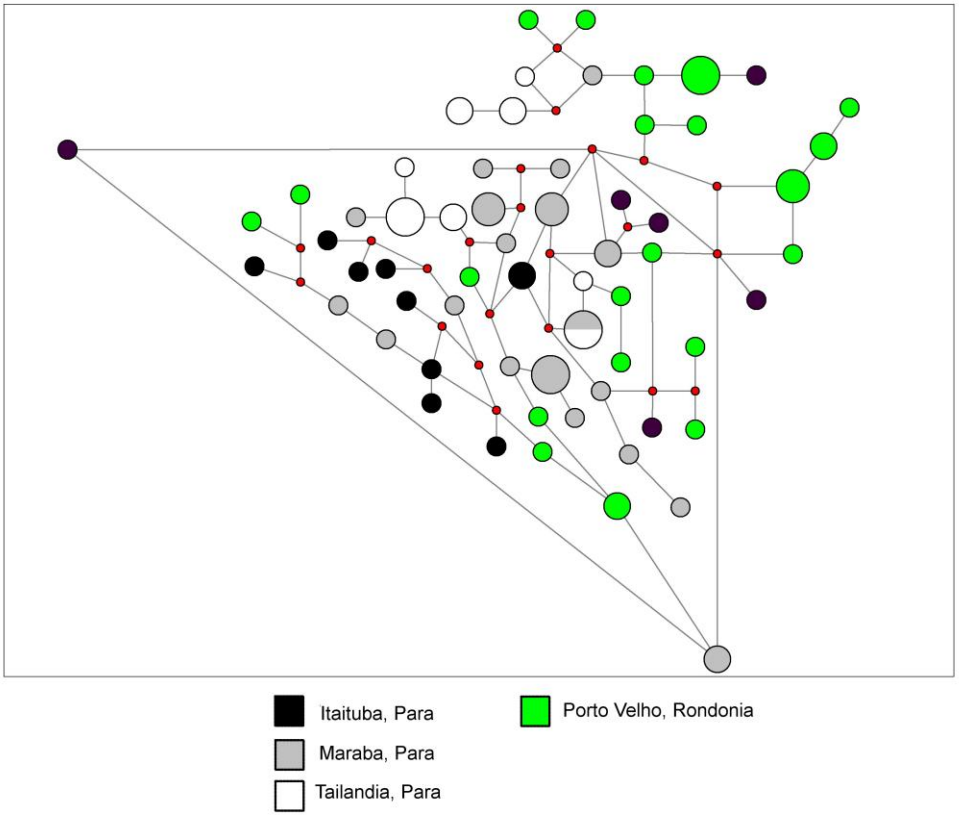
The figure shows variation in H_e surrounding *pfmdr1* during the 1980s (n=25) and 1990s (n=114). Only H_e for the quadruple mutant is shown because there are too few samples with other genotypes. There does not seem to have been an increase in H_e over time. The two flat, dashed lines represent two different estimates of neutral H_e in Brazil.

Figure 9.6 Network diagram of Brazilian data from the 1980s



In this Network diagram, we have included all samples collected in Brazil prior to 1996.

Figure 9.7 Network diagram of Brazilian data from the late 1990s



In this Network diagram, we have included all data that falls between 1996 and 1999.

Figure 9.8 Network diagram of all Brazilian data regardless of date
This network diagram shows the interrelatedness of samples collected in Brazil across all time points and locations.

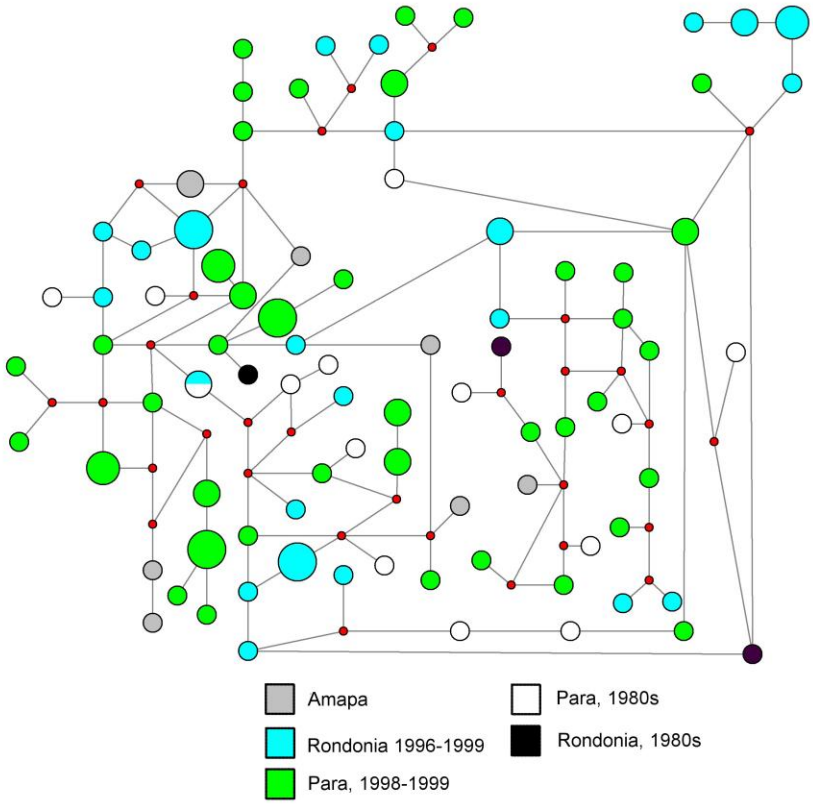


Table 9.1 Samples used in this study

Brazilian State	Location	Year	Number of Samples
Para	Itaituba	1983	2
		1984	1
		1985	4
		1986	2
		1999	23
	Marabá	1984	2
		1986	1
		1998	22
		1999	18
	Moju	1998	30
	Tucuruí	1983	1
		1984	2
	Paragominas	1983	2
		1984	2
		1985	1
Amapá		1983	1
		1984	2
		1985	9
		1986	1
Rondônia	Ariquemes	1984	1
		1988	2
		1996	2
	Porto Velho	1996	19
		1998	28
		1999	12

