Natural Malaria Infection in Anopheles gambiae Is Regulated by a Single Genomic Control Region

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Natural Malaria Infection in *Anopheles gambiae* Is Regulated by a Single Genomic Control Region

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We surveyed an *Anopheles gambiae* population in a West African malaria transmission zone for naturally occurring genetic loci that control mosquito infection with the human malaria parasite, *Plasmodium falciparum*. The strongest *Plasmodium* resistance loci cluster in a small region of chromosome 2L and each locus explains at least 89% of parasite-free mosquitoes in independent pedigrees. Together, the clustered loci form a genomic *Plasmodium*-resistance island that explains most of the genetic variation for malaria parasite infection of mosquitoes in nature. Among the candidate genes in this chromosome region, RNA interference knockdown assays confirm a role in *Plasmodium* resistance for *Anopheles Plasmodium-responsive leucine-rich repeat 1* (*APL1*), encoding a leucine-rich repeat protein that is similar to molecules involved in natural pathogen resistance mechanisms in plants and mammals.

The mosquito *Anopheles gambiae* is the major African vector of human malaria caused by *Plasmodium falciparum*. Genetically resistant and susceptible mosquitoes exist in nature, and that resistance can segregate as a simple Mendelian trait (*1*), but until now the prevalence, strength, and genomic location of such natural resistance loci in mosquitoes were not known. Genetic control of mosquito response to *Plasmodium* has been studied in an inbred laboratory strain of *A. gambiae* selected for the ability to melanize animal malaria parasites (*2*), for which *A. gambiae* is not a natural vector. However, associations uncovered in laboratory genetic mapping experiments may (*3*) or may not (*4*) hold when tested in nature.

We sampled genetic variation in a natural mosquito population by initiating isofemale pedigrees from wild mosquitoes captured in village dwellings in Mali, and we used the pedigrees to map allelic variants that have major effects on parasite development (*5*). Because female *A. gambiae* mate only once (*5*, *6*), each mosquito pedigree was the progeny of a single-pair cross that occurred in nature. Mosquito pedigrees were challenged by feeding on blood from natural human malaria infections in the same village. Thus, these experiments captured vector-parasite interactions of the evolutionary pair responsible for malaria transmission in the local population.

Each pedigree was fed malaria-infected blood from a single *P. falciparum* gametocyte carrier, and unfed mosquitoes were removed from the analysis. Thus, absence of oocysts resulted from failure of parasite development rather than lack of feeding. At 7 to 8 days after the infected blood meal, we dissected all mosquitoes and counted normal oocyst-stage parasites on the midgut. The number of oocysts constitutes the quantitative phenotype. If melanization of parasites was observed, which is an insect response to many pathogens (*2*), the fraction of melanized oocysts (melanized oocysts per total oocyst number) was also counted and used as a second, distinct quantitative phenotype. DNA extracted from each mosquito was genotyped with the use of 25 microsatellite markers spaced throughout the genome (*1*, *7*), enabling us to perform a genome-wide scan with ~9–centimorgan (cM) resolution. Linkage between DNA markers and either of the two quantitative phenotypes was detected with the use of nonparametric tests (*1*).

Of 101 pedigrees that were generated and challenged, 27 met criteria for genotyping: (i) >20 mosquitoes, and (ii) at least 30% of mosquitoes with normal oocysts, or any mosquitoes with melanized parasites (fig. S1). Notably, 22 pedigrees had no infected individuals despite feeding on blood that contained gametocytes. Such completely uninfected pedigrees are not suitable for genetic analysis, but their large number suggests that mosquito resistance to *P. falciparum* is common.

Among 17 genotyped pedigrees [including two from our previous work (*1*)], significant linkage between quantitative infection phenotype and DNA marker(s) was detected in seven pedigrees [genome-wide P value < 0.05 by permutation (*5*)]: five loci linked to *P. falciparum* infection intensity (Pfin) and two linked to *P. falciparum* melanization (Pfmel) (Fig. 1 and Table 1). Overall, 41% of pedigrees analyzed revealed a significant locus, indicating that loci with a major effect on parasite development in the field population are widespread. All analyzed pedigrees were independently generated from wild isofemales. Each named locus was the only significant locus found in the pedigree. Secondary loci with influence on *Plasmodium* may segregate in some of these pedigrees, but if so, none had a strong enough influence on parasite development to be independently identified as a distinct locus with genome-wide significance.

