Comparison of the knockdown resistance locus (*kdr*) in *Anopheles stephensi*, *An. arabiensis*, and *Culex pipiens s.l.* suggests differing mechanisms of pyrethroid resistance in east Ethiopia

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Abstract

The recent detection of the malaria vector mosquito species *Anopheles stephensi*, which is typically restricted to South Asia and the Middle East, in the Horn of Africa emphasizes the importance of continued vector surveillance in the region. Previous reports of the absence of the knockdown resistance mutations (*kdr*) in the voltage-gated sodium channel (*vgsc*) in pyrethroid resistant *An. stephensi* emphasize the need for further investigation of the *kdr*-mediated resistance mutations in other mosquitoes of east Ethiopia. In this study, the knockdown resistance locus (*kdr*) in the voltage gated sodium channel (*vgsc*) was analyzed in *An. stephensi*, *An. arabiensis*, and *Culex pipiens s. l*. collected in east Ethiopia between 2016 and 2017. A portion of both vsgc was sequenced and amplified in *An. arabiensis* and *Cu. pipiens s.l*. and compared with *An. stephensi* sequences previously generated. All of *Cu. pipiens s.l.* (n = 42) and 71.6% of the *An. arabiensis* (n=67) carried *kdr* L1014F alleles known to confer pyrethroid resistance. Analysis of the downstream *kdr* intron in all three species revealed nucleotide diversity only in *An. stephensi* (s = 6, h = 3) previously shown to have no 1014 mutations. In addition, no evidence of non-neutral evolutionary processes was detected for *An. stephensi kdr* intron. Finally, no association between the *An. stephensi kdr* intron haplotypes and permethrin or DDT phenotypic resistance was detected. Overall, these results reveal evidence for differing degrees of selection at *kdr* that may suggest differing mechanisms of resistance across these species. While the presence of the L1014F mutation suggests target-site resistance mechanisms in *An. arabiensis* and *Cu. pipiens* in east Ethiopia, the lack of signatures for selection at *kdr* for resistant and non-resistant *An. stephensi* does not support a target-site mechanism of resistance based on the *kdr* locus in *An. stephensi*. This evidence of differing mechanisms of resistance across vector species can inform the design of future integrated strategies for vector control.

Keywords: malaria; vector-borne disease; insecticide resistance; DDT; selective sweep

Synopsis

Growing insecticide resistance hinders efforts to control the spread of vector-borne diseases. This is further complicated with the evidence of vectors moving into new regions as is the case with the newly detected *Anopheles stephensi* in the Horn of Africa. With this dynamic situation, it is important to evaluate how different local vectors develop resistance to insecticides to improve the implementation of insecticide-based strategies meant to target multiple vectors species. The authors analyzed a known insecticide resistance locus, *kdr*, in the *An. stephensi* and long-established *An. arabiensis* and *Culex pipiens sl* populations in east Ethiopia for evolutionary signatures of the common mechanism of resistance known as knockdown (target-site) resistance. The authors show that while *An. arabiensis* and *Culex pipiens sl* carry evolutionary signatures of the knockdown resistance mechanism, the *An. stephensi* do not. Additional comparisons of *kdr* genetic diversity and insecticide resistance status in the *An. stephensi* from Ethiopia further supports that the *kdr* and neighboring loci likely do not play a role in resistance in this *An. stephensi* population. These findings support the notion of that vectors in the same general region can have different modes for which they evolve resistance in east Ethiopia.

INTRODUCTION

Vector-borne diseases are major public health concern, of which malaria remains a leading threat with 228 million cases reported in 2018 (1). In Ethiopia, where both *Plasmodium vivax* and *P. falciparum* are prevalent and multiple *Anopheles* vector populations are present,1.5 million malaria cases were reported in 2017 (2). Malaria control in Ethiopia and the rest of the continent is now challenged with the recent finding of *An. stephensi*, a malaria vector, which is typically restricted to South Asia and the Middle East, in the Horn of Africa (3-6). With the recent evidence of *An. stephensi* demonstrated ability to transmit both major *Plasmodium* species (7), concerns are raised that this vector may contribute to the growing number of *P. vivax* cases being reported in Africa (Twohig et al. 2019). In addition, the number of dengue infections has recently increased in eastern Ethiopia (8, 9) and lymphatic filariasis in west Ethiopia (10). Given the multiple lines of evidence suggestive of vector-borne disease challenges, continued evaluation of mosquito vector populations in the region is needed to guide interventions.