Table 9.2 Temporal and geographic distribution of *dhfr* alleles in Brazil

Site	Year	N=	Genotype-Haplotype
Amapá	1983	1	51/108-A1
Paragominas, Para	1983	1	51/108-D1
Paragominas, Para	1983	1	51/108-D1
Paragominas, Para	1984	1	51/108-D1
Paragominas, Para	1985	1	51/108-D1
Tucuruí, Para	1983	1	51/108-D1
Tucuruí, Para	1984	1	51/108-D1
Marabá, Para	1984	1	51/108-D1
Marabá, Para	1984	1	51/108-A1
Porto Velho, Rondônia	1999	2	51/108-D1

Site	Year	N=	Genotype-Haplotype
Amapá	1984	5	51/108/164-A1
	1986	1	51/108/164-A1
Itaituba, Para	1985	1	51/108/164-A1
Itaituba, Para	1986	1	51/108/164-A1
	1999	1	51/108/164-A1
Porto Velho, Rondônia	1996	3	51/108/164-A1
	1998	1	51/108/164-D1
	1999	1	51/108/164-A1
Tailândia, Para	1998	1	51/108/164-A1
Marabá, Para	1999	4	51/108/164-A1

Site	Year	N=	Genotype-Haplotype
Itaituba, Pará	1983	1	50/51/108-D1
	1983	1	50/51/108-A1
	1984	1	50/51/108-D1
	1985	2	50/51/108-D1
	1985	1	50/51/108-D1
	1999	16	50/51/108-D1
Ariquemes, Rondônia	1984	1	50/51/108-D1
	1986	1	50/51/108-D1
	1996	1	50/51/108-D1
Paragominas, Para	1984	1	50/51/108-D1
Amapá	1985	5	50/51/108-D1
Porto Velho, Rondônia	1996	6	50/51/108-D1
	1998	16	50/51/108-D1
	1999	4	50/51/108-D1
Marabá, Para	1998	16	50/51/108-D1
Tailândia, Para	1998	8	50/51/108-D1

This table, broken into three pieces for readability, describes the genotypes of *dhfr* seen over time at different sites in Brazil.

Table 9.3 Triple mutant *dhfr* 51/108/164

Site	Year	n=	*dhfr*	*dhps*	*pfcrt*	*pfmdr1*
Amapá	1984	1	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
-	1985	1	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1985	2	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α2*
-	1986	1	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Itaituba, Para	1985	1	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
-	1999	3	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Porto Velho, Rondônia	1996	2	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1999	1	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Marabá, Para	1999	1	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1999	2	51/108/164-A1	437/540/581-A1	S_{AGT}VMNT-A2	184/1034/1042/1246-α
Tailândia, Para	1998	1	51/108/164-A1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α

This table describe the multidrug resistant lineages seen in Brazil. This table will be expanded before this chapter is published as a paper after we have additional data for *dhfr*. * Parasites carrying "-α2" had an allele of 200 bp -1.40 kb from the gene.

Table 9.4 Triple mutant *dhfr* 51/108

Site	Year	n=	*dhfr*	*dhps*	*pfcrt*	*pfmdr1*
Tucuri, Para	1983	1	51/108-D1	437/581-A1	S_{AGT}VMNT-A2	184/1034/1042/1246-α
	1984	1	51/108-D1	437/581-A1	S_{AGT}VMNT-A2	184/1034/1042/1246-α
Amapá	1983	1	51/108-D1	437/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α2
Paragominas, Para	1983	1	51/108-A3	437/581-A1	S_{AGT}VMNT-A2	184/1034/1042/1246-α2
	1983	1	51/108-D1	437/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α2
Paragominas, Para	1984	1	51/108-D1	437/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Paragominas, Para	1985	1	51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Porto Velho, Rondônia	1999	1	51/108-D1	437/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α

This table describes the multidrug resistant lineages seen in Brazil. This table will be expanded before this chapter is published as a paper after we have additional data for *dhfr*. The S_{AGT}VMNT is highlighted to make it easier to find in the table.

Table 9.5 Triple mutant *dhfr* 50/51/108

Site	Year	n=	*dhfr*	*dhps*	*pfcrt*	*pfmdr1*
Paragominas, Para	1984	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Amapá	1985	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1985	3	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α2
	1985	1	50/51/108-D1	437/540/581-A1	S_{AGT}VMNT-A2	184/1034/1042/1246-α2
Itaituba, Para	1985	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Ariquemes, Rondônia	1988	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1996	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Porto Velho, Rondônia	1996	3	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1998	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1998	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1998	2	50/51/108-D1	437/540/581-A3	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1998	1	50/51/108-D1	437/540/581-A3	S_{AGT}VMNT-A2	184/1034/1042/1246-α
	1998	3	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1042/1246-α
	1998	1	50/51/108-D1	437/540/581-A3	S_{TCT}VMNT-A2	184/1042/1246-α
	1998	1	50/51/108-D1	437/540/581-A1	S_{AGT}VMNT-A1	184/1042/1246-α
	1999	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1999	4	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1999	2	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1999	3	50/51/108-D1	437/540/581-A1	S_{AGT}VMNT-A2	184/1034/1042/1246-α
	1999	1	50/51/108-D1	437/540/581-A1	S_{AGT}VMNT-A2	184/1034/1042/1246-α
	1999	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1042/1246-α
Tailândia, Para	1998	6	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
Marabá, Para	1998	18	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1998	1	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1998	2	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α
	1999	2	50/51/108-D1	437/540/581-A1	S_{TCT}VMNT-A2	184/1034/1042/1246-α

This table describe the multidrug resistant lineages seen in Brazil. This table will be expanded before this chapter is published as a paper after we have additional data for *dhfr*.

