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

Tamar E. Carter[1]*, Araya Gebresilassie[2], Shantoy Hansel[3], Lambodhar Damodaran[4], Callum Montgomery[3], Victoria Bonnell[5], Karen Lopez[3], Daniel Janies[3]**, Solomon Yared[6]**

1) Department of Biology, Baylor University, Waco, TX, USA
2) Department of Zoological Sciences, Addis Ababa University, Addis Ababa, Ethiopia
3) Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, NC, USA
4) Institute of Bioinformatics, University of Georgia, Athens, GA, USA
5) Department of Molecular Biology and Biochemistry, Pennsylvania State University, State College, PA, USA
6) Department of Biology, Jigjiga University, Jigjiga, Ethiopia

TC: tamar_carter@baylor.edu
AG: arayagh2006@yahoo.com
SH: shansel@uncc.edu
LD: Lambodhar.Damodaran@uga.edu
CM: cmontg24@uncc.edu
TB: vab18@psu.edu
KL: klopez1@uncc.edu
DJ: djanies@uncc.edu
SY: solyar2005@yahoo.com

*Corresponding author: tamar_carter@baylor.edu
** Contributed equally

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

Figures: 3, **Tables:** 3

Abstract

The malaria vector, *Anopheles stephensi*, which is typically restricted to South Asia and the Middle East, was recently detected in the Horn of Africa. Controlling the spread of this vector could involve integrated vector control that considers the status of insecticide resistance of multiple vector species in the region. Previous reports indicate that the knockdown resistance mutations (*kdr*) in the voltage-gated sodium channel (*vgsc*) are absent in both pyrethroid resistant and sensitive variants of *An. stephensi* in east Ethiopia but similar information on other vector species in the same areas is limited. In this study, *kdr* and the neighboring intron was analyzed in *An. stephensi*, *An. arabiensis*, and *Culex pipiens s. l.* collected in east Ethiopia between 2016 and 2017. Sequence analysis revealed that all of *Cx. pipiens s.l.* (n = 42) and 71.6% of the *An. arabiensis* (n=67) carried *kdr* L1014F known to confer target-site pyrethroid resistance. Intronic variation was only observed in *An. stephensi* (segregating sites = 6, haplotypes = 3) previously shown to have no kdr mutations. In addition, no evidence of non-neutral evolutionary processes was detected at the *An. stephensi kdr* intron which further supports target-site mechanism not being a major resistance mechanism in this *An. stephensi* population. Overall, these results suggest differences in evolved mechanisms of pyrethroid/DDT resistance in populations of vector species from the same region. Variation in insecticide resistance mechanisms in East Ethiopian mosquito vectors highlight possible species or population specific biological factors and distinct environmental exposures that shape their evolution.

BACKGROUND

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 Africa is now challenged with the recent discoveries of *An. stephensi*, a malaria vector, which is typically restricted to South Asia and the Middle East, in the Horn of Africa and recently demonstrated to transmit local *Plasmodium* 3, 4, 5, 6. Among several approaches to mitigating the *An. stephensi* is integrated vector control that target multiple vectors. Integrated vector control has the benefits of cutting costs and while minimizing adverse outcomes of single-target vector control on non-target species populations. 7

Integrated vector control strategies based on insecticides should account for insecticide resistance status of the different vectors. In Ethiopia, insecticides like pyrethroids have been deployed through indoor residual spraying and long-lasting insecticidal nets (LLIN). Exacerbated by the use of insecticides in the agricultural industries, widespread insecticide resistance has been reported across multiple vector species 8. In Culicidae, the main mechanisms of resistance to pyrethroids include target-site and metabolic-based resistance 9. Pyrethroid based target-site resistance is caused by mutations in the voltage-gated sodium channel leading to altered neurological response to insecticides in mosquitoes [i.e. knockdown resistance (*kdr*), reviewed in 10]. Knockdown resistance is broadly studied and is widely reported across species of Culicidae including *Anopheles* spp. 9 and *Culex pipiens s.l.* 11. In *Anopheles*, *kdr* involves the substitution of leucine (TTA) with phenylalanine (TTT) or serine (TCA) in the voltage gated sodium channel protein, commonly known as *kdr* mutations L1014F and L1014S 12. Similar mutations that confer resistance to pyrethroids (also known as L1014F and L1014S) are observed in the *vgsc* of *Culex* mosquitoes.

For metabolic resistance, the insecticide is degraded, sequestered or exported out of the cell before it can bind to its target 9. Metabolic resistance has not been linked to a single trackable genetic

69 variant in most species. However, previous functional studies have found the over-expression of
70 detoxification enzymes such as cytochrome P450s lead to metabolic resistance 9, 13.

