Plasmodium evasion of mosquito immunity and global malaria transmission: The lock-and-key theory

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Plasmodium falciparum **malaria originated in Africa and became global as humans migrated to other continents. During this journey, parasites encountered new mosquito species, some of them evolutionarily distant from African vectors. We have previously shown that the *Pfs47* protein allows the parasite to evade the mosquito immune system of *Anopheles gambiae* mosquitoes. Here, we investigated the role of *Pfs47*-mediated immune evasion in the adaptation of *P. falciparum* to evolutionarily distant mosquito species. We found that *P. falciparum* isolates from Africa, Asia, or the Americas have low compatibility to malaria vectors from a different continent, an effect that is mediated by the mosquito immune system. We identified 42 different haplotypes of *Pfs47* that have a strong geographic population structure and much lower haplotype diversity outside Africa. Replacement of the *Pfs47* haplotypes in a *P. falciparum* isolate is sufficient to make it compatible to a different mosquito species. Those parasites that express a *Pfs47* haplotype compatible with a given vector evade antiplasmodial immunity and survive. We propose that *Pfs47*-mediated immune evasion has been critical for the globalization of *P. falciparum* malaria as parasites adapted to new vector species. Our findings predict that this ongoing selective force by the mosquito immune system could influence the dispersal of *Plasmodium* genetic traits and point to *Pfs47* as a potential target to block malaria transmission. A new model, the "lock-and-key theory" of *P. falciparum* globalization, is proposed, and its implications are discussed.**

malaria globalization | immune evasion | anopheles immunity | *Plasmodium* selection | *Pfs47*

The most deadly form of malaria in humans is caused by *Plasmodium falciparum* parasites. Malaria originated in Africa (1, 2) and is transmitted by anopheline mosquitoes. The disease became global as humans migrated to other continents and parasites encountered different mosquito species that were sometimes evolutionarily distant from African vectors (3). For example, anophelines of the subgenus *Nyssorhynchus* (malaria vectors in Central and South America, such as *Anopheles albimanus*) diverged from the subgenus *Cellia* (malaria vectors in Africa, India, and South Asia) about 100 Mya (4). *P. falciparum* parasites are transmitted by more than 70 different anopheline species worldwide (3), but compatibilities differ between specific vector–parasite combinations (5). For example, *P. falciparum* NF54 (*Pf* NF54), of putative African origin, effectively infects *Anopheles gambiae*, the main malaria vector in sub-Saharan Africa; but *A. albimanus* is highly refractory to this strain (6–8); whereas Asian *P. falciparum* isolates infect *Anopheles stephensi* (Nijmegen strain), a major vector in India, more effectively than *A. gambiae* (9). Similar differences in compatibility have been reported between *Plasmodium vivax* and different anopheline species (10, 11). The *A. gambiae* immune system can mount effective antiplasmodial responses mediated by the complement-like system that limit infection (12). We have previously shown that some *P. falciparum* lines can avoid detection by the *A. gambiae* immune system (13) and identified *Pfs47* as the gene that mediated immune evasion (14). Here, we present direct evidence of selection of *P. falciparum* by the mosquito immune system and show that providing *P. falciparum* with a *Pfs47* haplotype compatible for a given anopheline mosquito is sufficient for the parasite to evade mosquito immunity. The implications of *P. falciparum* selection by mosquitoes for global malaria transmission are discussed.

Results

Differences in Compatibility Between *P. falciparum* Isolates from Diverse Geographic Origin and Different Anopheline Species. The compatibility between *P. falciparum* isolates from different continents and mosquito vectors that are geographically and evolutionarily distant was investigated by simultaneously infecting major malaria vectors from Africa (*A. gambiae*), Southeast Asia (*Anopheles dirus*), and the New World (*A. albimanus*) with *P. falciparum* isolates from humans living in those regions. African *P. falciparum* isolates (NF54, MRA-1181) rendered higher infection intensity ($P < 0.001$) and prevalence in *A. gambiae* than in *A. dirus* mosquitoes (92% vs. 60%, $P < 0.01$) (Fig. 1*A* and *SI Appendix*, Table S1 and Fig. S1). Infections were even lower in *A. albimanus* ($P < 0.0001$), with an infection prevalence of only 30% ($P < 0.0001$). Isolates from Southeast Asia (MRA-1241, MRA-1236) led to higher infection intensity in *A. dirus* compared with *A. gambiae* ($P < 0.0001$) (Fig. 1*A* and *SI Appendix*, Table S1 and Fig. S1); whereas *A. albimanus* was highly refractory to these Asian isolates, with an infection prevalence of ≤13% ($P < 0.0001$) (Fig. 1*A* and *SI Appendix*, Table S1 and Fig. S1). In contrast, a South American isolate (7G8) infected *A. albimanus* with a higher intensity ($P < 0.05$) than *A. gambiae*

Significance

Plasmodium falciparum **malaria originated in Africa but became global as humans migrated around the world. It is now transmitted by many different anopheline mosquito species, but little is known about the adaptation of *Plasmodium* to different vectors. Here, we show that the mosquito immune system is a major barrier for some *P. falciparum* isolates to infect mosquitoes from a different continent. *Pfs47* is a protein that makes parasites "invisible" to the mosquito immune system. We found that parasites expressing a *Pfs47* haplotype compatible with a given vector species can evade mosquito immunity. These findings suggest that *Pfs47*-mediated evasion of the mosquito immunity was critical for malaria globalization and may be a key target to disrupt disease transmission.**

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Data deposition: Pfs47 sequences obtained from a *P. falciparum* collection at NIH have been deposited in the GenBank database, www.ncbi.nlm.nih.gov/nuccore/ (accession nos. KT892015–KT892060).

