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Quantitative Trait Loci Controlling Refractoriness to *Plasmodium falciparum* in Natural *Anopheles gambiae* Mosquitoes From a Malaria-Endemic Region in Western Kenya

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ABSTRACT

Natural anopheline populations exhibit much variation in ability to support malaria parasite development, but the genetic mechanisms underlying this variation are not clear. Previous studies in Mali, West Africa, identified two quantitative trait loci (QTL) in *Anopheles gambiae* mosquitoes that confer refractoriness (failure of oocyst development in mosquito midguts) to natural *Plasmodium falciparum* parasites. We hypothesize that new QTL may be involved in mosquito refractoriness to malaria parasites and that the frequency of natural refractoriness genotypes may be higher in the basin region of Lake Victoria, East Africa, where malaria transmission intensity and parasite genetic diversity are among the highest in the world. Using field-derived F_2 isofemale families and microsatellite marker genotyping, two loci significantly affecting oocyst density were identified: one on chromosome 2 between markers AG2H135 and AG2H603 and the second on chromosome 3 near marker AG3H93. The first locus was detected in three of the five isofemale families studied and colocalized to the same region as *Pen3* and *pfin1* described in other studies. The second locus was detected in two of the five isofemale families, and it appears to be a new QTL. QTL on chromosome 2 showed significant additive effects while those on chromosome 3 exhibited significant dominant effects. Identification of *P. falciparum*-refractoriness QTL in natural *An. gambiae* mosquitoes is critical to the identification of the genes involved in malaria parasite transmission in nature and for understanding the coevolution between malaria parasites and mosquito vectors.

MALARIA is the most important parasitic disease in the world. It continues to place tremendous health and economic constraints on a large percentage of the world's population despite efforts to contain it; hence, there is a tremendous need to develop new means of control (World Health Organization 1998; Breman *et al.* 2004; Snow *et al.* 2005). Among the novel approaches being pursued is the proposed genetic manipulation of the malaria vector to disrupt parasite transmission (Curtis 1994; Aultmann *et al.* 2001) by taking advantage of observed natural refractoriness. Although natural refractoriness is not a new observation (Vargas 1949), its underlying mechanisms remain unknown (Vernick *et al.* 2005). The realization that the immune system of mosquitoes has the potential to kill malaria parasites at several developmental stages (Richman and Kafatos 1996) has inspired several studies aimed at unraveling the molecular mechanisms of the mosquito's immune responses against malaria parasites. Previous studies have identified a number of molecules inhibiting malaria parasite development in mosquitoes, including antimicrobial peptides (Gwadz *et al.* 1989; Lambrechts *et al.* 2004), signaling pathways and pattern recognition peptides (Barillas-Mury *et al.* 2000; Dimopoulos *et al.* 2001), monoclonal antibodies (De Capurro *et al.* 2000), and SM1 (Ito *et al.* 2002; Jacobs-Lorena 2003).

In parallel with efforts to understand the mosquito's immune mechanisms are genetic approaches aimed at identification and characterization of refractoriness. Such approaches have been used to define melanotic encapsulation of oocysts (Collins *et al.* 1986) and ookinete penetration failure (Vernick *et al.* 1995). Several quantitative trait loci (QTL) conferring encapsulation and melanization of malaria parasites and negatively charged sephadex beads in mosquitoes have been localized (Gorman *et al.* 1997; Zheng *et al.* 1997; Thomasova *et al.* 2002). Natural *Anopheles gambiae* populations in Africa do not normally show the encapsulation/melanization phenotype to Africa-origin *Plasmodium falciparum*; however, a substantial proportion of *An. gambiae* individuals in a population do not support successful parasite development in the midguts. Identification of a genetic mechanism of natural refractoriness (*i.e.*, failure of ookinetes to develop into oocysts in mosquito midgut) is particularly interesting. Niare *et al.* (2002)

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used linkage analysis approaches and identified two genomic regions on chromosome 2 that confer *An. gambiae* mosquito natural refractoriness to *P. falciparum* parasites in Mali, West Africa.

