

Malaria Molecular Epidemiology: An Evolutionary Genetics Perspective*

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ABSTRACT Malaria is a vector-borne disease that involves multiple parasite species in a variety of ecological settings. However, the parasite species causing the disease, the prevalence of subclinical infections, the emergence of drug resistance, the scale-up of interventions, and the ecological factors affecting malaria transmission, among others, are aspects that vary across areas where malaria is endemic. Such complexities have propelled the study of parasite genetic diversity patterns in the context of epidemiologic investigations. Importantly, molecular studies indicate that the time and spatial distribution of malaria cases reflect epidemiologic processes that cannot be fully understood without characterizing the evolutionary forces shaping parasite population genetic patterns. Although broad in scope, this review in the *Microbiology Spectrum* Curated Collection: Advances in Molecular Epidemiology highlights the need for understanding population genetic concepts when interpreting parasite molecular data. First, we discuss malaria complexity in terms of the parasite species involved. Second, we describe how molecular data are changing our understanding of malaria incidence and infectiousness. Third, we compare different approaches to generate parasite genetic information in the context of epidemiologically relevant questions related to malaria control. Finally, we describe a few *Plasmodium* genomic studies as evidence of how these approaches will provide new insights into the malaria disease dynamics. *This article is part of a curated collection.

INTRODUCTION

Malaria is a vector-borne parasitic disease endemic in tropical and subtropical regions worldwide. Despite progress on reducing its burden, nearly 40% of the world's population remains at risk of infection (1). Malaria is caused by protozoa of the genus *Plasmodium*

(Apicomplexa: [Plasmodiidae](#)), a diverse group that infects a variety of vertebrate hosts, including primates (2–4). Such diversity has led to a disease involving multiple parasites and vector species across various ecosystems worldwide (1).

There are four species of *Plasmodium* that commonly infect humans: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*. Of these, *P. falciparum* and *P. vivax* cause most cases of malaria morbidity, and mortality (1). These parasites emerged independently as human pathogens during the radiation of a *Plasmodium* clade associated with nonhuman primate and rodent hosts (3, 4) and that appears to be older than the origin of hominids (4–7) (Fig. 1). Such complex evolutionary history may explain the biological differences between parasite species causing human malaria (2–7).

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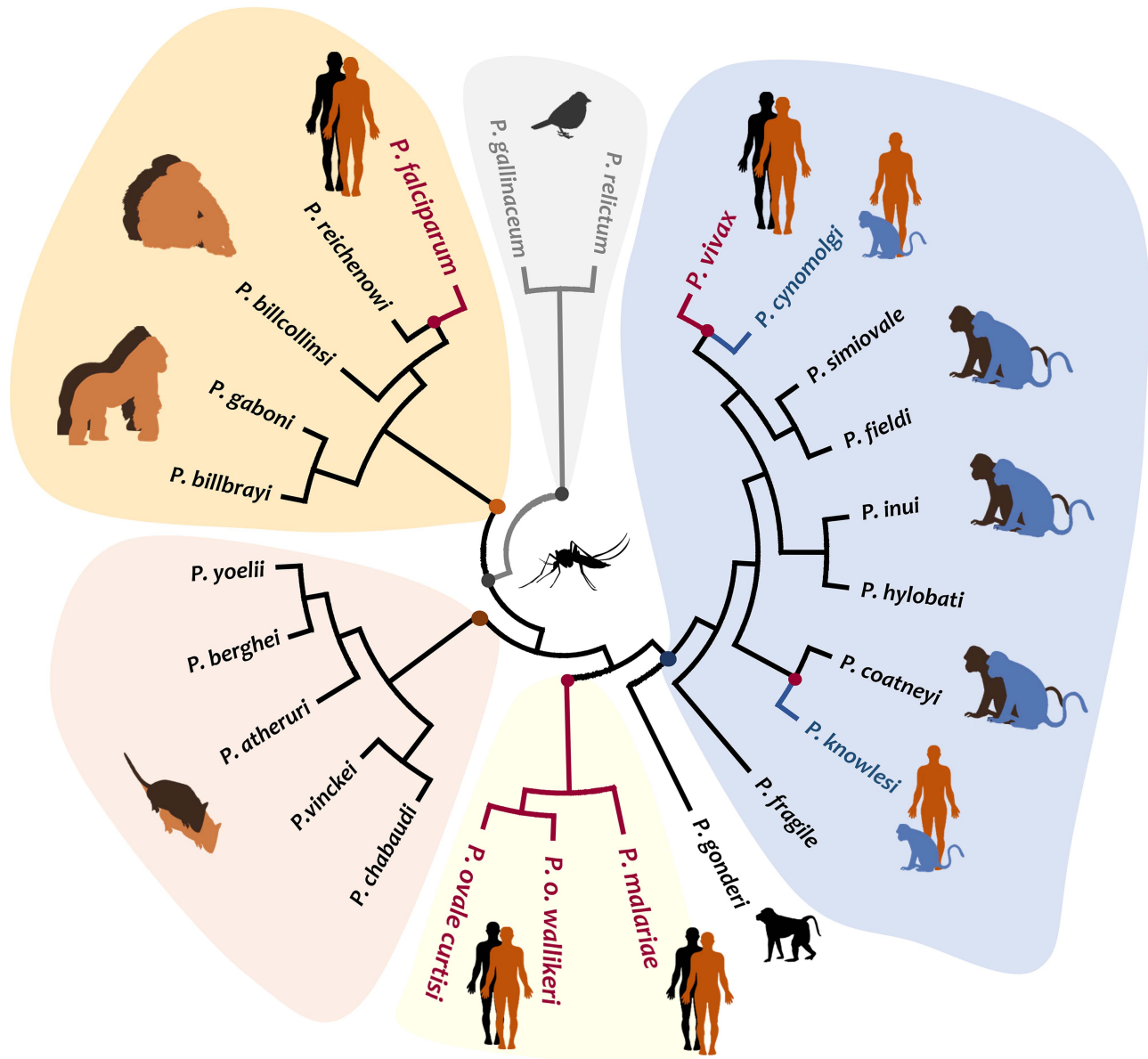


FIGURE 1 Phylogeny of *Plasmodium* parasites based on the mitochondrial genome. The phylogenetic tree shows all the *Plasmodium* species parasitic to humans, including those that cause zoonotic malaras. Although not a comprehensive phylogeny, it evidences that parasites causing human malaria are not a monophyletic group.