71 In Ethiopia, pyrethroid and DDT resistance have been reported in much of the northern and
72 western portion of the country in the primary malaria vector *An. arabiensis* 14, 15, 16, 17. In *An.*
73 *arabiensis,* both target-site and metabolic resistance play a role in pyrethroid and DDT resistance. In
74 eastern Ethiopia, a recent investigation revealed *An. stephensi* were resistant to pyrethroids, although, the
75 L1014F and L1014S mutations were absent 18. *An. arabiensis* insecticide resistance in eastern Ethiopia
76 has not been well characterized. Even more so, the status of insecticide resistance in *Cx. pipiens s.l.* (most
77 likely *Cx. quinquefasciatus*) is unknown throughout most of the country.

78 Knowing the status of resistance to pyrethroids across vector species in a region can provide insight
79 into the effectiveness of particular insecticides used to target multiple species. Genetic analyses of putative
80 insecticide resistance loci across local vector populations, can provide information on the range of
81 mechanisms of insecticide resistance in a region. While *kdr* L1014F and L1014S mutation frequencies
82 provide preliminary evidence of target-site resistance to pyrethroids, analysis of the variation in neighboring
83 intronic region provides information of the long-term impact of pyrethroids on the evolution of the mosquito
84 populations. Tests for neutrality, such as Tajima's D 19, can be used to evaluate the genetic diversity of
85 the kdr locus including the intronic region to determine if the patterns differ from expectations under neutral
86 evolution. It is expected that if the *kdr* locus was under selection due to pressure from the pyrethroids, then
87 we hypothesize that a selective sweep would have led to decreased nucleotide diversity of linked alleles
88 20, 21. Thus, these analyses are helpful in clarifying the mechanisms of resistance, the current status of
89 pyrethroid resistance, and predicting the risk of resistance emerging locally. Here we examine the
90 nucleotide diversity surrounding the *kdr* locus to test for the hypothesis of selective sweeps in *An. stephensi,*
91 *An. arabiensis*, and *Culex pipiens s. l.* collected in east Ethiopia.

92

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 18 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 were collected from Kebri Dehar in 2016 as part the first detection of this species in Ethiopia 4. Mosquitoes were collected as larvae and lab-reared for testing for resistance to insecticides as previously detailed 18. *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 22. *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 Harewe (northeast) in 2017. Harewe and Meki are about 350 km northwest and 600 km west of Kebri Dehar, respectively (Fig 1).

Fig. 1 Collection sites.

Culex specimens were collected using CDC light traps in Kebri Dehar in 2017. Morphological key and sequencing of *ITS2* locus were used for *Culex* identification using a previously published PCR protocol 4. All amplicons were cleaned using Exosap and sequenced using Sanger technology with ABI BigDyeTM Terminator v3.1 chemistry (Thermofisher, Santa Clara, CA) according to manufacturer recommendations and run on a 3130 Genetic Analyzer (Thermo Fisher, Santa Clara, CA). Sequences were cleaned and analyzed using CodonCode Aligner Program V. 6.0.2 (CodonCode Corporation, Centerville, MA). ITS2 sequences from *Culex* specimen were submitted as queries to the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) for species identification 23.

Amplification and sequencing of kdr loci

117 Once species or species complex identification was complete, samples were processed. For *kdr* mutation
118 analysis, polymerase chain reaction (PCR) was used to amplify the region of the *vgsc* gene that housed the
119 homologous *kdr* 1014 and a neighboring downstream intron in all specimens (reference sequences used for
120 *An. stephensi*, *An. arabiensis* and *Culex pipiens sl*. were JF304952, GU248311, and BN001092,
121 respectively). One leg from each mosquito specimen or extracted DNA was used as individual templates
122 for PCR. Each species required a different PCR protocol. DNA extraction were performed using DNEasy
123 Qiagen kit (Qiagen, Valencia, USA). All PCR reactions were performed at 25µl total with 12.5 ul 2X
124 Promega Hot Start Master Mix (Promega Corporation, Madison, USA) and the primer conditions listed in
125 Tab 1. *An. stephensi kdr* amplification was completed according to Singh et al., 24 with modifications as
126 detailed in Yared et al., 18. Temperature cycling was as follows: 95°C for 5 min, followed by 35 cycles of
127 95°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, and a final extension of 72 °C for 7 min. Amplifications
128 of the kdr fragment from *An. arabiensis* were completed according to methods in Verhaeghen et al 25.
129 Temperature cycling was as follows: 95°C for 1 min, followed by 30 cycles of 95°C for 30 sec, 52°C for
130 30 sec, 72°C for 1 min, and a final extension of 72°C for 10 min. Amplifications of the kdr fragment from
131 *Culex pipiens* s.*l* were completed according methods in Chen et al 26. Temperature cycling was as follows:
132 94°C for 5 min, followed by 30 cycles of 94°C for 40 sec, 58°C for 30 sec, 72°C for 40 sec, and a final
133 extension of 72°C for 8 min.

