

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

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26 **Abstract**

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

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

50 **Synopsis**

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

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71 INTRODUCTION

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

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

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

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

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

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

130 **METHODS**

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

135 **Sample collection and species identification**

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

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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 BigDye™ 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

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

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

Assay	Primer	Sequence	Annealing	Final Primer
			Temperature (°C)	Concentration (μ M)
<i>An. stephensi</i>	<i>KdrF</i>	GGACCAYGATTTGCCAAGAT	50	1.25
	VGS_1R	CGAAATTGGACAAAAGCAAGG	50	1.25
<i>An. arabiensis</i>	<i>Agd1</i>	ATAGATTCCCCGACCATG	52	1.25
	<i>Agd2</i>	AGACAAGGATGATGAACC	52	1.25
<i>Culex</i>	<i>Cpp1</i>	CCTGCCACGGTGGA ACTTC	58	1
	<i>Cpp2</i>	GGACAAAAGCAAGGCTAAGAA	58	1

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174 **Sequence analysis**

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

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

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

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

198 ***An. stephensi* *kdr* Intron and resistance phenotype association analysis**

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

206 **RESULTS**

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

218 *Kdr analysis*

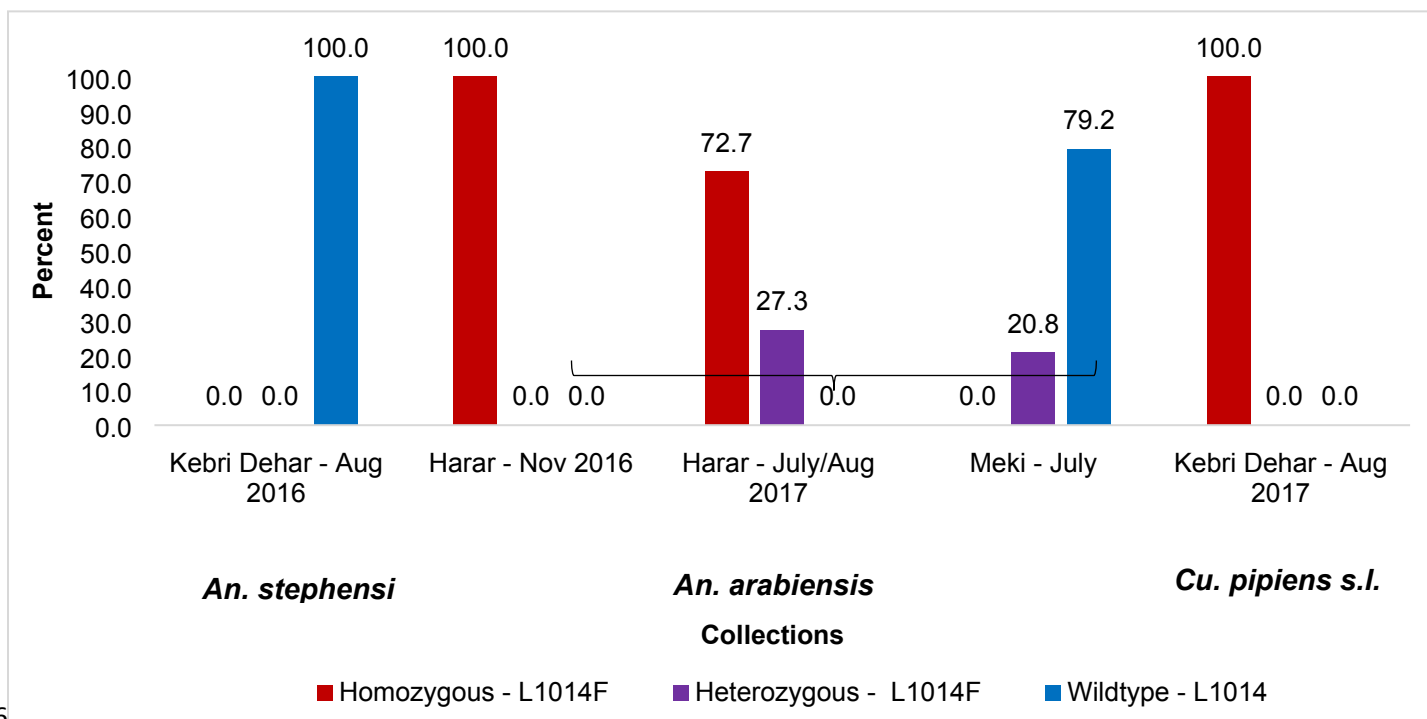
219 The *kdr* fragments were sequenced for *An. stephensi*, *Cu. pipiens s.l.*, and *An. arabiensis*.
220 The sequencing resulted in 184, 452, and 290 base pair fragments for *An. stephensi*, *Cu. pipiens*
221 *s. l.* and *An. arabiensis*, respectively. The percent of each *kdr* genotype observed by species is
222 shown in Fig 1. A total of 131 *An. stephensi* were analyzed in this study, including 80 newly
223 reported sequences. None of the *An. stephensi* analyzed in this study carried a mutation at the
224 *kdr* 1014. All 42 *Cu. pipiens s.l.* specimens collected at the same site carried *kdr* L1014F
225 mutations as homozygous. Of the 67 *An. arabiensis*, 71.6% carried the *kdr* L1014F mutation
226 (heterozygous and homozygous). The allele frequency of L1014F mutation varied across *An.*
227 *arabiensis* collections, where the highest frequency was observed in Harawe in November 2016
228 (100%). L1014F allele frequency for Harawe July/August 2017 and in Meki July 2017 collections
229 were 86.4% and 10%, respectively. None of the *Cu. pipiens s.l.* or *An. arabiensis* collected at
230 these localities carried the L1014S allele.