Table 9.6 Pairwise F_{ST} of different sites in Brazil

	Itaituba, Para	Marabá, Para	Moju, Para	Rondônia
Itaituba, Para				
Marabá, Para	0.04*			
Tailândia, Para	0.27	0.21		
Rondônia	0.09	0.07	0.30	

This table shows pairwise F_{ST} of different sites using samples collected during 1996 to 2003. Rondônia is made up almost entirely of samples collected in Porto Velho, with one additional sample coming from Ariquemes. *this is the only result in this table which was not statistically significant.

Table 9.7 Tests for bottlenecks

Location	Period	Wilcoxin H_e deficiency	Wilcoxin H_e excess.	Allele Frequency Mode Shift
Marabá, Para	1998-99	1.00	0.01	Yes
Tailândia, Para	1998	0.99	0.02	Yes
Tailândia, Para	1999	1.00	0.01	Yes
Porto Velho, Rondônia	1996-99	0.84	0.18	Normal L dist.
Porto Velho, Rondônia	1996	0.88	0.14	Yes
Porto Velho, Rondônia	1998-99	0.90	0.12	Yes

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CHAPTER 10

CONCLUSIONS

The goal of this dissertation was to describe the influence of malaria control on the population structure of *P. falciparum* and the origin and spread of drug resistant genes in South America. To achieve this goal, I combined a historical overview of malaria control with extensive molecular data from Bolivia, Brazil, Peru, and Venezuela. At the beginning of my research, I hypothesized that CQ and SP resistance developed multiple times and spread throughout South America, rather than as single events in the Amazon basin. I also proposed that there was not free gene flow across South America, but rather that the Andes Mountains acted as a barrier to the movement of resistant alleles. Futhermore, I hypothesized that the inbreeding, extensive linkage disequilibrium, and low genetic diversity would influence how resistance developed and spread in South America and lead to the spread of multidrug resisitant lineages. To examine these issues, I focused on four genes known to contribute to drug resistance in *P. falciparum*: *pfcrt, pfmdr1, dhfr,* and *dhps* and ~59 surrounding microsatellite loci, as well as an additional 12 neutral markers from various chromosomes for comparisons to neutral expectations of H_e and population structure.

In Sifontes, Venezuela, we found that all parasites carried chloroquine resistant *pfcrt* alleles, though there were two similar alleles, S_{tct}VMNT (91%) and S_{agt}VMNT (9%). We also found that there were only two *pfmdr1* alleles circulating, Y184F/N1042D/D1246Y (37%) and Y184F/S1034C/N1042D/D1246Y (63%), with shared ancestry. When we combined these findings with earlier work by our lab group regarding *dhfr* and *dhps* genotypes/haplotypes [1], we found that there were three major multidrug resistant haplotypes: S_{agt}VMNT *pfcrt*/quadruple mutant *pfmdr1*/double mutant *dhfr* /double mutant *dhps*; S_{tct}VMNT *pfcrt* /triple mutant *pfmdr1*/triple mutant *dhfr*/triplemutant *dhps*; and S_{tct}VMNT *pfcrt*/quadruple mutant *pfmdr1*/triple mutant *dhfr*/triple mutant *dhps*. This was the first indication that multidrug resistant lineages could be a reality in South America.

We concluded that a bottleneck took place in Sifontes. Since the paper was published, I began to use the Bottleneck program to statistically test for bottlenecks and rapid populations expansions. Using this tool, and additional data for the 11 microsatellites previously described for use in this test, it appear that a recent bottleneck had occurred in Sifontes (Sign test assuming MBE: p=0.04; Wilcoxin 1-tailed test for H_e deficiency: p= 0.97; Wilcoxin 1-tailed test for H_e excess: p=0.03; and the allele frequency distribution was skewed). In addition, the S_{agt}VMNT *pfcrt* multidrug resistant haplotype appeared to extend to neutral markers (Figure 2), which may indicate it has recently been introduced to the Venezuelan population. We also found that *pfmdr1* duplication had occurred, which might indicate a regional shift towards mefloquine resistance.

In Peru, the population structure of *P. falciparum* was restricted to five clonal lineages in the four different geographical regions examined (the Pacific Coast, Western Amazon, Central Amazon and Eastern Amazon). These clonal lineages were designated as clonets A, B, C, D, and E. The distribution of these clonets, lack of genetic diversity, and different drug resistance profiles and alleles suggested that:

1) Two (A and B) were very recent introductions from the greater Amazon basin where CQ and SP resistant dominated

2) One clonet (C) was likely an earlier introduction from the Amazon based on its SP sensitivity, yet shared haplotype diversity with *dhfr*, *dhps*, and *pfcrt* in clonets A and B.

3) One clonet (E) appeared to be either a residual coastal population that had expanded in Peru or, more likely a population that had recently been introduced to Peru.

4) One clonet (D) was possibly an older coastal introduction to the interior based on its shared gene haplotypes with clonet E and SP sensitivity

5) Clonets C and D may have been in Peru for the longest time as they appeared to be undergoing recombination, particularly where the largest epidemics took place (Padre Cocha/Iquitos).

6) It appears Clonets A, B and E may have expanded during the peak malaria epidemic in the 1990s.

Given the highly effective Peruvian malaria control efforts of the 20th century, retrospectively the finding that *P. falciparum* populations were extremely clonal is perhaps unsurprising. Although other parts of

South America also had effective malarial control programs, the population structure of Peru is unique, based on the sites examined in this dissertation (though it is possible other areas like coastal Ecuador and Colombia might have a similar clonal structure). From this study, it is clear that that Peruvian Amazon appears to be a melting pot of drug resistance lineages from the Pacific coast and the Amazon interior. This unique population structure gave us the opportunity to hypothesize how malaria parasites reinvaded an area where malaria control had reduced autochthonous cases to the extent that they were not reported at all for a number of years.