Although our experiments detect significant linkage to loci on all three chromosomes, the loci with the strongest effect colocalize in a small region of chromosome arm 2L (Fig. 1 and Table 1). Four of the seven independent loci mapped to a 15–megabase (Mb) region of chro-

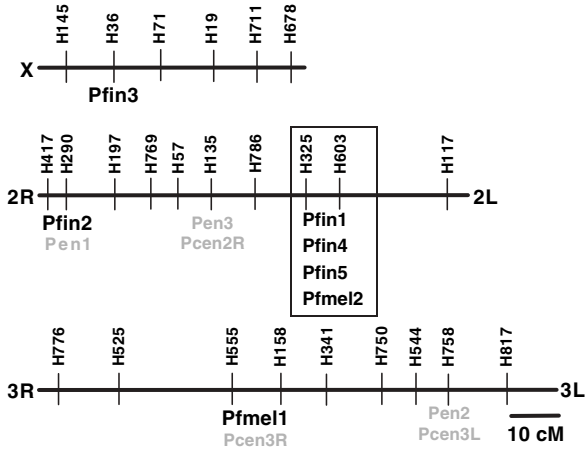

Fig. 1. Genome-wide *A. gambiae* linkage map. Genomic location of loci linked with Pfin (number of normal oocysts following infected blood meal) or Pfmel (fraction of melanized parasites) in wild isofemale pedigrees from Mali, West Africa. Each locus shows significant linkage in a single F1- or F2-generation pedigree to indicated marker locus. Horizontal lines indicate chromosomes (R, right arm; L, left arm), and vertical lines indicate microsatellite markers. Naturally occurring *P. falciparum* resistance loci are shown in black. Pen and Pcen loci (gray) indicate the genomic location of melanization loci mapped in a laboratory-selected *Plasmodium*-melanizing line of *A. gambiae* challenged with two different strains of the simian model parasite, *P. cynomolgi* (Pen, *P. cynomolgi* B; Pcen, *P. cynomolgi* Ceylon) (*15*, *16*). The box highlights the genomic cluster of loci with linkage to *P. falciparum* infection outcome in four independent mosquito pedigrees.

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mosome 2L, 5 Mb on either side of markers H603 and H325 [$P \leq 0.014$ for clustering of loci (*5*)]. For fine mapping of the clustered loci, six to ten additional markers (*5*) were tested in the four pedigrees (Fig. 2). Three of the four clustered loci (Pfin1, Pfin4, and Pfin5) stand out among all mapped loci, because they have by far the lowest *P* values and the greatest influence on parasite numbers (Table 1). For all three Pfin loci, the presence of a resistance allele explains at least 89% of parasite-free mosquitoes within a pedigree; the fourth locus in the cluster, Pfmel2, explains 100% of mosquitoes with melanized parasites within the pedigree. The four clustered loci display dominant or semidominant effects on parasite infection. In contrast, Pfin2 and Pfin3, located outside this cluster, display recessive inheritance of resistance and explain little of the variation in infection phenotype. Given the data, linkage in this region could be due to four distinct loci in the population, different allelic forms of the same locus, or a single causative mutation. Regardless, a specific chromosome region, which controls the majority of naturally segregating variation for *P. falciparum* infection detected in this study, is a major *Plasmodium*-resistance island (PRI) of the *A. gambiae* genome. Finding linkage in independent pedigrees over multiple years, with each pedigree fed on infected blood from a different gametocyte carrier, further suggests that the resistance effect controlled by the mosquito PRI is largely independent of parasite genotype.