134 All amplicons were cleaned using Exosap and sequenced using Sanger technology with ABI BigDyeTM
135 Terminator v3.1 chemistry (Thermofisher, Santa Clara, CA) according to manufacturer recommendations
136 and run on a 3130 Genetic Analyzer (Thermo Fisher, Santa Clara, CA).

137

138 *Sequence analysis*

139 Sequences were submitted as queries to the National Center for Biotechnology Information's
140 (NCBI) Basic Local Alignment Search Tool (BLAST) to confirm correct loci were amplified. Sequences
141 were then aligned in CodonCode (CodonCode Corp., Dedham, MA, USA) by species or species complex

142 to identify *kdr* L1014F or L1014S mutations based on reference sequence details from previous reports 18,
143 24, 25. Heterozygous genotypes at kdr was determined based on the number of peaks observed in the
144 chromatogram, with each peak indicate a different alleles. The *kdr* allele and genotype frequencies were
145 then calculated and compared across species.

146 We determined the level of diversity in the neighboring intron downstream of the *kdr* 1014 in *Culex*
147 spp., *An. arabiensis, and An. stephensi* for additional evidence of selection on that locus. In addition to the
148 sequences generated in this study, we included sequences from resistant and non-resistant *An. stephensi*
149 analyzed in a previous study on insecticide resistance in *An. stephensi* 18. We calculated the number of
150 segregating sites, nucleotide diversity, the estimated number of haplotypes, and haplotype diversity using
151 the program DNAsp v5 27. Haplotypes were reconstructed using Phase 2.1 28, HAPAR, and fastPHASE
152 29 algorithms in DNAsp. The neighboring downstream intron was also tested for neutrality using Tajima's
153 D 19, Fu's F 30, and Fu and Li's D* and F* tests 31.

154 **RESULTS**

155 Prior to insecticide resistance genotyping, all *Culex* ITS2 sequences were analyzed to identify
156 species. All sequences were identical and had equivalent high matching scores for two members of the *Cx.*
157 *pipiens* complex: *Cx. p. quinquefasciatus* and *Cx. p. pipiens*. Because we could not identify these mosquitos
158 to species, we will refer to these specimens by the broader taxonomic classification, *Cx. pipiens s. l.* (i.e.,
159 *Cx. pipiens* complex) in this study. *An. arabiensis* species identification was detailed in previous study 22.
160 In total, 10, 33, and 24 *An. arabiensis* were collected in Harewe in November 2016, Harewe in July/August
161 2017, and Meki in July 2017 collections, respectively.

162 *Kdr analysis*

163 The *kdr* fragments were sequenced for *An. stephensi*, *Cx. pipiens s.l.,* and *An. arabiensis.* The
164 sequencing resulted in 184, 452, and 290 base pair fragments for *An. stephensi*, *Cx. pipiens s. l.* and *An.*
165 *arabiensis*, respectively. The percent of each *kdr* genotype observed by species is shown in Fig 2. A total

166 of 131 *An. stephensi* were analyzed, including 80 newly reported sequences. None of the *An. stephensi*
167 analyzed in this study carried a mutation at the *kdr* 1014. All 42 *Cx. pipiens s.l*. specimens collected at the
168 same site carried *kdr* L1014F mutations as homozygous. Of the 67 *An. arabiensis*, 71.6% carried the *kdr*
169 L1014F mutation (heterozygous and homozygous). The allele frequency of L1014F mutation varied across
170 *An. arabiensis* collections, where the highest frequency was observed in Harewe in November 2016
171 (100%). L1014F allele frequency for Harewe July/August 2017 and in Meki July 2017 collections were
172 86.4% and 10%, respectively. No L101S mutations were detected in *Cx. pipiens* s.l. or *An. arabiensis*.

173 **Fig. 2** Frequency of *kdr* 1014 genotypes in *An. stephensi, Culex pipiens s.l.*, and *An. arabiensis*
174 collections.

175 A portion of the neighboring downstream intron for each species was analyzed to evaluate the
176 level of diversity (Fig 3). Intron analysis revealed no polymorphisms for either *Cx. pipiens* or *An. arabiensis*
177 (for both L1014F and L1014 wild type specimens). Of the 131 *An. stephensi* specimens from Kebri Dehar
178 examined for *kdr* mutations, six segregating sites were detected, and three haplotypes predicted. Genetic
179 diversity estimates are reported in Tab 2.