We then revisited the central Peruvian Amazon site (Iquitos) using samples that had been collected between 2006-7. At this time, Iquitos was still a major reservoir for malaria transmission in Peru. Our study showed that important changes had occurred in these clonets over the intervening years. Among isolates from this period, only a few samples maintained the four clonets found in the Central Amazon in 1999. Indeed, a majority of the parasites were hybrid lineages of B and C or C and D. While some isolates with B and C clonets have survived, clonet A had almost disappeared and there were only a few isolates that appeared to be D clonets. There has been significant recombination in the area since 1999 and this implies that there was sufficient transmission to breakdown clonal propagation. Our findings are consistent with a recent report that has examined 15 parasite isolates from the same region in a high throughput genomic study, which found that the parasites were clonal and had at most four parental haplotypes [2]. Most of the clonets survived in 2006-7 and carried CQ resistant, but SP sensitive/low resistant genotypes. This finding suggests that the replacement of SP with artemisinin-based combination therapy (ACT) (artesunate + mefloquine) in 2001 as the primary drug for *P. falciparum* treatment in this region has probably given these new hybrid clonets higher fitness than existing clonets. It appears that, in the absence of SP, there was no selective advantage for highly SP resistant clonets and this observation is consistent with two other previous reports from our laboratory demonstrating the decline in the SP resistant genotypes since 2005 in this region [3,4]. The evidence for earlier clonets, admixture, and clonal expansion had disappeared in this sample set. This implies that the evidence for such events can rapidly

disappear if there are multiple clonets present, sufficient sexual reproduction, and selection pressure for particular multidrug resistant profiles.

Brazil's population structure differed from these previous findings. There were only a few alleles for each one of the genes which therefore led to only a few multidrug resistant haplotypes. In constrast, an examination of the population structure of parasites collected from various Brazilian localities did not indicate as simple a scenario. Rather, our network diagrams indicated a parasite population with internal migration, admixture, and some reassortment of chromosomes. Retrospectively, this is again unsurprising. Historically, Brazil has reported *P. falciparum* resistance to many drugs quite quickly, giving resistance alleles a long period to spread and recombine with local parasite lineages throughout the country. Additionally, Brazil has made an effort to colonize the Amazon interior since the 1960s and this, along with a migratory population of miners and agricultural workers, most likely acted to circulate parasites throughout the Amazon basin. It is likely that the parasite introductions and admixture occur in Brazil much in the way they do in Peru. However, parasite populations seemed to have a larger portion of diversity explained within sites, perhaps because there are so many more cases in this region than Peru (even at its nadir control only reduced *P. falciparum* cases to 28,557 in 1974). I exhaustively examined network diagrams based on neutral haplotypes, and also divided the dataset by *dhfr* genotypes in search of Brazilian clonets, but I was unable to find evidence that Brazil had a clonal population structure similar to Peru. At least in the eastern Brazilian states of Amapá and Pará, it appears that the double mutant *dhfr* and *dhps* alleles were supplanted by triple mutants sometime during the mid 1980s. In addition, our preliminary results suggest that isolation by distance will be rejected for this region and that, at least in Pará, there have been recent population bottlenecks. Though neutral population structure does not show the sort of linkage we reported in Peru, the apparent fixation of a few highly resistant alleles of *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* in the Brazilian Amazon effectively leads to linkage among the four chromosomes they reside upon.

Based on early research, it had already been proposed that highly resistant *dhfr* alleles had spread from the southern Amazon basin along with highly resistant *dhps* alleles (and potentially CQ

resistant *pfcrt* alleles). It was also argued that there had been two major CQ resistant *pfcrt* origination events with one on the coast and the other in the Amazon interior. Finally, there were indications that there might be regional variation in the geographic distribution of *pfmdr1*. My research provides further insight into these hypotheses.

General Conclusions

As mentioned in the introduction, few studies have examined the overall population structure of *P. falciparum* in South America. The most comprehensive study found that parasites collected in Bolivia and Brazil clustered, while parasites collected in Colombia were more distantly related [5]. Another study found that coastal and interior parasites in South America had significant allele sharing across all chromosomes and that CQ resistance had developed locally using limited samples from the interior and the coast of South America [6]. Yet another suggested that, at least in the Brazilian Amazon basin, there did not appear to be any bottlenecks or epidemic expansions of clones. They argued that there was little gene flow throughout the Brazilian Amazon Basin and that it did not have the low genetic diversity and linkage disequilibrium reported elsewhere in South America [7]. Our Brazilian study is in disagreement with many of this study's findings, possibly because we used more neutral microsatellite loci from different parasite chromosomes. In addition a number of papers suggest that South American parasites had limited genetic diversity and even linkage that extended to multiple chromosomes due to inbreeding [1,5,8,9,10,11,12,13,14]. They also suggest that, while local populations were often different from each, these differences were not due to isolation by distance.

There were also additional studies that examined drug resistance genes which gave insight into South American population structure. Another concluded that Brazil and Guyana were quite similar, but that Colombia differed based on a few microsatellites surrounding *pfcrt* and *pfmdr1* and samples from Brazil, Colombia, and Guyana. It was also concluded that S_{agt}VMNT and S_{tct}VMNT were closely related [15]. Still another study concluded that the S_{tct}VMNT allele represented the original CQ resistant allele in the Brazilian Amazon and was responsible for the sweep of CQ resistance in the Amazon Basin using 15 microsatellites on 4 chromosomes. However, these conclusions were a hybrid of general population

structure and the history of the *pfcrt* chromosome because 7 of the microsatellites used were on Ch. 7, which carries *pfcrt* [16]. A study of coastal and interior samples collected in Colombia found that double and triple mutant *dhfr* and *dhps* seen in both regions originated only once and grouped with these genotypes in the Amazon interior. They also found that migration had occurred over the Andes in both directions [14]. Another study examined only a few parasites on the Pacific Coast and interior, but used an extensive set of microsatellites. It concluded that SVMNT in the Amazon interior (which was similar to a Peruvian CVMNT allele) and CVMET on the coast (which was similar to a Ecuadorian CVMNT allele) had independently evolved [6]. One of the main messages to take from these studies is that the Pacific Coast of South America have different CQ resistance histories.