There are definite similarities in the life cycles among all *Plasmodium* species. In all species, a fraction of the circulating parasites in the blood (merozoite) differentiates into sexual stages (gametocytes) that are then taken up by the mosquito vector (Fig. 2). However, there are also marked differences between these parasite species in terms of their life histories. For example, *P. falciparum* infection produces gametocytes after a longer period than does *P. vivax* infection, but *P. falciparum* gametocytes are infectious longer than those in *P. vivax* (8). Such

differences may affect their transmission and how interventions affect their fitness (8, 9).

Plasmodium vivax and *P. ovale* develop a dormant liver stage (hypnozoite) that reactivates, causing relapse (infection of the red blood cells) after several weeks (to months or years) of the primary infection. Thus, a radical cure of a malaria patient with any of these two parasites requires eliminating those dormant stages not found in *P. falciparum* or *P. malariae* (1, 2). On the other hand, *P. falciparum*-infected erythrocytes can

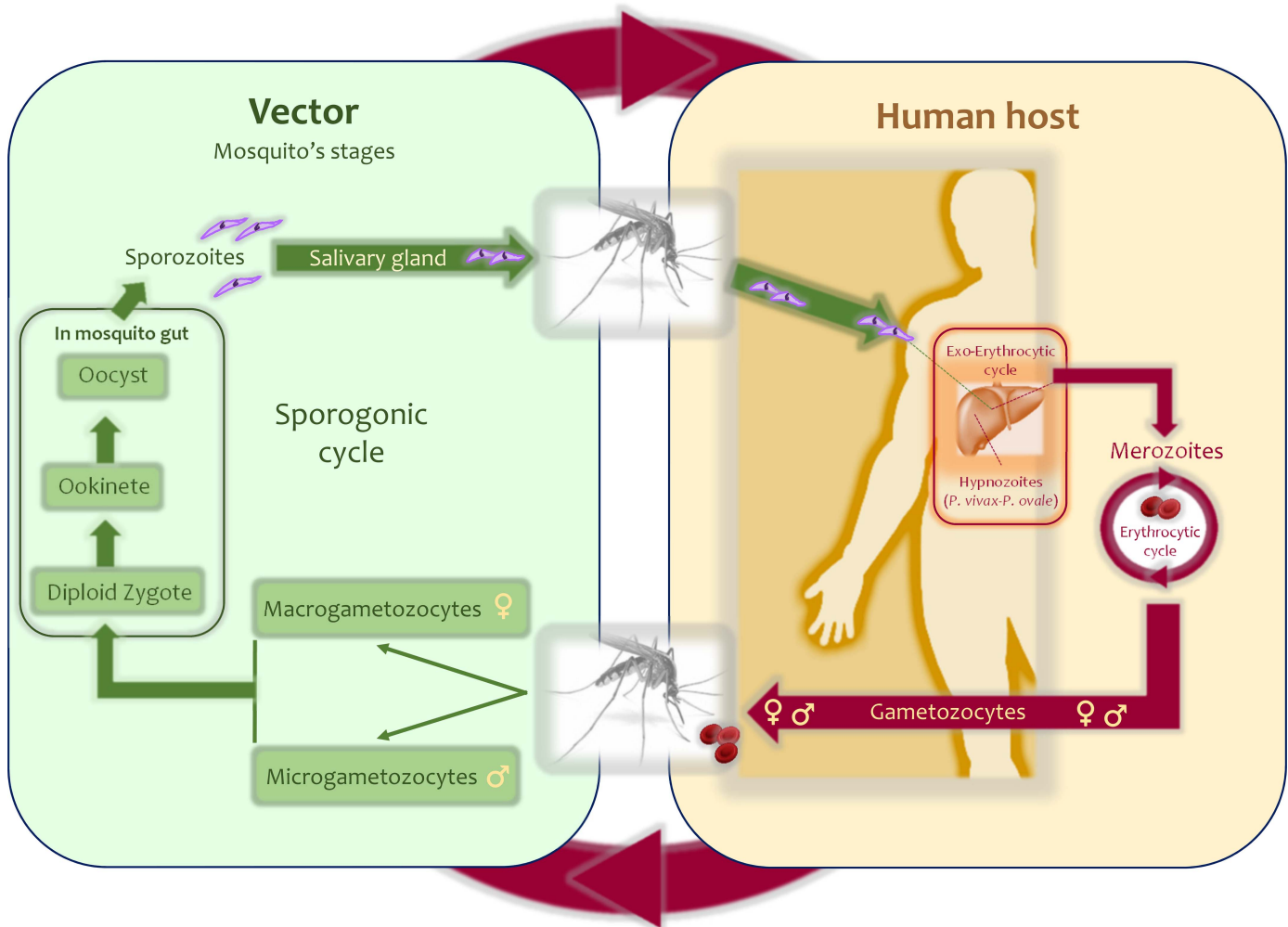


FIGURE 2 *Plasmodium* life cycle. The *Plasmodium* haplontic life cycle comprises a vertebrate host and a dipteran vector. In human malarias, an infected female *Anopheles* mosquito inoculates haploid sporozoites into the host. These sporozoites invade the liver cells and mature into schizonts. The schizonts rupture, releasing merozoites that infect the red blood cells. Some species develop dormant liver stages or hypnozoites that can produce merozoites at a later time (relapse). A fraction of merozoites differentiates into gametocytes (micro- and macrogametozytes). All these stages are haploid. These gametocytes are then taken up by an *Anopheles* mosquito, in which zygote formation takes place (diploid stage). Due to the nature of the cycle, inbreeding is common. The zygote differentiates into ookinetes and oocysts, the latter with a [syncytial](#) cell or [sporoblast](#) containing thousands of nuclei in which meiosis takes place, producing haploid sporozoites.

adhere to the endothelium of capillaries and venules, a process mediated by a gene family without clear orthologs in the other human malarias (10). This process, called sequestration, is linked to severe clinical presentations that are seldom observed in other non-*falciparum* malaria infections (11, 12). In contrast, *P. malariae*, if untreated, can produce a chronic infection that remains latent in blood for years (2).