Because of large genetic differentiation between West Africa and East Africa *An. gambiae* populations (Lehman *et al.* 2003), different *P. falciparum*-refractoriness QTL may be involved in the East Africa mosquito populations. In addition, malaria transmission in the western Kenya region is perennial, and the area experiences the highest malaria transmission intensity in the world (annual entomological inoculation rate in the order of hundreds) (Beier *et al.* 1999), while Mali exhibits seasonal malaria transmission with much lower intensity (Kleinschmidt *et al.* 2001). We hypothesize that the frequency of natural refractoriness genotypes is higher, as would be expected under a higher selection pressure by the malaria parasites (Schwartz and Koella 2001). The goal of this study is to determine whether new *P. falciparum*-refractoriness QTL, in addition to the QTL previously identified by Niare *et al.* (2002), can be detected and whether *P. falciparum*-refractoriness QTL are common in the western Kenya *An. gambiae* populations. Our study identified two QTL, one detected in three of the five isofemale families studied and colocalized to the same region as *Pen3* and *pfin1* described previously (Zheng *et al.* 1997; Niare *et al.* 2002); the second, located on chromosome 3 and detected in two of five isofemale families, appears to be a new QTL.

MATERIALS AND METHODS

Breeding mosquito pedigrees and experimental design: The mosquitoes used in this study were obtained from gravid female *An. gambiae* individuals collected from Mbita, a village on the shores of Lake Victoria in western Kenya where high malaria prevalence and high transmission intensity were reported (Mutero *et al.* 1998; Shililu *et al.* 2003). Gravid wild-caught females were kept in the insectary at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology in Mbita Point. Temperature and humidity in the insectary were not regulated. The mosquitoes were supplied with a 6% glucose solution on cotton wicks for two days. They were then put in individual oviposition cups, and eggs from each individual were reared separately to generate F_1 isofemale families. Individuals of each F_1 family were allowed to mate within their family, and females were given a blood meal to enable them to lay eggs. Eggs from each F_1 family were reared together to give rise to F_2 families. Mature 5- or 6-day-old F_2 females were given infectious blood meals and then dissected 8 days postinfection to obtain the quantitative phenotype data. After oviposition, legs from each wild-caught mosquito were used to identify *An. gambiae* spp. by species identification PCR (Scott *et al.* 1993). The carcasses of the wild parents and the F_1 females used to generate F_2 families were saved for microsatellite genotyping. Because the first mating renders *An. gambiae* females refractory to subsequent insemination (Craig 1967; Gwadz *et al.* 1971), and the majority (>97%) of female *An. gambiae* mosquitoes mate only once in nature (Tripet *et al.* 2001), each of the F_2 isofemale families is likely a product of a single pair cross.

Gametocyte carrier screening: *P. falciparum* carriers were recruited from 2- to 10-year old children in the rural area around Mbita using a protocol approved by the institutional review board of the State University of New York at Buffalo and the ethical review board of the Kenya Medical Research Institute. Finger-prick blood samples were collected and thick blood smears were air dried, stained with 8% Giemsa for 15 min, and microscopically examined for the presence of *P. falciparum* trophozoites and gametocytes. The gametocyte densities were assessed by counting against 500 leukocytes and converted to number of parasites per microliter by assuming a standard leukocyte count of 8000/μl. Asymptomatic gametocyte-positive children were recruited as donors of infectious blood meals for mosquito membrane feeding.

Experimental infections and phenotyping: Experimental infections took place in the afternoon following the morning finger-prick examinations. A clinician from Mbita Health Center drew 2 ml of venous blood into heparinized tubes prewarmed to 37°. Prewarming was necessary to guard against a lower temperature that might mimic temperatures in the mosquito gut and thus induce exflagellation of macrogametocytes before they could be ingested by the mosquitoes. To reduce human factors such as transmission-blocking immunity (Mulder *et al.* 1994) the blood was centrifuged at 37° for 3 min at 2000 × *g* and the serum was replaced with the same volume of nonimmune AB serum purchased from Sigma. The mixture was used to feed 5- or 6-day-old mosquitoes that had been starved for 12–16 hr using parafilm membrane feeders that were prewarmed to 37° (Tchuinkam *et al.* 1993). Mosquitoes were allowed to feed for 30 min, after which the fed mosquitoes were placed into cages 15 × 15 × 15 cm^3 in size. These mosquitoes were then maintained in the insectary on 6% glucose in cotton wicks that were changed daily to avoid bacterial contamination. Eight days after blood feeding, mosquitoes were dissected and their mid-guts were stained with 2% mercurochrome in distilled water to determine the number of oocysts with light microscopy. The number of oocysts in the midguts of individual mosquitoes constituted targeted phenotype.