Few authors have suggested how drug resistance could have spread throughout South America. One paper suggests that highly resistant alleles of *dhfr*, *dhps*, and *pfcrt* could have spread together across the Amazon, originating in the Southwest based on molecular data [16]. Another paper proposes how CQ resistance could have spread in South America based on historical reports [17]. While it also erroneously suggests that CQ resistance originated outside of South America (though some rare CVIET alleles did spread from Africa [16]), its hypothesis regarding how CQ resistance spread through South America is a good starting point. It posits that CQ resistance originated on the border of Colombia and Venezuela, rapidly spread south throughout the Amazon basin in less than seven years (with the exception of Peru, French Guiana , Guyana, and Suriname). Furthermore, CQ resistance was thought to have spread south along the Pacific coast, but at a much slower rate, reaching Ecuador in 1976 and then spreading over the Andes in Peru in 1980. Others suggested that were two major CQ resistant *pfcrt* origination events with one on the coast and the other in the Amazon interior. There were indications that there might be regional variation in the geographic distribution of *pfmdr1*, but no one has explored this to any great depth. For SP resistance, there was limited historical reporting and molecular data and the existing literature proposed that highly resistant *dhfr* alleles had spread from the southern Amazon basin along with highly resistant *dhps* alleles (and potentially CQ resistant *pfcrt* alleles).

Based on my literature review, it appeared that while the first well known reports of CQ resistance were reported along the Colombian/Venezuelan border [18,19], the first reports of refractory *P. falciparum* isolates were actually from hospitals in Belém and Santarém, Brazil in 1946-1947 [20] and later in Rondônia, Brazil in 1954 [21]. The sudden reporting of CQ resistance throughout the Brazilian Amazon, after the publication of papers reporting resistance in the north, suggests that the Brazilian reports may represent the medical establishment putting a name to a preexisting problem.

In addition, the hypothesis that CQ resistance originally developed twice in South America needs to be critically examined. The gold standard paper which first examined the underlying *pfcrt* haplotypes engendering this resistance could be interpreted differently than suggested by the authors. CVMNT on the Pacific Coast and the CVMNT in the interior were grouped respectively with CVMET and SVMNT. It was concluded that these two CVMNT alleles therefore represented different origination events. However, a comparison of the CVMNT allele reported in the Ecuadorian S\strain and the CVMNT reported in Padre Cocha suggests they may be more closely related than argued; 6 out of the surrounding 15 microsatellites were shared between these two alleles and two that differed could represent either one-repeat-sized slippage events or sequencer variation. If this is accepted as plausible, then only slightly less than half of the microsatellites around this gene differed between the two CVMNT haplotypes. In our Peruvian study, a comparison of the coastal and interior CVMNT alleles showed that five out of the eleven surrounding microsatellite alleles were shared and another three could have been very simple slippage events. The remaining alleles could be argued to be slightly larger slippage events. A more extensive comparison of these two CVMNT may suggest they originated in a single event between 1954-1959 and diverged on the Pacific Coast and Interior [6].

Regardless, if the CQ resistance CVMNT allele originated twice in South America, then my dissertation data and the available historical data would suggest it occurred once on the border of Venezuela and Colombia and once in Rondônia. However, CVMNT could also have originated once in the Brazilian Amazon in the mid-1950s and spread north and east, with subsequent changes to its microsatellite profile over the next 50 years as it spread along the Pacific Coast. Even if CVMNT

developed in both regions in two separate events, the limited distribution of the CVMET allele to Colombia implies that it developed in that region later. While the support is somewhat circumstantial, we argue that the SVMNT alleles developed also developed later and swept along with resistant *dhfr* and *dhps* alleles. S_{AGT}VMNT may represent the first allele as it seemed to be more often associated with double mutant *dhfr* and *dhps* in the Eastern Brazil in our data. S_{TCT}VMNT could have swept through the Amazon by sweeping along with highly resistant triple mutant *dhfr* and *dhps*.

In the case of SP resistance, it may be naïve to state that resistance to this drug combination occurred whole-cloth. Indeed, field use of pyrimethamine in the late 1950s led to apparent resistance by 1959 in western Venezuela [22]. In 1968, strains of *P. falciparum* collected throughout Brazil had varying levels of pyrimethamine resistance, with parasites in Colombia, Venezuela, and Pará, Venezuela showing moderate resistance. While RII resistance was reported in Southern Brazil in the same study, the distribution of the moderately pyrimethamine resistant parasites gives circumstantial evidence that pyrimethamine resistant parasites had spread from these early Venezuelan strains. It also suggests that some mutations in *dhfr*, pyrimethamine's target, may be older than could be expected based on implementation of SP as a first line drug in South America. For these reasons, it may be difficult to tease apart whether the spread of the two major triple mutant *dhfr* alleles 50/108/164 and 51/108/164 occurred contemporaneously or as two successive events. However, in our study, the presence of double mutants in the eastern states of Amapá and Pará suggest that these triple mutants may have still been spreading east through the Amazon basin at that time, which may suggest that they had developed relatively recently.

Sulphadoxine resistance caused by mutations in *dhps* may have occurred slightly later than the initial *dhfr* mutations, as the first reports of SP resistance in Brazil came from Goiás, Brazil in 1972. More reports of SP resistance would occur in 1977 in Venezuela [23], and 1978 in the eastern Brazilian state of Maranhão [21,24], and later still in Colombia in 1981, when SP was first used in that region [25]. In the case of Colombia, there is no indication that the SP resistant lineages with multiple mutations are novel to that area [14]. Based on our research in Peru, it seems more likely that neighboring populations in Venezuela or Brazil with SP resistant genes spread rapidly into the country once SP drug pressure made

them of higher fitness than local parasites. Parasites with RIII cases were first reported between Manaus and Porto Velho, Brazil in1982 [24] and, by 1988, 22% of parasites in neighboring Bolivia carried RIII resistance [26]. It is plausible to conjecture that parasites in Goiás already had preexisting *dhfr* mutations and rapidly accrued other mutations to *dhps* which then spread to where RIII resistance was first reported in Brazil. These resistant parasites could then have spread east, west, and north along with colonists and minors, spreading the resistant strains throughout Brazil and the greater Amazon basin.