Efforts to control vector populations in Ethiopia and abroad have relied heavily on the use of insecticides such as pyrethroids deployed through indoor residual spraying, insecticide-treated bed nets (ITNs), and long-lasting insecticidal nets (LLIN). As a result, widespread insecticide resistance has been reported across multiple vector species (11). In Culicidae, the main mechanisms of resistance to pyrethroids include target-site and metabolic-based resistance (12). Target-site resistance is based on altered neurological response to insecticides in mosquitoes and is caused by mutations in the voltage-gated sodium channel leading to knockdown resistance (*kdr*) [reviewed in (13)]. This mechanism is broadly studied and is widely reported across species of Culicidae including *Anopheles* spp. (12) and *Culex pipiens s.l.* (14). *Culex pipiens* s.l. is a complex of mosquito species that includes vectors for lymphatic filariasis and multiple arboviruses including the Rift Valley fever virus (RVFV) and West Nile virus. *Culex pipiens* complex also includes vectors for avian *Plasmodium*, filarioid helminths (Dirofilaria spp.), and Usutu virus.

In *Anopheles*, *kdr* involves the substitution of leucine (TTA) to phenylalanine (TTT) or to serine (TCA) at the 995 codon in the voltage gated sodium channel, commonly known as *kdr* L1014F and L1014S (15). A similar mutation is observed in the *vgsc* of *Culex* mosquitoes (GeneID:CPIJ007596) (16, 17). For metabolic resistance, the insecticide is degraded, sequestered or exported out of the cell before it can bind to its target (12). Metabolic resistance has not been linked to a single trackable genetic variant in most species. However, previous functional studies have found the over-expression of detoxification enzymes such as cytochrome P450s lead to metabolic resistance. (12, 18).

In Ethiopia, pyrethroid and DDT resistance has been reported in much of the northern and western portion of the country in the primary malaria vector *An. arabiensis*, (19-22). In *An. arabiensis,* both target-site and metabolic resistance to play a role in pyrethroid and DDT resistance. In eastern Ethiopia, a recent investigation revealed *An. stephensi* were resistant to pyrethroids however the L1014F and L1014S mutations were absent (23). *An. arabiensis* insecticide resistance in eastern Ethiopia has not been well characterized. Even more so, the status of insecticide resistance in *Cu. pipiens s.l.* (most likely *Cu. quinquefasciatus*) is unknown throughout most of the country. Understanding the molecular bases for resistance in these vector populations in Ethiopia is important for vector control planning. Effective vector control strategies are moving toward integrated approaches that take into account the ecology and behavior of multiple vector species (24). Knowing the variety of mechanisms of resistance to pyrethroids across vector species in a region can guide expectations of response to insecticides and the appropriate molecular markers for tracking insecticide resistance each species.

Genetic analysis of putative resistance loci across local vector populations, including those that spread malaria, can provide information on the range of mechanisms of resistance in a region. While *kdr* 1014 mutation frequencies provide preliminary evidence of target-site resistance to pyrethroids, analysis of the variation in neighboring intronic region provide information of the long-

term impact of pyrethroids on the evolution of the mosquito populations. Tests for neutrality, such as Tajima's D (25), can be used to evaluate the genetic diversity the intronic region to determine if the patterns differ from expectations under neutral evolution. It is expected that if the *kdr* locus was under selection due to pressure from the pyrethroids, then a selective sweep would cause low to no nucleotide diversity at the intron. (26, 27) . Thus, these analyses are helpful in clarifying the mechanisms of resistance, the current status of pyrethroid resistance, and predicting the risk of resistance emerging locally. Here we examine the diversity surrounding the *kdr* locus for evidence of selective pressure for insight into the mechanism of resistance in *An. stephensi, An. arabiensis*, and *Culex pipiens s. l.* collected in east Ethiopia.

METHODS

The study involved sequencing of a portion of the *vgsc* gene that contains loci that when mutated can confer resistance to pyrethroids. For *An. stephensi,* data came from sequences generated in a previous study (23) and generated in the present study. *An. arabiensis* and *Culex* sequence data was also generated in this study as detailed below.