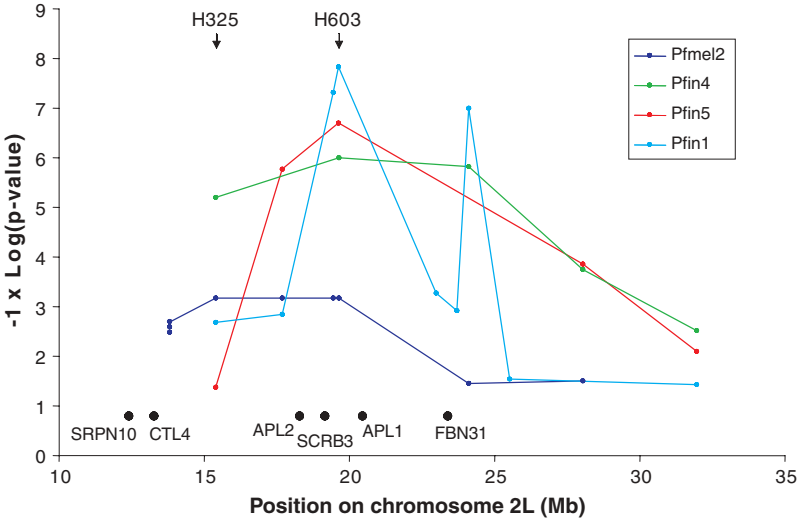

Fig. 2. Fine mapping of the *Plasmodium* resistance island. Nominal *P* values are shown as a function of position for the four pedigrees defining the PRI. The map shows consistent evidence of significant linkage over closely spaced microsatellite markers (points on graphed lines) in all four pedigrees with significant linkage to two distinct infection phenotypes, oocyst intensity (Pfin) and parasite melanization (Pfmel) (See (*5*) for markers). The locations of the markers yielding significant linkage signal in the initial 9-cM scan, H325 and H603, are indicated by arrows. Only the most informative markers are shown (≥3 genotypes in a pedigree). For clarity, the labeled candidate genes above the *x* axis are those annotated immune genes that were also either transcriptionally regulated after *P. berghei* infection or present in an immune-enriched EST library. Predicted functions or functional domains: SRPN10, serpin; CTL4, c-type lectin; SCRB3, scavenger receptor; and FBN31, fibrinogen. All candidate genes are shown in fig. S2.

The small size of the PRI makes feasible a search for candidate genes. Three filters were applied to the 976 Ensembl-predicted transcripts in this 15-Mb interval (Fig. 3 and fig. S2) (*5*). First, the interval contained 39 predicted members of mosquito immune gene families by sequence annotation, including putative functions in pathogen recognition, immune signal modulation, immune effectors, and signal transduction. Second, 13 genes in the interval were transcriptionally regulated on whole-genome microarrays after infection with *P. berghei*. Third, 29 genes were represented in an enriched *A. gambiae* immune expressed sequence tag (EST) library containing a wide spectrum of host defense genes, including transcriptional response to *Plasmodium*. Taking into account overlaps between categories, the filters highlight 72 genes. Despite caveats of the filters (e.g., causative genes might not belong to known families of immune genes or be transcriptionally regulated by *Plasmodium* infection), these genes represent a plausible set of candidate genes for initial screening.

Genes for two novel leucine-rich repeat (LRR)–containing proteins (Ensembl *A. gambiae* genome database accession codes ENSANGG00000012041 and ENSANGG00000019333) were the only ones to satisfy all filter criteria (Fig. 3). These genes are named *Anopheles Plasmodium-responsive leucine-rich repeat 1* (*APL1*) and *APL2* (respective to the accession codes above). Interestingly, the 39 immune genes by annotation in the PRI included 13 genes for LRR proteins, among them APL1 and APL2, and the numerically largest genomic cluster of LRR genes coincides with the PRI (fig. S3). LRR proteins have known roles in innate immunity in many phyla as direct or indirect pathogen recognition factors such as NACHT-LRR proteins, Toll-like receptors, and plant R genes (*8*–*10*). Plant R genes form genomic clusters of rapidly evolving homologs (*11*).

