REVIEW

Malaria epidemiology: Insights from the genome of the malaria parasite

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ABSTRACT

Malaria continues to be one of the most prevalent human infectious diseases. It is caused by protozoan parasites of the *Plasmodium* genus that are transmitted from person to person by the bite of an infected female *Anopheles* mosquito. Studies of malaria epidemiology have traditionally used the microscope to measure the prevalence of infection within the human population and density of parasites within each human host. This method allows the distinction between species but not individuals within a species. Now there is unlimited access to a complete genome sequence for *P. falciparum* and several other genomes are almost complete. This genome information has allowed malariologists to examine the molecular epidemiology of malaria using a variety of polymorphic genetic markers. Consequently, pictures are emerging of the genomic diversity, evolution and population genetics of malaria parasites. This review is an account of these studies and their impact on malaria epidemiology.

KEYWORDS: *Malaria,* Plasmodium*, epidemiology, genomics, diversity, evolution, population genetics*

INTRODUCTION

Malaria continues to be one of the most devastating infectious diseases of our time, rivaling HIV and tuberculosis as a killer disease in tropical and subtropical regions ((WHO, 2005), Figure 1). Around 3.2 billion people are at risk of malaria each year (WHO, 2005), with around 500 million people proceeding to clinical disease, and 2-3 million deaths occurring (Snow et al, 2005). Over 90% of these deaths occur in sub-Saharan Africa (WHO, 2005). The burden of morbidity and mortality is biased towards young children, not yet immune to clinical symptoms (Snow et al, 2005), and pregnant women where parasites are sequestered in the placenta (Rowe and Kyes, 2004). Despite much suffering and many years of research there is no effective vaccine and drugs are either too expensive for the majority of people that are at risk of disease, or no longer effective due to extensive drug resistance of the malaria parasite. Hence, there is a shortage of effective interventions for malaria.

The parasites that cause malarial disease are protozoan organisms that also infect many animal species including primates, lizards and birds. Four *Plasmodium* species are responsible for human malaria: *P. falciparum*, *P. vivax, P. ovale and P. malariae*. *P. falciparum* is the most virulent parasite, and is responsible for the majority of malaria-related mortality. It is found in all malaria endemic regions of the world and is the most common human malaria parasite in Africa (WHO, 2005). *P. vivax* is rarely found in Africa, but is the most common species outside Africa (Mendis et al, 2001; Carter and Mendis, 2002).

The malaria parasite has a complex lifecycle involving both asexual and sexual stages with obligatory phases in both humans and the female *Anopheles* mosquito (Box 1). For most of its lifecycle the parasite has a haploid genome, but following sexual reproduction in the mosquito it undergoes a brief period of diploidy. Here, recombination occurs with a high crossover rate (Su et al, 1999) resulting in the re-assortment of alleles. Recombination allows the

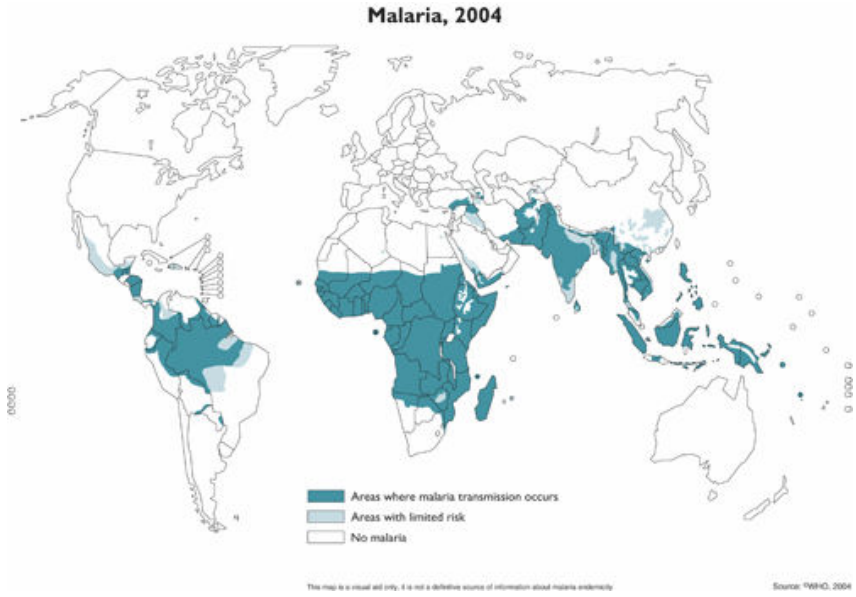
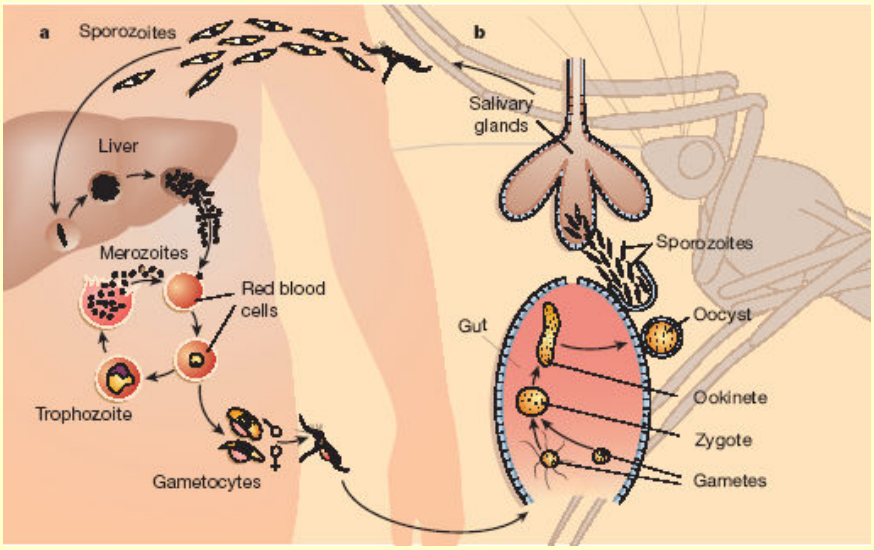