180 To further evaluate the potential functional significance of the *kdr* locus in *An. stephensi* based on
181 evidence of positive selection, we performed tests for neutrality at the *An. stephensi kdr* intron. No evidence
182 of non-neutral processes was detected in *An. stephensi* for the *kdr* locus (Tab 3). The absence of variation
183 in *An. arabiensis* and *Cx. pipiens* s.l. *kdr* introns precluded tests for neutrality.

184 **Fig. 3** Summary of *kdr* haplotypes across three Culicidae species in east Ethiopia. Solid lines
185 depict the exon housing the *kdr* locus and dotted lines depict the downstream intron. Green square
186 indicates the presence of the *kdr* L1014F. Triangles denote single nucleotide polymorphisms
187 (SNPs) found in the intron relative to the most prevalent intron haplotype.

188

189

190 **DISCUSSION**

191 Our results reveal variation at the *kdr* locus across different vector species found in east Ethiopia
192 suggesting different mechanisms of pyrethroid/DDT resistance. Notably, the *kdr* L1014F mutation was not
193 observed consistently across the species included in this study. Unlike the *An. stephensi*, that carried no
194 L1014F mutations, both *Cx. pipiens sl.* and *An. arabiensis* carried the L1014F. Based on these findings, it
195 is likely that *Cx. pipiens s.l.* and *An. arabiensis* should not share the same mechanisms of pyrethroid
196 resistance as *An. stephensi*. We also observed differences in the nucleotide diversity of the neighboring
197 intronic region of the three species. While *An. stephensi* exhibited multiple segregating sites and resultant
198 haplotypes, only a single intronic haplotype is observed for *An. arabiensis* and *Cx. pipiens s.l.* These data
199 may point to distinct differences in biological and environmental factors that shape each species/population.
200 From a species standpoint, behaviors shaped by both their biology and environment, like feeding and resting
201 preferences may impact the degree of exposure to insecticides. *Cx. pipiens sl* tend toward exophilic behavior
202 and feed generally during the day up to the early evening outdoors. *An. stephensi* and *An. arabiensis* both
203 feed at night, however, *An. stephensi* is mostly endophilic while *An. arabiensis* is exophilic.

204 In addition to species level differences, the different patterns of *kdr* variation may be explained by
205 multiple evolutionary processes acting on each population sampled: 1) The data may reflect different levels
206 of selective pressure occurring at each location, such that the populations that were under selective pressure
207 from insecticides exhibited *kdr* mutations and no intronic variation. 2) The variation could also reflect
208 previous demographic events, like recent drops in population size or population introductions resulting in
209 a bottleneck and a decline in intronic variation. We can best evaluate these possibilities in the context of
210 variation at other regions of the genomes in these mosquitoes. The COI have been previously analyzed in
211 the *An. arabiensis* and *An. stephensi* (Carter et al. 2018, Carter el 2019). While multiple COI haplotypes
212 were observed for each *An. arabiensis* collection, only a single COI haplotype was identified in the *An.*
213 *stephensi*. The higher level of diversity in COI in *An. arabiensis* relative to the *kdr* intronic region supports
214 that selective pressure rather than population bottleneck has shaped the variation at the *kdr*. The opposite

215 pattern observed in the *An. stephensi* gives greater support for the absence of selection on that locus in that
216 species. The degree of variation at kdr in *An. stephensi* may also reflect the likely notion of this species
217 being a recent introduction to that region, so it would not have had the same years of exposure to the local
218 pressure that would cause evolved target-site resistance in the local vector populations. No COI data was
219 available for the *Cx. pipiens* s.l. in this study, and both population bottleneck and/or selection on the *kdr*
220 locus remain plausible explanations for the lack of variation.

221 The multiple collections that comprised our *An. arabiensis* sample set provide preliminary insight
222 into the basis for population *kdr* variation within a species. We observed a range of *kdr* allele frequencies
223 across the *An. arabiensis* sample collections. The collections differ by location and/ or date of collection,
224 suggesting the geography or timing could play a role in the variation in kdr L014 frequencies observed.
225 Additional surveillance in a larger sample size is needed to verify the importance of geographic and
226 temporal factors shaping the frequency of the mutation. Another notable observation, was the shared intron
227 haplotype between the *An. arabiensis* that carried the L1014F mutation to those that did not. The
228 mosquitoes that carried the once advantageous allele may suffer fitness costs in the absence of the selective
229 pressure, which would result in a rebound of the wild-type allele at that locus. These findings underline the
230 value of investigating the *kdr* intronic variation for evidence of fluctuating selective pressures and the
231 potential for the emergence of insecticide resistance in the future.

