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Malaria Epidemiology at the Clone Level

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Abstract

Genotyping to distinguish between parasite clones is nowadays a standard in many molecular epidemiological studies of malaria. It has become crucial in drug trials and to follow individual clones in epidemiological studies, and to understand how drug resistance emerges and spreads. Here, we will review the applications of the increasingly available genotyping tools and whole genome sequencing data, and argue for a better integration of population genetics findings into malaria control strategies.

Keywords

Microsatellite; SNP; whole-genome sequencing; parasite migration; relapse; drug resistance

Genotyping malaria parasites: Lessons learned and the way forward

Genotyping of malaria parasites, i.e. distinguishing between **clones** (see glossary) and assessing the relationship among them by typing **polymorphic genetic markers** such as **microsatellites** (see glossary), **antigens**, or **SNPs**, has become a standard tool in epidemiological research in the last two decades. A widely-used panel of *Plasmodium falciparum* microsatellite markers was published in 2000 [1], and genome-wide SNP panels have been used in numerous studies [2–4]. Whole genome sequencing of *P. falciparum* field isolates was published in 2012 [5], with over 3000 genomes available to-date (<https://www.malariagen.net/>). Development of genotyping protocols for *Plasmodium vivax* has been slower, but two panels of *P. vivax* microsatellite markers published a decade ago [6, 7] have triggered a large number of studies on its diversity and **population structure**. The field is now at a transition point with increasing whole-genome *P. vivax* data being published. While until recently only approx. 25 *P. vivax* genomes had been available [8–12], studies

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published in 2016 have increased this number to over 500 [13–15]. Genome-wide *P. vivax* SNP assays as well as **amplicon**-based genotyping protocols were recently developed [16–18]. Table 1 gives an overview of the different types of genotyping markers, and their benefits and limitations.

Across almost all genotyping studies, *P. falciparum* and *P. vivax* were found to be highly diverse [11, 19–21]. For instance, using a panel of a few microsatellites, or sequencing a parasite antigen such as AMA-1 [22, 23], in most populations nearly every parasite sampled was different. While fewer studies were done on the population genetic composition of the other malaria parasite species infecting humans – *Plasmodium ovale* [24], *Plasmodium malariae* [25], and *Plasmodium knowlesi* [26] – they revealed important aspects about their biology. This included the finding that *P. ovale* is in fact two distinct parasite species – *P. ovale curtisi* and *P. ovale wallikeri* – which co-occur in many countries [24].

The recent advancements of genotyping methods and the increasing availability of data demands for a discussion on their potential to support malaria control programs, and to identify fields where future studies can enhance our understanding of malaria epidemiology.

The molecular force of blood-stage infection to assess exposure at the individual level

Individuals in malaria endemic regions are often co-infected with overlapping infections of different clones. Thus, while these individuals test positive by PCR during many months, the underlying infections dynamics – i.e. the number and timing of the acquisition of new infections – remains unknown. Genotyping allows us to assess the **molecular force of blood-stage infection** ($_{\text{mol}}\text{FOB}$), i.e. the incidence of genetically distinct parasite clones acquired over time. $_{\text{mol}}\text{FOB}$ has proven to be a very sensitive marker to assess the impact of factors such as age or season on the number of acquired infections [27, 28]. In particular, when transmission intensity is high, prevalence reaches saturation, while $_{\text{mol}}\text{FOB}$ still reflects changes in transmission [29]. Importantly, while *P. falciparum* $_{\text{mol}}\text{FOB}$ is closely related to the number of infectious mosquito bites [27], *P. vivax* $_{\text{mol}}\text{FOB}$ represents the sum of primary infections and relapses [28].