Figure 1. The global distribution of malaria in 2004 (WHO 2005). Reproduced from WHO website (WHO 2005; http://www.who.int/malaria/malariaendemiccountries.html); reprinted with permission.

Box 1. The lifecycle of malaria parasites. When an infected mosquito feeds on the human host, it injects a small number of sporozoites from its salivary glands into the blood. (a) Sporozoites then travel to the host liver and invade hepatocytes. Importantly, *P. vivax* and *P. ovale* parasites can form dormant stages known as hypnozoites in the liver meaning that relapse is possible for many years after initial infection. After around two weeks of maturation to exo-erythrocytic schizonts, all four species, now in the merozoite stage, burst out of the hepatocytes and invade the erythrocytes (red blood cells). Here, they go through several stages from rings to trophozoites to erythrocytic schizonts, a process that takes around 2 days (3 days for *P. malariae*). The mature schizonts again rupture the cell to release merozoites which re-invade new erythrocytes. Parasites can continue to replicate asexually using this mechanism, but some can also form transmission stages, known as gametocytes. The mosquito stages take approximately two weeks and begin with gametocytes ingested with the mosquito blood meal. (b) Ingested gametocytes form gametes that fuse in the mosquito midgut to become zygotes. Zygotes develop into ookinetes that can traverse the gut wall to develop into oocysts. The oocyst then bursts and releases sporozoites that can then migrate to the salivary gland, and thus begins a new lifecycle.



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generation of novel parasite genomes if the blood meal contains two or more genetically distinct clones. Out-crossing occurs commonly in regions of high *Plasmodium* transmission (high rates of infection) such as Africa and Papua New Guinea (PNG) because there are many multiple infections (Babiker et al, 1994; Paul et al, 1995). This creates genetic diversity and provides opportunities for the dissemination of advantageous alleles among parasites of a population.

Epidemiology can be described as the study of disease patterns. Such studies can be applied to the control of that disease. The epidemiology of malaria is described as endemic (stable) when a consistent pattern of *Plasmodium* transmission is found in humans over a number of years. Malaria epidemiology in areas of stable transmission is typified by an age dependent pattern of non-sterilizing immunity in the human host (Cattani et al, 1986; Molineaux, 1988). This is thought to be due to the gradual acquisition of antibodies to diverse parasite surface antigens (Bull et al, 1998). Stable malaria is generally characterized by a genetically diverse parasite population (or many novel genomes). At the other extreme, epidemic (unstable) malaria occurs when there is a large increase in the prevalence of cases at any period in time. In unstable malaria, parasites are generally less genetically diverse. This is due to clonal expansion of single genomes in the absence of immune pressure (Arez et al, 1999; Laserson et al, 1999; Hoffmann et al, 2003). Unstable malaria may also be associated with diverse parasites where infections have been able to persist sub-patently in the host between transmission seasons (Babiker, 1998). Globally, a spectrum of epidemiologies of malaria showing varying transmission intensities can be found (Molineaux, 1988; Gilles, 1993; Snow et al, 2005). Transmission differences were shown to occur within continents and within countries (Snow et al., 2005; Guerra et al., 2006, in press). Variations can also occur locally, for example, in PNG, varying malaria epidemiology was observed between villages and even between households within the same village (Cattani et al, 1986).

A complex interplay between history, environment, vector, host and parasite has resulted in the varying patterns of malaria epidemiology across the globe. We are only just beginning to understand how parasite genetic diversity relates to these patterns. The advent of molecular genetic technologies combined with a complete genome sequence for *P. falciparum* (Gardner et al, 2002) has allowed the measurement of genetic diversity using a variety of markers throughout the genome. In this article I will review the post-genomic epidemiology of malaria by discussing the Malaria Genome Project, and studies of genome wide diversity, evolution and population genetics of the most studied human malaria parasites, *P. falciparum* and *P. vivax*. The impact of the Malaria Genome project on investigations into two parasite survival characteristics that are issues for disease control, namely drug resistance and variant antigens will also be discussed. (For a review on malaria epidemiology, see Greenwood et al, 2005)