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



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

243

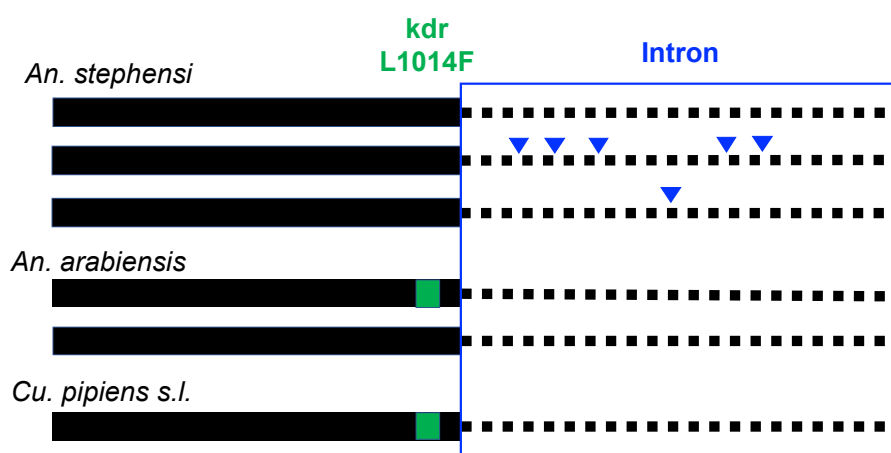
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248 **Fig. 2** Summary of *kdr* haplotypes across three Culicidae species in east Ethiopia. Solid lines
 249 depict the exon housing the *kdr* locus and dotted lines depict the downstream intron. Green
 250 square indicates the presence of the *kdr*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