Studies assessing the association between antibody titers and protection from clinical malaria are frequently confounded by pronounced heterogeneity of exposure. Even at small geographical scales, some individuals are at a much higher risk of getting bitten by a mosquito than others, and thus they are at a higher risk of getting clinical malaria. In contrast to studies where parameters of transmission intensity are only available at the population level, inclusion of $_{\text{mol}}\text{FOB}$ as a marker of exposure at the individual level greatly improves the ability to predict the effect of antibody titers on protection from clinical malaria. Such studies are essential to evaluate vaccine candidates [30, 31]. Based on the same concept, the association between sickle-cell disease – a mutation in human hosts – and protection from clinical malaria was explored [32].

Studies on $_{\text{mol}}\text{FOB}$ differentiating between any two clones by genotyping can be done reliably using a single highly polymorphic marker. Importantly, the imperfect detection of

minority clones due to day-to-day fluctuations in clone densities or template competition in PCR reactions impact genotyping outcomes more than imperfect resolution of markers [33–35].

Assessing population structure to inform and guide malaria control

Population genetic structure in relation to malaria control is being studied based on different concepts, and at different spatial scales. A first concept aims to identify genetically isolated parasite populations, suggesting limited gene flow among them (Figure 1A). These populations could be preferentially targeted for elimination, as the risk for parasite re-introduction is considered to be small. Such studies are usually conducted at countrywide spatial scales, aiming to identify provinces or districts where malaria control could be scaled up towards elimination [36]. On the other hand, assuming that parasites spread from transmission foci to surrounding areas, clusters of genetically similar parasites are identified (Figure 1B), expecting that control efforts targeting foci may also have beneficial effects on surrounding areas [37]. The spatial scale of such studies is usually smaller. A transmission focus could be as small as a homestead or a village, and parasite spread is expected to extend only for a few kilometers. Lastly, in regions with previously no malaria reported, genotyping can help to distinguish between local outbreaks caused by imported infections (i.e. clonal population structure), and ongoing, but previously undetected transmission (Figure 1C) [38]. If transmission had been ongoing, diversity is expected to be maintained. In the case of *P. vivax*, a genetically diverse **hypnozoite** reservoir, acquired during times of higher transmission, is another source of diversity in blood-stage infections even when mosquito-borne transmission is very low or absent.

Microsatellites [6, 39] as well as SNPs [3, 16, 40] have shown pronounced genetic differences between parasites from different continents, both for *P. vivax* and *P. falciparum*. It is thus possible to identify the origin of parasites imported from distant regions. The ability to track parasite migration on a national or regional level, and in parallel to identify isolated parasite populations that could be targeted for elimination, heavily depends on local context, and often differs for *P. falciparum* and *P. vivax* in the same country. In Cambodia, a *P. vivax* SNP barcode as well as whole genome sequencing failed to find population structure – most likely due to ongoing transmission throughout the country [15, 17]. Yet Cambodian *P. falciparum* infections exhibit strong population structure, which is mediated by geography as well as levels of resistance to artemisinin [41].

The identification of small-scale transmission foci and quantification of gene flow to surrounding areas requires dense sampling within space and time, and high-resolution genotyping markers to estimate **relatedness** of parasites. Owing to these requirements, relatively few studies have been conducted. In Ghana, Kenya, Solomon Islands, and the Peruvian Amazon, relatedness of parasites collected across a range of a few km was found, suggesting gene flow at small scales but limited parasite migrations at larger scales [37, 42, 43]. It remains to be shown under what conditions, and at what geographical scale, transmission foci can be identified that are stable over time and thus could be targeted for malaria control. In eastern Kenya, relatedness of parasites sampled within less than one kilometer suggested high levels of gene flow [37]. In the same site, transmission foci,

characterized by high numbers of individuals carrying antibodies against *P. falciparum*, were identified as the most probable source of parasites in their near surroundings [44]. Yet, a trial conducting vector control and mass drug administration in transmission foci failed to observe a change in parasite prevalence in surrounding areas within a radius of 500 meters [44]. This example highlights the challenges to translate population genetic findings into malaria control interventions.