THE MALARIA GENOME PROJECT

The Malaria Genome Project began as an initiative in 1996 by a consortium of genome centers including the Sanger Institute, The Institute for Genomic Research (TIGR)/Naval Medical Research Centre and Stanford University. The aim was to sequence the genome of the *P. falciparum* (clone 3D7). The majority of the genome was finished and annotated in 2002 (Gardner et al, 2002) and is now complete, with the exception of a small number of repetitive regions that have been difficult to sequence. The 14 chromosome nuclear genome was found to consist of 23 Mb of DNA sequence containing 5268 predicted genes (Gardner et al, 2002). *P. falciparum* also contains a mitochondrial genome of approximately 6 kb, and a plastid genome of 35 kb, as do the other malaria species. Approximately 60% of the predicted genes did not match any sequence in the public databases and were thus annotated as "hypothetical" proteins. This shows how much more we have to learn about the biology of this parasite, and the large potential for identifying new drug and vaccine targets.

All genome information is readily accessible online via the databases PlasmoDB (www.plasmodb.org) or GeneDB (www.genedb.org) (reviewed in Aslett et al, 2005). For each predicted gene a number of parameters were determined using bioinformatic tools. These include homology-based functional annotations, protein motifs and domains. Queries can be made using keywords, regular expressions (a string of characters eg: a sequence motif) or by DNA or protein sequence homology using the Basic Local Alignment Search Tool (BLAST) (Altschul et al, 1990). Expressed sequence tags (ESTs), microarray (Bozdech et al, 2003; Le Roch et al, 2003) and proteomic (Florens et al, 2002; Lasonder et al, 2002) data have also been aligned to the *P. falciparum* genome to reveal the stage-specificity of each gene. Gene ontology (biological processes), paralogs (homologs within the same species), orthologs (homologs within different species), and syntenic regions with other species (conserved order of genes) can be viewed. PlasmoDB also shows the locations of the identified single nucleotide polymorphisms (SNPs) (Mu et al, 2002; Mu et al, 2005a). Researchers are able to contribute their own data to the databases to enhance these features.

More recently, the Sanger Institute, the Institute for Genomic Research (TIGR) and the Broad Institute have been sequencing the genomes of a number of additional *Plasmodium* species including rodent and primate malaria parasites (Table 1). These include four additional *P. falciparum* genomes: a fresh clinical isolate originating in Ghana; a well-studied clone, known as IT, from Brazil; and HB3 and Dd2, the parents of a genetic cross to map chloroquine resistance (Su et al, 1997). Clone 3D7 is assumed to be of African origin, although its precise origin was unknown because it was isolated from Amsterdam airport (Walliker et al, 1987). The additional genomes will complement the completed 3D7 genome, and reveal any loss of genetic material which is known to occur after long periods of parasite culture (Day et al, 1993). Sequencing of the *P. vivax* (clone SalI), genome is almost complete and the manuscript is due to be submitted in early 2006 (J. Carlton, personal communication), while the *P. ovale* and *P. malariae* genome projects are in the planning stages (M. Berriman, personal communication). Sequencing of additional non-human parasites will aid genetic studies in model organism parasites as well as to complete synteny

Table 1. Summary of *Plasmodium* genome projects

Species	Strain	Host	Genome sequence	Institution	Reference
P. berghei	ANKA	rodent	8X	Sanger	http://www.sanger.ac.uk/Projects/Protozoa
P. chabaudi	AS	African thicket rats	8X	Sanger	http://www.sanger.ac.uk/Projects/Protozoa
P. yoelii yoelii	17XNL	African thicket rats	5X	TIGR, NMRC	Carlton et al, 2002
P. falciparum	3D7	human	18X	Sanger, TIGR, Stanford	Gardner et al, 2002
P. falciparum	Ghana clinical	human	8X	Sanger	http://www.sanger.ac.uk/Projects/Protozoa
P. falciparum	IT	human	1X	Sanger	http://www.sanger.ac.uk/Projects/Protozoa
P. falciparum	HB3	human	8X	Broad Institute	http://www.broad.mit.edu/seq/msc
P. falciparum	Dd2	human	in progress	Broad Institute	http://www.broad.mit.edu/seq/msc
P. vivax	SalI	human	10X	TIGR	Carlton 2003; http://www.tigr.org/tdb/e2k1/pva1/
P. malariae		human	planned	Sanger	M. Berriman, personal communication
P. ovale		human	planned	Sanger	M. Berriman, personal communication
P. knowlesi		Monkey	8X	Sanger	http://www.sanger.ac.uk/Projects/Protozoa
P. reichenowi	Dennis	Chimpanzee	in progress	Sanger	http://www.sanger.ac.uk/Projects/Protozoa
P. gallinaceum		Bird	3X	Sanger	http://www.sanger.ac.uk/Projects/Protozoa

maps revealing insights into genome structure and evolution (Hall and Carlton, 2005). Comparative genomics of non-human primate species that are closely related to human malaria species may reveal genetic traits associated with host switch and co-evolution.