Table 1. Pedigrees with significant linkage. We examined two *P. falciparum* resistance phenotypes in *A. gambiae* 8 days after a malaria-infected blood meal: (i) normal parasite count and (ii) rate of parasite melanization. Nominal *P* values are derived from two-tailed Wilcoxon-Mann-Whitney tests, whereas genome-wide *P* values result from 10^5 to 10^8 simulations per pedigree, as appropriate. Neither of the pedigrees yielding Pfmel loci gave Pfin loci, nor did the Pfin4 pedigree give a Pfmel locus. Gen, generation after wild mother. *N*, number of females in the pedigree. Prevalence, percentage of mosquitoes in pedigree with at least one normal oocyst per midgut. Melanization rate, fraction of total malaria parasites melanized in individuals with at least one parasite melanized. Allele effect, dominant or codominant inheritance; genotype effect, recessive inheritance.

Locus	Location	Gen	*N*	Prevalence (%)	Oocyst range		Melanization rate (%)	*P* value		Effect
					Normal	Melanized		Nominal	Genome-wide	
Pfin1	2L	F2	83	67	0–116	0	0	1.47×10^{-8}	1.20×10^{-7}	Allele
Pfin2	2R	F2	82	96	0–66	0	0	7.56×10^{-4}	2.66×10^{-2}	Genotype
Pfin3	X	F2	152	72	0–31	0	0	1.12×10^{-4}	2.59×10^{-3}	Genotype
Pfin4	2L	F1	45	80	0–69	0–51‡	46 (24–91)	9.78×10^{-7}	3.02×10^{-6}	Allele
Pfin5	2L	F1	62	53	0–37	0	0	1.37×10^{-7}	5.60×10^{-7}	Allele
Pfmel1	3R	F1	40	100*	4–151	0–117	39 (2–85)	1.84×10^{-4}	4.98×10^{-3}	Allele
Pfmel2	2L	F1	21	100†	1–87	0–43	60 (31–77)	2.30×10^{-4}	1.35×10^{-3}	Allele

*Melanization prevalence (fraction of individuals in pedigree with at least one melanized parasite) is 42.5%. †Melanization prevalence is 29%. ‡Melanization prevalence is 22%.

The roles of APL1 and APL2 were functionally tested by RNA interference (RNAi)–mediated reductions of gene expression in laboratory colony mosquitoes infected with *P. berghei* (*5*). APL1 knockdown mosquitoes carried significantly higher numbers of oocysts (by a factor of 6 to 15, $P < 0.007$) than did controls treated with green fluorescent protein (GFP) double-stranded RNA (Fig. 4 and fig. S4). Few or no melanized parasites were observed in the APL1 and APL2 knockdown mosquitoes, similar to controls. In contrast, reduction of APL2 did not affect oocyst number ($P = 0.515$). Thus, APL1 mediates significant protection from *Plasmodium* infection and could underlie, at least in part, the phenotypic effect of the PRI. However, APL1 is only a candidate that must await further genetic and functional studies for confirmation of a role in natural transmission. In this regard, the phenotypes in the APL1 knockdowns and controls (high versus moderate oocyst number) do not precisely reproduce those observed in susceptible and resistant Pfin genotypes after natural infection (many versus zero oocysts). Exact replication of the field phenotype may require specific aspects of the genetic background found in field mosquitoes or the specific APL1 allele present in the natural pedigrees. Also, response to *P. berghei* may be different from response to *P. falciparum*. Resolving the exact role, if any, of APL1 in the natural transmission system will require additional fieldwork.

There is no previous information on genetic control of malaria parasite melanization in nature. Our study shows that control of melanization maps to two different loci, Pfmel1 and Pfmel2. Melanization was inefficient as a resistance mechanism because parasite removal by melanization was only partial, as compared with complete elimination by the Pfin loci. Moreover, pedigrees segregating Pfmel alleles displayed 100% prevalence of normal parasites despite the ability to melanize (Table 1). The particularly high prevalence of normal parasites could indicate that natural melanization alleles, while killing the melanized parasites, may also cause defects in other mechanisms that otherwise limit parasite numbers. On balance, the observed melanizing genotypes would likely result in more, not less, efficient transmission of *Plasmodium* in nature. Given the data from these pedigrees, melanization of parasites in *A. gambiae* is not a major component of natural *Plasmodium* resistance.