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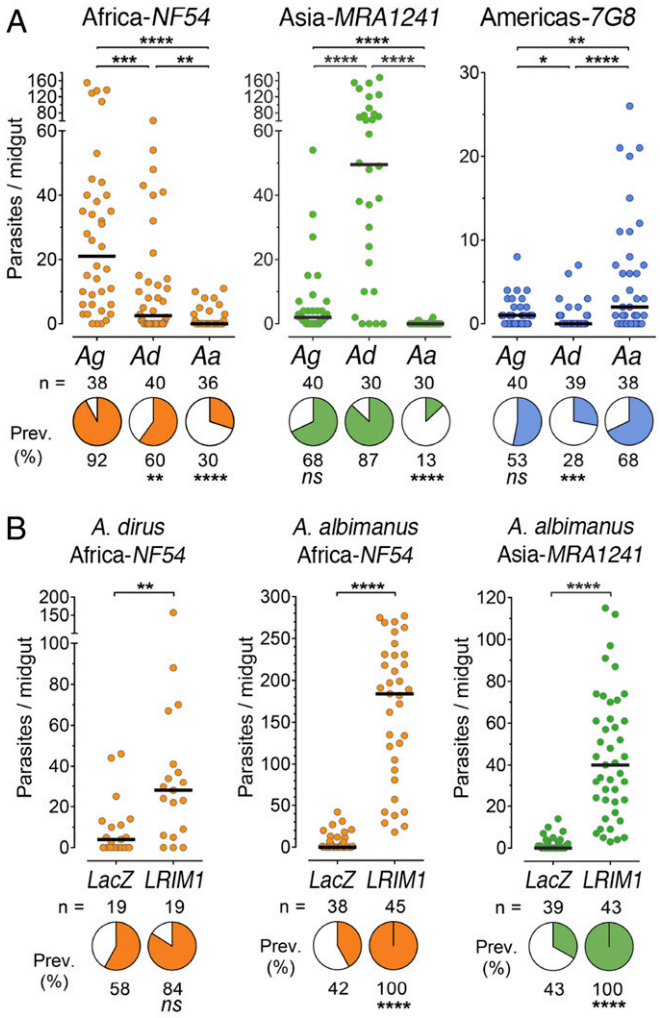

Fig. 1. Compatibility between *P. falciparum* isolates from diverse geographic origin and different anopheline species. (*A*) Infection of *A. gambiae* (*Ag*), *A. dirus* (*Ad*), and *A. albimanus* (*Aa*) mosquitoes with *P. falciparum* NF54 (African), MRA-1241 (Asian), and 7G8 (South American) lines. (*B*) Effect of disrupting the complement-like system by silencing the leucine-rich repeat immune protein 1 (LRIM1) on susceptibility of *A. dirus* and *A. albimanus* to infection with incompatible *P. falciparum* strains. Each circle represents the number of oocysts present in individual mosquitoes, and the medians are indicated by the line (*n* = number of midguts). Pie charts represent the prevalence of infection. All phenotypes were confirmed in two or three independent experiments (*SI Appendix*, Tables S1 and S2). Medians were compared using the Mann–Whitney test, and prevalences using the χ^2 test: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; *ns*, no significant difference.

(Fig. 1*A* and *SI Appendix*, Table S1). *A. dirus* was highly refractory to this South American line, and the infection prevalence was low (28%, $P < 0.001$) (Fig. 1*A* and *SI Appendix*, Table S1). These results are consistent with the hypothesis that, when malaria-infected humans migrate to new geographic regions, *P. falciparum* parasites are selected as they adapt to new mosquito vectors, because only those parasites that are able to infect local anopheline species can continue to be transmitted.

Mosquito Antiplasmodial Immunity Mediates the Incompatibility Between Some *P. falciparum*–Vector Combinations. The thioester-containing protein TEP1 is a key effector of the *A. gambiae* mosquito complement-like system that mediates highly effective antiplasmodial responses (12). TEP1 is stabilized by forming a complex with the leucine-rich repeat proteins LRIM1 (leucine-rich immune protein 1) and APL1C (*Anopheles Plasmodium*-responsive leucine-rich repeat 1-C) (15, 16). Active TEP1 (TEP1-cut) disappears from *A. gambiae* hemolymph when either LRIM1 or APL1C are silenced (15), resulting in a functional depletion of TEP1. As a result, silencing TEP1, LRIM1, or APL1C in *A. gambiae* have a similar effect, en-hancing the infectivity of *P. falciparum* (13) and *Plasmodium berghei* (15).

Plasmodium invasion of the mosquito midgut causes irreversible damage and leads to apoptosis and nitration responses (17, 18) through activation of JNK signaling (18, 19) in the injured cells. We have shown that there is a functional link between epithelial nitration and activation of the complement-like system (17). The nitration response, as the parasite traverses the epithelial cell, "tags" the parasites and results in TEP1 binding to the parasite's surface and activation of a complex that damages the parasite (17). The *Pfs47* gene allows some African *P. falciparum* strains to evade the mosquito complement-like system of *A. gambiae* (14). *Pfs47* protein is expressed on the surface of female gametocytes and ookinetes (14, 20). African parasites (NF54 line) expressing *Pfs47* avoid detection by the *A. gambiae* immune system by suppressing JNK signaling, avoiding the induction of epithelial nitration (14, 18). In contrast, a NF54 *Pfs47KO* line triggers a strong midgut nitration response that leads to TEP1-mediated parasite killing (14, 18).