The distributions of these malaria parasites also vary worldwide (1); there could be only one species in some areas where malaria is endemic, while all four *Plasmodium* species can coexist in others. *Plasmodium vivax* can be present in temperate zones, whereas the other human parasites are generally restricted to tropical and subtropical regions (1, 2). *Plasmodium falciparum* and *P. vivax* may have overlapping distributions in many

areas of endemicity outside sub-Saharan Africa but could differ in their temporal and spatial occurrence at a local scale (13–15). In addition to these four *Plasmodium* species, zoonotic infections and potential animal reservoirs further complicate malaria epidemiology.

The most recognizable zoonotic malaria agent is *Plasmodium knowlesi*, a parasite found in nonhuman primates from Southeast Asia (Fig. 1) (4, 16, 17). Although human-to-human transmission has not been demonstrated, the incidence of this zoonosis is increasing in some areas compared to the more common human malarias (1, 18). How this zoonosis affects the goal of eliminating the disease is a matter that requires some discussion in countries such as Malaysia (1). In addition, there is growing evidence indicating that another nonhuman primate parasite from Southeast Asia, *Plasmodium cynomolgi*, could naturally infect humans, even as an asymptomatic infection (19, 20). The actual prevalence of human *P. cynomolgi* infections seems to be low, and there are no indications of human-to-human transmission (20).

The possibility of anthrozoönotic malaria cycles may also hamper elimination efforts in regions such as South America, as there are studies indicating that nonhuman primates may act as reservoirs (21, 22). Two previously accepted nonhuman primate species, *Plasmodium brasilianum* and *Plasmodium simium*, are likely synonyms of *P. malariae* and *P. vivax*, respectively, found in humans (4, 23). The interpretation of these findings, however, remains elusive. It has not been ruled out that nonhuman primates could be actively infected by human parasites without spillback to humans. This has been observed in Africa in a process in which the vectors may play a critical role (24, 25).

All these factors, together with the uneven scale-up of control interventions across regions of endemicity, drive a constantly changing malaria epidemiology. Unveiling such complex dynamics requires the use of molecular approaches and parasite population genetics.

MOLECULAR TOOLS IN MALARIA EPIDEMIOLOGY

Detecting parasite genetic material is critical when traditional epidemiologic and clinical data, on their own, cannot inform about processes leading to the observed incidence and distribution of cases. Studying parasite genetics allows us to (i) estimate the real incidence rate and prevalence by detecting subclinical infections or submicroscopic peripheral parasitemia, (ii) identify potentially infectious individuals by targeting genes expressed in gametocytes, (iii) differentiate a recrudes-

cent case from a new infection, (iv) evaluate interventions (e.g., surveillance of mutations associated with drug resistance or alleles of an antigen targeted by a vaccine), (v) assess how spatial connectivity sustains malaria transmission by measuring the parasite gene flow-migration-colonization patterns, (vi) link parasite loci with phenotypes of interest such as drug resistance or genes that affect pathogenesis, and (vii) detect zoonotic or anthrozoönotic infections (16, 20, 22, 26–30).

Given the broad application of molecular technologies in malaria epidemiology (Fig. 3), in this review, commonly used methods are discussed intertwined with population biology and population genetics questions wherever these connections are relevant. In order to assist the reader, Fig. 3 shows a diagram linking molecular techniques in the context of problems that are commonly addressed.

DETECTING THE PARASITE USING MOLECULAR METHODS

Although confirmation of a *Plasmodium* infection is a necessary step in managing a malaria case, scaling up the use of molecular diagnostic techniques in the context of elimination and control remains a work in progress (26, 31). A precise diagnosis (detection) allows for studying how risk factors drive incidence and the distribution of cases (27, 28). It is also essential to assess the efficacy of deployed interventions (27, 30–32). Given its importance, testing and developing molecular diagnostic methods constitute an active research area. Those readers interested in this issue could check one of the many reviews available (32–34). Here the focus is limited to some aspects of diagnostics that affect population-level investigations.

The examination of thick blood smears using light microscopy remains the standard for malaria laboratory confirmation (1, 31). Unfortunately, microscopy shows reduced sensitivity when parasitemias are low; such is the case for asymptomatic patients (26, 34–36). It also lacks the power to detect zoonotic and anthrozoönotic infections that may be important in some settings (16, 20–22). In addition, sustaining dependable microscopists is challenging in underserved areas or wherever malaria transmission is no longer perceived as a threat. These problems have led to the development of rapid diagnostic tests (RDTs) based on immunochromatographic detection by monoclonal antibodies against specific parasite proteins (32).

