

Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of *Anopheles gambiae*

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Insecticide resistance is an ideal model to study the emergence and spread of adaptive variants. In the African malaria mosquito, *Anopheles gambiae*, this is complemented by a strong public health rationale. In this insect, resistance to pyrethroid and DDT insecticides is strongly associated with the mutations L1014F and L1014S within the para voltage-gated sodium channel (VGSC). Across much of West Africa, 1014F frequency approaches fixation. Here, we document the emergence of a mutation, N1575Y, within the linker between domains III–IV of the VGSC. In data extending over 40 kbp of the VGSC 1575Y occurs on only a single long-range haplotype, also bearing 1014F. The 1014F–1575Y haplotype was found in both M and S molecular forms of *An. gambiae* in West/Central African sample sites separated by up to 2,000 km. In Burkina Faso M form, 1575Y allele frequency rose significantly from 0.053 to 0.172 between 2008 and 2010. Extended haplotype homozygosity analysis of the wild-type 1575N allele showed rapid decay of linkage disequilibrium (LD), in sharp contrast to the extended LD exhibited by 1575Y. A haplotype with long-range LD and high/increasing frequency is a classical sign of strong positive selection acting on a recent mutant. 1575Y occurs ubiquitously on a 1014F haplotypic background, suggesting that the N1575Y mutation compensates for deleterious fitness effects of 1014F and/or confers additional resistance to insecticides. Haplotypic tests of association suggest the latter: The 1014F–1575Y haplotype confers a significant additive benefit above 1014F–1575N for survival to DDT (M form $P = 0.03$) and permethrin (S form $P = 0.003$).

kdr | selective sweep | inactivation particle

The impetus to eradicate malaria has yielded a significant reduction of malaria mortality and morbidity via antiparasite artemisinin combination therapies (1) and scaling up of coverage with insecticide-based interventions (2). Vector control interventions are based primarily around either provision of long-lasting insecticide-treated nets (LLINs) or indoor residual spraying of insecticide onto surfaces where mosquitoes rest after blood feeding. A major threat to the success of these interventions is the development of insecticide resistance in malaria vectors (3, 4). Resistance is a particular threat to LLINs, as there is currently only one class of insecticides, the pyrethroids, approved by WHO for impregnation of bednets. Resistance is typically evaluated through phenotypic bioassays, although DNA-based diagnostics are supplementing such assays as part of resistance monitoring strategies (3).

In Sub-Saharan Africa, the primary vectors of malaria are *Anopheles gambiae sensu stricto* and *Anopheles arabiensis*. In *An. gambiae s.s.*, two molecular forms, M and S, are recognized, occurring in sympatry throughout West and Central Africa (5). Interform gene flow is geographically variable (6) but sufficient to allow introgression and spread of selected alleles (7). A series of knockdown resistance (*kdr*) mutations in the sodium channel are the best characterized resistance mechanisms in *Anopheles*. The presence of *kdr* has been conclusively linked to reduced mortality following exposure to both DDT and pyrethroids in

a large number of studies (3, 8). Pyrethroids and DDT target the insect voltage-gated sodium channel (VGSC), binding to the open (activated) sodium channel pore and preventing inactivation (9). Several mutations within the sodium channel have been identified in an array of insects and cause varying degrees of resistance (reviewed in ref. 10). Many of these mutations occur at key residues within the so-called binding pocket enclosed by the IIS4–S5 linker and IIS5/III6 helices (9). In *An. gambiae s.s.*, two single-base-pair substitutions occur at codon 1014 within segment 6 of domain II (numbering according to the housefly *para* sequence, GenBank accession no. X96668) resulting in substitution of leucine with either phenylalanine or serine (11, 12). The ready availability of assays for the *kdr* 1014 mutations has led to their routine screening as partial resistance diagnostics in *An. gambiae*. However, insecticide resistance is a rapidly evolving trait (13, 14) and, particularly in large populations, new mutations can arise frequently (15).

Identifying adaptive mutations in natural populations poses a significant challenge. Alleles positively selected from standing variation are difficult to detect owing to the time it usually takes for new variants to reach sufficient frequencies, during which period recombination will break down linkage disequilibrium (LD) with marker loci (16). This is particularly problematic for organisms such as *An. gambiae*, where LD is very short (17–19).

For de novo mutations, reduced heterozygosity at linked sites represents a signature of strong selection and can be used as a means to identify variants at selective advantage. In human genetics, for example, strong evidence exists for signatures of adaptation associated with malaria resistance genes, including the Duffy antigen protein (20) and Glucose-6-phosphate dehydrogenase (21).