Sample collection and species identification

An. stephensi analyzed in this were study collected from Kebri Dehar in 2016 as part the first detection of this species in Ethiopia (4). Specimens were larvae collected and lab-reared for testing for resistance to insect ides as previously detailed (23). *An. arabiensis* and *Culex* specimens collected in east Ethiopia in 2017 were included in this study. *An. arabiensis* species identification was based on morphological keys and molecular analysis of internal transcribed spacer 2 (*ITS2*) and cytochrome oxidase I (*COI*) loci as reported previously (28). *An. arabiensis* were collected using CDC light traps (John W. Hock, Gainesville, FL, USA) over four different collection times at two sites, Meki (east-central Ethiopia) and Harawe (northeast) in 2017. Harawe and Meki are about 350 km northwest and 600 km west of Kebri Dehar, respectively.

145 *Culex* specimens were collected using CDC light traps in Kebri Dehar in 2017. Morphological key
146 and sequencing of *ITS2* locus were used for *Culex* identification using a previously published
147 PCR protocol (4). All amplicons were cleaned using Exosap and sequenced using Sanger
148 technology with ABI BigDyeTM Terminator v3.1 chemistry (Thermofisher, Santa Clara, CA)
149 according to manufacturer recommendations and run on a 3130 Genetic Analyzer (Thermo
150 Fisher, Santa Clara, CA). Sequences were cleaned and analyzed using CodonCode Aligner
151 Program V. 6.0.2 (CodonCode Corporation, Centerville, MA). ITS2 sequences from *Culex*
152 specimen were submitted as queries to the National Center for Biotechnology Information's
153 (NCBI) Basic Local Alignment Search Tool (BLAST) for species identification (29).

154

155 **Amplification and sequencing of *kdr* loci**

156 Once species or species complex identification was complete, samples were processed. For *kdr*
157 mutation analysis, polymerase chain reaction (PCR) was used to amplify the region of the *vgsc*
158 gene that housed the homologous *kdr* 1014 and a neighboring downstream intron in all
159 specimens. One leg from each mosquito specimen or extracted DNA was used as individual
160 templates for PCR. Each species required a different PCR protocol. *An. stephensi kdr*
161 amplification was completed according to Singh et al (30) with modifications as detailed in Yared
162 et al (23). *An. arabiensis kdr* amplifications were completed according to Martinez-Torres et al
163 (17). *Culex pipiens* s.l *kdr* amplifications were completed according to Chen et al (16). All PCR
164 reactions were performed at 25µl total with 2X Promega Hot Start Master Mix (Promega
165 Corporation, Madison, USA) and the primer conditions listed in Tab 1. All amplicons were cleaned
166 using Exosap and sequenced using Sanger technology with ABI BigDyeTM Terminator v3.1
167 chemistry (Thermofisher, Santa Clara, CA) according to manufacturer recommendations and run
168 on a 3130 Genetic Analyzer (Thermo Fisher, Santa Clara, CA). The *An. stephensi kdr* sequences

were further analyzed for association with pyrethroid resistance. The Fisher-exact test was performed to determine whether *kdr* intron haplotypes were associated with pyrethroid resistance.

Tab 1. List of primer and conditions used for PCR amplification of portions of the voltage gated sodium channel gene.

Assay	Primer	Sequence	Annealing Temperature (˚C)	Final Primer Concentration (µM)
An. stephensi	*Kdr*F	GGACCAYGATTTGCCAAGAT	50	1.25
	VGS_1R	CGAAATTGGACAAAAGCAAGG	50	1.25
An. arabiensis	Agd1	ATAGATTCCCCGACCATG	52	1.25
	Agd2	AGACAAGGATGATGAACC	52	1.25
Culex	Cpp1	CCTGCCACGGTGGAACTTC	58	1
	Cpp2	GGACAAAAGCAAGGCTAAGAA	58	1

Sequence analysis

Sequences were submitted as queries to the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) to confirm correct loci were amplified. Sequences were then aligned by species or species complex to identify *kdr* mutations. The *kdr* allele and genotype frequencies were then calculated and compared across species. *Aedes aegypti vgsc* sequence taken from NCBI was used as an outgroup.