232 Several limitations to these studies should be considered. The *An. stephensi* were collected as larvae
233 and pupae and the *An. arabiensis* and *Cx. pipiens s.l.* were collected as wild-caught adults. This method of
234 collection may pose a concern that the immature specimen set would not reflect the natural diversity of the
235 wild-caught adult population. Concerns with clonality however are lowered when considering the level of
236 diversity observed at the *An. stephensi kdr* locus and at the ace-1R locus (3 haplotypes detected; data not
237 shown). In addition, while *An. stephensi* phenotypic resistance has been reported for east Ethiopia,
238 phenotypic data on *An. arabiensis* and its association with *kdr* has only been studied for portions of the
239 country outside of east Ethiopia. Also, the association of *kdr* mutations and phenotypic resistance in *Cx.*

240 *pipiens sl* observed in other parts of the world have not been confirmed in Ethiopia. Follow-up studies
241 would benefit from additional bioassay tests for *An. arabiensis* and *Cx. pipiens* in east Ethiopia in junction
242 with the molecular analysis of *kdr*. Finally, given the geographic variation in kdr mutation frequencies
243 observed in *An. arabiensis*, future studies should look at the frequency of *kdr* mutations of these vectors in
244 other regions in Ethiopia to confirm the status of target-site pyrethroid/ DDT resistance.

245 In conclusion, the different patterns of diversity at the *kdr* loci across species indicate that Culicidae
246 in east Ethiopia likely have different mechanisms of resistance profiles. Both *An. arabiensis* and *Cx. pipiens*
247 sample sets revealed notable L1014F allele frequencies that confer target-site resistance and absence of
248 intron variation that tells of selective pressure on that locus in those species. Additional investigations are
249 needed to determine the mechanisms and genetic basis of pyrethroid resistance (metabolic, cuticle, or
250 another undiscovered mechanism) in *An. stephensi*. These finding emphasize the need for careful
251 consideration of molecular approaches used to evaluate insecticide resistance status across multiple species
252 and will inform the development and future implementation of novel integrated vector control strategies.

253 **Abbreviations**

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

255 **DNA:** Deoxyribonucleic Acid

256 **FMOH:** Federal Ministry of Health

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

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

259 **PCR:** Polymerase chain reaction

260 ***KDR*:** knockdown resistance

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

COI: Cytochrome c oxidase subunit 1 gene

CDC: Centers for Disease Control and Prevention

WHO: World Health Organization

Declarations

Competing interests

The authors declare that they have no competing interests and the manuscript has not been published before or submitted elsewhere for publication.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its supplemental files. Sequences have been submitted to NCBI Genbank database.

Funding

This study was financially supported by Jigjiga University. This project was also funded by Baylor University and the University of North Carolina at Charlotte Multicultural Postdoctoral Fellowship.

Acknowledgements

Our gratitude goes to Mr. Negib Abdi and Habtamu Atlaw for their facilitating financial and arranging the car for the field work. We would also like to thank Mr. Geleta Bekele for his technical support in rearing mosquito at field laboratory. We would like to also thank Dr. Jason Pitts and Ms. Jeanne Samake for the helpful comments on the manuscript. We acknowledge the support of various entities of the University of North Carolina at Charlotte including: the Department of Bioinformatics and Genomics of the College of Computing and Informatics and the Department of Biological Sciences of the College of Literature Science and the Arts. We are grateful for the support of the Belk Family. We would also like to

acknowledge the support of Baylor University Department of Biology of the College of Arts and Sciences.

285 **Tables**

286 **Tab 1**. List of primer and conditions used for PCR amplification of portions of the voltage gated sodium
287 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

288

289

290 **Tab 2.** Genetic diversity estimates for *kdr* neighboring downstream intron in the *vgsc* for *An. stephensi*,
291 *An. arabiensis*, and *Cx. pipiens s.l.,* where n = number of genes (two per individuals), S = number of
292 polymorphic (i.e., segregating) sites, K = average number of pairwise nucleotide differences, Pi =
293 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
Cx. pipiens s.l.	84	0	0	0	1	0