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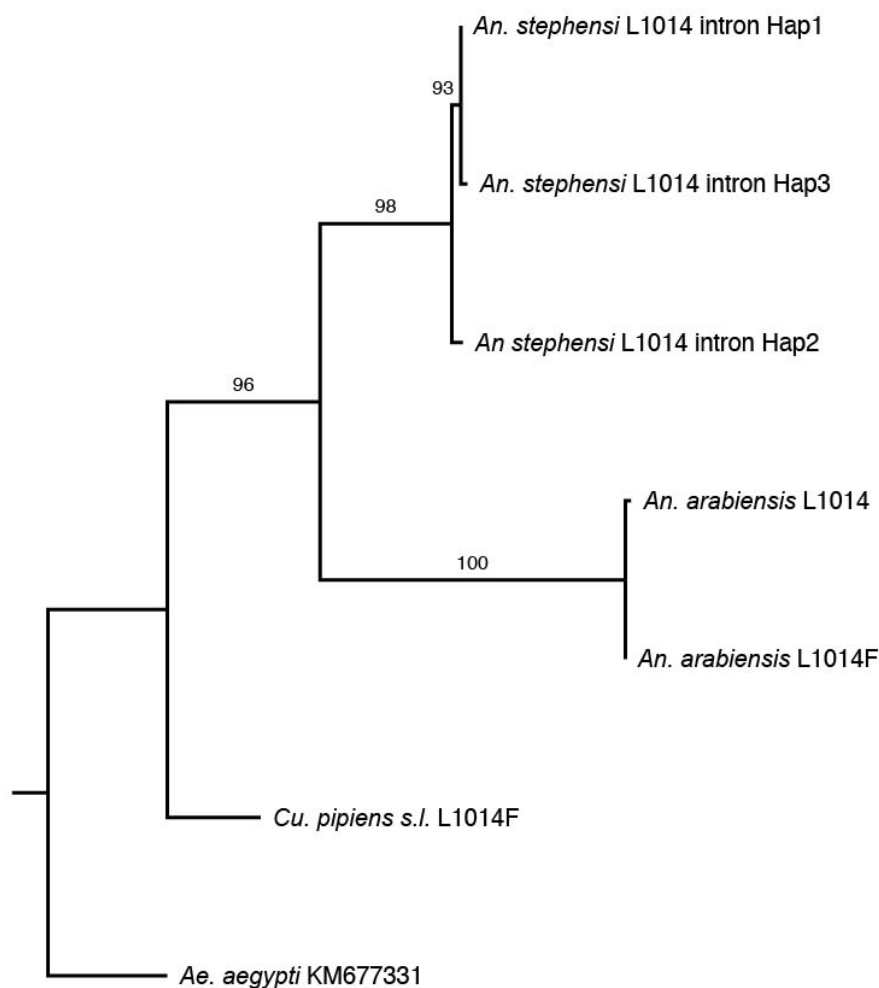
254 **Tab 2.** Genetic diversity estimates for *kdr* neighboring downstream intron in the *vgsc* for *An.*
 255 *stephensi*, *An. arabiensis*, and *Cu. pipiens s.l.*, where n = number of sequences used, S =
 256 number of polymorphic (ie segregating) sites, K = average number of pairwise nucleotide
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

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

258

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

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



268

269 To further evaluate the potential functional significance of the *kdr* locus in *An. stephensi*
270 based on evidence of positive selection, we conducted tests for neutrality at the *An. stephensi kdr*
271 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-
272 neutral processes was detected in *An. stephensi* for the *kdr* locus. The absence of variation in
273 *An. arabiensis* and *Cu. pipiens* s.l. *kdr* introns precluded tests for neutrality.

274 **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

275

276 *Comparing An. stephensi kdr intron haplotype vs. phenotype data*

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

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

291

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

Insecticide	Status	N	<i>kdr</i> 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

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295

296 DISCUSSION

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

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

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

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

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

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

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

353 **Abbreviations**

354 **BLAST:** Basic Local Alignment Search Tool

355 **DNA:** Deoxyribonucleic Acid

356 **FMOH:** Federal Ministry of Health

357 **ITS2:** Internal transcribed spacer 2 region

358 **NCBI:** National Center of Biotechnology Information

359 **PCR:** Polymerase chain reaction

360 **KDR:** knockdown resistance

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

362 **COI:** Cytochrome c oxidase subunit 1 gene

363 **CDC:** Centers for Disease Control and Prevention

364 **WHO:** World Health Organization

365

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476

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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