We are interested in comparing the level of diversity in the neighboring intron downstream of the 1014 mutations in *Culex* spp. and *An. arabiensis to An. stephensi.* The cross-species comparison provides evidence for past insecticide use in east Ethiopia and the mechanisms of resistance across species. In addition to the sequences generated in this study, we included

sequences from resistant and non-resistant *An. stephensi* analyzed in a previous study on insecticide resistance in *An. stephensi* (23). We calculated the number of segregating sites, nucleotide diversity, the estimated number of haplotypes, and haplotype diversity using the program DNAsp v5 (31). Haplotypes were reconstructed using Phase 2.1 (32), HAPAR, and fastPHASE (33) algorithms in DNAsp. The neighboring downstream intron was also tested for neutrality using Tajima's D (25), Fu's F (34), and Fu and Li's D* and F* tests (35).

We also performed phylogenetic analysis of the 1014+intron sequences. Alignments were created with MAFFT version 7 (36) and ragged ends were trimmed using Mesquite 3.51 (37). Phylogenetic relationships using the sequenced portion of *vgsc* were inferred using RAxML (38) which is based on a maximum likelihood (ML) approach. The GTRGAMMA option that uses GTR model of nucleotide substitution with gamma model of rate of heterogeneity was applied. A thousand replicates were performed with the strategy searching for the heuristically-best-scoring tree and bootstrap analysis in one run. Best scoring trees under ML with bootstrap values from RAxML were viewed and rooted under the outgroup criterion (*Aedes aegypti*) in FigTree (39).

An. stephensi kdr Intron and resistance phenotype association analysis

Comparison of phenotype and haplotype data can provide additional clarification of the role of the *vgsc* in pyrethroid or DDT resistance in *An. stephensi.* Intron haplotypes may be linked to proximal mutations not covered in the regions sequenced that confer resistance. WHO bioassay insecticide resistance data were previously generated for the *An. stephensi* included in this study (23). We tested the association between the *kdr* intron haplotypes and resistance status (resistant vs. non-resistant) based on permethrin or DDT WHO bioassay results. Fisher Exact test with alpha = 0.05 was used to evaluate the association.