The goal of this dissertation was to examine the population structure of *P. falciparum* in South America and describe the origin and spread of drug resistant alleles throughout the continent. Our sampling was sufficient to begin to describe the population structure of *P. falciparum* within the interior, though conclusions regarding Pacific coast parasite populations are hampered by limited sampling. However, network diagrams of all data from contemporaneous sites (Figure 10.2), contemporaneous sites outside of Brazil connected to historical samples in Brazil (Figure 10.3), and all isolates examined during this dissertation (Figure 10.4) indicate that:

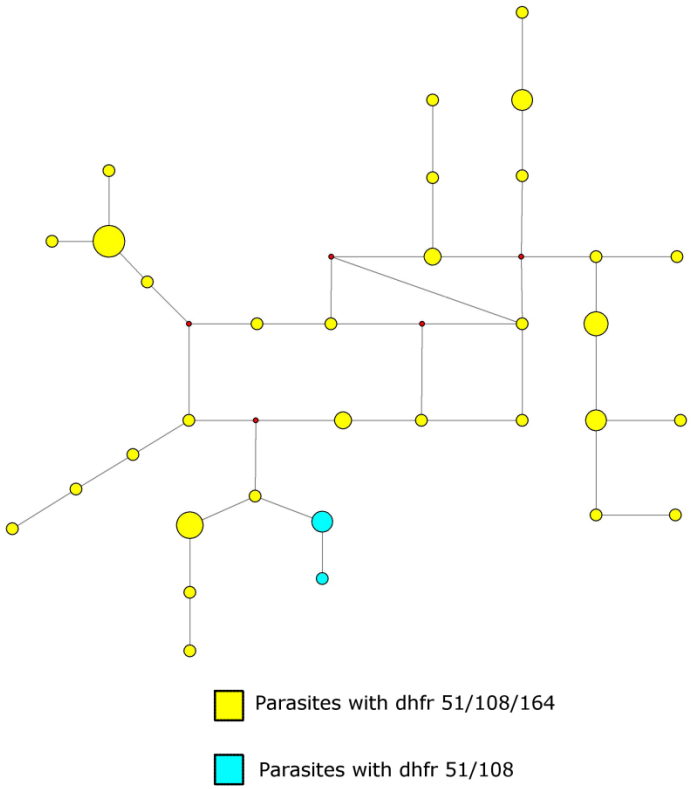
1) The highly clonal structure reported in Peru is somewhat of an outlier amongst the countries examined in this dissertation.
2) Brazil appeared to have the most interconnected population amongst the countries (see also Table 10.1 for a comparison of F_{ST} values calculated within Peru to other sites in this dissertation).
3) Venezuela and Bolivia appeared to have multiple groupings of parasites, which may indicate that they represent sites where admixture had previously occurred and been masked by partial recombination.

We had also proposed that that the Andes Mountains would act as a barrier to parasite population. The presence of a coastal parasite lineage invading the Western Peruvian Amazon, reports by other researchers of potentially the same process in Colombia [14], and other Peruvian parasites that appear to have been migrants that spread over the Andes during the late 1970s suggests that the Andes Mountains are more of a permeable barrier for parasite migration than we originally assumed. Regardless, it appears

that the coast of South America is acting in relative isolation from the parasite populations of the interior as evidenced by the lack of SP resistance in Peru when it was rampant throughout the interior, the differing population structure of coastal Peruvian parasites and Peruvian Amazon parasites, and numerous papers which suggest Colombian coastal isolates do not group with those collected in the interior [5,6,15].

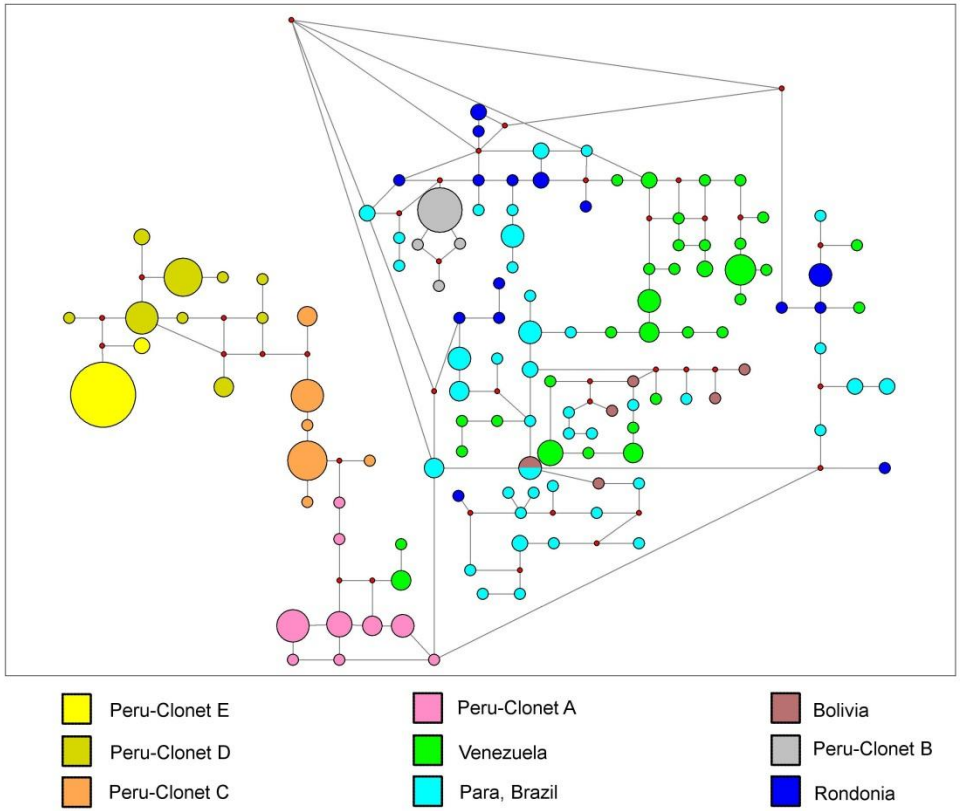
In the future, it is hoped that future studies will use the same markers we applied to Bolivia, Brazil, Peru and Venezuela in the coastal countries of Colombia and Ecuador in order to fully describe the dynamics of theses populations and their interrelatedness. While this dissertation did not touch upon the northeastern countries of South America (Guyana, Suriname and British Guiana), there is some evidence that the dynamics of these countries may be different from the remainder of the Amazon interior and deserve examination as well. By highlighting the level of genetic diversity, potential migratory patterns, and historical process of *P. falciparum* populations in the countries described in this dissertation, I hope that I have created a resource for future researchers interested in the dynamics of this parasite in South America. If and when ACT resistance is first reported in South America, perhaps my findings will influence the public health response in some small manner.