GENOMIC DIVERSITY OF MALARIA PARASITES

Genetic polymorphisms have been used as molecular markers to determine the evolution and the population genetics of the malaria parasite. Knowledge of the genomic diversity of *P. vivax* is not as broad as that for *P. falciparum.* This is mostly due to the lack of a complete genome sequence, and an existing method for its *in vitro* culture. But as the sequencing of the *P. vivax* genome proceeds, more studies will be possible. An investigation of the genetic diversity in *P. ovale* showed the existence of much variation with two major lineages (Win et al, 2004; reviewed in Collins and Jeffrey, 2005), however the analysis of polymorphisms in large stretches of genomic DNA are yet to be done for this species, and for *P. malariae*, so they will not be discussed further.

Genetic polymorphisms that have been used to study diversity in the *P. falciparum* and *P. vivax* genomes fall into three major categories: (1) single nucleotide polymorphisms (SNPs); (2) microsatellites and other repeats; and (3) indels.

SNPs

SNPs confer point mutations in the nucleotide sequence that may or may not encode amino acid polymorphisms. These are known as nonsynonymous (or amino acid) and synonymous polymorphisms respectively. In the genomes of both *P. falciparum* and *P. vivax* the pattern of SNPs is described as a mosaic, consisting of long stretches of monomorphic DNA interspersed with islands of high density SNPs (Volkman et al, 2001; Mu et al, 2002; Volkman et al, 2002; Feng et al, 2003). The majority of genes are relatively monomorphic (few differences among genomes), whilst only a few are highly polymorphic (many differences among genomes). Predominantly nonsynonymous polymorphisms are found in *P. falciparum* genes (Rich et al, 1998). A study of chromosome 2 showed that the genes with many SNPs (both nonsynonymous and synonymous) contained transmembrane domains. This suggests that they are exported to the surface and that their abundance of SNPs is due to exposure to selection by the host immune system (Volkman et al, 2002). By mapping SNPs throughout the genome, novel antigens, that may be vaccine candidates, or other functionally important genes (under selection), may be discovered.

Microsatellites and other repeats

Microsatellites are small (1-4 base pair (bp)) tandem repeats, while minisatellites can be classified as consisting of repeat units of more than 5 bp. Repeats diversify through the expansion and contraction of repeat arrays. They change at a faster rate than SNPs due to replication slippage (Su et al, 1999; Anderson et al, 2000a). Amongst even the most monomorphic *P. falciparum* sequences lay polymorphic microsatellites and minisatellites. Microsatellite polymorphisms are widespread throughout non-coding sequences of *P. falciparum* (Volkman et al, 2001). The majority of microsatellites consist of 1-3 bp TA repeats (*e.g.*, T, TA or TAA), although some more complex repeats have been found (Anderson et al, 2000b; Volkman et al, 2001; Mu et al, 2002). This may in part be a consequence of the ~ 80% AT rich genome of *P. falciparum* (Gardner et al, 2002). In contrast, the *P. vivax* genome contains around 55% AT nucleotides (Carlton, 2003) and polymorphic microsatellites appear to be less diverse (Leclerc et al, 2004) and less common (Feng et al, 2003; Leclerc et al, 2004). On the other hand, polymorphic minisatellites were found to be more abundant in the *P. vivax* genome than in *P. falciparum* which may also be a consequence of their different base compositions (Feng et al, 2003).

Indels

Indels are inserts or deletions of 1 or more base pairs in non-repetitive DNA sequence. A 2 nucleotide deletion, and 1 nucleotide insert were found over 4106 bp of sequence in a study of introns of *P. falciparum* (Volkman et al, 2001). Only 3 single nucleotide indels were found in over 100 kb of *P. vivax* DNA, but these were observed within repetitive sequences (Feng et al, 2003).

EVOLUTION OF MALARIA PARASITES

Investigations into the origins and history of malaria parasites have given insights into what conditions may have led to the origin and spread of disease. This was done through comparison with the natural population history of the Anophelene vector, humans (and our ancestors) and non-human primates. This knowledge may show how we could avoid similar situations in the future, and explain the complex epidemiology of present day malaria. Furthermore, a parasite of ancient origin will be more genetically diverse and perhaps pose a greater challenge for control, whereas homogeneous parasite populations may be more equally susceptible to antimalarial interventions. Both *P. falciparum* and *P. vivax* have been successful at adapting to new environmental challenges. They contain polymorphic surface antigen and drug resistance genes (see sections below), and as discussed above *P. falciparum* has an abundance of polymorphic microsatellites (Su et al, 1999; Anderson et al, 2000a). This led to the assumption that malaria parasites were of ancient origin.

To determine the origin of an organism, the time to the most recent common ancestor (MRCA) can be calculated using SNPs among a few isolates with distinct global origins. However, the time to MRCA should be calculated using only synonymous polymorphisms because they are considered to accumulate randomly, and are neutrally evolving (i.e. a type of molecular clock), whereas nonsynonymous polymorphisms are more likely to have accumulated under selection. Alternatively, other putatively neutral SNPs such as those within non-coding sequences or neutrally selected genes (eg: housekeeping genes) can be used.