Clonal *P. falciparum* outbreaks have been reported from Solomon Islands [45], the highlands of Papua New Guinea [46] or Latin America [38, 47, 48], and in some cases were tracked back to regions of ongoing transmission [38, 48]. Genotyping thus confirmed that cases in previously malaria-free regions were caused by a few imported parasites. In South Korea, *P. vivax* has been re-emerging since 1993. Genotyping of samples collected over 15 years suggested continuous introduction of parasites, likely from North Korea [49, 50]. In Papua, Indonesia, genotyping has identified two co-circulating *P. falciparum* populations, one being closely related to populations on other Indonesian islands [51]. Possibly, this population was imported by migrant workers. The studies in South Korea and Papua showed that local malaria elimination efforts are endangered by importation of infections across international borders or islands. Screening of military personnel and people living near the demilitarized border zone (DMZ) in South Korea, and of migrant workers in Indonesia, will likely be needed to interrupt parasite importation.

Despite the description of population genetic structure from local to global scales, empirical evidence for the relationship between observed patterns of parasite population structure and actual numbers of infected hosts migrating remains scarce. Studies assessing whether genotyping is more informative to identify imported cases than evaluating the travel history of patients are lacking. The risk of parasite re-introduction within a certain period of time after successful local elimination has not been quantified. Such numbers are urgently needed to formulate recommendations for malaria control strategies based on population genetics findings. Several challenges need to be met if genetic surveillance is to trigger appropriate programmatic action. A fast turnaround time of genotyping is required, i.e. information must be available within weeks to guide a response once infections are classified as imported or locally transmitted. While control of ongoing transmission might require an upscaling of activities such as indoor residual spraying or bed net distribution, outbreaks caused by imported cases might demand a strengthening of screening activities at borders, or control targeted towards specific groups such as migrants from endemic areas. In addition, identifying the origin of imported infections requires comparing genotypes to country-wide or global databases. Such readily accessible genetic repositories are currently lacking. Geospatial surveillance systems to map index cases in real time are in place e.g. in Solomon Islands [52] or along the Ecuador-Peruvian border [53], and potentially could be expanded to include data on the origin of parasites based on genotyping.

Genetic diversity and population structure as surrogate markers for transmission intensity

Given the need for reliable estimates of malaria transmission levels to design and evaluate control strategies, parasite genetic diversity and population structure have been assessed as surrogate markers for transmission intensity; with lower diversity, less gene flow and more **sub-structure** expected once transmission is low. While overall a trend towards lower diversity was observed in regions of low and focal transmission, often differences in diversity were minimal despite substantial differences in transmission intensity [3, 6, 39, 54]. For example, *P. vivax* microsatellite diversity in Papua New Guinea and Madagascar was nearly identical [39], even though endemicity in the former is very high, while it is low-moderate in the latter [55].

P. falciparum clonal parasite populations, consistent with outbreaks in regions with very low transmission, occurred in several countries [38, 45–48]. In contrast, clonal or near-clonal *P. vivax* population structure was only reported in a few cases and the findings are difficult to interpret. While a near-clonal *P. vivax* population structure was found in Tajikistan, where malaria has been virtually eliminated with the exception of imported cases [39], or Iran, where transmission is low [56], it has also been reported from Ethiopia, where transmission is considered to be high [57]. Thus, the occurrence of clonal or closely related parasites cannot generally serve as a marker for low *P. vivax* transmission. On the other hand, Sri Lanka has been declared malaria-free in 2016 after no locally acquired case had been diagnosed for over three years (<http://www.searo.who.int/mediacentre/releases/2016/1631/en/>), yet in the years just preceding elimination *P. vivax* diversity had remained high [58].