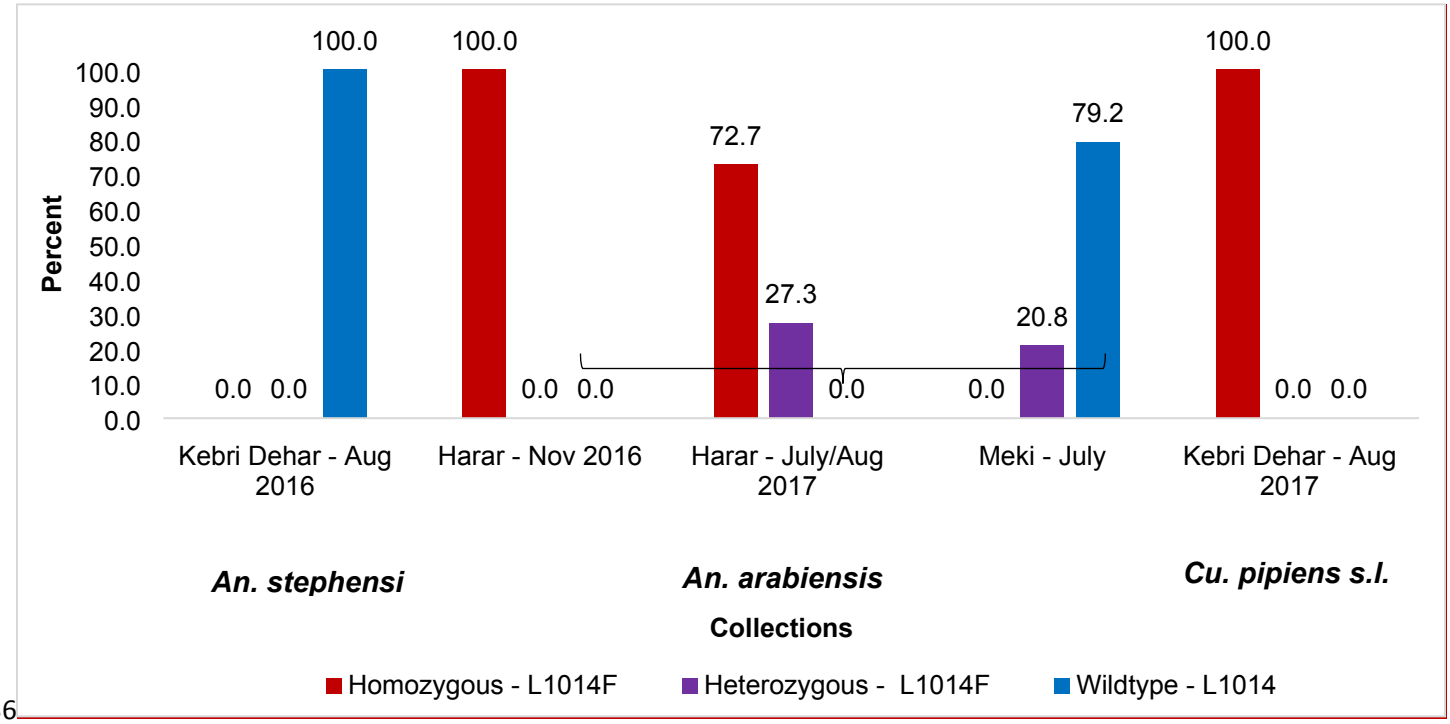
RESULTS

To provide greater ecological context to the mechanism behind insecticide resistance in *An. stephensi*, we also examined the frequency of the insecticide resistance mutations in other Culicidae species collected in eastern Ethiopia, including *Cu. pipiens s.l* and *An. arabiensis*. Prior to insecticide resistance genotyping, all *Culex* ITS2 sequences were analyzed to identify species. All sequences were identical and had equivalent high matching scores for two members of the *Cu. pipiens* complex: *Cu. quinquefasciatus* and *Cu. pipiens pipiens*. Because we could not determine which of these two species these specimens belong to, we will refer to these specimens by its broader taxonomic classification, *Cu. pipiens s. l.* (*ie Cu. pipiens* complex) in this study. *An. arabiensis* species identification was detailed in previous study (28). In total, 10, 33, and 24 *An. arabiensis* were collected in Harawe in November 2016, Harawe in July/August 2017, and Meki in July 2017 collections, respectively.

Kdr analysis

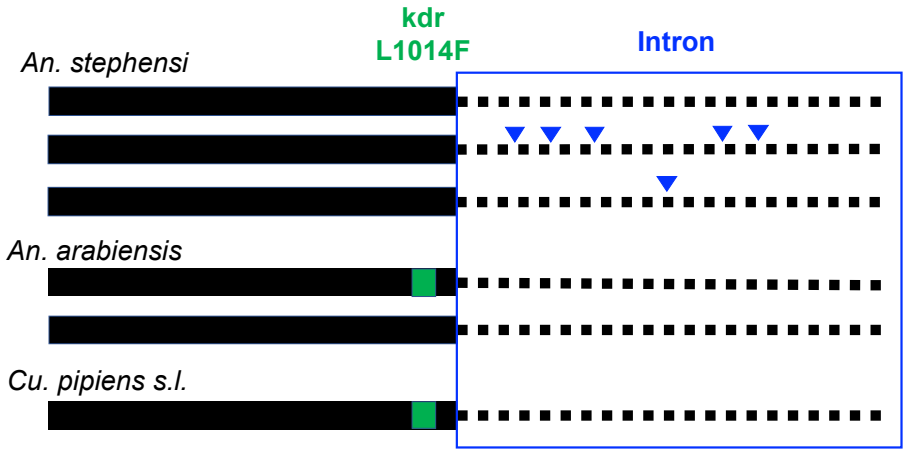
The *kdr* fragments were sequenced for *An. stephensi*, *Cu. pipiens s.l.,* and *An. arabiensis.* The sequencing resulted in 184, 452, and 290 base pair fragments for *An. stephensi*, *Cu. pipiens s. l*. and *An. arabiensis*, respectively. The percent of each *kdr* genotype observed by species is shown in Fig 1. A total of 131 *An. stephensi* were analyzed in this study, including 80 newly reported sequences. None of the *An. stephensi* analyzed in this study carried a mutation at the *kdr* 1014. All 42 *Cu. pipiens s.l*. specimens collected at the same site carried *kdr* L1014F mutations as homozygous. Of the 67 *An. arabiensis*, 71.6% carried the *kdr* L1014F mutation (heterozygous and homozygous). The allele frequency of L1014F mutation varied across *An. arabiensis* collections, where the highest frequency was observed in Harawe in November 2016 (100%). L1014F allele frequency for Harawe July/August 2017 and in Meki July 2017 collections were 86.4% and 10%, respectively. None of the *Cu. pipiens s.l*. or *An. arabiensis* collected at these localities carried the L1014S allele.

Fig. 1 Frequency of *kdr* 1014 genotypes in *An. stephensi, Culex pipiens s.l.*, and *An. arabiensis* collections.