A whole-genome comparative analysis of the TEP protein family from *A. gambiae*, *Aedes aegypti*, and *Drosophila melanogaster* revealed that there are species-specific expansions in this family and that there is no clear ortholog of *A. gambiae* TEP1 in these other species (21). In contrast, LRIM1 has clear 1:1 orthologs in dipterans, including culicine and anopheline mosquitoes (*SI Appendix*, Fig. S2). The involvement of the mosquito immune system in the incompatibility between certain parasite–vector combinations was explored by identifying orthologs of *A. gambiae* LRIM1 in *A. dirus* and *A. albimanus* and evaluating the effect of disrupting the complement-like system on *P. falciparum* infection. Silencing LRIM1 enhanced the ability of the African *Pf* NF54 line to infect *A. dirus* ($P < 0.01$) (Fig. 1 and *SI Appendix*, Table S2). The effect was even more dramatic in *A. albimanus*, where silencing LRIM1 greatly increased the intensity of infection with the African *Pf*NF54 and the Asian *Pf* MRA-1241 lines (Fig. 1*B* and *SI Appendix*, Table S1; $P < 0.0001$), increasing the infection prevalence from 42% to 100% ($P < 0.0001$), and from 43% to 100% ($P < 0.0001$), respectively (Fig. 1*B*). These findings indicate that the mosquito immune system is an important barrier for adaptation of *P. falciparum* to new vectors and could have limited the introduction of some *P. falciparum* isolates to Asia or the Americas.

***Pfs47* Is Polymorphic and Has a Strong Geographic Structure.** Analysis of *Pfs47* in 364 worldwide *P. falciparum* isolates (Dataset S1) allowed us to identify 47 different DNA haplotypes (Dataset S2) that exhibit a high ratio of nonsynonymous/synonymous (dN/dS = 30) substitutions, in agreement with previous reports (22) and indicative of selection. *Pfs47* has three s48/45 domains and most polymorphisms are present in the second domain (D2) (*SI Appendix*, Fig. S3). Phylogenetic analysis of the 42 *Pfs47* protein haplotypes identified formed two main clades. One clade includes 32 haplotypes that are more frequent in Africa (orange), one unique to Papua New Guinea (brown), and three haplotypes that are the only ones present in the New World (blue); whereas the second clade consists of six haplotypes that are more frequent in Asia (green) (Fig. 2). These data confirm previous reports (23) that *Pfs47* has a strong geographic differentiation, with very high index of fixation (*Fst* values from 0.60 to 0.88; *SI Appendix*, Table S5), and is among the *P. falciparum* genes with the highest geographic differentiation (24). We explored the hypothesis that *Pfs47* haplotypes are responsible for parasite adaptation to local mosquito vectors by allowing them to escape immune detection.

Genetic Replacement of *Pfs47* Haplotypes Is Sufficient to Change Parasite–Vector Compatibility. The *Pfs47* gene was deleted from the African NF54 line and complemented with haplotypes from

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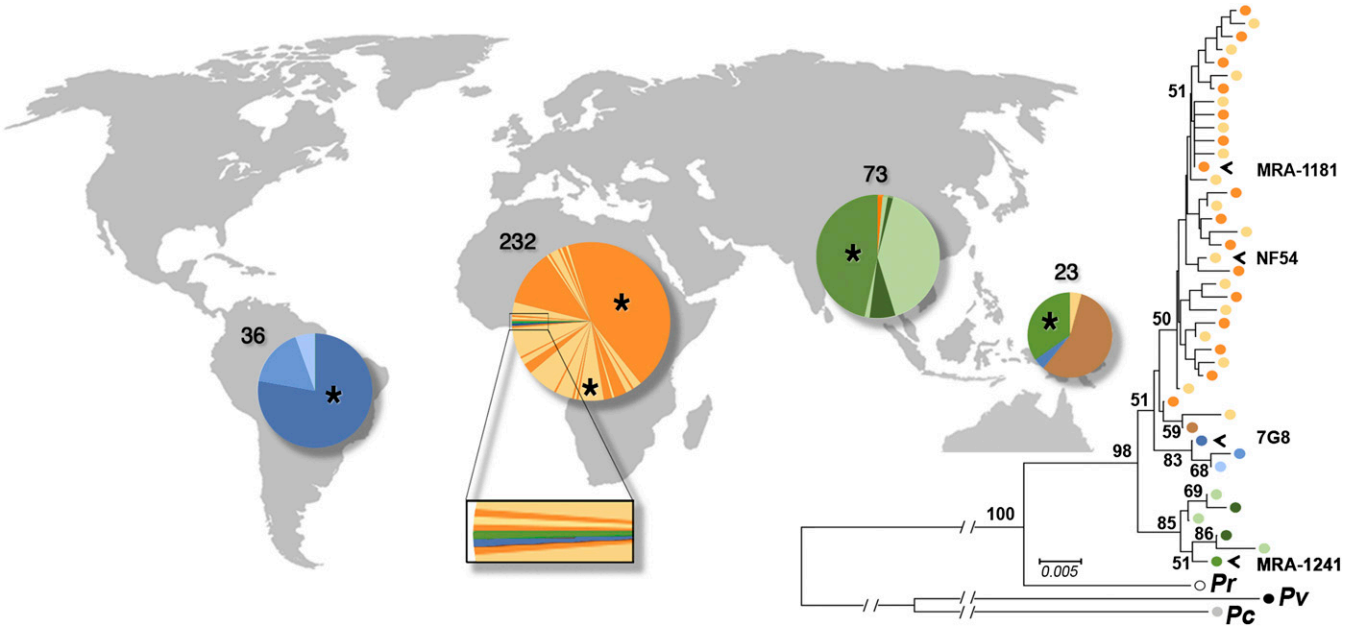

Fig. 2. Geographic distribution and phylogenetic relationship of *Pfs47* haplotypes. The predicted *Pfs47* protein sequence of 364 isolates was analyzed. Pie charts indicate the proportion *Pfs47* haplotypes found in isolates from the Americas (36), Africa (232), South East Asia (73), and Papua New Guinea (23). A neighbor-joining tree was generated with the *Plasmodium reichenowi, Plasmodium vivax, and Plasmodium cynomolgi* P47 sequences as outgroups; nodes with statistical support >50% (bootstrap analysis) are indicated. The main clades identified were given different colors. The haplotype of isolates tested in laboratory infections is indicated in the map with asterisks (*) and the name and position in the tree with arrowheads (<).