Microsatellite genotyping and linkage analysis: Genomic DNA was extracted individually from all the parents, F_1, and F_2 isofemale families following the phenol/chloroform method of Serverson (1997). DNA from wild-caught female parents used as isofemale family founders and DNA from each F_1 pedigree were used to establish the segregation pattern of the markers used in molecular typing. Molecular typing used 20 polymorphic microsatellite markers selected from published linkage maps (Zheng *et al.* 1996; Wang *et al.* 1999) to cover the two autosomal chromosomes and the X chromosome for broad genome coverage. Microsatellite primers for PCR labeled with fluorescent M13 dye were obtained from Integrated DNA Technologies (Coralville, IA) and used to amplify microsatellite alleles according to the supplier's recommendations. PCR products were loaded and separated on polyacrylamide gels using a Li-Cor sequencer (Li-Cor). The size of microsatellite alleles was determined with Li-Cor GeneImagIR software and genotypes were assigned to individual mosquitoes on the basis of allele sizes.

Data analysis: For each F_2 isofemale family, mating occurred in nature and the fathers were not available for genotyping. Thus, paternity genotypes of the wild parents were inferred from allele separation patterns on polyacrylamide gels using multipoint analysis (Lander and Green 1987; Kruglyak *et al.* 1996; Markianos *et al.* 2001). Determination of paternal genotypes was necessary because linkage maps based on interval mapping require knowledge of the *cis*- or *trans*-relationships between alleles at different loci. Paternal reconstruction is straightforward for any family of >10 progeny, a condition met

TABLE 1

Prevalence and intensity of oocyst infections in F_2 isofemale *Anopheles gambiae* families

Isofemale family	No. of F_2 females[a]	Parasite/μl[b]	Prevalence[c]	Infection intensity range[d]	Mean intensity[e]
101	38	352	0.53	28	8.58 (0.95)
102	41	352	0.39	33	11.26 (1.10)
201	58	736	0.19	11	6.15 (0.90)
202	69	480	0.32	16	7.83 (0.86)
203	47	480	0.23	14	4.60 (1.18)

[a] F_2 individuals of each isofemale family were from a single wild-caught female.
[b] Number of gametocytes per microliter at the time of feeding.
[c] Proportion of F_2 individuals infected with malaria parasites per family.
[d] Infection intensity range refers to the difference between the highest and lowest number of oocysts in the infected mosquitoes.
[e] Mean intensity refers to the average infection rates among infected mosquitoes per family. SE values are shown in parentheses.

by all of the isofemale families in the study. Any families that were contaminated or that resulted from multiple matings were clearly evident because of the presence of aberrant nonparental genotypes, and they were discarded from the analysis. Analysis of the data to locate QTL for each family was done by least-squares interval mapping (Seaton *et al.* 2002) using QTL Express (http://qtl.cap.ed.ac.uk), with the thresholds for statistical significance determined by a permutation test (1000 permutations) for each chromosome. Confidence intervals for QTL location were obtained by bootstrapping (Visscher *et al.* 1996). For each isofemale family in which a significant QTL was detected, each of the three mosquito chromosomes was analyzed separately. Significant QTL were identified and the analyses were then repeated for each significant QTL while holding all other significant QTL as cofactors. This determines epistatic interactions between the significant QTL and any other loci on the whole genome. The data were also analyzed by maximum likelihood composite interval mapping (Wang *et al.* 2001–2005) using QTL Cartographer version 2.5 (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm).

RESULTS

Experimental infections and phenotyping: Five F_2 isofemale families of *An. gambiae* were developed and successfully infected and genotyped (Table 1). The prevalence (percentage of infected mosquitoes) among the isofemale families varied from 19 to 53% whereas the mean oocyst counts varied from 4.60 to 11.26. Two families, coded 101 and 102, were developed and phenotyped during the short rainy season in October 2003. A *t*-test on the mean number of oocysts as a measure of parasite load in the two isofemale families gave a significant difference ($P < 0.05$). Because these two families were reared under identical environmental conditions and exposed to an infectious blood meal from the same gametocyte donor, the difference in oocyst density was caused by genetic variations between mosquito families. Three families, coded 201, 202, and 203, were developed and phenotyped during the long rainy season, May through June 2004. Of these three families, 202 and 203 were reared under identical environmental conditions and given an infectious blood meal from the same gametocyte donor. Similarly, *t*-test on the mean number of oocysts between the families gave a significant difference ($P < 0.05$), indicating significant genetic variation in mosquito susceptibility to malaria parasite infection.