A portion of the neighboring downstream intron for each species was analyzed to evaluate the level of diversity (Fig 2). Intron analysis revealed no polymorphisms for either *Cu. pipiens* or *An. arabiensis* (for both L1014F and L1014 wild type specimens). Of the 131 *An. stephensi* specimens from Kebri Dehar examined for *kdr* mutations, six segregating sites were detected, and three haplotypes predicted. Genetic diversity estimates are reported in Tab 2.

Fig. 2 Summary of *kdr* haplotypes across three Culicidae species in east Ethiopia. Solid lines depict the exon housing the *kdr* locus and dotted lines depict the downstream intron. Green square indicates the presence of the *kdr* L1014F. Triangles denote single nucleotide polymorphisms (SNPs) found in the intron relative to the most prevalent intron haplotype.

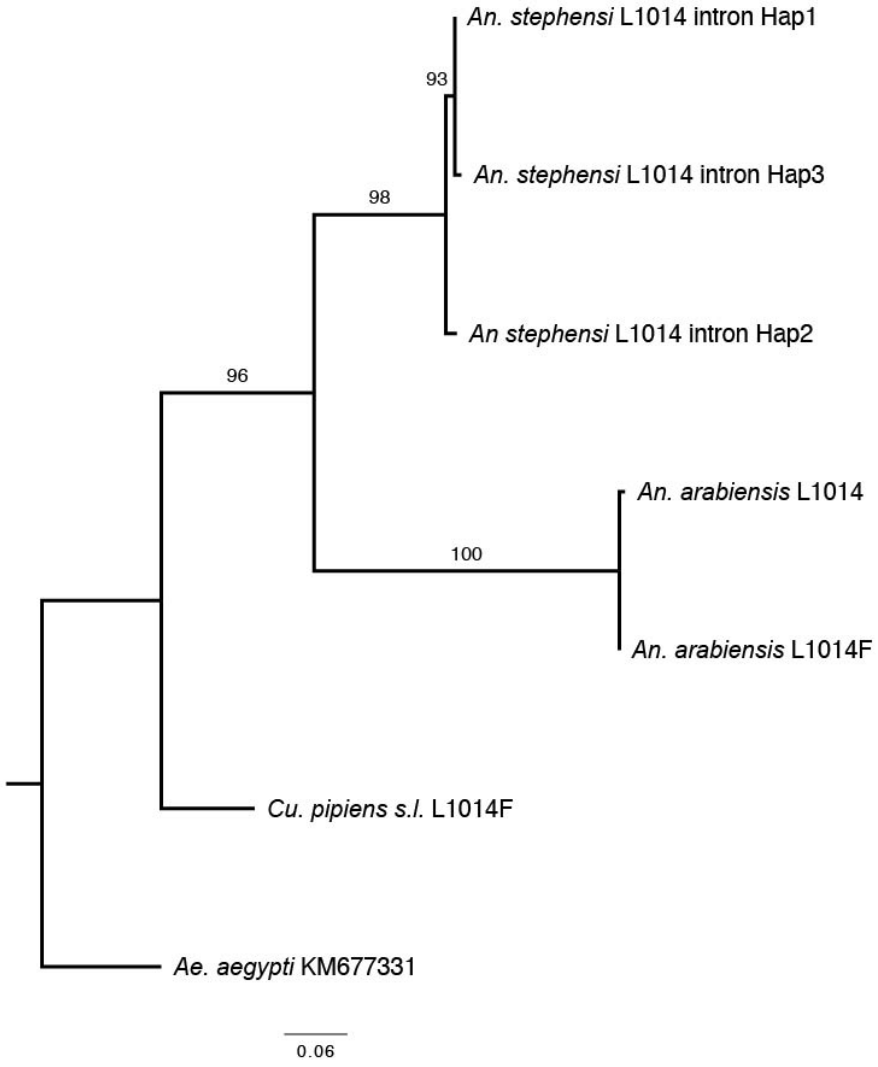


Tab 2. Genetic diversity estimates for *kdr* neighboring downstream intron in the *vgsc* for *An. stephensi*, *An. arabiensis*, and *Cu. pipiens s.l.,* where n = number of sequences used, S = number of polymorphic (ie segregating) sites, K = average number of pairwise nucleotide differences, Pi = nucleotide diversity, h = number of Haplotypes, Hd = haplotype diversity.