294

295

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

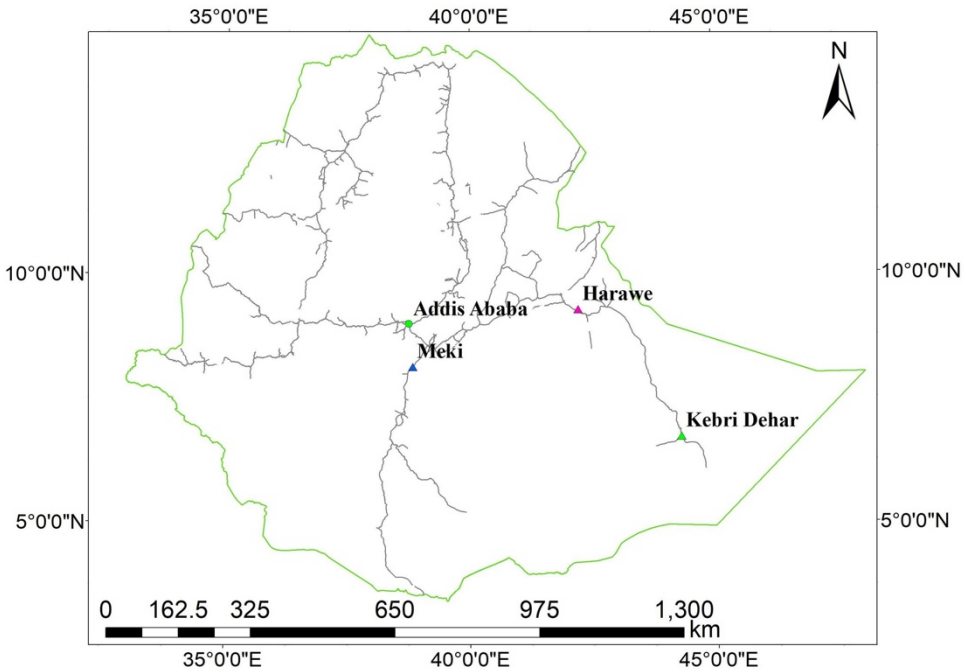
Test	**Estimate**
n	258
Tajima's D	0.03839
Fu's F	3.556
Fu and Li's D	1.04354
Fu and Li's F	0.82943

297

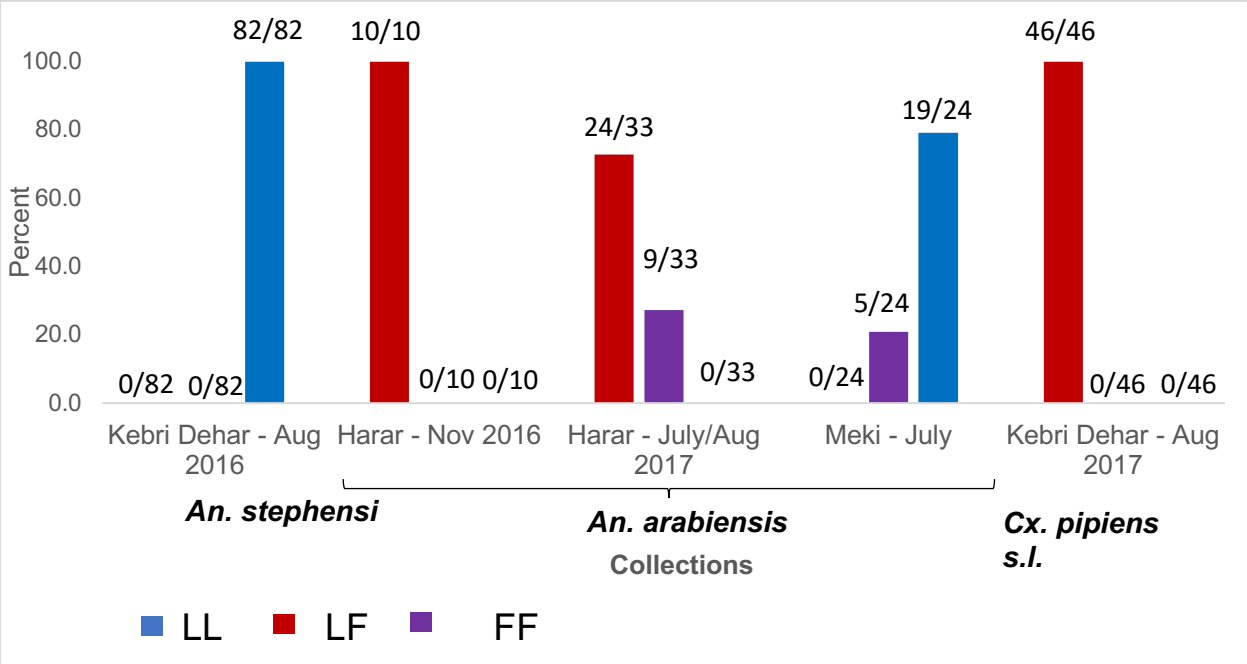
298

Figures

Fig. 1 Collection sites.