P. falciparum

An examination of diversity in a collection of genes from *P. falciparum* revealed a high ratio of nonsynonymous to synonymous polymorphism (Rich et al, 1998). Using the synonymous polymorphisms, the time to MRCA was calculated to approximately 6,000 years ago. Similar studies suggested the MRCA was much more ancient at around 150-200,000 years (reviewed in Hughes and Verra, 2002). The authors examined mostly antigen and drug resistance encoding loci, known to be under selection, but that were available from the GenBank database prior to the genome project. It was therefore possible that there were biased ratios of nonsynonymous to synonymous polymorphisms, and errors in the GenBank data (Barry et al, 2003). Further studies were carried out to clarify this paradox using genes under putatively neutral selection and careful sequencing procedures.

A study of the introns of putative housekeeping genes described a time to MRCA of 10,000-25,000 years (Volkman et al, 2001). In contrast, a further examination of coding and non-coding regions in selected and neutral genes across chromosome 3, predicted a much older MRCA of 100,000-180,000 years ago (Mu et al, 2002). The difference in MRCA estimates was partially resolved by analysis of the 6 kb mitochondrial genome. This confirmed *P. falciparum* was likely to be widespread in the human population for more than 50,000 years but had undergone a major recent expansion around 10,000 years ago (Joy et al, 2003).

A recent expansion is supported by human migration patterns and behavior (reviewed in Hume et al, 2003) and a time to MRCA of around 10,000 years, is coincident with the advent of agriculture and humans living in large populations (Coluzzi, 1999; reviewed in Hartl et al, 2003). *P. falciparum* appears to have experienced this rapid expansion and spread around the world in parallel with its human host and with the speciation of *Anopheles* mosquitoes (Coluzzi, 1999). Behavior changes of the human host may have contributed to an increase in parasite population size. For example, the development of large sedentary agricultural settlements could have allowed the expansion of host, vector, and consequently *Plasmodium* populations. A number of major bottlenecks that would reduce genetic diversity may have also occurred when the parasite adapted to the different species of *Anopheles* worldwide (Hartl et al, 2003; Hume et al, 2003; Joy et al, 2003).

P. vivax

P. vivax appears to have undergone a different mode of evolution to that of *P. falciparum*. The fact that *P. vivax* can relapse may have resulted in the wider distribution (outside Africa). The higher frequency of SNPs (Feng et al, 2003; Escalante et al, 2005) indicated that *P. vivax* would have an older MRCA (Escalante et al, 2005). It was previously suggested that the emergence of Duffy antigen negativity in humans resulted in the near-eradication of *P. vivax* from Africa (Miller et al, 1976). Two studies examined SNPs in the mitochondrial DNA in *P. vivax* and reference strains for primate malarias to investigate evolution in *P. vivax* (Jongwutiwes et al, 2005; Mu et al, 2005b). Both described a history of *P. vivax* consistent with an origin in Asia and horizontal transfer from Old World monkeys (Asian macaques) between 45,000-300,000 years ago. These data support an argument where *P. vivax* migrated into, not out-of Africa (Jongwutiwes et al, 2005; Mu et al, 2005b). An investigation of the circumsporozoite protein (CSP) gene suggested that host transfer occurred very recently between humans and New World monkeys (South American platyrrhines) and that it involved at least two independent events (Lim et al, 2005).

The *P. vivax* story still needs some resolution, through analysis of larger regions of genomic sequence as was done for *P. falciparum*, plus sequencing of more isolates from both human (including *P. vivax* isolates from Africa) and monkeys. Although, results from these studies show that changes in human behavior thousands of years ago contributed at least to the widespread expansion and distribution of *P. falciparum*.

POPULATION GENETICS OF *P. FALCIPARUM*

Examining parasite populations in depth (many isolates) and comparing them to distant populations permits the

analysis of the geographic structure (*i.e.*, how populations from different parts of the world relate to each other). Population genetics can be used to predict and monitor the effects of disease interventions, especially if specific loci are monitored. Measuring the diversity gives an indication of the range (*e.g.*, number and characteristics of alleles), organization (*e.g.*, allele prevalences), outbreeding (*e.g.*, random or self mating within the mosquito midgut) and natural history, since high levels of polymorphism would suggest a long history of the population. A reduction in these parameters following interventions would be indicators that the intervention was successful. Whereas, the increased prevalence of an allele in association with resistance (eg: drug resistance, see section below) would indicate that a new intervention should be implemented. Comparisons among populations such as measuring gene flow (differentiation) could predict how quickly resistance may be spread to other populations, as well as its natural history, such as whether it is the origin or a subpopulation of other populations. Of course, the calculation of population genetic parameters is much more complicated than outlined here (Hartl and Clark, 1997).

To examine the worldwide population structure of *P. falciparum* a limited number of studies have examined *P. falciparum* population genetics on a global or local level using haplotypes of putatively neutral microsatellites, previously developed for defining recombination parameters and genetic crosses (Su et al, 1999), or they have used SNPs in nuclear genes (Mu et al, 2005a).