Microsatellite genotyping and linkage analysis: In all isofemale families, six loci were genotyped on the X chromosome and seven loci were genotyped on each of the second and third chromosomes. Given that the genome size of *An. gambiae* is 215 cM (Zheng *et al.* 1996), the resolution of our map scan is 10.75 cM. This marker density is appropriate for detecting and mapping of *An. gambiae* QTL at F_2 recombination (Darvasi *et al.* 1993; Broman 2001). Genotypes of the F_2 isofemale progeny were tested for Mendelian segregation distortion at each locus within each family. Significant segregation distortions were found for some loci, but the affected loci were not clustered on any particular chromosomal region and thus could not affect QTL analysis. Significant QTL for refractoriness were detected on chromosomes 2 and 3 in some isofemale families. None were detected on chromosome X. The two methods of QTL analysis gave similar results for QTL position and effects. Table 2 shows a summary of LOD score peaks and their locations, additive and dominance effects, and the variance explained by respective QTL. Figure 1 shows the QTL profiles for all studied isofemale families on chromosomes 2 and 3. Markers flanking peak LOD scores and 95% confidence intervals for QTL-containing regions are given in Table 3. The results given in Tables 2 and 3 and Figure 1 are from least-squares analysis by QTL Express, with the exception of the variance explained by the QTL that were derived from the maximum likelihood analysis with QTL Cartographer.

For chromosome 2, three of five isofemale families studied showed significant QTL for the number of oocysts in F_2 populations, whereas two isofemale families showed significant QTL on chromosome 3. The

TABLE 2

Summary of location and effects of detected QTL for refractoriness against *Plasmodium falciparum* infection in *Anopheles gambiae* isofemale families

Chromosome	Statistic	Isofemale family				
		101	102	201	202	203
2	LOD score	2.26*	0.73	2.41**	1.69*	0.90
	Location (cM)[a]	44	20	44	51	46
	Additive effect[b]	−2.12 (0.62)	0.31 (0.63)	−1.93 (0.62)	−0.74 (0.28)	0.54 (0.49)
	Dominance effect[c]	−0.78 (0.95)	0.54 (1.2)	−1.25 (0.95)	0.45 (0.41)	0.36 (0.19)
	Variance (%)[d]	10.2	1.0	11.0	8.3	1.3
3	LOD score	1.97*	0.79	0.85	0.81	3.53**
	Location (cM)[a]	6	36	67	48	5
	Additive effect[b]	−1.24 (0.86)	0.09 (0.14)	−0.02 (0.26)	0.16 (0.73)	−1.6 (0.95)
	Dominance effect[c]	−2.42 (1.03)	0.13 (0.61)	0.75 (0.38)	0.05 (0.12)	−3.23 (1.02)
	Variance (%)[d]	9.1	2.1	4.1	3.8	14.2

* $P < 0.05$; ** $P < 0.01$.

[a] Location (in centimorgans) was estimated at the position of maximum LOD score.

[b] The negative additive effect indicates genetic effects for refractoriness. Numbers in parentheses are SE.

[c] The negative dominance effect suggests that heterozygotes had lower parasite intensity when compared with homozygotes. Numbers in parentheses are SE.

[d] Variance is the proportion of phenotypic variance explained by each QTL.

significant QTL detected on chromosome 2 in this study exhibited a significant additive genetic effect, but the respective dominance effects were not significant. In addition, these QTL consistently showed negative additive effects, suggesting that the QTL detected in the three isofemale families on chromosome 2 are likely to be the same loci and affect malaria parasites in a similar manner. Conversely, the significant QTL detected in two isofemale families for chromosome 3 appear to be acting dominant as indicated by their respective standard errors. The dominance effect measures the degree to which heterozygous individuals express the trait above or below the average for homozygous individuals. A positive sign will indicate that heterozygous mosquitoes exhibit a higher value than the average for homozygous mosquitoes, while a negative value will indicate that heterozygous mosquitoes have a lower average number of oocysts. In this study, the significant QTL on chromosome 3 had a negative dominance effect implying that they are associated with refractoriness to *P. falciparum* infection. From the genetic effects of the detected QTL and their relative positions on the two chromosomes, the most parsimonious interpretation of these results is that there is one additive QTL on chromosome 2 and one dominant QTL on chromosome 3 and that these control refractoriness in families in which significant QTL were detected, rather than multiple QTL occurring in different families.