We report a comprehensive population survey of *A. gambiae* in Mali using a study design that allows identification and genetic mapping of loci with major effect on *P. falciparum* development in the field population. We detected genetic loci responsible for two distinct outcomes of *Plasmodium* infection, variation in numbers of normal parasites and rate of parasite killing by melanization. Most loci with major effect were clustered, forming a significant PRI in the *A. gambiae* genome. Annotation information and laboratory-based experiments were used to extract candidate genes for functional testing.

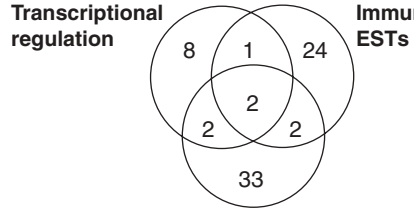

Fig. 3. Candidate gene filtering. Venn diagram of candidate genes in the 15-Mb interval surrounding the PRI, 5-Mb outside markers H325 and H603 [nucleotide coordinates 2L:10398172 to 24623620 on the 2La inverted chromosome (*5*)]. Venn segments indicate numbers of genes with changes in transcript abundance 24 hours after *P. berghei* infection by whole-genome microarray (false discovery rate = 0.05), predicted immune genes by annotation, and genes represented by ESTs in a subtracted immune-enriched library (*5*). Intersection of the three filtering criteria within the PRI contains the two predicted LRR-containing proteins APL1 and APL2.

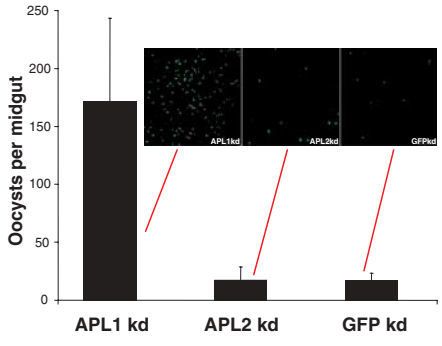

Fig. 4. Silencing of APL1 gene expression increases *Plasmodium* infection. APL1 significantly limits the efficiency of malaria parasite infection in four independent knockdown experiments. APL1 kd, oocyst numbers in mosquitoes 7 to 8 days after RNAi-mediated reduction of APL1 transcript abundance followed by infection with *P. berghei*; APL2 kd, mosquitoes treated with APL2 double-stranded RNA; GFP kd, control mosquitoes treated with GFP double-stranded RNA. The APL1 and GFP data are averages of the mean oocyst number from four independent experiments, and error bars indicate standard error. APL2 data comes from three replicate experiments that tested APL2 along with APL1 and GFP control. The APL1 kd results in oocyst loads 6 to 15 times as high as those of GFP controls ($P < 0.007$). The APL1 kd effect was significant by Wilcoxon-Mann-Whitney, Student's *t* test, and Kolmogorov-Smirnov when analyzed for pooled data across replicates or in analysis of individual replicates. The APL2 kd had no significant effect on parasite number ($P = 0.515$). (Inset) Fluorescence micrographs of oocysts [constitutively fluorescent line PbGFPCON (*17*)] in midguts of APL1 kd, APL2 kd, and GFP kd mosquitoes, respectively.

Examination of malaria transmission in wild populations is essential to understand and ideally capitalize on the efficient malaria conrol strategies already implemented by *A. gambiae* in nature. For example, one possible malaria control approach would increase the population frequency of pre-existing natural resistance alleles like those described here by elevating the fitness cost of malaria parasite infection (*12*). Recently reported entomopathogenic fungi can disproportionately kill *Plasmodium*-infected mosquitoes as compared with uninfected ones (*13*, *14*), and thus could have the properties required of a selective agent to transform vector populations to *Plasmodium* resistance. Such an evolutionary engineering strategy would not require introduction of new genetic information into natural vector populations.

The most notable feature of the observed mosquito resistance is that it segregates as a simple Mendelian trait of major effect at reasonably high frequency in randomly sampled natural genotypes. Interestingly, many mosquito pedigrees completely eliminated the parasite despite feeding on infected blood. We speculate that the "wild-type" mosquito phenotype is resistance and that susceptibility should be attributed to specific points of failure or loss of function in the mosquito immune system.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5773/577/DC1
Materials and Methods
Figs. S1 to S4
References

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