Insecticide resistance provides an ideal model to study the adaptation of newly emerged alleles. First, resistance emerges over a relatively short period, and second, because we know when synthetic insecticides were introduced for insect control, we can estimate when positively selected alleles may have arisen in a population and trace their ancestry. For the *An. gambiae s.s.* VGSC, we have shown previously that two mutations at codon 1014 have been subject to strong selection, have risen to high frequency, and show extended long range LD indicative of a selective sweep (7). However, the observation of putative recombination at the telomeric end of 1014F carrying haplotypes

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Data deposition: The SNPs reported in this paper have been deposited in the dbSNP Short Genetic Variations database, www.ncbi.nlm.nih.gov/projects/SNP (submitter SNP accession nos. are listed in Dataset S1).

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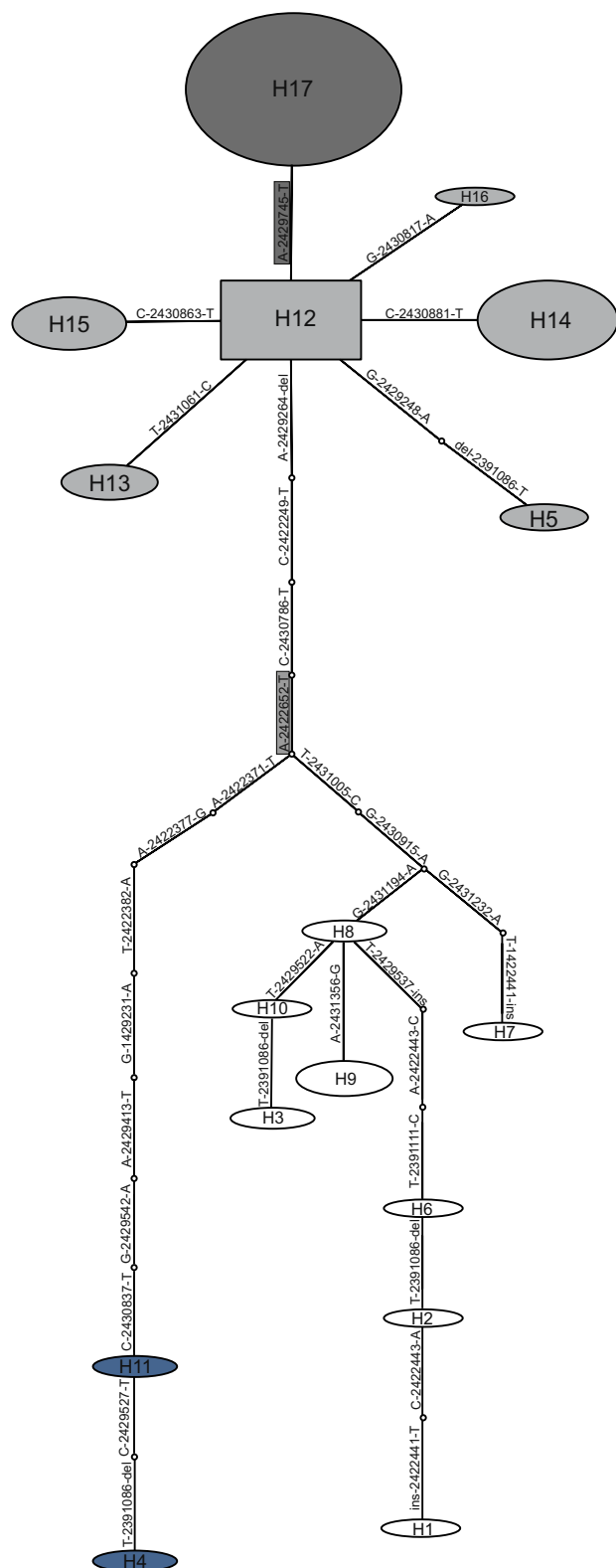


Fig. 3. Network of *An. gambiae sensu lato* VGSC haplotypes. Haplotypes bearing 1014F are shaded gray with the 1575Y haplotype (H17) shaded dark gray. The core 1014F-containing haplotype is depicted in a rectangle. Two haplotypes from *An. arabiensis* (H4 and H11 shaded blue) were included, but their position is equivocal: An alternative network exists in which the positions of all *An. gambiae* haplotypes are identical, but the *An. arabiensis* haplotypes are joined to a node between haplotype 12 and the 1014L *An. gambiae* sequences by a reticulation. Polygon size corresponds to the sample

at a low frequency in the subsample from this population (0.179; 95% CI = 0.079–0.356). 1575Y was present in 2006 S form populations from Ghana with an allele frequency of 0.162 (95% CI = 0.112–0.23), whereas 1014F was at high frequency (0.938; 95% CI = 0.892–0.965).