While genetic diversity markers may help to better understand the transmission scenario, they can only complement, but not replace, measurements such as **entomological inoculation rate** or force of infection, the number of clinical cases, or prevalence of infection by microscopy or PCR. Likewise, parasite population structure is a poor surrogate marker for transmission intensity. While distinct parasite population clusters in regions of low or moderate endemicity were observed, some of them now aiming for malaria elimination [57, 59], the effective population size is large in many endemic countries [39]. Thus, once gene flow is interrupted it will take many parasite generations until populations exhibit pronounced genetic differences (www.nature.com/scitable/topicpage/genetic-drift-and-effective-population-size-772523). As a result, population sub-structuring might serve as an indicator about past transmission in regions where little data is available, but not necessarily as a measure for recent changes. Importantly, vector distribution might have a pronounced impact on parasite population structure, further confounding its relationship to transmission intensity (Box 1).

Genotyping to elucidate *P. vivax* relapse patterns

Unlike *P. falciparum*, *P. vivax* (and *P. ovale*) can remain latent in the liver as hypnozoites for months to years before relapsing to develop new blood-stage infections. The epidemiology of relapses is little understood – e.g. the timing and triggers of relapses [60–62], the spectrum of clinical symptoms caused by relapses, or gametocyte carriage and thus the

transmission potential of relapses. Studying these questions is hampered by a lack of methods to distinguish between primary infections and relapses.

Individuals in moderate-to-high transmission areas accumulate a diverse reservoir of liver hypnozoites, originating from numerous mosquito bites and/or genetically diverse inocula (Figure 2). As hypnozoites likely reactivate in a stochastic manner [63], after several infective bites a complex pattern of new infections and relapses emerges (Figure 2). Relapses in adults in high-transmission settings are thus rarely homologous (i.e. genetically identical) to the last blood-stage infection (Figure 2: relapse of inoculum 1 during ongoing primary blood-stage infection of inoculum 2) [64].

When a mosquito feeds on an individual with multiple clone infection or successively on different humans, it takes up genetically diverse gametocytes, leading to recombination. Resulting sporozoites (the form of the parasite that is transmitted from the mosquito to the human host) are **meiotic siblings** that share 50% of their genome (Figure 2). Thus, primary infections and relapses of parasites of a single mosquito inoculum are often related. Indeed, whole genome sequencing of a single individual presenting with multiple relapses over a period of 33 months found them to share large parts of their genome [65]. Likewise, in infants free of hypnozoites from previous infections [66], and in individuals living in regions of low transmission [59, 67], suspected relapses were mostly of the same genotype as the first blood-stage infection (homologous).

These observations can be exploited to identify relapses in longitudinal studies. Assuming that clones that share a substantial part of their genome with previous infections in the same individual are relapses originating from the same inoculum, and using microsatellites and amplicon sequencing for genotyping, in Peru 90% of all positive follow-up samples were identified as relapses [68], and >50% in Cambodia [18]. The same concept was also applied in a trial of tafenoquine, a new drug targeting hypnozoites. Genotyping showed that most recurring parasites were unrelated to the initial infection and thus not relapses, and thus that the drug was effectual in clearing hypnozoites [69].

Data on the proportion of relapses among all blood-stage infections in a population provides a basis for estimating the benefits of treating the hypnozoite stage. Due to potentially severe side effects individuals with reduced activity of the Glucose-6-phosphate dehydrogenase (G6PD) enzyme [70], and the 14-day treatment regimen required [70], primaquine (the only licensed drug eliminating hypnozoites) is not routinely administered in many *P. vivax* endemic countries. Better estimates of the reduction of relapses and thus overall transmission could be used to assess the benefits of G6PD-deficiency screening and primaquine administration. Understanding the seasonality of relapses [71, 72] could guide the design of mass-screen and treatment strategies, i.e. the diagnosis and treatment of infections in asymptomatic carriers. Importantly, most studies to-date typed only 2 or 3 *P. vivax* markers [67, 69] or a single amplicon [18], and their power to detect related relapses was limited. Specifically selected genome-wide markers would enable a more detailed classification.

