1   *Anopheles stephensi* **as an emerging malaria vector in the Horn of Africa with high**

2   **susceptibility to Ethiopian *Plasmodium vivax* and *Plasmodium falciparum* isolates**

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30   **Summary of the article**: *An. stephensi,* a metropolitan malaria vector that recently expanded

31   to the Horn of African, was highly susceptible to local *P. falciparum* and *P. vivax* isolates

32   from Ethiopia and may increase malariogenic potential of rapidly expanding urban settings in

33   Africa.

34   **Running title:** *An. stephensi* is an emerging vector in Africa

35   **Keywords:** *An. stephensi*, urban, vector competence, membrane feeding, emerging,

36   outbreak, transmission

37

38 **Abstract**

39 *Anopheles stephensi*, an efficient Asian malaria vector, recently spread into the Horn of

40 Africa and may increase malaria receptivity in African urban areas. We assessed occurrence,

41 genetic complexity, blood meal source and infection status of *An. stephensi* in Awash Sebat

42 Kilo town, Ethiopia. We used membrane feeding assays to assess competence of local *An.*

43 *stephensi* to *P. vivax* and *P. falciparum* isolates from clinical patients. 75.3% of the examined

44 waterbodies were infested with *An. stephensi* developmental stages that were

45 genetically closely related to isolates from Djibouti and Pakistan. Both *P. vivax* and *P.*

46 *falciparum* were detected in wild-caught adult *An. stephensi*. Local *An. stephensi* was more

47 receptive to *P. vivax* compared to a colony of *An. arabiensis*. We conclude that *An.*

48 *stephensi* is an established vector in this part of Ethiopia, highly permissive for local *P.*

49 *vivax* and *P. falciparum* isolates and presents an important new challenge for malaria

50 control.

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Background

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55   With expanded global malaria control efforts there have been two decades of substantial

56   declines in malaria cases and deaths. These successes were mainly attributable to wide-scale

57   deployment of vector control tools and availability of efficacious treatment [1]. Control

58   programs in Africa traditionally focus on rural settings, which is where most infections occur

59   [2] although malaria transmission is also a health concern in some urban settings [3, 4].

60   In 2015, 38% of Africans were living in urban settings; the number of Africans residing in

61   urban areas is expected to double in the coming 25 years [5]. Urban settings can be sinks of

62   malaria transmission primarily associated with importation of malaria from (rural) areas of

63   intense transmission due to movement of people at the urban-rural interface [6]. With the

64   adaptation of existing vectors to urban environments [7] and emerging vectors such as

65   *Anopheles stephensi* in urban areas [8], malaria transmission in urban settings is becoming

66   more likely. Urban areas can thereby form foci of active malaria transmission [9]. *An.*

67   *stephensi* is an efficient vector for both *Plasmodium vivax* and *P. falciparum* in Asia and is

68   the dominant malaria vector in India and the Persian Gulf [10]. *An. stephensi* predominantly

69   breeds in urban settings with a preference for human-made water storage containers [11].

70   Recent reports indicate that *An. stephensi* is spreading in the Horn of Africa (Djibouti [13],

71   Ethiopia [14] and the Republic of Sudan [15]). *An. stephensi* emergence has been

72   epidemiologically linked to an unusual resurgence in local malaria cases in Djibouti city [16].

73   A recent technical consultative meeting convened at the World Health Organization (WHO)

74   identified that there is potential for spread of *An. stephensi* across Africa and urged for more

75   data on its distribution to allow monitoring of potential spread of *An. stephensi* from the

76   currently affected areas and on the vector's susceptibility to local *Plasmodium* isolates [15].

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78   In the present study, we examined the abundance of *An. stephensi* in an urban setting in

79   Ethiopia, characterized its aquatic habitats, biting and resting behavior, and, for the first time,

80   examined its competence to transmit local *P. vivax* and *P. falciparum* isolates.

81

82   **Methods**

83   **Description of study site**

84   This study was conducted in Awash Sebat Kilo town (916 meters above sea level;

85   8°58'59.99" N 40°10'0.01" E), on the main transportation corridor from Addis Ababa (220km

86   southeast) to Djibouti (Figure 1). The town has an estimated total population of 24,700 [17];

87   the semi-arid climate is dominated by a major rainy season (July-August) and short

88   intermittent rains (April/May) and an average temperature of 25.8^oC (17.3^oC-33.6^oC) [18].

89   The Awash River Valley is the most irrigated area in the country with extensive river-fed

90   agriculture. Malaria transmission is perennial in the area surrounding the town with annual

91   parasite incidence of 536 per 1000 population in 2019 (five-year trend summarized in

92   supplemental notes). Entomological surveys conducted in 2018 detected the occurrence of

93   *An. stephensi* in Awash Sebat Kilo town [19].