Current RDTs detect *Plasmodium*-specific antigens such as *falciparum*-specific histidine-rich protein 2 and

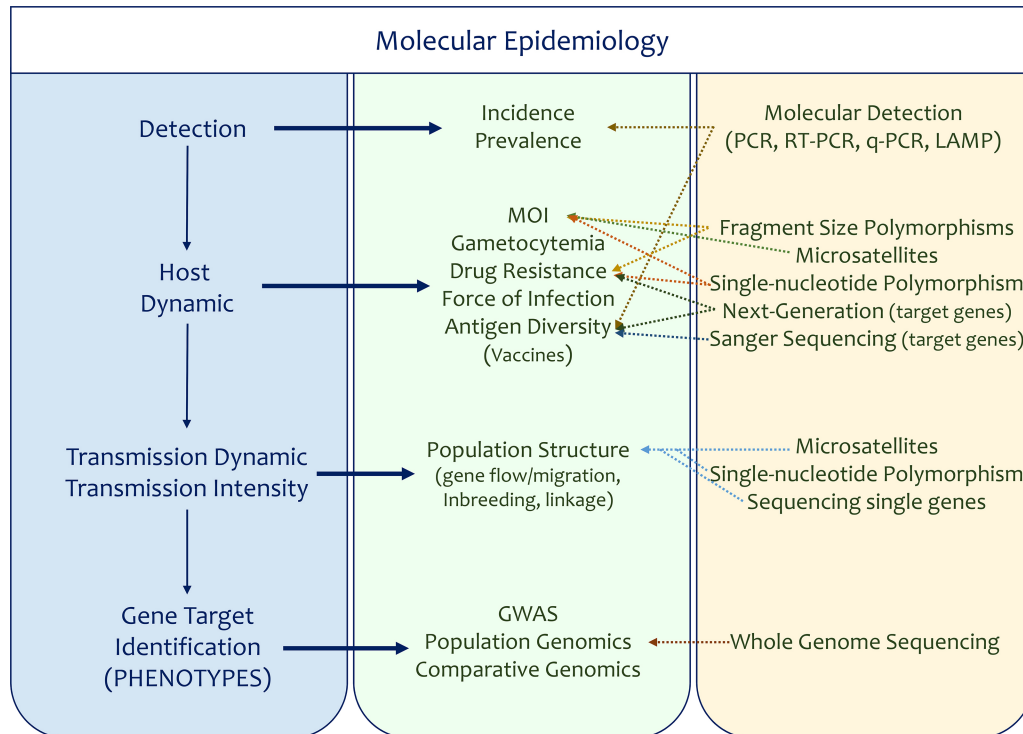


FIGURE 3 Approaches in malaria molecular epidemiology. Shown are techniques and approaches commonly used to generate molecular information in the context of epidemiologic investigations.

histidine-rich protein 3 (HRP-2 and HRP-3, respectively) or *Plasmodium*-specific lactate dehydrogenase and aldolase (1, 26, 31, 32). RDT results are reproducible and rapidly available, and RDTs require minimal training and no instrumentation or dedicated facilities (31, 32). There are still technical limitations, such as reduced sensitivity with samples from malaria patients with low parasitemias (e.g., *P. vivax* and *P. malariae*), as well as handling problems that cause denaturation of antibodies. Importantly, the *Pfhrp-2* and *Pfhrp-3* genes are located in subtelomeric regions, areas of the *Plasmodium* genome that undergo frequent recombination and rearrangements. As a result, there are *Pfhrp-2* gene polymorphisms (37), including different forms of *Pfhrp-2* and *Pfhrp-3* deletions in *P. falciparum*, affecting the efficacy of RDTs. Such deletions were initially discovered in Peru, but there are reports of their occurrence worldwide (34, 37–41). It has been proposed that the prevalence of these *Pfhrp-2* and *Pfhrp-3* deletions could be selected for by the deployment of the RDTs, as those parasites are “undetected” and not treated (28, 37, 38). A stochastic simulation model suggested that the use of *Pfhrp-2*-detecting RDTs was sufficient to select for *Pfhrp2* and -3 double deletions in this parasite species,

prompting the WHO to recommend implementation of surveillance activities in identified regions of concern (40).

In addition to RDTs, there are a variety of molecular methods, including conventional PCR, quantitative reverse transcription-PCR (qRT-PCR), RT-PCR, and loop-mediated isothermal amplification. These methods target the 18S rRNA, mitochondrial genes, and repetitive sequences (20, 26, 27, 32–34, 37, 42–44). Their commonality is that these methods target parts of the parasite genome found in multiple copies, increasing their sensitivity when parasitemias are low. However, these methods are costly, and some require dedicated infrastructure and a high level of technical training, limiting their use mostly to research projects.

Although still limited compared with the use of microscopy or RDTs (1, 26, 31, 34), the use of molecular diagnostic methods has changed our perception of malaria transmission. There is overwhelming evidence indicating that a significant proportion of the malaria incidence rate is comprised of asymptomatic infections (13, 26, 34–36, 44–46). Thus, the attention is shifting from solely focusing on passive case detection (clinical cases) to understanding how asymptomatic subclinical infections may compromise control and elimination

efforts by sustaining transmission (8, 26, 30, 35, 45–47). In addition, the actual prevalence of non-*falciparum* malarias, such as those caused by *P. malariae* and *P. ovale*, is now considered higher than previously thought (48), particularly as part of mixed infections. Finally, the discovery of zoonoses such as those caused by *P. knowlesi* and *P. cynomolgi* has been possible because of the use of molecular methods (16–23).

INFERRING THE PREVALENCE OF INFECTIOUS PATIENTS

Understanding malaria epidemiological patterns requires deconstructing the incidence rate and clinical outcomes in terms of population-level processes. There are multispecies/multistage approaches to perform blood stage and gametocyte quantification by qPCR and qRT-PCR (8, 49, 50). Such data are helpful in parameterizing mathematical models developed to understand transmission and/or inform interventions (for examples, see references 9 and 51). As an example, many control strategies were deployed under the assumption that acquired immunity renders a person noninfectious, so asymptomatic infections in semi-immune individuals do not have a meaningful role in transmission (52, 53). Ascertaining differential patient infectiveness (e.g., those with clinical manifestations compared to asymptomatics) requires detecting and quantifying gametocytes (8, 49, 54).