Africa (NF54 as positive control), Asia (MRA-1241 and MRA-1236), or the New World (7G8). The NF54 *Pfs47KO* line had low infectivity in *A. gambiae*, *A. dirus*, and *A. albimanus* (Fig. 3*A* and *SI Appendix*, Table S3) that can be rescued by disrupting the mosquito complement-like system (Fig. 3*B* and *SI Appendix*, Table S3). As expected, complementation of the NF54 *Pfs47KO* line with the same *Pfs47* (NF54) haplotype enhanced infectivity of *A. gambiae*, but not of *A. dirus* and *A. albimanus* (Fig. 3*A* and *SI Appendix*, Table S3). Complementing the NF54 *Pfs47KO* line with a frequent Asian *Pfs47* haplotype (MRA-1241) drastically changed vector compatibility, as this line infected *A. dirus* at a significantly higher intensity than *A. gambiae* ($P < 0.01$), and much higher intensity than *A. albimanus* ($P < 0.0001$) (Fig. 3*A* and *SI Appendix*, Table S3). Complementation of the NF54 *Pfs47KO* line with a South American *Pfs47* haplotype (7G8) greatly enhanced infectivity of *A. albimanus*. Interestingly, parasites with a NF54 genetic background expressing the 7G8 *Pfs47* haplotype also infected *A. dirus* and *A. gambiae* efficiently, indicating that some gene(s) besides *Pfs47* are also responsible for the low infectivity of the 7G8 line in *A. dirus* and *A. gambiae* (Fig. 3*A* and *SI Appendix*, Table S3). However, one should also consider that there are significant differences in the prevalence of infection with the *Pfs47KO* line between the different mosquito species. For example, complementation with the 7G8 *Pfs47* haplotype greatly enhanced the prevalence of infection in *A. albimanus* relative to the *Pfs47KO* line (from 0% to 63%), it had a moderate effect increasing the prevalence in *A. gambiae* from 22% to 55%, whereas the effect in *A. dirus* was more modest, with an increase from 34% to 43%.

When a given mosquito species was infected with a line complemented with a compatible *Pfs47* haplotype (*NF54 Pfs47* haplotype in *A. gambiae*, *MRA-1241 Pfs47* haplotype in *A. dirus*, and *7G8 Pfs47* haplotype in *A. albimanus*), disruption of the complement-like system no longer enhanced infection (Fig. 3*B* and *SI Appendix*, Table S4). These results show that replacement of *Pfs47* haplotypes is sufficient to change the compatibility of *P. falciparum* to evolutionarily diverse anopheline vectors, by allowing the parasite to evade the mosquito complement-like system.

Discussion

We present direct experimental evidence that *P. falciparum* isolates vary in their compatibility to anopheline vectors, being more compatible to a vector from the same geographic origin. These experiments involved simultaneous laboratory infections of three evolutionarily distant anopheline malaria vectors (*A. gambiae*, *A. dirus*, and *A. albimnanus*) with *P. falciparum* isolates from their corresponding geographic regions. Differences in susceptibility to *P. falciparum* within a single vector species have been observed (25, 26) but tend to be modest.

Four independent lines of evidence indicate that *Pfs47* plays a central role in *Plasmodium* adaptation: (*i*) *Pfs47* mediates *Plasmodium falciparum* evasion of the *A. gambiae* complement system; (*ii*) genome-wide SNP analysis indicates that some SNPs in *Pfs47* have one of the strongest geographic differentiation in the whole *P. falciparum* genome (24); (*iii*) replacing the *Pfs47* gene of NF54 with a different haplotype is enough to change the compatibility to different vectors; (*iv*) once a parasite expresses a compatible *Pfs47* haplotype, disrupting mosquito immunity no longer enhances infection, indicating that the parasite is no longer targeted by the mosquito complement-like system. However, other *P. falciparum* genes are probably also involved in parasite adaptation to new vectors and may interact with *Pfs47*. This is suggested by the fact that parasites expressing the 7G8 *Pfs47* haplotype in a NF54 genetic background infected *A. dirus* more efficiently (Fig. 3*A*) than when this haplotype was present in a 7G8 genetic background (Fig. 1*A*). There is also evidence of differences in the interaction of *Pfs47* with different mosquitoes, because although the level of infectivity of the NF54 line lacking *Pfs47* (*Pfs47KO*) is greatly reduced in the three species tested, there are differences in the basal level of infection (Fig. 3 *A* and *B*). For example, the *Pfs47KO* line cannot infect *A. albimanus*, whereas a consistent low level of infection is observed in *A. gambiae* and *A. dirus* (Fig. 3 *A* and *B*).