Global population structure

The first study to examine the global population structure of *P. falciparum* was performed using microsatellites (Anderson et al, 2000a), while a second used SNPs from chromosome 3 and transporter genes (Mu et al, 2005a). Parasite populations sampled included Africa, Asia, PNG, and Latin America. *P. falciparum* was found to have a complex global population structure. Diversity was correlated with the endemicity and transmission intensity of each population in both studies (Anderson et al, 2000a; Mu et al, 2005a) as were levels of linkage equilbrium (Anderson et al., 2000a) and recombination rates (Mu et al, 2005a). African parasites were the most diverse followed by PNG parasites. Asian parasites had a moderate level of diversity and Latin American parasites showed very low levels of diversity and high levels of linkage disequilibrium. Populations were found to be structured according to their continental origins (Anderson et al, 2000a; Mu et al, 2005a). There was some gene flow between Asian and PNG parasites when microsatellites were analyzed (Anderson et al, 2000a), but it was not possible to distinguish between them using the chromosome 3 SNPs (Mu et al, 2005a), demonstrating the importance of using a variety of genetic markers. Within Latin America, populations were significantly differentiated in the microsatellite study. Such differences may be attributed to multiple independent introductions of the parasite into Latin America with the African slave trade around 500 years ago (Anderson et al, 2000a).

Local population structure

The above-described studies generalize the levels of global population structure into trends observed in low (*e.g.*, Asia and Latin America) and high transmission regions (eg: Africa and PNG). However, there was not extensive sampling within regions to examine populations in relatively close proximity to each other. It is not unusual to find micro-variation with mosaic patterns of malaria epidemiology within regional or local areas, as measured by parasite prevalence and density (Cattani et al, 1986). This presumably also affects the genetic structure of parasite populations. Variation among different parasite populations in the Congo and Kenya reflect spatial population genetic heterogeneity and inbreeding in stable malarious areas (Durand et al, 2003, Razakanrainibe et al, 2005). Further studies are required to examine local population structure in endemic regions.

Local population genetic investigations have also been performed within countries with unstable malaria. Malaria control efforts resulted in a ten-fold decrease in the incidence of *P. falciparum* malaria in Malaysian Borneo from 1995-1999 (Singh and Cox-Singh, 2001). Population genetic parameters were measured in eight *P. falciparum* foci in two regional areas (three and five populations each) separated by a geographic barrier (Anthony et al., 2005). Seven populations showed evidence of clonal expansion with high levels of linkage disequilibrium. The remaining population showed high levels of diversity and no linkage disequilibrium. However, the time of sample collection may have biased the results for this latter population. Malaria control efforts in this region appear to have affected parasite diversity and the population genetic data suggests that it may be easier to maintain low prevalence (Anthony et al, 2005). A study in Brazil, described low levels of diversity and significant linkage disequilibrium for three of five populations studied, and indicated a reduced recombination rate due to inbreeding rather than epidemic expansion. The remaining two populations showed no linkage disequilibrium suggesting that, because resistance alleles are common throughout the region, there may be an increased spread of multilocus drug resistance phenotypes (Machado et al, 2004).

Levels of differentiation in Malaysian Borneo were correlated with distance between populations (Anthony et al, 2005). However, in the Brazilian Amazon, population genetic analysis of five parasite populations in three states showed no isolation by distance (Machado et al, 2004). The multiple origins of Latin American parasite populations, described above, may be the reason for this disparity (Anderson et al, 2000a).

DRUG RESISTANCE

Monitoring the emergence and spread of drug resistance will influence drug policies so that public health bodies can take appropriate action. This may involve switching to a second line drug when resistance arises or the use of combination therapies (White, 1999). Drug resistance genes can be examined for the prevalence of mutations conferring resistance before, during and following the use of a drug in the community.

The potential for differential levels of drug resistance phenotypes to be encoded by additional genes is well recog-

nized. But at present major drug resistance markers have only been identified for chloroquine (*P. falciparum* chrloroquine resistance transporter (*pfcrt)*) and antifolates (dihyrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*)) discussed below. Amplification of the multidrug resistance transporter (Pf*mdr1*) has been implicated in the increased transport of drug from the parasite (reviewed in Duraisingh and Cowman, 2005).

Chloroquine resistance (CQR)

Chloroquine resistant (CQR) parasites were originally discovered in Asia and Latin America in the late 1950's and subsequently spread to all malaria endemic areas (Payne, 1987). This was disastrous for the control of malaria as chloroquine was the most effective, safe and cheap treatment available at the time. The mode of action of chloroquine is not known and therefore an obvious target was not available to study the molecular mechanism for CQR. The range of CQR levels suggested that resistance was encoded by multiple genes, but genetic cross and association studies revealed the gene *pfcrt* to be a significant contributor (Su et al, 1997; Fidock et al, 2000). There are two known allelic variants of *pfcrt*, African/Asian and Latin American (Fidock et al, 2000). The Latin American *pfcrt* allele was also found in PNG which suggested an independent origin of CQR in PNG to that in Asia (Mehlotra et al, 2001). This was surprising as it was expected that the Asian allele would be found, because that there is gene flow between Asian and PNG parasites (Anderson et al, 2000).