Molecular assays (e.g., qRT-PCR) have been developed to detect and measure genes expressed in gametocytes, such as *Pfs25* in *P. falciparum* and *Pvs25* in *P. vivax* (8, 46, 49, 50, 55–61). Using such approaches, it has been shown that asymptomatic *P. falciparum* patients can infect mosquitoes from Africa (57). There are only a handful of studies from areas with low transmission outside Africa, or on any of the other malarial parasites. However, pioneering investigations with *P. vivax* have found comparable expression levels of *Pvs25* in symptomatic and asymptomatic patients in South America (55, 58). There is also progress in assessing the differential contributions of lineages to gametocyte production, as well as possible within-host interactions, by targeting gametocyte-expressed polymorphic loci in *P. falciparum* (e.g., *pfs230* and *pfg377*) (8, 59, 60). Analogous approaches for other malarial parasites have not been reported yet.

Nonetheless, gametocytemia and infectiousness are the results of a process that is still poorly characterized (50, 60–62); it is not a static parameter that can be estimated by sampling at a given time point and that is directly comparable across studies (50, 60, 61). Thus, developing gametocyte markers and approaches that

identify groups of individuals that disproportionately sustain transmission (e.g., asymptomatic patients) is becoming a critical issue in the context of malaria elimination (8, 60–62).

STUDYING PARASITE GENETIC DIVERSITY

Beyond the detection of asexual or sexual stages (infected and infectious patients), many molecular epidemiologic investigations follow or characterize parasite genetic variants. Parasite genotyping initially focused on the prevalence of mutations conferring resistance to antimalarial drugs or on the diversity of genes encoding antigens considered vaccine candidates (27, 28). The controversies around the so-called “clonal theory of parasitic protozoa,” however, energized the incorporation of population genetics concepts into malaria epidemiology (63).

At the time that the clonal theory was proposed, many considered sexual reproduction as a simile to lack of linkage disequilibrium and high genetic diversity (63). As a result, finding population structures (nonrandom mating) (64) in *Plasmodium* somehow was not expected (63). The observation of linkage disequilibrium (association of alleles segregating in different loci) in *P. falciparum* demonstrated that population expansion of linked groups (also called clonal expansions) was a relatively common phenomenon. This early work led to a better understanding of how linkage disequilibrium in malaria parasites was driven by inbreeding or selection (27, 28, 64–66) and also brought attention to characterizing parasite genetic diversity in order to understand disease dynamics. Nowadays, measurements of parasite genetic polymorphisms are part of many epidemiologic studies covering topics ranging from assessing how transmission intensity affects the complexity of infection to identifying/following polymorphisms on genes linked to drug resistance or other forms of selection, such as antigenic variation (10, 27–29).

There are several methods available for parasite genotyping (27, 28), including single (or targeted)-gene, multilocus, and genomic approaches (Fig. 3). Researchers need to consider the advantages and limitations of each method in the context of their specific studies. An essential task in any molecular epidemiologic study, however, is to envision the expected molecular pattern under a specific epidemiologic outcome or set of assumptions.

Traditionally, the sampling in epidemiologic investigations focuses on collecting specimens in a defined population of patients in terms of time and space (27, 31). However, the time and spatial scales where the

malaria cases occur will not fully account for the evolutionary time and processes behind the genetic patterns in the parasite populations. Leveraging on the differences between the evolutionary time scale in the parasite and the observed epidemiology informs about the disease dynamics in ways that cannot be achieved by using solely traditional metrics (27, 28, 65–70).

There are two sources of variation in any epidemiologic study using parasite genetics. One is the sampling of cases that is addressed by estimating the number of specimens to be collected given the question; this source of variation is considered by epidemiologists when they estimate their sample sizes. The other source of variation comes from the evolutionary processes that affected the sampled parasite loci that also need to be inferred or modeled. Those processes are genetic drift (related to historical demographic changes in transmission and random uneven reproduction of parasite lineages), type of mutations (point mutations or changes in the number of repeats), mutation rates (given by the loci functional constraints and mode of evolution), or whether such allelic variants affect the parasite fitness (genetic variants encoding phenotypes with different degrees of reproductive success or natural selection) (27–29, 64, 65, 67). These processes may also affect a set of loci differently depending on other factors, such as inbreeding (self-fertilization) and their relative locations in the chromosome (64).

As a result, a study design requires thinking about the sampling at those two levels: the number of individuals and number and type of loci. If the question involves estimating a frequency (e.g., a mutation) in a population or any other metric that consists of reporting the frequency of parasite variants at the level of infections (e.g., the multiplicity of infection; see below), the sampling should focus on individuals (cases). However, many questions require also focusing on the number of loci and whether they are linked (alleles at those loci do not segregate independently, likely due to their proximity in the chromosome) or unlinked (recombination allows alleles at those loci to segregate independently) (64). This is important when a study aims to compare whether two infections are generated by the same parasite (e.g., separating recrudescence from new infections), patterns in the parasite genetic diversity (e.g., population structure or geographic differentiation), or contrasting cases and controls given a phenotype (e.g., discovering mutations linked to a particular phenotype such as drug resistance; see below), among other investigations (27–29, 65, 71, 72). In all cases, the quality of phenotypic and epidemiologic metadata associated with the specimens will

limit the information derived from the parasite genetic data. In the following sections, we describe different types of studies that interrogate parasite molecular data. Those studies will progressively show, following a historical trend, the link between epidemiology and population genetics.

Antimalarial Drug Surveillance

A method that was widely used incorporated PCR and restriction fragment length polymorphism to detect *P. falciparum* mutations associated with drug resistance to antimalarial drugs such as sulfadoxine-pyrimethamine and chloroquine (27, 73, 74). These methods and others follow the frequency of each mutation as an independent locus. This approach has limitations when the goal is to follow alleles with multiple mutations (e.g., double or triple mutants) that differ in their levels of resistance (69, 75). In particular, alleles sometimes cannot be adequately reconstructed from single-mutation reports because they are derived from patients infected by multiple parasite lineages that differ in their mutation profiles. A classic example is the three mutations in the *Pfdhfr* gene conferring resistance to pyrimethamine in *P. falciparum*. Those mutations could be found in a given sample; however, whether this indicates an infection with two alleles (two parasite lineages), each containing mutations with intermediate resistance (two mutations), or a single highly resistant triple mutant allele is difficult to ascertain from a table with the frequency of individual mutations (69, 75). Although the technology has changed, the approach of tracking individual mutations is still prevailing, as in the case of those reported in the *Pfk13* gene associated with delayed artemisinin parasite clearance in *P. falciparum* (70).