DISCUSSION

The identification of chromosomal regions associated with resistance against infection by *P. falciparum* in *An. gambiae* is the starting point for efforts to clone and characterize the actual genes that confer refractoriness. Identification of such genes is important not only for understanding the biological mechanisms of refractoriness but also for insights into the coevolutionary relationships between mosquitoes and malaria parasites in different ecological regions where malaria is endemic. A number of studies have been undertaken to map chromosomal regions associated with different mechanisms of refractoriness to malaria parasites by mosquito vectors. Zheng *et al.* (1997) identified one major QTL named *Pen1* and two minor QTL named *Pen2* and *Pen3* for encapsulation of *P. cynomolgi* B by *An. gambiae*. Similarly, Gorman *et al.* (1997) identified a QTL, which appeared to be identical to *Pen1*, for melanin deposition on charged sephadex beads and encapsulation of *P. berghei* by *An. gambiae*. In further analyses, *Pen1* has been fine mapped to a region spanning 1.5 Mb of DNA, allowing comparison of *An. gambiae* and *Drosophila melanogaster* at the sequence level. This has revealed unusually high polymorphism at this locus (Thomasova *et al.* 2002). A study on *P. cynomolgi* Ceylon encapsulation by *An. gambiae* identified two chromosomal regions similar to *Pen2* and *Pen3* on chromosome 2 and another new QTL that mapped to chromosome 3; this implied variation of QTL for the encapsulation mechanism that varies with parasite species (Zheng *et al.* 2003). Despite the identification of major QTL for encapsulation using laboratory and field experiments, no single causative locus has yet been isolated. In addition, encapsulation of African-origin *P. falciparum* is not efficient in its natural vector, *An. gambiae*. Studies with *An. gambiae* mosquitoes from Tanzania

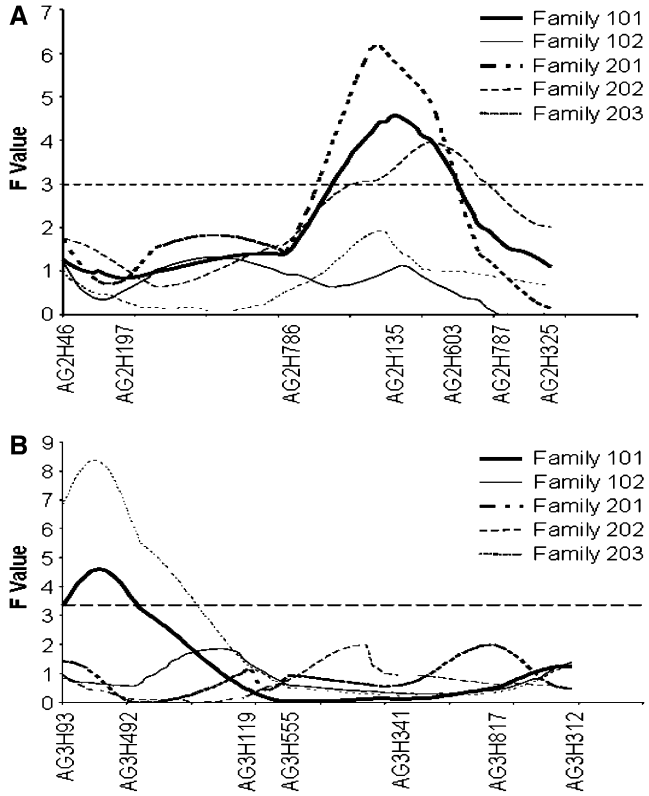

FIGURE 1.—Profiles of quantitative trait loci mapping on chromosome 2 (A) and 3 (B) for different isofemale families. The markers listed on the *x*-axis are microsatellite markers. The *F*-values were derived from least-squares analyses and the horizontal line indicates the significant linkage association at $P < 0.05$ level.

have shown that <1% of the mosquitoes could encapsulate *P. falciparum*, but 90% of them could melanize sephadex beads (SCHWARTZ and KOELLA 2002). More studies using natural vector–parasite interactions with the tools and knowledge acquired from studies with inbred strains are needed. Continued dissection of the mosquito genome, finer-scale mapping, increased functional characterization of genes and their protein products, and continued candidate gene analysis of mosquitoes' innate immunity genes will lead to more informed efforts to identify malaria refractory genes.