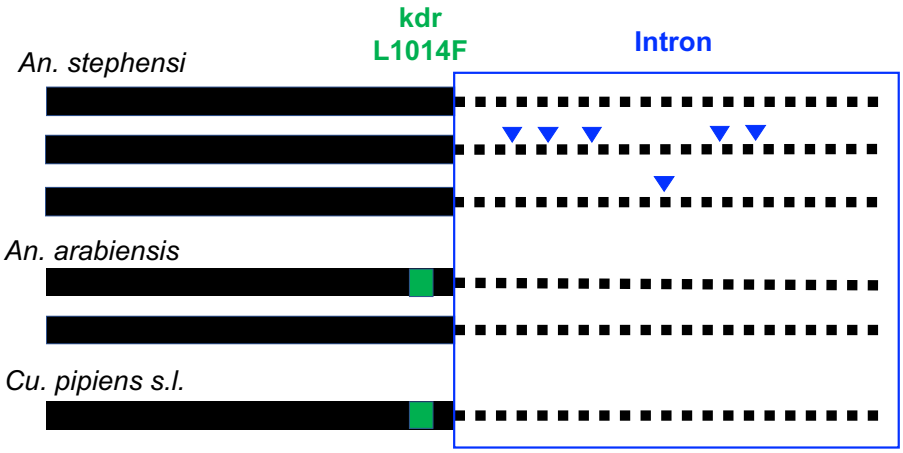
311 **Fig. 2** Frequency of *kdr* 1014 genotypes in *An. stephensi, Culex pipiens s.l.*, and *An. arabiensis* collections.



312

313

314 **Fig. 3** Summary of *kdr* haplotypes across three Culicidae species in east Ethiopia. Solid lines depict the
315 exon housing the *kdr* locus and dotted lines depict the downstream intron. Green square indicates the
316 presence of the *kdr* L1014F. Triangles denote single nucleotide polymorphisms (SNPs) found in the
317 intron relative to the most prevalent intron haplotype.



318

319

320

321

322 **References**

323 1. Organization WH, 2019. World malaria report 2019.

324 2. Organisation WH, 2018. World malaria report 2018.

325 3. Balkew M, et al., 2020. Geographical distribution of *Anopheles stephensi* in eastern Ethiopia.
326 Parasit Vectors 13: 35.

327 4. Carter TE, Yared S, Gebresilassie A, Bonnell V, Damodaran L, Lopez K, Ibrahim M,
328 Mohammed S and Janies D, 2018. First detection of *Anopheles stephensi* Liston, 1901
329 (Diptera: culicidae) in Ethiopia using molecular and morphological approaches. Acta Trop
330 188: 180-186.

331 5. Faulde MK, Rueda LM and Khaireh BA, 2014. First record of the Asian malaria vector
332 *Anopheles stephensi* and its possible role in the resurgence of malaria in Djibouti, Horn of
333 Africa. Acta Trop 139: 39-43.

334 6. Seyfarth M, Khaireh BA, Abdi AA, Bouh SM and Faulde MK, 2019. Five years following first
335 detection of *Anopheles stephensi* (Diptera: Culicidae) in Djibouti, Horn of Africa:
336 populations established-malaria emerging. Parasitol Res 118: 725-732.

337 7. Golding N, Wilson AL, Moyes CL, Cano J, Pigott DM, Velayudhan R, Brooker SJ, Smith DL,
338 Hay SI and Lindsay SW, 2015. Integrating vector control across diseases. BMC Med 13:
339 249.

340 8. Ranson H and Lissenden N, 2016. Insecticide Resistance in African *Anopheles* Mosquitoes: A
341 Worsening Situation that Needs Urgent Action to Maintain Malaria Control. Trends in
342 Parasitology 32: 187-196.