The availability of the *P. falciparum* genome sequence allowed the CQR selective sweep to be studied in more detail. The genomic region surrounding *pfcrt* was examined by genotyping 15 flanking microsatellite loci spanning 200 kb, in 87 worldwide isolates from each of the major malaria endemic regions (Wootton et al, 2002). Microsatellite haplotypes were different for PNG and Latin American parasites. This confirmed independent origins of CQR in these two locations. A third *pfcrt* allele was also found in other parts of Latin America suggesting an additional origin of CQR in the Americas. Furthermore, Asia and Africa shared a common microsatellite haplotype, but the Asian CQR haplotype was more diverse. This suggested that it spread from an origin in Asia to Africa. Together this data revealed that there were at least four origins of CQR world-wide (Wootton et al, 2002). The Asian haplotype has also been identified in the Amazon basin further highlighting the impact of travel in the spread of drug resistance (Vieira et al, 2004). A genome wide study also reported reduced allelic diversity on chromosomes 1, 5, 6, 7, 10 and 12 that were not associated with CQR, suggesting other selective sweeps in the global population of *P. falciparum*. The molecular mechanism behind these putative sweeps is yet to be determined (Wootton et al, 2002).

Antifolate resistance

The antifolate drug combination sulfadoxine-pyrimethamine (SP) was introduced in the late 1970s-80s following the widespread failure of chloroquine. This drug disrupts the folate synthesis pathway of *P. falciparum* by targeting DHPS (sulfadoxine), and DHFR (pyrimethamine). Previously these two drugs had been used independently, with the use of sufonamides during World War II, and pyrimethamine in the 1950s. Consequently, point mutations arose in the genes encoding these enzymes and allowed the rapid development of resistance to the combination of the two drugs (Plowe et al, 1997; Wang et al, 1997; Sims et al, 1999). Single (S108N), double (N51I:S108N or C59R:S108N), triple (N51I:C59R:S108N or C59R:S108N:I164L) or quadruple (N51I:C59R:S108N:I164L) point mutations in the *dhfr* gene are responsible for increasing levels of resistance respectively (Plowe et al, 1997). Single (A437G) or double (S436F:A437G or A437G:K534E) mutations in the *dhps* gene result in a similar phenotype (Wu et al., 1996; Triglia et al., 1997; Triglia et al., 1998). By examining flaking microsatellite haplotypes in African parasites, three independent origins of *dhfr* double mutants (two for the N51I:S108N allele and one for the C59R:S108N allele), and one independent origin of the *dhfr* triple mutant (N51I:C59R:S108N) were found. The other *dhfr* triple mutant allele (C59R:S108N:I164L) was not found in Africa. For *dhps*, multiple origins of the single mutant allele were found in Africa, while only one origin of one of the double mutant alleles (A437G:534E) was identified (Roper et al, 2003). The *dhfr* triple mutant alleles found in Africa were shown to have originated in Asia where both triple mutant alleles and the quadruple mutant allele are common (Nair et al, 2003; Roper et al, 2004). The distribution of *dhps* and *dhfr* mutant alleles in South America indicated that SP resistance had a single origin (Cortese et al, 2002). Further examination of flanking microsatellites in the South American isolates is needed to ascertain whether these alleles also originated in Asia.

Genetic studies on the genes encoding both CQR and antifolate resistance and the flanking genomic regions show that gene flow as a consequence of human migration rather than the emergence of new mutations are a major factor in spreading anti-malarial drug resistance. This has implications for the implementation of drug policies. For example, introducing combination therapies may slow the evolution of resistance because multiple mutations do not occur as often as single mutations do (White, 1999; Mackinnon, 2005), thus appearing to be mainly spread by human migration (Nair et al, 2003; Roper et al, 2004).

VARIANT ANTIGENS

Parasites express antigens on their surface during the extracellular stages (sporozoite, merozoite) and on the surface of the host cell during the intracellular stages (liver, intraerythrocytic and gametocyte stages). Such antigens are being investigated as vaccine candidates (reviewed in Ballou et al, 2004), but they are highly polymorphic due to diversifying selection by the host immune system. This has hampered efforts to develop an effective vaccine, but identifying all the variants in a population through population genetics (see section above) and including them in the vaccine preparation may show promise (Genton et al, 2002). Because they are so diverse these variant antigens can be used to determine the molecular epidemiology of malaria. For example they have been used to monitor multiple infection rates, and to follow individual genomes throughout the course of an infection (reviewed in Greenwood, 2002).