In M form samples from Benin, 1575Y was found at a frequency of 0.146 and was highest in the northern site of Malanville (0.321, $n = 56$ alleles), whereas in the south, 1575Y was detected only from Houeyiho (1 of 54 alleles) (Table 1). 1575Y frequency was somewhat higher in S form (0.262), but not significantly so ($P = 0.053$).

Haplotype Analysis. The 1575Y variant was inextricably linked to 1014F, and 1575Y homozygotes were detected exclusively in 1014F homozygotes (Table S1) suggesting these mutations occur on the same haplotype. Confirmation came through sequencing 55 individuals for exons 7–10, 21, 28–30 and 32–33. The haplotype network was based on 49 individuals with six excluded due to missing data or uncertainty in haplotype construction ($n = 98$ haplotypes). Seventeen haplotypes (*An. gambiae* $n = 15$; *An. arabiensis* $n = 2$) were recovered using PHASE with only a single 1575Y bearing haplotype regardless of molecular form (M or S) or sampling location (Fig. 3). The same 15 *An. gambiae* haplotypes were recovered in 10 of 10 runs of PHASE. Seven haplotypes carried 1014F (including the 1575Y-bearing haplotype) and form a distinct cluster in the statistical parsimony network (Fig. 3) separated from the other eight *An. gambiae* haplotypes carrying 1014L by a minimum of seven mutations.

Sequencing revealed a further 31 VGSC SNPs ([Dataset S1](#)), of which 16 were intronic, nine synonymous and six nonsynonymous. Of the nonsynonymous SNPs, one (*I1532T*) encodes an isoleucine to threonine change at position 1532. The remaining five are found within the variable intracellular carboxyl terminus loop 3' of the IVS6 helix, a region where no resistance-associated mutations have been detected previously in insects (23). For *I1532T*, 3 of 40 M form samples from Goundry were C/T heterozygotes (frequency = 0.038; 95% CI 0.013–0.105).

Sequencing of Exon 20, the Site of Super *kdr*. The two copies of duplicated exon 20 (20c and 20d) were sequenced from both *An. gambiae* s.s. M and S forms carrying 1014-FF and 1575-YY to screen for additional mutations within the sodium channel binding pocket. No additional variation was detected.

Extended Haplotype Homozygosity (EHH). EHH analysis was used to compare patterns of LD of *1575N*, *1014F*, and *1575Y*, assessed across 17 SNPs. For *1014F*, LD is complete in the centromeric direction but EHH decays in the telomeric direction (Fig. 4A), consistent with previous observations (7). For *1575N*, EHH decays rapidly in both directions (Fig. 4B), whereas there is complete LD for *1575Y*. It should be noted that the 95% CIs of EHH are entirely nonoverlapping just a few base pairs away from the core for wild type versus mutant comparisons at both *1014* (telomeric) and *1575* (both telomeric and centromeric) (Fig. 4A and B). The single haplotype of *1575Y* and decay of LD for both *1014F* and *1575N* suggest that *1575Y* is currently undergoing a selective sweep in *An. gambiae* s.s. populations from West Africa.

Association of *N1575Y* with Resistance. Because *1575Y* occurs solely on a *1014F*-bearing haplotype, we applied haplotypic association tests (Fig. 5) to investigate whether there is an additive benefit of *1575Y* over and above the resistance benefit conferred by *1014F*. Additive benefits of *1575Y* were detected for M form samples phenotyped with DDT (OR = 2.6; 1.05–6.48) and S form samples phenotyped with permethrin (OR = 1.93; 1.24–3.0).