The *Pfs47* protein is expressed on the surface of female gametocytes and has a large number of haplotypes, suggesting that the human immune system may be driving diversity as it selects

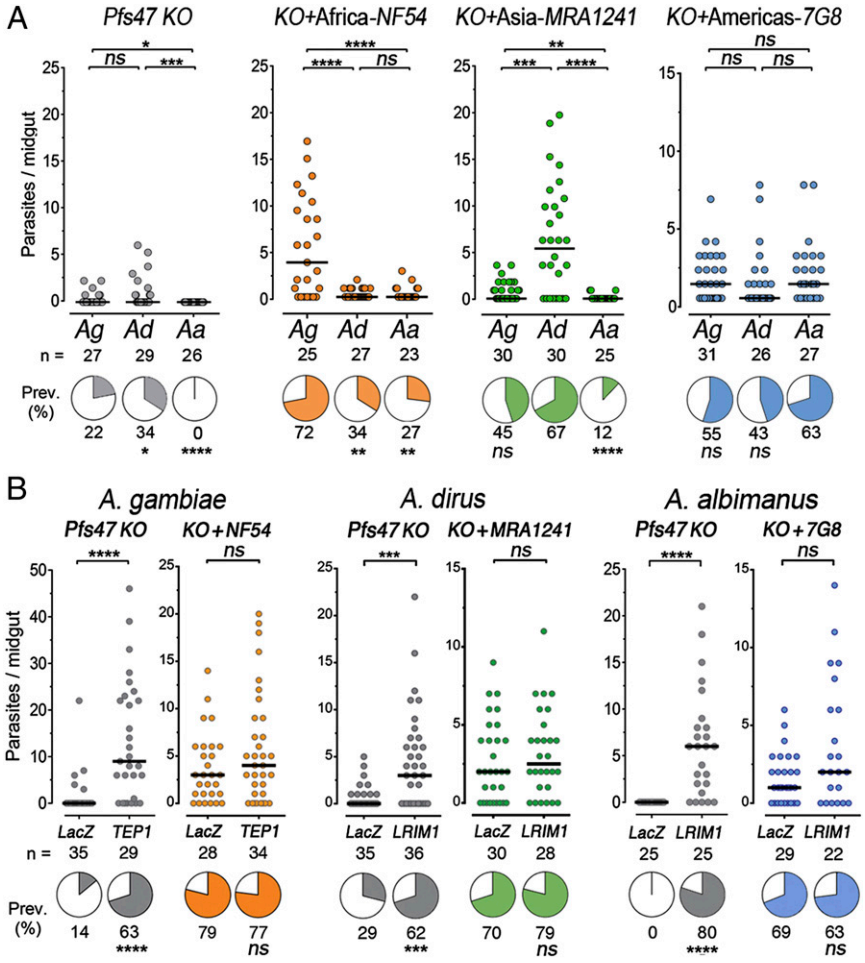

Fig. 3. Effect of complementing NF54 *Pfs47KO* parasites with different *Pfs47* haplotypes on infectivity to different anopheline mosquito species. (*A*) Infection of *A. gambiae* (*Ag*), *A. dirus* (*Ad*), and *A. albimanus* (*Aa*) mosquitoes with *P. falciparum* NF54 *Pfs47KO* line and complemented derivatives expressing *Pfs47* haplotypes from *P. falciparum* NF54 (African), MRA1241 (Asian), and 7G8 (South American) strains. (*B*) Effect of silencing the leucine-rich repeat immune protein 1 (LRIM1) in *A. dirus* (*Ad*) and *A. albimanus* or the thioester-containing protein 1 (TEP1) of *A. gambiae* (*Ag*) on infection with a NF54 *Pfs47KO* line or complemented derivatives. Each circle represents the number of oocysts in individual mosquitoes. Medians are indicated by the line (n = number of midguts). Pie charts represent the prevalence of infection. All phenotypes were confirmed in two or three independent experiments (*SI Appendix*, Tables S3 and S4). Medians were compared using the Mann–Whitney test, and prevalences using the χ^2 test: *ns*, no significant difference; $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

for parasites that avoid immune detection. Antibodies against *Pfs47* might be deleterious to the parasite, as they could block transmission to mosquitoes. At the same time, our results indicate that the mosquito immune system is an important barrier for *P. falciparum* infection in some anopheline species that selects for compatible *Pfs47* haplotypes, resulting in purifying selection.

Based on our findings, we propose the "lock-and-key theory" of malaria globalization. We can think of *Pfs47* as a "key" that allows the parasite to "turn off" the mosquito detection system by interacting with some mosquito receptor protein(s) (the lock). There are different haplotypes of this key, and the parasite needs to have the right key for the "lock" present in a given mosquito species to survive, continue to be transmitted, and become established in a new region (Fig. 4). As humans migrate around the globe, parasites are selected as they adapt to local mosquitoes that are sometimes evolutionarily distant from those vectors present in their place of origin. This model raises many questions: How universal is it, in terms of *P. falciparum* interactions with other mosquito vector species? Does it also apply to other *Plasmodium* species, such as *P. vivax*? Our model predicts that, if this is the case, a global analysis of *P. vivax* whole-genome sequences should reveal *Pvs47*, the *P. vivax* ortholog of *Pfs47*, as one of the genes with the strongest geographic differentiation and very high fixation indices. The biochemical nature of the mosquito receptor (the lock) and how the interaction of *Pfs47* with this protein(s) disrupts JNK signaling also remain to be defined.

This model has important implications. For example, the close physical proximity (151 kb) between *Pfs47* and a recently discovered determinant of artemisinin resistance in humans, the kelch propeller domain protein (27), is expected to result in strong genetic linkage disequilibrium. Therefore, selection of *Pfs47* haplotypes by different mosquito species could affect the probability of dispersal of drug resistance. In fact, the Asian *P. falciparum* MRA-1241 line used in this study is resistant to artemisinin (27) and has a *Pfs47* haplotype that infects poorly *A. albimanus* (Fig. 1). One would predict that this resistance gene is less likely to spread to regions in the New World, where *A. albimanus* is the main mosquito vector. We propose that the immune system of anopheline vectors limits the dispersal of some *P. falciparum* haplotypes around the world, and this could influence the parasite's population structure and the epidemiology of malaria. These studies also point to *Pfs47* as a potential key target to block malaria transmission. We present this initial version of the lock-and-key theory of malaria globalization as a reference point to be challenged and modified as our understanding of these complex vector–parasite interactions at a local and global level evolves.