Species	n	S	k	Pi	h	Hd
An. stephensi	262	6	0.996	0.00545	3	0.225
An. arabiensis	134	0	0	0	1	0
Cu. pipiens s.l.	84	0	0	0	1	0

The phylogenetic analysis of the *kdr* and downstream intron sequences reveals significant differentiation between species (Fig 3, bootstrap > 93). Alignments revealed that the region surrounding the *kdr* locus is conserved while the intron region is highly divergent across species (Supplemental File 1 and 2).

Fig. 3 Maximum-likelihood tree of *An. stephensi*, *An. arabienis*, and *Cu. pipines s.l. vgsc* sequences from east Ethiopia. Analysis is based on a portion of the *vgsc* containing *kdr* 1014 and portions of the downstream intron (185 bp) with *Ae. aegypti* outgroup. Final ML Optimization Likelihood: -664.487198.



To further evaluate the potential functional significance of the *kdr* locus in *An. stephensi* based on evidence of positive selection, we conducted tests for neutrality at the *An. stephensi kdr* intron in using Tajima's D, Fu's F, Fu and Li's D, and Fu and Li's F (Tab 3). No evidence of non-neutral processes was detected in *An. stephensi* for the *kdr* locus. The absence of variation in *An. arabiensis* and *Cu. pipiens* s.l. *kdr* introns precluded tests for neutrality.

Tab 3. Tests for neutrality for downstream *kdr* intron for *An. stephensi*. All p-value > 0.10.

Test	Estimate
n	258
Tajima's D	0.03839
Fu's F	3.556
Fu and Li's D	1.04354
Fu and Li's F	0.82943

Comparing An. stephensi kdr intron haplotype vs. phenotype data

To further elucidate the significance of the *kdr* locus for insecticide resistance in *An. stephensi*, we tested the association between the haplotypes that were observed in the *kdr* intron and the phenotypic resistance to either the pyrethroid insecticide permethrin or DDT [phenotype data from (23)]. While the intron variation itself is likely of no functional significance, it may be linked to upstream or downstream variants in coding regions of the *vgsc*. The *kdr* locus was sequenced in 88 *An. stephensi* that were tested for permethrin or DDT sensitivity, previously (Tab 4). In this analysis, we focused on the *kdr* intron haplotypes with the highest frequency, designated Hap1 and Hap2. Specimens that had one copy of the rarer third haplotype Hap3 (n=4)

were excluded from the analysis. The remaining samples were divided based on whether they had at least one copy of the *kdr*H2 haplotype (n=24, 27.3%) or not (n=64, 72.7%). Of the 84 *An. stephensi* used of this analysis, 55 (62.5%) were classified as pyrethroid sensitive and 33 (37.5%) were classified as resistant. The results of the Fisher exact test revealed no significant difference in frequency of insecticide resistance between carriers of the *kdr* intron Hap2 carriers and non-carriers (two-sided prob p-value = 0.8052).

Tab 4. Comparison of *An. stephensi kdr* intron haplotypes and insecticide resistance status. No *An. stephensi* carried two copies of Hap3 (ie Hap3/Hap3).

Insecticide	**Status**	**N**	***kdr* Intron Haplotypes**				
			Hap1/Hap1	*Hap1/Hap2*	*Hap1/Hap3*	*Hap2/Hap3*	*Hap2/Hap2*
Permethrin	Resistant	17	13	3	0	0	1
	Sensitive	41	26	12	2	0	1
DDT	Resistant	16	11	3	1	0	1
	Sensitive	14	11	2	0	1	0
Combined	Resistant	33	24	6	1	0	2
	Sensitive	55	37	14	2	1	1
Total		88	61	20	3	1	3

DISCUSSION

Our results reveal key differences between insecticide resistance loci across east Ethiopian Culicidae. In particular, the *kdr* L1014F mutation was not observed consistently across the species included in this study. The difference in the genetic diversity at the *kdr* loci observed

between *Cu. pipiens complex* specimens and *An. stephensi,* both collected at the same location and likely exposed to similar degrees of insecticides leading up to their detection and collection, was an unexpected finding. Unlike the *An. stephensi*, all the *Culex pipiens s.l.* carried the L1014F mutation. Similarly, *An. arabiensis* sample sets had a high frequency of L1014F mutations, supporting the relevance of *kdr* based target-site mechanism of resistance for those species.