343 9. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z and Corbel V, 2011. Pyrethroid resistance
344 in African anopheline mosquitoes: what are the implications for malaria control? Trends
345 in Parasitology 27: 91-98.
346 10. Davies TG, Field LM, Usherwood PN and Williamson MS, 2007. A comparative study of
347 voltage-gated sodium channels in the Insecta: implications for pyrethroid resistance in
348 Anopheline and other Neopteran species. Insect Mol Biol 16: 361-75.
349 11. Scott JG, Yoshimizu MH and Kasai S, 2015. Pyrethroid resistance in *Culex pipiens*
350 mosquitoes. Pestic Biochem Physiol 120: 68-76.
351 12. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J and Collins FH, 2000. Identification
352 of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles*
353 *gambiae* associated with resistance to DDT and pyrethroids. Insect Molecular Biology 9:
354 491-497.
355 13. David JP, Ismail HM, Chandor-Proust A and Paine MJ, 2013. Role of cytochrome P450s in
356 insecticide resistance: impact on the control of mosquito-borne diseases and use of
357 insecticides on Earth. Philos Trans R Soc Lond B Biol Sci 368: 20120429.
358 14. Alemayehu E, et al., 2017. Mapping insecticide resistance and characterization of resistance
359 mechanisms in *Anopheles arabiensis* (Diptera: Culicidae) in Ethiopia. Parasit Vectors 10:
360 407.
361 15. Asale A, Getachew Y, Hailesilassie W, Speybroeck N, Duchateau L and Yewhalaw D, 2014.
362 Evaluation of the efficacy of DDT indoor residual spraying and long-lasting insecticidal
363 nets against insecticide resistant populations of *Anopheles arabiensis* Patton (Diptera:
364 Culicidae) from Ethiopia using experimental huts. Parasit Vectors 7: 131.

365 16. Balkew M, Ibrahim M, Koekemoer LL, Brooke BD, Engers H, Aseffa A, Gebre-Michael T
366 and Elhassen I, 2010. Insecticide resistance in *Anopheles arabiensis* (Diptera: Culicidae)
367 from villages in central, northern and south west Ethiopia and detection of kdr mutation.
368 Parasit Vectors 3: 40.
369 17. Yewhalaw D, Van Bortel W, Denis L, Coosemans M, Duchateau L and Speybroeck N, 2010.
370 First Evidence of High Knockdown Resistance Frequency in *Anopheles arabiensis*
371 (Diptera: Culicidae) from Ethiopia. American Journal of Tropical Medicine and Hygiene
372 83: 122-125.
373 18. Yared S, Gebressielasie A, Damodaran L, Bonnell V, Lopez K, Janies D and Carter T, 2020.
374 Insecticide Resistance in *Anopheles stephensi* in Somali Region, Eastern Ethiopia.
375 19. Tajima F, 1989. Statistical method for testing the neutral mutation hypothesis by DNA
376 polymorphism. Genetics 123: 585-95.
377 20. Chang X, et al., 2016. Landscape genetic structure and evolutionary genetics of insecticide
378 resistance gene mutations in *Anopheles sinensis*. Parasit Vectors 9: 228.
379 21. Weill M, Chandre F, Brengues C, Manguin S, Akogbeto M, Pasteur N, Guillet P and Raymond
380 M, 2000. The kdr mutation occurs in the Mopti form of *Anopheles gambiae* s.s. through
381 introgression. Insect Molecular Biology 9: 451-455.
382 22. Carter TE, Yared S, Hansel S, Lopez K and Janies D, 2019. Sequence-based identification of
383 *Anopheles* species in eastern Ethiopia. Malar J 18: 135.
384 23. Madden T, 2002. The BLAST Sequence Analysis Tool. Bethesda, MD
385 24. Singh OP, Dykes CL, Lather M, Agrawal OP and Adak T, 2011. Knockdown resistance (kdr)-
386 like mutations in the voltage-gated sodium channel of a malaria vector *Anopheles stephensi*
387 and PCR assays for their detection. Malaria journal 10: 59.

388 25. Verhaeghen K, Van Bortel W, Roelants P, Backeljau T and Coosemans M, 2006. Detection of
389 the East and West African kdr mutation in *Anopheles gambiae* and *Anopheles arabiensis*
390 from Uganda using a new assay based on FRET/Melt Curve analysis. Malar J 5: 16.
391 26. Chen L, et al., 2010. Molecular ecology of pyrethroid knockdown resistance in *Culex pipiens*
392 pallens mosquitoes. PLoS One 5: e11681.
393 27. Rozas J, Sanchez-DelBarrio JC, Messeguer X and Rozas R, 2003. DnaSP, DNA polymorphism
394 analyses by the coalescent and other methods. Bioinformatics 19: 2496-7.
395 28. Stephens M, Smith NJ and Donnelly P, 2001. A new statistical method for haplotype
396 reconstruction from population data. Am J Hum Genet 68: 978-89.
397 29. Scheet P and Stephens M, 2006. A fast and flexible statistical model for large-scale population
398 genotype data: applications to inferring missing genotypes and haplotypic phase. Am J
399 Hum Genet 78: 629-44.
400 30. Fu YX, 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking
401 and background selection. Genetics 147: 915-25.
402 31. Fu YX and Li WH, 1993. Statistical tests of neutrality of mutations. Genetics 133: 693-709.

403

404