Newly discovered antigen genes within the parasite genome offers hope in the search for an effective malaria vaccine (reviewed in Doolan et al, 2003). Parallel genomic technologies have proven successful in the search for novel vaccine candidates. For example, proteomic analysis of proteins cleaved from the surface of infected erythrocytes resulted in the discovery of novel surface proteins. This was achieved by mapping the protein sequences obtained back to the genome sequence, followed by the examination of expression patterns using immunofluorescence (Florens et al, 2004; Winter et al, 2005). Furthermore, the analysis of polymorphisms across whole chromosomes using oligonucleotide arrays provided SNP maps across the genome (Volkman et al, 2002; Carret et al, 2005). Regions of high SNP density correlated with well known antigen genes in both *P. falciparum* (Volkman et al, 2002; Carret et al, 2005) and *P. vivax* (Feng et al, 2003). Therefore, novel antigen genes may be discovered when this technology is expanded. In other studies, a motif was discovered that predicts export from the parasite during the asexual stages. Genome wide searches revealed around 400 genes within the *P. falciparum* genome involved in remodeling of the host erythrocyte, including 160 proteins not previously known to be exported (Hiller et al, 2004; Marti et al, 2004). Some of these may also be vaccine candidates if also found to be on the parasite or infected erythrocyte surface. Accumulating credentials from post-genomic technologies such as those described here have great utility in the search for new vaccine candidates.

The majority of well known *P. falciparum* surface antigens are encoded by single copy genes, with the exception of PfEMP1 and RIFIN, encoded by up to around 60 and around 177 genes respectively (Gardner et al., 2002). The PfEMP1 coat switches by differential expression of *var* genes, allowing antigenic variation and sequestration within the host to avoid detection by the host immune system (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). The Malaria Genome Project allowed an entire *var* gene repertoire to be described for the first time. Three major *var* gene groups (A, B and C) were defined, each having a specific structural and genomic organization (Kraemer & Smith, 2003; Lavstsen et al., 2003). Analysis of a small (~100 kb) region of the *P. vivax* genome revealed a multigene family (approximately 600 genes) that encode diverse immunogenic parasite surface antigens known as VIR (reviewed in del Portillo, 2004). The publication of the *P. vivax* genome will reveal an entire *vir* repertoire that will uncover the structure and organization of this multigene family similar to that for the *P. falciparum var* genes.

CONCLUDING REMARKS

Global populations of *P. falciparum* and *P. vivax* show signatures of their natural history in their genomes. Their genomes are composed of relatively monomorphic sequences with highly polymorphic "islands" that are concentrated at the telomeres (Volkman et al, 2002; Carret et al, 2005). Overlaying the genomes of the four additional *P. falciparum* genome sequences will further contribute to a genome wide map of genetic diversity within this species. Such genome wide polymorphism analyses have been carried out using comparative genome hybridization (CGH) microarrays (Volkman et al, 2002; Carret et al, 2005). Examining the polymorphic regions of the genome in many isolates from distinct populations has already begun to uncover the relationships between parasite genetic diversity and the epidemiology of malaria across the globe (Anderson et al., 2000; Mu et al., 2005a). These studies have shown that parasite populations display varying degrees of diversity and differentiation that reflect the evolutionary history of the parasite and its human host, and the epidemiology of malaria. Within localized regions, micro-variation was observed (Durand et al., 2003, Razakanrainibe et al. 2005), but the extent of this needs further investigation. Equally important to study, are unusually monomorphic regions. These indicate selective sweeps due to linkage disequilibrium, such as the CQR locus discussed above. A large-scale SNP mapping project known as HapMap is also currently underway to identify regions of linkage disequilibrium across the genome (http://www.broad.mit.edu/infect). Combining this information with genome wide functional annotations may thereby enhance possibilities of finding novel drug and vaccine candidates, and the genes associated with specific phenotypes such as drug resistance and virulence.

Descriptions of the genomic diversity, evolution and population genetics of *P. falciparum* using SNPs could not have been done without the large regions of annotated genomic sequence provided by the Malaria Genome Project. It will now be necessary to carefully monitor each distinct parasite population as disease interventions (eg: drugs and vaccines) are being applied, to monitor and predict outcomes. We now have a wide-range of polymorphic markers for *P. falciparum* with which to do this, and genome wide markers for *P. vivax* are imminent. Further investigations into the genomic diversity, evolution and population genetics of the other human malaria parasites will be possible when their genome projects are complete.

In addition to the parasite genomes described, the entire human (HGPConsortium, 2004) and the African mosquito vector, *Anopheles gambiae* (Holt et al, 2002) genomes have been sequenced. There is now great opportunity for investigations into host-parasite interactions and co-evolution (Hoffman et al, 2002). The epidemiology of malaria has been well defined using traditional tools such as the microscope, but now we have the opportunity of examining each species on an entirely new level. The completion of the *P. falciparum* genome has undoubtedly resulted in a new approach to malaria epidemiology by producing the molecular tools to map, characterize and monitor its genes in natural populations of parasites. Monitoring genes that are important to parasite survival as well as neutral markers will assist in implementing the appropriate disease control measures.

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STATEMENT OF COMPETING INTERESTS

The author declared no competing interests.

LIST OF ABBREVIATIONS

PNG; Papua New Guinea
BLAST; Basic Local Alignment Search Tool
SNP; single nucleotide polymorphism
MRCA; most recent common ancestor
CQR; chloroquine resistance
Pfcrt; *Plasmodium falciparum* chloroquine resistance transporter
SP; Sulfadoxine-pyrimethamine
dhfr; Dihydrofolate reductase
dhps; Dihydropterate synthase

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