Figure 10.1.Network diagram of Venezuelan isolates



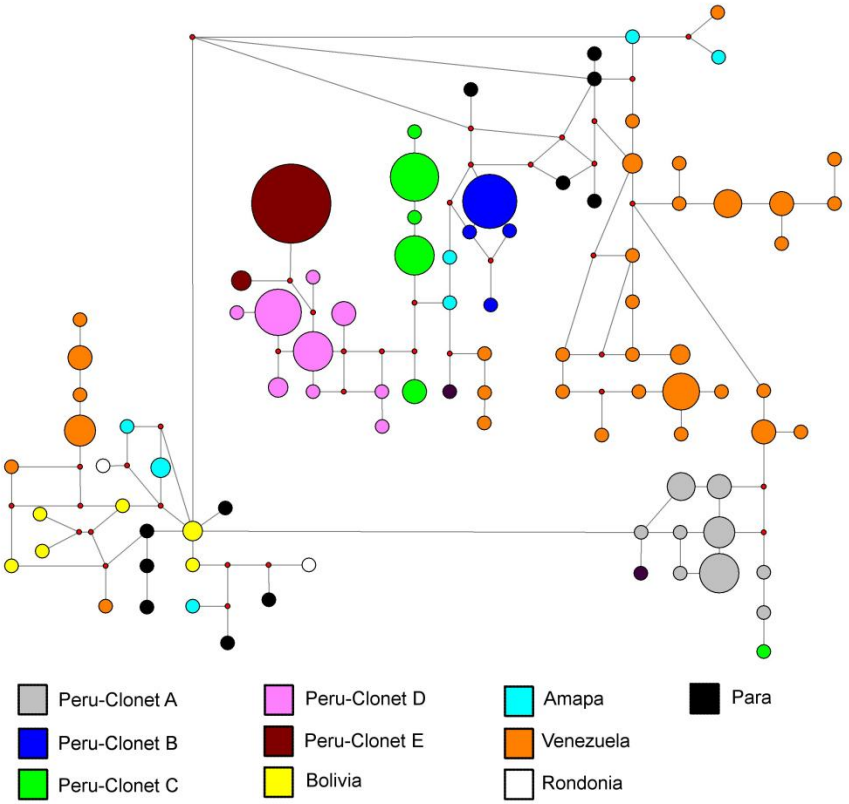
This network diagram shows the population structure of Venezuela. Samples carrying the two multidrug resistant profiles this population are colored differently using dhfr as a frame of reference. The 51/108 *dhfr* allele is associated with the minor S_{AGT}VMNT allele and a Y184F/S1034C/N1042D/D1246Y *pfmdr1 allele*.

Figure 10.2 Network diagram of all dissertation data that collected between 1998 and 2003