Materials and Methods

***Anopheles gambiae* Mosquitoes and *Plasmodium* Parasites.** The *Anopheles gambiae* G3, *Anopheles dirus* A *s.s.*, and *Anopheles albimanus* strains were used. Mosquitoes were reared at 27 °C and 80% humidity on a 12-h light–dark cycle under standard laboratory conditions. The *Plasmodium falciparum* strains used—NF54, SenTh015.04 (MRA-1181) from Senegal, 7G8 from Brazil, IPC 3445 Pailin Cambodia 2010 (MRA-1236), IPC 4912 Mondolkiri Cambodia 2011 (MRA-1241), NF54-Pfs47KO and Pfs47KO complemented lines (Pfs47 Africa-Nf54, Pfs47 Asia MRA-1241, and Pfs47 America-7G8)—were maintained in O+ human erythrocytes using RPMI medium 1640 supplemented with 25 mM Hepes, 10 mg/L hypoxanthine, 25 mM $NaHCO_3$, and 10% (vol/vol) heat-inactivated type O+ human serum (Interstate Blood Bank) at 37 °C and with a gas mixture of 5% O_2, 5% CO_2, and balance N_2 (28, 29).

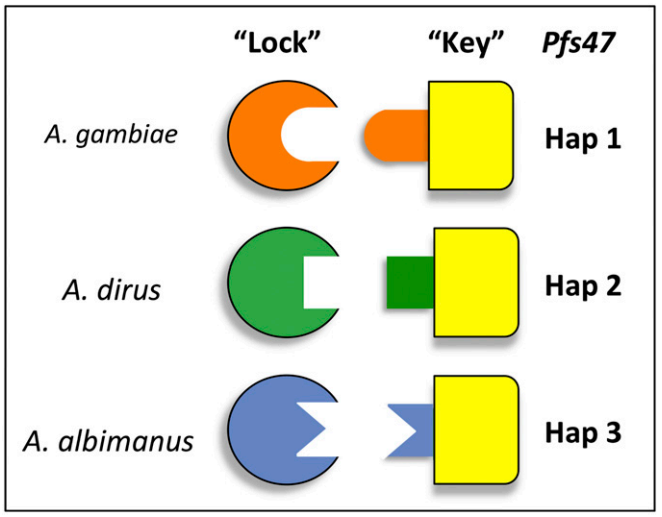

Fig. 4. Diagram of the lock-and-key theory of malaria globalization. We can think of *Pfs47* as a key, with a constant region (yellow) and a variable one (different colors) that allows the parasite to turn off the mosquito detection system by interacting with some mosquito receptor protein(s) (the lock). There are different haplotypes (Hap) of this key, and the parasite needs to have the right key for the lock present in a given mosquito species to evade mosquito immunity and survive. This ability to avoid detection by the mosquito allows the parasite to continue to be transmitted and to become established in a new geographic region.

Experimental Infection of Mosquitoes with *P. falciparum*. Mosquito females were infected artificially by membrane feeding with *P. falciparum* gametocyte cultures. Gametocytogenesis was induced as previously described (30). Mature gametocyte cultures (stages IV and V) that were 14–16 d were used to feed mosquitoes using membrane feeders at 37 °C for 30 min. Midguts were dissected 8–10 d after feeding, and oocysts were stained with 0.1% (wt/vol) mercurochrome in water and counted by light microscopy. The distribution of parasite numbers in individual mosquitoes between control and experimental groups was compared using the nonparametric Mann–Whitney test. All parasite phenotypes were confirmed in at least two independent experiments.