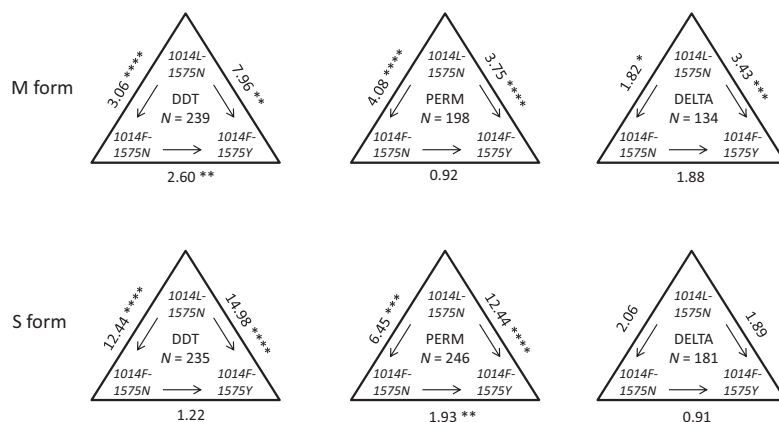


Fig. 5. Results of haplotypic association tests for the three 1014–1575 haplotypes encountered in this study (1014L-1575N, 1014F-1575N, 1014F-1575Y) with resistance phenotype to DDT, permethrin (perm), and deltamethrin (delta). Phenotype (alive/dead) was determined following 1-h exposure to 4% DDT, 0.75% permethrin, or 0.05% deltamethrin followed by 24-h recovery. Population mortality data are provided in Table S3. Odds ratios (ORs) are given with significance indicated with asterisks. The arrow within the triangle indicates the direction of OR calculation (e.g., M form DDT phenotyped individuals carrying the 1014F-1575Y haplotype are 2.60 times more likely to be resistant than individuals carrying the 1014L-1575N haplotype).

1329 (equivalent to 1410 in housefly numbering) in domain III SIV-V disrupts fast inactivation, but normal inactivation is restored by a compensatory opposing charge mutation within the linker at position 1489 (1565 housefly numbering and part of the IFM inactivation particle motif) (30). Although we provide evidence for an additive benefit of 1575Y, we cannot preclude the possibility that in wild populations, N1575Y may compensate for fitness costs of 1014F in the absence of insecticide exposure which would also result in the signals of positive selection that we have detected.

An additional I-T mutation at position 1532 was also detected at low frequency in M form samples. Position 1532 is located within the III S6 helix which forms one side of the pyrethroid/DDT binding site. Three nearby residues (F1534, G1535 and F1538) have already been implicated in resistance in other insect species (23). In *Aedes aegypti*, F1534C is correlated with both permethrin and DDT resistance (22). However, the I1532 side-chain actually points away from the binding pocket toward the channel pore and so at present the role of this mutation in resistance awaits further investigation.

Given the recognized role of L1014F and L1014S in conferring resistance phenotypes in *An. gambiae* (8) it is understandable that studies have focused overwhelmingly on genotyping these *kdr* markers in studies of insecticide resistance. However, this approach neglects other resistance mutations which may be present with the sodium channel. The identification of a mutation involved in resistance suggests that there is merit in exploring the sodium channel for additional resistance mutations. Detection of the mutation at an early stage presents an ideal opportunity for modeling studies predicting spread and estimating selection coefficients (31). The N1575Y TaqMan assay will facilitate this.

Materials and Methods

Sample Collections. *An. gambiae* s.s. females were collected from 10 sites across West/Central Africa, and *An. arabiensis* females were collected from 4 sites in Burkina Faso (Fig. 1). Burkinabe samples (342 M form, 711 S form and 272 *An. arabiensis*) were collected in 2008–2010 from four locations (detailed sample site information in ref. 32). Mosquitoes from Burkina Faso were phenotyped for permethrin (0.75%), deltamethrin (0.05%), or DDT (4%) in WHO susceptibility tests (33).

Both M and S molecular forms of *An. gambiae* were collected in 2006 from Accra, Ghana, and Yaoundé, Cameroon (18). At the time of collection, the 1014F allele frequency in these populations was high in *An. gambiae* s.s. S form (0.98 and 0.87, respectively) and rare in M form (~0.01) (18). More recent collections in Ghana show a marked increase in 1014F allele frequency in M form samples (7). A subset of the 2006 samples were included in this study: 88 S form and 16 M form from Ghana, and 20 S form and 20 M form from Cameroon were screened.

A total of 112 *An. gambiae* s.s. from four sites in Benin were included in the analysis. M and S forms of *An. gambiae* s.s. were collected from three sites in the south: Houeyiho (M form; $n = 30$), Bohicon (S form; $n = 20$; M

form $n = 4$), and Tori Bossito (S form; $n = 23$, M form $n = 6$) and one site in the north, Malanville (29 M form) (Fig. 1).

All samples were distinguished using SINE PCR (34).

Targeted Sequencing of Exon 30 of the Voltage-Gated Sodium Channel. A total of 330 bp of exon 30 was amplified (for primers, see Table S2) from S form *An. gambiae* s.s. from Kuinima ($n = 12$) and Soumouso ($n = 9$) in Burkina Faso where DDT and pyrethroid resistance is widespread, and two samples from the insecticide susceptible Kisumu laboratory-colony. One nonsynonymous mutation was observed in the resistant mosquitoes, an asparagine to tyrosine at position 1575 (N1575Y).