Multiplicity of Infection

The first genotyping methods relied on agarose electrophoresis to detect genes encoding surface antigens with a variable number of tandem repeats (76). These methods are still in use around the world (27, 28). The loci more commonly sampled are *Pfmsp2* and *Pfglurp* in *P. falciparum* (there are no orthologs of these loci in *P. vivax*), *Pvmsp3a* in *P. vivax* (with no ortholog in *P. falciparum*) (77, 78), and *msp1* and *csp* in both species (27, 71, 76, 79). Some of these antigen genes, such as *Pfmsp2* and *Pfmsp1* (*P. falciparum*), have two or more groups of alleles that share particular motifs (24, 71). These groups are referred to as allele families. Usually, fragment size polymorphisms are studied after identifying the allele family as part of the genotyping process (71). This approach has proven useful to determine the

number of different parasite genotypes coinfecting a single patient, or multiplicity of infection (MOI) (80).

MOI reflects coinfections (two or more genotypes being transmitted simultaneously by a mosquito) and superinfections (multiple but independent infections). As a result, the frequency of infections by multiple lineages is expected to decline as transmission decreases, and superinfections/coinfections become less likely (28, 65, 80–83). However, the relationship between MOI and transmission is far from linear (68, 80–84).

MOI is a summary of several processes, including the genealogy of the circulating parasites (e.g., a superinfection by identical parasites at a given locus cannot be detected) and the characteristics of the infection (e.g., superinfections may be more likely in asymptomatic patients with a long time of exposure). Studies also indicate that the prevalence of multiple infections is affected by a variety of other factors, including age and immunity (27, 28, 82). In addition, there is variance in transmission driven by spatial differences in mosquito biting rates (28, 65). Thus, large-scale geographic associations between MOI and transmission may be difficult to observe because of a combination of these factors (84, 85).

MOI can provide valuable information in the context of following changes in transmission longitudinally in a specific location (86). Indeed, cross-sectional and longitudinal studies indicate that the prevalence of *P. falciparum* single clonal infections increases with a reduction in malaria transmission (28, 66, 87–89). However, these trends in MOI have not been observed for *P. vivax* (28, 65, 84, 90, 91), for which multiclonal infections remain common even in low-transmission areas (28, 65, 84, 90). This could be the result of hypnozoites from prior infections accumulating in the liver and thus causing multiple relapses of distinct genotypes (28, 65). Unfortunately, there are no data published on *P. ovale* and *P. malariae*.

It is worth noting that MOI has also been used as a proxy for intrahost dynamics under the hypothesis that competition between parasite lineages may relate to disease severity (reviewed in reference 82). Like in the case of transmission, there is conflicting evidence on how MOI is associated with disease severity in human malarias. Part of the problem is the variety of genotyping methods used (not only fragment size polymorphism; see below) and that often studies do not include suitable controls (82, 86, 90, 91). Thus, understanding and measuring MOI require well-planned epidemiologic investigations.

Single-Gene Studies

Many studies rely on sequencing partial or complete genes from the nuclear or organelle genomes of *Plas-*

modium spp. These investigations aim to characterize the diversity of a vaccine candidate, genes involved in pathogenesis, or genes encoding drug targets that may harbor point mutations that result in a drug-resistant phenotype (10, 27, 78, 92–95). In addition, this approach has been used to understand global patterns of diversity, including the malaria parasites' evolutionary histories, gene flow, and/or population structure (94, 96–102).

These types of investigations have contributed to our knowledge of malaria genomic epidemiology. In the context of genes encoding antigens, single-copy genes expressed in the infective stage inoculated from the mosquito vector, the sporozoite, and the erythrocytic state (merozoite) tend to be more polymorphic than those expressed in the gametocyte or in the mosquito vector (10, 27, 94, 96). Among orthologous genes, *P. falciparum* and *P. vivax* show differences in terms of how natural selection drives the observed pattern of polymorphism (94, 103–105). Finally, patterns from the mitochondrial genome (considered a single-locus approach because of lack of recombination) and housekeeping genes indicated that *P. vivax* is far more diverse than *P. falciparum* (97–99, 101). This pattern was also evidenced whenever orthologous genes encoding antigens were studied.

The target gene approach is enriched by the use of next-generation sequencing (NGS) technologies. In particular, NGS on targeted amplification can detect the relative abundance of different alleles in a single infection by counting hundreds of overlapping reads in polymorphic regions. Thus, coupled with appropriate sampling, NGS can inform intrahost dynamics in a way that Sanger sequencing and other genotyping methods cannot. These technologies have been used in the context of evaluating vaccine efficacy, following dynamics of mutations associated with drug resistance, measuring the complexity of infections (MOI), and differentiating recrudescence from new infections (91, 106–110).

Multilocus Genotyping

Multilocus genotyping studies seek to understand processes that deviate from the expectation of random mating (population structure), as well as the dispersion of alleles linked to particular phenotypes of interest (e.g., drug resistance). The genome is sampled by genotyping several loci so linkage disequilibrium and other population-level parameters can be characterized. Linkage disequilibrium (LD), however, can emerge for a variety of reasons. First, loci could be physically close in the genome. This can be very informative if such loci are linked to mutations that confer resistance to an antimalarial

drug. In such cases, characterizing multilocus haplotypes can be used to establish whether drug resistance originated one or more times. Second, linkage can emerge because of processes that affect random mating, such as inbreeding, reduction of the effective population size, epidemic expansions, and geographic isolation (28, 64, 65). The available evidence indicates that population structures are commonly observed in malarial parasite populations; the use of these patterns to deploy and evaluate the efficacy of interventions remains an area of extensive research (28, 65, 69, 83).