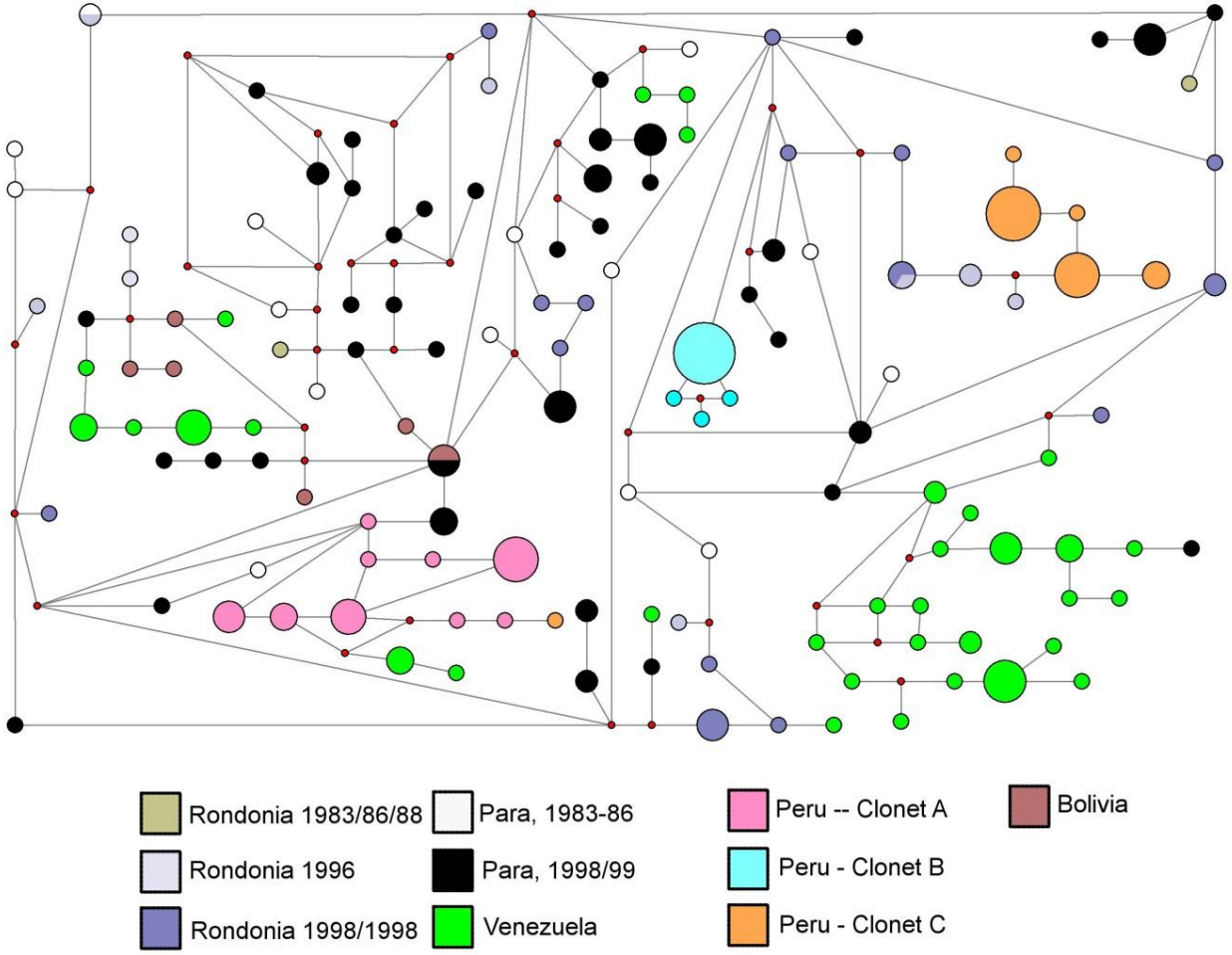
In this Network diagram, we have included all data collected between 1998 and 2003. As suggested by our previous paper, Peruvian coastal parasites have markedly different haplotypes than the rest of the samples collected in the interior. Peruvian clonet A appears most like Peruvian clonet C (proposed as the ancestral type). However, it is also connected to a cluster of samples collected in Pará, Brazil, Bolivia, and Venezuela. It is also only one marker away from a sample collected in Rondônia. We previously assumed clonet A was introduced in the late 1990s based on its SP resistance, as Bolivia and Rondônia are the closest collection sites to Peru, and it seems possible it came from one of these populations. Interestingly, clonet B's two closest neighbors were collected in Rondônia and more distantly with samples collected in Pará. Assuming it was recently introduced, it appears it may have come from Rondônia. The mixing of various haplotypes from Pará and Rondônia, Brazil, as well as Venezuela suggests that there is greater population connectivity in the remainder of the Amazon basin.

Figure 10.3 Network diagram of historical data from Brazil with all data outside of the country



In this Network diagram, we have included all samples collected in Brazil prior to 1996. We have also included our later samples collected in Peru, Bolivia, and Amapá, Brazil between 1998 and 2003. Note that 4/5 of the Peruvian clonets group together, while clonet A groups with samples collected in Venezuela in 2003. Bolivia groups with samples collected in all sites except Peru.

Figure 10.4 Figure 10.4 Network diagram of almost all data used in this dissertation



This network diagram includes almost all data ignoring dates of collection. Clonets D and E were not included in order to simplify the figure. Samples from Amapá were not included because they generated hypothetical haplotypes which made the figure unreadable. Peruvian Clonets B and C's closests neighbors were collected in Rondônia in 1998/1999. Clonet A's closest neighbors came from Pará or Venezuela. Venezuela fragmented into four groups in this figure. One group looked most like parasites collected in Pará and Rondônia around 1999. Another group looked like parasites collected in Pará in 1999, as well as parasites from Bolivia. The third group associated with Peruvian Clonet A and the final group was connected to samples collected in Pará. While parasites collected in Pará and Rondônia did appear to separate into different groups, there was not an obvious overall pattern, possibly suggesting these sites have strong internal migration.

Table 10.1 Pairwise F_{ST} of varios sites and clonets in South America

	Itaituba, Para	Marabá, Para	Tailândia, Para	Rondônia	Peru Clonet A	Peru Clonet B	Peru Clonet C	Peru Clonet D	Peru Clonet E	Bolivia
Itaituba, Para										
Marabá, Para	0.04*									
Moju, Para	0.27	0.21								
Rondônia	0.09	0.07	0.30							
Peru Clonet A	0.46	0.47	0.58	0.42						
Peru Clonet B	0.50	0.45	0.71	0.44	0.77					
Peru Clonet C	0.69	0.69	0.75	0.65	0.78	0.86477				
Peru Clonet D	0.63	0.61	0.75	0.60	0.74	0.81854	0.71061			
Peru Clonet E	0.79	0.74	0.67	0.71	0.87	0.94180	0.86767	0.77893		
Bolivia	0.15	0.10	0.22	0.18	0.58	0.70251	0.75606	0.64140	0.87295	
Venezuela	0.19	0.25	0.33	0.23	0.35	0.39024	0.60010	0.57141	0.68887	0.26479

This table shows pairwise F_{ST} of different sites or clonets using samples collected during 1996 to 2003. Rondônia is made up almost entirely of samples collected in Porto Velho, with one additional sample coming from Ariquemes. Samples with very low F_{ST} values are highlighted in red. Relatively low F_{ST} values are highlighted in yellow. Within Brazil, it appears that three Pará sites (Itaituba and Marabá) share more in common with Rondônia than each other. They are share low F_{ST} values with Bolivia. Bolivia also share a low F_{ST} with Rondônia. Venezuela, is somewhat similar to Itaituba, Marabá, and Rondônia. The majority of the remaining comparisons are higher than 0.45 and suggest strong differentiation. While the Peruvian clonets are generally quite distinct from the other collection sites, clonets A and B share the lowest reported F_{ST} values with samples collected in two sites in Pará and Rondônia. The remaining clonets, argued to be either ancestral (clonet C) or coastal (clonet D and E) were even more differentiated from other populations.

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