We also observed differences in the nucleotide diversity of the neighboring intronic region of the three species. While *An. stephensi* exhibited multiple segregating sites and resultant haplotypes, only a single intronic haplotype is observed for *An. arabiensis* and *Cu. pipiens s.l.* Although neutrality tests could not be performed for these samples sets due to the absence of nucleotide variation, the absence of variation itself could be evidence of a hard-selective sweep, indicative of a locus under selective pressure. Tests for neutrality of the *An. stephensi* reveals no evidence of non-neutral processes, such as positive selection, shaping that variation. Taken together, the high frequency of the L1014F mutation in *Cu. pipiens* and *An. arabiensis* and absence of the variation in the neighboring intron supports the notion that these east Ethiopian mosquitoes have undergone selective pressure on the *vgsc* from pyrethroids that likely would lead to target-site resistance. However, the absence of 1014 mutations and presence of intronic variation in pyrethroid resistant *An. stephensi* suggest the opposite and point to the possibility of different mechanisms of resistance across the eastern Ethiopian Culicidae. Additional analysis of the permethrin and DDT sensitivity phenotype and *kdr* intron haplotype data reveal no significant association with the intron and insecticide resistance status. This further supports the notion that the *kdr* locus and proximal loci in the *vgsc* likely play no role in pyrethroid resistance in *An. stephensi* in Ethiopia and other loci associated with metabolic resistance or other mechanisms of resistance may be more relevant. Analysis of the full *vgsc* gene sequence is need and ongoing to confirm the role of this gene in *An. stephensi* insecticide resistance.

Interestingly, when we compare the intronic regions of the *An. arabiensis* that carried the L1014F mutation to those that did not, the same signature of lack variation of in the intron (i.e. a single haplotype) was observed. The mosquitoes that carried the once advantageous allele may suffer fitness costs in the absence of the selective pressure, which would result in a rebound of the wild-type allele at that locus. However, the intronic haplotype associated with resistance remains in the population. These findings underline the limitations of relying solely on *kdr* 1014 allele frequencies for evidence of the degree of impact of selective pressure on the mosquito population and the determination of mechanism of resistance in a particular species. Broader sequence analysis including neighboring introns can provide context of the historical impact of insecticide resistance.

Several limitations to these studies should be considered. The *An. stephensi* were collected as larvae and pupae and the *An. arabiensis* and *Cu. pipiens s.l.* were collected as wild-caught adults. This method of collection may pose a concern that the immature specimen set would not reflect the natural diversity of the wild-caught adult population. Concerns with clonality however are lowered when considering the level of diversity observed at the *An. stephensi kdr* locus and at the ace-1R locus (3 haplotypes detected; data not shown). In addition, while *An. stephensi* phenotypic resistance has been reported, *An. arabiensis* and *Cu. pipiens s.l.* phenotypic resistance data is still lacking for east Ethiopia. Though pyrethroid resistance in *An. arabiensis* and *Cu. pipiens s.l.* is widely reported in Africa, complementary bioassay and genetic data can confirm the molecular-based findings observed in this study.

In conclusion, the different patterns of diversity at the *kdr* loci across species does support the potential for differing mechanisms of resistance across Culicidae in east Ethiopia. Both *An. arabiensis* and *Cu. pipiens* sample sets revealed notable L1014F allele frequencies that confer target-site resistance and absence of intron variation that tells of selective pressure on that locus in those species. Additional investigations are needed to determine the mechanisms and genetic

basis of pyrethroid resistance (metabolic or another undiscovered mechanism) in *An. stephensi*. These finding emphasize the need for careful consideration of molecular approaches used to evaluate insecticide resistance status across multiple species and will inform the development and future implementation of novel integrated vector control strategies.

Abbreviations

BLAST: Basic Local Alignment Search Tool

DNA: Deoxyribonucleic Acid

FMOH: Federal Ministry of Health

ITS2: Internal transcribed spacer 2 region

NCBI: National Center of Biotechnology Information

PCR: Polymerase chain reaction

***KDR*:** knockdown resistance

***VGSC*:** voltage-gated sodium channel

COI: Cytochrome c oxidase subunit 1 gene

CDC: Centers for Disease Control and Prevention

WHO: World Health Organization

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477 **Supporting Information**

478 S1 Figure. Image of alignment used for phylogenetic analysis.

479 S2 Table. Text file of alignment used for phylogenetic analysis.

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