Two types of loci are commonly used in multilocus genotyping: microsatellites (65, 67, 68, 82, 83, 111, 112) and single nucleotide polymorphisms (SNPs) (113, 114). These loci can be used separately or in combination; they document similar processes (69, 75, 115–118). Microsatellites are abundant in *Plasmodium* spp., but their characteristics change across species. While the *P. falciparum* genome has an average of one microsatellite locus per 2 to 3 kb of sequence, the *P. vivax* genome has far fewer microsatellites, and they usually occur in more complex patterns (112). An advantage of microsatellites is that they have a higher mutation rate than SNPs, which allows detection of recent events with fewer loci (65, 72). A limitation, however, is that microsatellites evolve according to complex evolutionary models and are not equally suitable worldwide (67, 68, 112).

Using these loci, researchers can study different aspects of population structure, such as linkage disequilibrium and gene flow (65, 66, 72, 83). We know that *P. falciparum* and *P. vivax* have undergone clonal expansions as evidenced by extensive linkage disequilibrium (28, 65, 66, 69, 87–89, 118). Indeed, inbreeding seems to be critical to explain many of the observed patterns (65, 66, 119). Furthermore, clonal lineages tend to replace each other through time and space (65). When *P. falciparum* populations are compared across continents, it is found that genetic diversity and recombination rates are highest in areas of holoendemicity and lowest in regions of hypoendemicity of Central and South America (28, 65, 66, 87–89, 118). In contrast, *P. vivax* genetic diversity was found to vary worldwide and does not follow the same pattern (28, 65, 111, 120, 121). While transmission affects the inbreeding rate, diversity in the parasite population by itself can be affected by (among other factors) historical processes or selective sweeps (28, 64, 66, 69, 96–101). In such cases, polymorphisms physically linked to mutations that are increasing in frequency because they are being selected for (e.g., conferring drug resistance) leave a specific pattern involving a reduction of genetic diversity in the

chromosome region harboring such mutations (64, 69, 116, 117, 122–125). In such investigations, microsatellites have been used to understand the multiple geographic origins and dispersion of haplotypes with mutations conferring drug resistance in *P. falciparum* (69, 75, 115–117, 124).

SNPs are starting to replace microsatellites in several contexts given their high reproducibility, which allows exploration of patterns over long periods and spatial scales. However, they have some limitations. Given their relatively low mutation rate compared to that of microsatellites, usually a larger number of loci is required to find the same patterns (72). In addition, ascertainment bias is a problem in some contexts. In particular, SNPs may be identified in relatively small sample sizes and may be more likely to reflect common rather than rare alleles (126), affecting assumptions in some population genetic analyses (e.g., inferences about parasite demographic history or selection). SNP data, however, are starting to yield patterns similar to those found with microsatellites, such as strong population structure explained by geographic isolation and inbreeding (91, 113, 114, 127, 128).

There are efforts directed to develop SNP typing protocols for *P. falciparum* and *P. vivax*. Their goal is to identify SNPs that can distinguish between parasites from different geographic areas (113, 114) or separate new infections from a recrudescence (91, 129). Many of the SNPs currently used in *P. falciparum* have been selected from different chromosomes so that demographic processes, including infection origin, can be studied (113, 114). These sets of standardized SNPs are usually referred to as a molecular barcode (113, 114). However, recombination between different parasite lineages will break such multilocus genotypes, so the term barcode used literally could be misleading. As in other approaches, the use of SNPs is challenged by multiclonal infections (28, 90, 91).

In addition to gene flow and population structure, multilocus genotyping has been used to assess changes in transmission intensity. However, the parasite genetic diversity is likely affected by events across multiple transmission events (years), whereas a change in malaria incidence may occur within weeks. Like in the case of MOI, studies seek to identify patterns of genetic diversity consistent with changes in transmission, such as a bottleneck in the parasite population as a result of scaling up interventions that should decrease the parasite population (63, 64, 127, 128). This will lead to changes in allele frequencies, with the expected outcome that genetic variation will be lost in response to the declining

transmission. A first approximation is detecting changes in linkage disequilibrium. As stated earlier in the context of MOI, the proportion of infections comprised of a single genotype (monoclonal) is expected to increase when transmission declines, so the inbreeding rate is expected to rise (28, 68). Thus, the number of infections by identical nonsegregating genotypes (linkage disequilibrium or LD) is expected to increase (28, 65, 68, 113, 127, 128, 130). However, in settings where a dramatic increase in transmission occurred after a sustained decrease in malaria incidence, many infections were shown to be caused by identical or highly related parasites (a so-called clonal expansion identified by multilocus genotypes that are stable in time and space) (68, 130). Thus, the observed high LD reflects the past population reduction (clonal expansion of a few lineages) rather than the ongoing increase in transmission. Measuring LD, however, is challenging in areas where multiclonal infections are common simply because the circulating lineages may be difficult to reconstruct (28, 83, 113, 114, 130).

Alternatively, other studies have attempted to follow changes in genetic diversity (heterozygosity). However, heterozygosity may show even more complex patterns than LD since they depend on the effective population size (see below) and the mutation rate of the loci under study (28, 120). Changes in genetic diversity could be almost undetectable if there are a few genetically divergent inbred lineages coexisting in an area. The parasite heterozygosity seems to be less affected by a reduction of malaria incidence of short duration (28, 68, 83, 131).

Lastly, some studies have aimed to estimate changes in the parasite effective population size, N_e (68, 120, 131). N_e measures the uneven reproductive success of parasite lineages, so it directly measures genetic drift (64). While a reduction in the effective population size is expected if transmission is reduced (120, 131), there are many factors that could make such an outcome difficult to detect in the context of epidemiologic investigations. First, there are multiple ways to estimate N_e , each one focusing on different aspects (e.g., number of parents or differences in the number of progeny) (64, 68, 131). Second, an increase in malaria incidence could be driven by closely related parasites and/or high variance in the number of secondary infections generated by infected individuals (super-spreaders) that will yield a low N_e (68). Third, N_e estimates might be inflated by migration or population substructure (131). Finally, the number of infections that sustains the parasite population between transmission seasons may have a greater impact on N_e than the total

number of cases in a given year or the number of cases during the high-transmission season (131).