Assessing drug efficacy and understanding the spread of drug resistance

One of the best examples of how genotyping and studies on population structure have had direct impact on malaria control is their role in our understanding of drug resistance – both to estimate the level of treatment failure in drug trials, and to identify new markers of resistance. Drug resistance has been a major concern for malaria control since chloroquine-resistant *P. falciparum* emerged in South-East Asia and Africa in the 1950s and 1960s [73]. *P. falciparum* resistance against nearly every drug in use has been reported, including artemisinin [74], the current first-line drug in most countries. *P. vivax* chloroquine and mefloquine were described later, in 1991 and 2014, respectively [75].

Drug efficacy trials are routinely conducted in epidemic countries and form the basis for the selection and eventual switch of treatment strategies. Genotyping of samples collected before treatment and at the day of recurrent parasitemia is crucial to distinguish between new infection and **recrudescence** [76–78]. A high proportion of individuals with recurrent parasitemia that were caused by new infection shows that the drug remains effectual, yet reinfection is frequent [76]. In contrast, detection of the same clone before and after treatment indicates treatment failure. *P. vivax* trials of drugs targeting blood-stage parasites are complicated by relapses. Relapses of homologous or closely related hypnozoites might be falsely counted as recrudescence, i.e. drug failure, when drugs do not target hypnozoites.

Population genetics has also helped to identify novel markers of resistance, such as SNPs and copy number variations (summarized in [79]), and to understand how these mutations spread across populations. Combining data from neutral microsatellites and from SNPs known to cause resistance, it was shown that *P. falciparum* chloroquine resistance had developed independently in several countries [80]. More recently, Kelch13 was identified as the marker for artemisinin resistance in *P. falciparum*. After its identification in culture [81], detailed population genetic analysis showed that artemisinin resistance has evolved several times independently in Cambodia and Southeast Asia [41, 82]. This had dramatic impacts for control: while the first artemisinin resistance containment plan focused on building a ‘firewall’ to avert migration of resistant parasites [83], control has shifted to push towards elimination in regions where levels of artemisinin resistance are high [84], and monitoring emergence of novel resistant populations [85]. Research to understand the underlying genetic background that facilitates emergence of drug resistance is ongoing, thus hoping to identify geographic regions where the risk of de-novo artemisinin resistance development is highest [82, 86]. In parallel, in Southeast Asia the frequency of the Kelch13-C580Y SNP is increasing, showing the selective advantage and spread of artemisinin-resistant clones [87].

Many markers of *P. vivax* drug resistance have been identified by screening for *P. falciparum* **orthologues**, and later been confirmed in field studies [88]. The increasing availability of *P. vivax* genomes is a rich resource to identify novel markers through genotype-phenotype association studies. For example, several copy number variations were identified as well as previously unknown regions under selection [13, 14]. These mutations showed strong geographical clustering, and might reflect different histories of drug use. Further phenotype-genotype association studies thus are expected to identify *P. vivax* specific markers for drug resistance.

Towards SNPs and whole genome sequencing – or the right marker for the right question

With the rapid development of novel protocols for SNP typing, amplicon-sequencing, and whole genome sequencing (Table 1), it will be important to select the typing methodology and SNP subsets best suited for specific research questions.

If the molecular force of infection is of interest, or to genotype in drug trials, a single or a few size polymorphic markers will in most cases offer sufficient discrimination power. While diversity of *P. falciparum* microsatellites varies considerably among countries [1], several *P. vivax* microsatellites have shown expected **heterozygosity** levels of >0.95 across regions [39, 54], i.e. less than one in twenty unrelated clones will share the same allele. Protocols for genotyping are straightforward and widely applied. On the other hand, detectability of minority clones is limited due to template competition in the PCR reaction [35]. As a result, substantial day-to-day variation was observed, with up to 40% of all clones not detected in a single sample [33]. This can be overcome by deep-sequencing highly diverse amplicons containing several SNPs. Diversity of such amplicons is comparable to microsatellites [18, 89, 90], and for *P. falciparum*, sensitivity to detect minority clones was greatly improved compared to size polymorphic markers [90]. However, amplicon sequencing raw data analysis is more laborious than microsatellite analysis; particularly the distinction between sequencing errors and true minority clones can be challenging.