Pyrosequencing of N1575Y. Two genotyping assays for N1575Y were developed. The first, a pyrosequencing assay, interrogated the mutation containing sequence 5'-AT[A/T]ATGCAATGAA-3'. PCRs were performed using the primers listed in Table S2. Reactions (20 μ L) contained 0.4 μ M each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.4 U KapaTaq (KAPA Biosystems) and 1–5 ng of template DNA using cycling conditions of 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 10 s. PCR products (made up to 40 μ L with dH₂O) were used as templates for pyrosequencing. Reactions were performed using PyroMark Gold Reagents (Qiagen) according to the manufacturer's instructions with the sequencing primer AG_VGSC_EX29seq (Table S2) and run on the PyroMark Q96 System (Qiagen).

TaqMan Genotyping of the N1575Y Mutation. To complement the suite of TaqMan assays already developed for *Anopheles* research (35), a custom TaqMan assay was developed for N1575Y. Primer and probe sequences are provided in Table S2. TaqMan reactions were undertaken in 10- μ L volumes containing 1 \times SensiMix (Bioline), 800 nM each primer, and 200 nM each probe on an Agilent MX3005P with cycling conditions of 10 min at 95 °C followed by 40 cycles of 15 s at 92 °C and 1 min at 60 °C.

All specimens were genotyped for L1014F using a TaqMan assay (36). A subset of samples was also genotyped for L1014S; however, no *An. gambiae* s.s. individuals were found to carry this allele ($n = 119$).

Identification of Haplotypes. Long-range sodium channel haplotypes were determined for 55 individuals through amplification and direct sequencing of four regions of the sodium channel gene covering exons 7–10, 21 (site of the 1014 codon), 28–30, and 32–33 (numbered following Davies et al.; ref. 37). Amplification primers are given in Table S2. To facilitate phasing of haplotypes, only individuals homozygous at the 1014 and 1575 codons were included (with the exception of three N1575Y heterozygotes from Cameroon where 1575Y homozygotes were not found). Representative samples of 1014-LL 1575-NN, 1014-FF 1575-NN, and 1014-FF 1575-YY from both M and S form individuals were sequenced. Genotypes at variable positions served as input for PHASE (38). Haplotypes were identified following 10 runs of PHASE with seed values altered for each run. Ambiguous positions were resolved through cloning. Unambiguous haplotypes were used to create a haplotype genealogy in TCS (39) with default conditions (95% connection limit; gaps treated as a fifth state).

EHH Analysis. The pattern of LD for the 1575 and 1014 haplotypes was inferred using EHH analysis (40) on all unequivocal haplotypes from *An. gambiae* ($n = 69$ haplotypes for 1014F core and $n = 61$ for 1575N core). The significance of differences in EHH values between 1014 and 1575 haplotypes

were determined by nonoverlapping 95% CI at each SNP position calculated using a bootstrapping approach ($n = 1,000$). EHH analysis was performed in R (41). To visualize the breakdown of LD extending from the core region, haplotype bifurcation plots were created using SWEEP (40).

Sequencing of Exon 20 Mutations. Within the putative binding pocket of the insect VGSC, a series of key amino acid residues that interact with DDT and pyrethroids, have been identified and are associated with resistance to DDT and pyrethroids (9, 37). These include the so called super *kdr* mutation at codon 918, which exists as a double mutation with *1014F* in houseflies and confers additive resistance to permethrin (42). This region is therefore a prime candidate for the emergence of resistant mutations at codons other than 1014. To confirm that *N1575Y* is not a marker of an additional mutation within this site, a ~400-bp region of exon 20 was sequenced from one *An. gambiae* s.s. S and M form individual from all four sites in Burkina Faso carrying *1014-FF* and *1575-YY*. Exon 20 is present in two alternatively spliced copies (Exon 20c/d) in *An. gambiae* s.s. (37). Therefore, primers specific to each copy were designed in flanking introns.

115327 Frequency in Goundry M Form. An isoleucine to threonine substitution was detected at position 1532 in the VGSC (2L:2428617). Subsequently, 40 M form samples from Goundry were amplified with Ex28F and Ex28R and sequenced with Ex28R to ascertain the frequency of this mutation.

Data Analysis. Haplotypic association tests for *1014L-1575N*, *1014F-1575N*, and *1014F-1575Y* and insecticide resistance were conducted in Haploview v4.1 (43).

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