This study identified a QTL on chromosome 2 associated with microsatellite markers AG2H603 and AG2H135 in three isofemale families. The second locus detected in this study is near the proximal end of chromosome 3 near microsatellite marker AG3H93, and it appears to be a novel locus. The QTL detected on chromosome 2 was significant in three isofemale families, although these families were exposed to infectious blood meals from different gametocyte donors. Further, these QTL shared the same chromosomal regions and had similar genetic effects, suggesting the same gene or genes on chromosome 2 may be used against different parasite isolates by different mosquitoes. A previous linkage analysis study using F_2 isofemale families of *An. gambiae* developed from wild-caught females was done in Mali, West Africa, and identified two QTL named *pfin1* near AG2H603 and *pfin2* near AG2H290 (NIARE *et al.* 2002). In comparison to the West Africa study, our results suggest that the locus on chromosome 2 described here could be the same as *pfin1*. Interestingly, the region also encompasses the *Pen3* locus, a locus associated with the parasite encapsulation mechanism (GORMAN *et al.* 1997; ZHENG *et al.* 1997). This suggests that a common genetic factor determining mosquito vector response to malaria parasites is located in this chromosomal region. The second QTL, *pfin2*, detected by NIARE *et al.* (2002), was not detected in this study. The marker associated with *pfin2*, AG2H290, was consistently monomorphic in all the isofemale families used in this study. Failure to detect *pfin2* and detection of a new QTL on chromosome 3 in our study suggests that additional genes and different gene combinations are involved in refractoriness to malaria parasites in western Kenya. This raises the possibility that different QTL may be responsible for refractoriness in different Plasmodium–mosquito interactions. This observation is not unique, as ZHENG *et al.* (2003) have demonstrated that different QTL are responsible for encapsulation of different strains of *P. cynomolgi*. Such observations have also been made in *D. melanogaster*, where a fly strain that encapsulates eggs of the parasitic wasp *Leptopilina boulardi* L104 from Congo is susceptible to *L. boulardi* G317 from Tunisia and *L. heterotoma*, implying that expression of

TABLE 3

Location, confidence intervals, and flanking markers for significant refractoriness QTLs to *Plasmodium falciparum* infection in *Anopheles gambiae* isofemale families

Chromosome	Isofemale family	Position (cM)	95% confidence interval of the QTL position (cM)	Flanking markers
2	101	44	33.5–52.1	AG2H786–AG2H787
	201	44	38.0–49.2	AG2H786–AG2H787
	204	55	44.5–61.2	AG2H135–AG2H787
3	101	6	1–17.5	AG3H93–AG3H492
	203	5	1–22.4	AG3H93–AG3H492

resistance to parasites varies among host–parasite systems (Carton and Nappi, 1997; Lambrechts *et al.* 2006). The design of the present study does not, however, allow the evaluation of whether mosquitoes from East and West Africa exhibit different refractoriness QTL or whether the genetic difference in the parasites between East and West Africa is dealt with by different QTL in mosquitoes. Genetic characterizations of factorial combinations between mosquito genotypes and parasite genotypes from the two regions are needed to answer this question.

Since the QTL detected in this study were based on the phenotype of infection intensities, as established by number of oocysts on day 8 postinfection, the loci could be associated with several biological mechanisms that reduce the number of oocysts. Such mechanisms include destruction of oocysts in ways similar to intracellular lytic responses in the midgut (Vernick *et al.* 1995), humoral responses against oocysts (Paskewitz *et al.* 1988), and mosquito defensin, an antibacterial peptide that destroys malaria parasites if a concurrent bacteria infection primes the mosquito's immune system (Lowenberger *et al.* 1999; Lambrechts *et al.* 2004). Several functional candidate gene analyses have implicated a number of genes and mechanisms that affect the ookinete and oocyst stages of development. Examples include thio-ester-containing proteins, leucin-rich protein, and c-type lectins (CTL4 and CTLMA2) that have been shown to influence refractoriness (Blandin *et al.* 2004; Osta *et al.* 2004). The knowledge and tools developed in such studies are useful in analyzing natural vector–parasite systems.

In summary, we identified two QTL conferring *An. gambiae* refractoriness to the *P. falciparum* parasite. These two QTL exhibit different genetic effects. The QTL on chromosome 2 is additive, while the one on chromosome 3 shows a dominant effect. The QTL on chromosome 2 maps to a similar region as *Pen3* and *pfin1*, reported in previous studies. The QTL on chromosome 3 is novel. The two QTL explain ~20% of the variation in parasite density, suggesting that other not-yet-identified loci may be involved. Characterization of the natural mosquito populations in responses to infection and development of natural malaria parasites will provide valuable insights into the molecular basis of host resistance to malaria parasites and coevolution between mosquito vectors and malaria parasites. This will have important implications for the development of novel strategies for controlling malaria transmission.

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