In contrast to the distinction between same vs. different clones, genome-wide markers are needed to assess relatedness, i.e. the proportion of genome shared. This is of interest when studying small-scale hotspots of transmission where closely related parasites are expected, or to identify related *P. vivax* relapses in longitudinal studies. While panels of 9–12 microsatellites have been used to assess relatedness [43], the small number of markers limits the level of detail of the analysis. Meiotic siblings can be identified - e.g. when clones share 4–6 out of 10 markers - but more detailed analysis (e.g. identification of half-siblings, sharing 25% of their genome) are hampered by stochastic variation due to the small number of markers and possible independent evolution of the same allele multiple times. Genome-wide SNP assays, including several SNPs on each of the parasite's 14 chromosomes [17], will be better suited for such studies.

Different technologies for genotyping SNPs have their benefits and disadvantages. High-resolution melt assays measure differences in dissociation temperature of double-stranded DNA between a wild type amplicon, and an amplicon containing a SNP. They can be run on light cyclers that are increasingly available in laboratories, including in endemic countries. Raw data analysis requires minimal bioinformatics skills, but results can be confounded by previously unknown SNPs in the amplicon, or by multiple clone infections. SNP-chips and nanofluidic technology [91] allow typing of a SNP panel with minimal input DNA, yet the equipment is not as widespread as qPCR. Amplicon sequencing is the only technology that can identify previously unknown SNPs, which is of particular relevance in the light of the very high diversity of malaria parasites. It is also the only method that may allow reliable reconstruction of several multi-locus **haplotypes** in multiclonal infections, as read numbers represent the proportion of each clone in a mixed infection with high accuracy [89, 90]. This

is difficult with microsatellite data; the minority clone might not be detected due to template competition in the PCR, and due to preferred amplification of smaller alleles, peak height does not always reflect template density [35]. Given that the proportion of multiple clone infection is high in many populations, the ability to reconstruct minority haplotypes might be crucial.

Whole genome sequencing allows for the most detailed studies of parasite relatedness and diversity [92]. Its applications go well beyond classical genotyping – i.e. the distinction of clones – for example when identifying genes under selection [93]. Sequencing from blood samples is hampered by an >99% excess of human DNA. It is thus restricted to high-density infections with leucocyte depletion at the point of collection [94], or alternatively enrichment of parasite DNA over human DNA after extraction is required [95, 96].

Concluding remarks and future perspectives

Parasite genotyping has become a standard in drug trials and in many epidemiological studies. The development of novel assays to type genome-wide SNPs and whole-genome sequencing extends studies beyond the simple discrimination of clones. They allow detailed insights on longitudinal clone dynamics, and assessing different degrees of relationship among parasites, for example to understand small-scale parasite gene flow in elimination settings. In combination with data on human migration, vector distribution, and ecological factors, this will be crucial to target residual transmission (see Outstanding Questions).

To fully benefit from the findings of genotyping efforts, a better integration of genotyping with control programs will be needed. This will require identification of specific questions relevant to control programs, followed by setting-up systems to communicate genotyping data within short time frames, allowing for targeted control.

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Glossary

Amplicon

Amplification product of a PCR. Amplicon-sequencing is a genotyping method where a polymorphic region is PCR-amplified followed by deep-sequencing.

Antigen

Protein that induces an immune reaction. Antigens are often highly diverse, thus when clones infect a host consecutively, later infections are not recognized by the antibodies induced by the antigen of the first clone.

Clonal population structure

Population of parasites that are genetically identical

Clone

Cells (e.g. parasites) that derive from a common ancestor and are genetically identical

Entomological inoculation rate (EIR)

Number of infective bites per person per unit of time (e.g. year).