How to incorporate such concepts as N_e into a metric useful to those working on malaria control is a matter for discussion (68, 120, 131, 132). Nevertheless, genetic information and population genetic parameters can inform how increasing incidence may actually be driven by local parasites rather than an introduction due to migration, and these observations can be used by control programs to evaluate their efficacy in the context of elimination (28, 68, 131, 132).

Whole-Genome-Sequence-Based Methods

Population genomics is a growing field with a significant impact on malaria molecular epidemiology. However, eliminating human DNA contamination remains a challenge, especially for *P. vivax* given the absence of a reliable culturing system. Fortunately, there has been substantial technical progress in that regard (133, 134), leading to many genomic studies reported for malarial parasites (29, 116, 132, 135–144).

Of particular importance are genome-wide association studies (GWAS) used to determine the molecular bases of antimalarial drug resistance. The idea is to detect specific patterns in the genome indicating that a mutation has been selected for (directional selection) (122, 123). In particular, when the frequency of a mutation increases as a result of its selective advantage (e.g., it confers drug resistance), it is expected that the physically linked region of the genome will also increase in frequency via hitchhiking (122, 123), showing the same genetic background in high frequency; this process is commonly called a selective sweep. The size of the genome region affected by a selective sweep will be determined by the recombination rate, as in each generation, recombination may break up the otherwise linked sites (123). Selective sweeps also result in low genetic variation around the adaptive mutation (expected because all individuals will share the adaptive mutation and its linked genetic background) and high haplotype homozygosity. GWAS require specimens with phenotypic information so the genetic bases of such phenotypes can be characterized. Since other factors can generate LD patterns similar to what are found after a selective sweep, including epidemic/clonal expansions such as the extensive inbreeding observed in malaria (28), it is critical to consider such processes when sampling for control specimens or for specimens without the phenotype of interest (116, 135–138).

As an example, studies on the emergence of resistance against artemisinin combination therapy (ACT) in South

Asia were possible because there were well-characterized *P. falciparum* isolates from patients showing low parasite clearance rates (135). By comparing those samples with others from sensitive parasites, GWAS approaches were able to identify genes or genomic regions associated with altered sensitivities to ACT. In particular, a region on chromosome 13 was first implicated (116, 136, 137), and then a specific locus was later identified, the *kelch13* gene (138). Using a similar approach, genetic markers for dihydroartemisinin-piperaquine (an ACT) in *P. falciparum* have been identified (139).

Selective sweeps related to antimalarial drugs have also been found for *P. vivax*. A pattern consistent with a selective sweep was found in Colombia at the *dhps* gene, which is targeted by sulfadoxine, a drug that combined with pyrimethamine was used to treat *P. falciparum* but not *P. vivax* malaria (140, 141). These mutations have also been found in other *P. vivax* populations, indicating that this parasite is under drug-related selective pressure even when it is not the primary target of the drug.

One interesting finding from population genomics studies is that *P. vivax* populations are far more genetically diverse than *P. falciparum*. Even in a single population, *P. vivax* shows as much genetic diversity as a sample including worldwide *P. falciparum* isolates (140–142). This pattern was consistent with previous observations made by single-gene and multilocus approaches (90, 98–100, 103, 111). Population genomic approaches have also been used to detect patterns consistent with positive selection that may lead to the discovery of genes involved in parasite-host interaction, including novel antigens and genes involved in invasion (143, 144). These studies actually show how population genomic approaches allow modeling demographic processes (e.g., geographic structure) that may mask signatures consistent with balancing selection.

Scaling up population genomics will require, however, dealing with the high prevalence of multiple infections (145, 146). There are several promising approaches that attempt to identify chromosome fragments that are identical by descent (similar because they share a common ancestry) even as part of multiple infections (more than one lineage), and such approaches will likely improve our understanding of the role played by natural selection and demography in the variation emerging from population genomics investigations (145–147).

Genome studies of *P. falciparum* also include comparative approaches against *P. reichenowi*, a parasite from chimpanzees (5), while studies of *P. vivax* have been carried out using *P. cynomolgi*, a macaque parasite from Southeast Asia (29, 148), and a newly discovered

P. vivax-like parasite in chimpanzees (6, 7). Although such studies seem distant from the questions formulated by an epidemiologist, they improve our understanding of the host-parasite relationship. In particular, these studies are revealing that the success of *Plasmodium* parasites in humans seems to be mediated by the evolution of gene families that are related to pathogenesis (5–7, 10). These studies have also identified genome signatures consistent with positive selection on genes that could be targeted by novel interventions. An interesting result is that positive selection appears to be more effective on single-copy genes located near telomeres (148). It has long been known that gene families, many located close to telomeres, are highly polymorphic and likely under positive selection (5). However, this process was harder to appreciate in single-copy genes. This result (148) could be explained, at least in part, by the fact that recombination is more common in these chromosome regions, making natural selection more efficient. However, such results are still preliminary.

CONCLUSION

The use of parasite genetic information empowers malaria epidemiologic investigations by unveiling factors affecting transmission and clinical outcomes in new ways. Nowadays, it is possible to study the disease dynamics with a combination of RNA- and DNA-based assays that can assess the contribution of asymptomatic or subclinical infections to malaria transmission. However, the translation of population genetic concepts into actionable and epidemiologically relevant information for malaria control is still a work in progress. Likely future work will require the integration of parasite genotyping into prospective investigations that can train/test integrated population genetics and epidemiologic models. Such models can then be used by policy makers to better deploy and evaluate interventions, particularly in the context of elimination. Nevertheless, genome approaches will likely allow the identification of novel targets for interventions or biomarkers that can be used to understand malaria transmission better in the context of control and elimination efforts.

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We declare that no conflicts of interest exist.

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