dsRNA-Mediated Gene Knockdown. Individual female *A. gambiae* mosquitoes were injected 1–2 d after emergence as previously described (31). Briefly, mosquitoes were injected with 69 nL of a 3 μg/μL dsRNA solution 3–4 d before receiving a *Plasmodium*-infected blood meal. The control dsRNA (LacZ) and *A. gambiae* dsTEP1 were produced as previously described (31, 32). The dsRNA for *A. dirus* LRIM1 and *A. albimanus* LRIM1 were produced using the MEGAscript RNAi Kit (Ambion) using DNA templates obtained by nested PCR using cDNA from whole female mosquitoes. The *A. dirus* (ADIR008083-RA) and *A. albimanus* (AALB5865-RA) LRIM1 orthologous genes were identified by blast (Vector Base; https://www.vectorbase.org/) and phylogenetic analysis. The primers used to obtain the DNA template for *A. dirus* were as follows: for external PCR: LRIM1_Adi8083ExFB, 5′-CATCGAATTGATGCACAAGC-3′, and LRIM1_Adi8083ExRB, 5′-ATCGGCCAACACCATTTTAG-3′ (PCR conditions were 94 °C for 3 min; 25 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; final extension, 72 °C, 5 min); for the internal PCR (primers containing T7 promoter): LRIM1_Adi8083InFB, 5′-TAATACGACTCACTATAGGGGAGCAGGATTGCAGAGAAT-3′, and LRIM1_Adi8083InRB, 5′-TAATACGACTCACTATAGGGTTTCCTGCGTCAATCTGTTG-3′ (PCR conditions were 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; final extension, 72 °C, 5 min; using 1 μL of the external primer PCR). The primers used for *A. albimanus* LRIM1 were as follows: for the external PCR: LRIM1_Aal5865ExFA, 5′-AAGGTTGAGCCGAAGAATGA-3′, and LRIM1_Aal5865ExRA, 5′-GCACTTCCCATGCTGCTAAT-3′ (PCR conditions were 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; final extension, 72 °C, 5 min); for internal PCR (primers containing T7 promoter): LRIM1_Aal5865InFA, 5′-TAATACGACTCACTATAGGGCTGTACGGCACCGTTAACCT-3′, and LRIM1_Aal5865InRA, 5′-TAATACGACTCACTATAGGGAGCTTGTTGTGCGAAAGGTC-3′ (PCR conditions were 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min; final extension, 72 °C, 5 min; using 1 μL of a 1–20 dilution of the external primer PCR). *A. gambiae* TEP1, *A. dirus* LRIM1, and *A. albimanus* LRIM1 gene silencing was assessed in whole sugar-fed mosquitoes by quantitative real-time PCR (qPCR) using the S7 ribosomal protein gene as internal reference. The primers used for qPCR for *A. dirus* were as follows: LRIM1_Adi_qPF, 5′-CTGCTCACTGCATGATTTGG-3′; LRIM1_Adi_qPR, 5′-ATCCGCTTAGTTGTCCGAAA-3′; S7_Adi_qPF, 5′-ATCCTGGAGCTGGAGATGAA-3′; and S7_Adi_qPR, 5′-TGATCGCTTCTTGTTGTTG-3′. The primers used for qPCR for *A. albimanus* were as follows: LRIM1_Aal_qPF, 5′-GACAAAAGTGTGCGCTTTGA-3′; LRIM1_Aal_qPR; 5′-CACTCCCGGATTAGAGCTTG-3′; S7_Aal_qPf, 5′-ACCTGGACAAGAACCAGCAG-3′; and S7_Aal_qPR, 5′-GTTTTCTGGGAATTCGAACG-3′. The silencing efficiency in dsRNA-injected mosquitoes was 81–84% for TEP1 in *A. gambiae*, and 63–68% and 80–83% for LRIM1 in *A. dirus* and *A. albimanus*, respectively, relative to dsLacZ-injected controls.

Pfs47 Nucleotide Diversity and Phylogenetic Analysis. Pfs47 sequences analyzed (Dataset S1) were obtained from direct sequencing from a *P. falciparum* collection at NIH (GenBank accession nos. KT892015–KT892060), PlasmoDB (plasmodb.org/plasmo/) database and from Manske et al. (24). Direct sequencing was done using the following primers: F1, 5′-ATGTGTATGGGAAGAATGATC-3′; R1, 5′-AACGTAGGGTTGTTTATAGTAC-3′; F2, 5′-CCTACTAACTGTTTTGAATCTC-3′; R2, 5′-TTCCGTTTTACTATTATCACATC-3′; F3, 5′-GAAGAAACTATTGTAGAATCTGG-3′; R3, 5′-TTCGTAAAATCACACCCATC-3′; F4, 5′-GATATACCAAATGAAGAAGAACG-3′; R4, 5′-GCTAACATATACATGCCTTCC-3′; and R5, 5′-TCATATGCTAACATACATGT-3′. Nucleotide diversity analysis and identification of DNA haplotypes (Dataset S2) were done with DnaSp (33), and phylogenetic analysis of protein haplotypes (Dataset S3) was done with MEGA6 (34).

Generation of the *P. falciparum Pfs47*KO Line. The *Pfs47* gene (PF3D7_1346800) of *P. falciparum* NF54 was disrupted by single crossover recombination with the 5.7-kb pPfs47KOattB plasmid (*SI Appendix*, Fig. S4). A *Pfs47* 851-bp internal fragment (nucleotides 289–1139) was PCR amplified with the primers prFRTPfs47F [which includes a 34-bp-long *FRT* site (35)] and prattBPfs47R [which contains a 40-bp-long *Mycobacterium smegmatis attB* adaptor (36)] and cloned by In-Fusion into a NcoI-digested pCC1 (37), generating the pPfs47KO-attB-FRT plasmid. A 39-bp-long *Zygosaccharomyces bailii* B2 recombinase site (38), generated by annealing the oligonucleotides prB2F and prB2R, was cloned by In-Fusion between the NotI and SacII sites of the pPfs47KO-attB-FRT plasmid, generating the pPfs47KO-attB-FRT-B2 plasmid (pPfs47KOattB). In summary, the pPfs47KOattB is a plasmid designed to disrupt the *Pfs47* gene by single crossover recombination with the advantage of integrating several recombination sites (*attB*, *FRT*, and *B2*) for further stable complementation assays. Parasite transfection was done as previously described (39). Briefly, 150 μL of leukocyte-cleared red blood cells (RBCs) (Sepacell R-500II; Fenwall) were washed once with incomplete cytomix (120 mM KCl, 0.15 mM CaCl, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes, pH 7.6 adjusted with KOH) and resuspended in 400 μL of cytomix. The plasmid (100 μg at a concentration of 1 μg/μL in cytomix) was added to RBCs in a chilled electroporation cuvette (Bio-Rad; 0.2-cm electrode) under sterile conditions. Electroporation was done with a Bio-Rad Gene Pulser II at 310 V and 975-μF capacitance. Electroporated RBCs were washed three times with 10 mL of complete culture media and mixed with *P. falciparum NF54* schizonts purified by Percoll-Sorbitol gradient. Culture media was changed daily and selection drug (5 μM WR99210) was added 24 h after electroporation and maintained continuously in the asexual cultures unless stated otherwise. Once parasitemia was assessed (week 8), parasites were PCR-genotyped for integration and then cloned by minimal dilution in 96-well plates. On day 21, 96-well plates were thick smeared and checked for parasites. Positive wells were transferred to a 24-well plates and later to a 6-well plate.