Haplotype

Group of markers across a haploid genome (e.g. *Plasmodium* parasites in humans)

Heterozygosity

measure of genetic diversity. It represents the chance that an unrelated clone in the same population carries the same allele of a certain marker.

Hypnozoite

Latent liver stage of *P. vivax* and *P. ovale* that remains in an arrested state before a relapse results in a new blood-stage infection

Meiotic sibling

Clones resulting from recombination of two unrelated parent clones in the mosquito vector. Meiotic siblings share 50% of their genome, i.e. they will carry the same allele at approx. 50% of loci.

Microsatellite

short repeat sequence in the genome (usually 2–6 base pairs). Different numbers of repeats result in genetic diversity. Many (>10) alleles of a single microsatellite are often observed.

Minority clone

Clone that is present at a density of <50% of all clones in a multiclonal infection

Molecular force of blood-stage infection ($_{\text{mol}}\text{FOB}$)

Number of genetically distinct clones that appear as blood-stream infections in an individual over time, e.g. 1 year. *P. falciparum* clones are detectable in the blood stream <2 weeks after the infective mosquito bite, thus *P. falciparum* $_{\text{mol}}\text{FOB}$ is closely correlated to the number of clones entering the body. *P. vivax* $_{\text{mol}}\text{FOB}$ is the sum of primary infections and relapses.

Orthologue

Genes in different species (e.g. *P. falciparum* and *P. vivax*) that evolved from the same ancestral gene.

Polymorphic genetic marker

A stretch of DNA that differs among individuals of a population and is used for genotyping

Population structure

Pattern of the genetic makeup of different parasite populations (e.g. in different countries)

Recrudescence

Re-appearance of the same clone after drug administration due to treatment failure

Relapse

Blood-stage infection caused by a *P. vivax* or *P. ovale* parasite that resided latent in the liver as hypnozoite for an extended period of time (weeks to years).

Relatedness

Proportion of genome shared of 2 parasite clones

Single nucleotide polymorphism (SNP)

a single base pair showing genetic diversity. Most SNPs are bi-allelic (e.g. A or T).

Sub-structure

Genetic structure within a genetically separated population (e.g. within one country with a parasite population that is genetically different to other countries)

Box 1**Mosquito species distribution and parasite population structure**

Parasite population structure across wider spatial scales, e.g. across provinces and countries, is usually interpreted with respect to human migration patterns only. Indeed, as the flight radius of mosquitos is in the range of only a few kilometers, mosquito behavior is expected to have little impact beyond that scale. However, the geographical distribution of different *Anopheles* species, and their respective susceptibility to infection, might be an important cause of parasite population structure. *Plasmodium* spp. are transmitted by a large number of *Anopheles* species, and parasites are adapted to local vectors. In *P. falciparum* feeding assays, infection success was lower in mosquito sub-species from other countries [97], and it depended on the parasite's ability to evade the mosquito immune system [98]. Likely the same is true for *P. vivax*. For example, Southern Mexico *P. vivax* populations were structured in ways that reflect vector distribution [99]. Thus, vector species distribution is likely to have a pronounced impact on the ability of imported parasites to establish local transmission, and thus ultimately on parasite gene flow across large distances. Pronounced parasite population structure does thus not necessarily imply absence of migration of infected human hosts (and thus successful control), it could also show that imported infections cannot be transmitted by local vectors.

Outstanding Questions

1. What is the cause of the very high diversity of malaria parasites? What role do evolutionary history, mutation rate, and host immune selection play?
2. Can the relationship between parasite population structure and risk of reintegration be quantified? What is the timescale of re-establishment of transmission after local elimination of genetically separated populations?
3. What is the role of vector population structure, and the susceptibility of different vectors, to parasite population structure?
4. Will SNP typing and whole-genome sequencing reflect changes in prevalence and transmission intensity better than microsatellites?
5. To what geographical scale - village, province, or country - can genotyping identify the source of imported infections?
6. What is the speed of change of parasite genetic diversity over time in the same population?
7. What proportion of relapses are genetically homologous, related and unrelated to the initial blood-stage infection in different transmission settings and age groups?
8. Can the high *P. vivax* diversity in population at the brink of elimination be fully explained by a genetically diverse hypnozoite reservoir?

Trends Box

- Genotyping of malaria parasites to distinguish between clones has become a standard in epidemiological research and drug trials in the last two decades.
- The amount of whole genome sequencing data of field isolates is growing rapidly, and novel genotyping methods are being developed, allowing for a much more detailed understanding of parasite clone dynamics in space and time.
- The impact of studies on parasite genetic diversity and population genetics on malaria control programs has remained limited.
- A better integration of genotyping results with data on human migration and vector distribution will be needed to fully benefit of the findings and to inform control programs.

Figure 1. Parasite population structure and malaria control

Dots of similar colors represent genetically related parasites, while dots of different color represent unrelated parasites. Arrows represent suspected parasite migration patterns. A) Limited gene flow between parasite populations, and as the result genetically different populations, suggests that after successful malaria elimination in one region the risk of re-introduction is small. Such analysis is usually done on the region to country level, e.g. parasites populations in different provinces might be compared and provinces for elimination identified. B) Population structure assuming that parasites spread from foci of high transmission (represented by large dots) to surrounding areas. In the example malaria control targeted towards the first focus (in red) would be expected to reduce transmission to the close surroundings with genetically similar parasites, but would not have an impact on the second focus (in blue), containing genetically different parasites. Foci of transmission would be small (e.g. a homestead), and parasite spread is expected across a few kilometers. C) Outbreak caused by an imported case to a previously malaria-free region, resulting in clonal population structure (top), vs. ongoing transmission of a genetically diverse population (bottom).

Figure 2. Initial blood-stages and relapses of genetically diverse parasites

Possible pattern of blood-stage parasites in an individual with multiple mosquito bites and relapses. After the initial inocula of genetically related sporozoites resulting from meiotic recombination, the primary infection and subsequently the first relapse are related and share most alleles. After a second inocula of an unrelated clone, and a second relapse of the first inocula, two unrelated clones can be detected in the blood stream.

Table 1

Types of genotyping markers and their applications

Type of marker	Technology	Application	Throughput (including raw data analysis)	Proportion genome covered/possibility to assess clone relatedness	Sensitivity to detect minority clones, and ability to quantify relative clone density	Refs
1–3 size-polymorphic antigens or microsatellites	Capillary electrophoresis	Drug trial, molFOB	High	Minimal	Moderate sensitivity, relative quantification not possible due to preferred amplification of shorter fragments	[28, 35, 76]
Panel of microsatellites		Population structure, <i>P. vivax</i> relapses, origin of imported infections	Medium-high	Small-Medium (usually 10–20 markers)		[38, 39, 68]
Single amplicon with many SNPs	Sanger sequencing	Drug trial, molFOB, <i>P. vivax</i> relapses	Medium	Minimal	Limited sensitivity, quantification possible	[100]
	Deep sequencing		Medium		High sensitivity, highly precise quantification of relative clone density	[18, 90, 101]
Genome-wide SNP panel	SNP-chip / high resolution melt (HRM) / microfluidics	Population structure, relatedness, genes under selection, <i>P. vivax</i> relapses, origin of imported infections	Medium-high	Medium (usually <200 SNPs)	Limited sensitivity, relative quantification not possible with most assays	[37, 48]
	Deep Sequencing		Medium		High sensitivity, relative quantification possible, but reconstruction of multi-locus minority haplotypes challenging	[17, 102]
Whole genome	Next-generation sequencing	Population structure, relatedness, genes under selection, <i>P. vivax</i> relapses	Low	Complete	Limited sensitivity (as coverage usually insufficient), reconstruction of haplotypes requires advanced bioinformatics analysis	[5, 65, 93, 103]