Stable Genetic Complementation of *P. falciparum Pfs47*KO Line. The generated *Pfs47KO* line was complemented with different alleles of *Pfs47* using three different pPfs47attP plasmids. The 8.3-kb pPfs47attP plasmids were assembled following several modifications to the previously developed pCBM-BSD plasmid, which contains a full-length *Pfs47* from *P. falciparum* 7G8 surrounded by upstream (1 kb) and downstream (0.16 kb) noncoding regions of the *Pfs47* ORF (14) (*SI Appendix*, Fig. S5). FRT and B2 recombinase sites were added by annealing and extension of the designed oligonucleotides prB2FRTF and prB2FRTFR, and cloned by In-Fusion between the BamHI and SpeI sites of the pCBM-BSD plasmid. An attP adaptor was also integrated into the plasmid by annealing the oligonucleotides prattPF and prattPR and cloned by In-Fusion between the HindIII and XhoI sites generating the pPFS47attp-7G8 plasmid. The pPFS47attp-7G8 plasmid contains the *Pfs47* gene and regulatory elements from *P. falciparum* 7G8. To introduce different haplotypes of *Pfs47* into the pPFS47attp-7G8 plasmid, the second domain of *Pfs47* from the *P. falciparum* NF54 or the MRA-1241 lines were PCR-amplified with the In-Fusion–designed oligonucleotides prClaF and prClaR. They were cloned using the In-Fusion kit between the two ClaI sites surrounding the domain two, generating the pPFS47attp-NF54 and pPFS47attp-MRA-1241 plasmids. Cloning procedures were carried out in TOP10F bacteria (Invitrogen), and every construct was fully sequenced (Operon).

P. falciparum Pfs47KO was cotransfected with a pPfs47attP plasmid (Pfs47 haplotypes Africa-NF54, Asia-MR4 1241, or Americas-7G8) and the pINT plasmid that codes for a mycobacteriophage Bxb1 integrase (40) using modification of a procedure previously described (41). Briefly, the transfection mixture consisting of 20 μL of packed RBCs, 5 μg of each plasmid DNA (1 μg/μL) (Qiagen plasmid Maxiprep kit), and of 70 μL of Amaxa SE solution, resulting in a final volume of 100 μL, was prepared at room temperature (RT). The transfection mixture was transferred to an Amaxa Nucleocuvette (Lonza), and transfections were performed at RT using an Amaxa 4D-Nucleofector. After applying the CM-162 pulse, electroporated RBCs were processed as mentioned above. Culture media were changed daily and selection drugs (2.5 μM Blasticidin HCl and 200 μg/mL Geneticin) were added 24 h after electroporation and maintained continuously in the asexual cultures, unless stated otherwise. Once parasites were observed (day 18), parasites were cloned by minimal dilution in 96-well plates. On day 14, 5-μL aliquots of the 96-well plates were incubated with 2× SYBR-Green-1 (Invitrogen) and 165 nM MitoTracker Deep Red (Invitrogen) in PBS, and measure of live parasite was assessed using flow cytometry. Positive wells were transferred to 24-well plates and later to 6-well plate.

Genotyping Confirmation or *P. falciparum* Transformant Lines. Primers pairs prIA1F/prIA1R and prIA2F/prIA2R were used to detect both integration arms of *pPfs47KOattB* into the *Pfs47* locus on chromosome 13; whereas primers pairs prIA1′F/prIA1′R and prIA2′F/prIA2′R were used to detect both integration arms of *pPfs47attP*s into the *Pfs47KO* locus on chromosome 13 (*SI Appendix*, Figs. S3 and S4). A combination of these primers was also used to check the absence/presence of full-length *Pfs47* and the episomal plasmids (*SI Appendix*, Figs. S3 and S4). *Pfs47* gene copy number was estimated by testing each DNA sample by relative qPCR with the primers PF13_0248F and PF13_0248R, using the *P. falciparum* gene Pf10_0203 (ADP ribosylation factor) as an internal reference gene using the primers 0203F and 0203R (14) (*SI Appendix*, Fig. S6). Pfs47 mRNA expression was confirmed by qPCR in the *P. falciparum* lines used in this work using cDNA from stage IV–V gametocyte cultures with the mentioned primers as previously described (14) (*SI Appendix*, Fig. S6). Estimation of gene dosage and mRNA expression was calculated according to the ΔΔCt method. The complemented *Pfs47* gene was fully sequenced for each line after cloning. The sequence of primers used appears in *SI Appendix*, Table S7.

Western Blot Analysis of Pfs47 Protein Expression. Expression of *Pfs47* protein in gametocytes from different *P. falciparum* lines was detected by Western blot (*SI Appendix*, Fig. S6) as previously described (14). Briefly, gametocytes were isolated by saponin treatment and frozen at −70 °C until used. The frozen pellet was resuspended in 100 μL of water and 5 μL of it was mixed with NuPage LDS Sample Buffer, heated at 70 °C for 10 min, fractionated in a 4–12% NuPage Bis Tris gradient gel (Novex), and transferred to nitrocellulose using the iBlot dry blotting system (Invitrogen). The blot was blocked with 5% (wt/vol) milk in Tris-buffered saline with Tween 20 (0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, pH 8; 0.05% Tween 20) (TBST) overnight at 4 °C, followed by incubation with anti-Pfs47 rat monoclonal antibodies (1 mg/mL) diluted 1:500 in TBST-milk solution for 2 h at RT. Subsequently, the blot was incubated for 1 h at RT with anti-rat IgG alkaline phosphatase conjugate (1 mg/mL; Promega) diluted 1:10,000 in TBST-milk solution. Antibody staining was detected with Western Blue stabilized substrate (Promega).

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