94   **Characterization of aquatic habitat, resting, feeding and biting behavior**

95   Aquatic sites were examined for the presence of *Anopheles* developmental stages by standard

96   dipping (10x) for 5 consecutive days. Developmental stages were separated from culicines in

97   the field and transported to Adama malaria center for rearing to adults. The resting, feeding

98   and host-seeking behavior of *An. stephensi* was assessed using five conventional

99   entomological sampling techniques: i) Centers for Disease Control (CDC) miniature light

100   traps (Johns W. Hocks Company model 512) catches, ii) human landing catches (HLC), iii)

101   pyrethrum spray sheet collection (PSC), iv) aspiration from animal shelters, and v) cattle-

102   baited traps (Supplemental notes). Adult mosquitoes were identified morphologically using

103   standard identification keys [20]. Fully fed mosquitoes identified as *An. stephensi* were kept

104   in paper cups at Adama laboratory in ambient conditions and allowed to lay eggs on filter

105   papers soaked in water on a cotton roll for egg ridge counts (Supplemental notes).

106

107   **Mosquito rearing and membrane feeding assay**

108   *An. stephensi* were reared from larvae/pupae (from aquatic site examinations) to adult at

109   ambient temperature (26 ± 3°C) and relative humidity ($70\pm10\%$) and fed with fish food

110   (Cichlid Sticks; King British Fish Food, Tetra). *An. arabiensis*, the principal malaria vector

111   of Ethiopia, from an established colony were reared under identical conditions and

112   maintained with 10% sucrose solution. Following informed consent, patients who presented

113   to the Adama malaria clinic with microscopy confirmed *P. vivax* and *P. falciparum* mono-

114   and mixed-species infections were asked to donate venous blood sample (5mL) in lithium

115   Heparin tubes (BD Vacutainer®). Asexual parasite and gametocyte densities were quantified

116   by two expert microscopists on thick blood films prepared from finger prick blood samples,

117   screening against 1000 leukocytes. Thin blood films were examined to identify *Plasmodium*

118   species.

119   Four-to-seven day old adult *An. stephensi* and *An. arabiensis* were starved for 3 (*An.*

120   *stephensi*) or 12 hours (*An. arabiensis*) before feeding. One hundred and twenty mosquitoes

121   of each species, 40 in each of 3 paper cups, were fed fresh patient blood through membrane

122   in the dark for exactly 25 minutes (Supplemental notes). Fully fed mosquitoes were

123   maintained under the same laboratory conditions with 10% sucrose solution for 7 days post

124   feeding before being dissected for oocyst detection and for 12 days for sporozoite detection.

125   **Molecular detection of parasites and blood meal sources and targeted sequencing of**

126   **morphologically identified *An. stephensi* mosquitoes**

127   *Plasmodium* infection status of individual wild-caught morphologically-confirmed adult *An.*

128   *stephensi* mosquitoes was assessed using nested polymerase chain reaction (nPCR) targeting

129   the small 18S subunit [21] using genomic DNA extracted from homogenate of mosquito's

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130   head-thorax and abdomen separately [22], indicating sporozoite and oocyst-stage infections,

131   respectively. Multiplex PCR that targets the mitochondrial cytochrome b gene and produces

132   species-specific fragments of varying sizes was used to assess blood meal sources of

133   individual mosquitoes [23]. For confirmation of morphologically identified *An. stephensi*,

134   DNA was extracted from whole mosquito bodies using the DNeasy Blood and Tissue kit

135   (Qiagen, UK). PCR was performed for each individual mosquito, targeting the nuclear

136   internal transcribed spacer 2 region (ITS2) and the mitochondrial cytochrome oxidase

137   subunit 1 gene (COI) [24]. Following PCR clean-up (Source BioScience Plc, Nottingham,

138   UK), chain termination sequencing was performed to generate unambiguous consensus

139   sequences for each sample (Supplemental notes). Sequences were assembled manually in

140   BioEdit v7.2.5 [25] to create unambiguous consensus sequences for each sample. Consensus

141   sequence alignments per gene were generated in ClustalW and used to perform nucleotide

142   BLAST (NCBI) database queries [26]. *An. stephensi* ITS2 and COI sequences, from across

143   the vector's geographic range, were downloaded from GenBank for phylogenetic analysis in

144   MEGA X [27]. Additional outgroup ITS2 sequences were retrieved for *An. maculatus, An.*

145   *maculipalpis, An. sawadwongporni* and *An. willmori.* Alternate maximum-likelihood (ML)

146   phylogenies were constructed using the Jukes-Cantor (ITS2; final tree *ln*L=-916.913) or

147   Tamura-Nei (COI; final tree *ln*L=-732.248) models, following appropriate nucleotide

148   substitution model selection in MEGA X. Bootstrap support for clade topologies was

149   estimated following the generation of 1000 pseudoreplicate datasets.

150   **Statistical analysis**

151   Analyses were performed in STATA version 13 (StataCorp., TX, USA) and GraphPad Prism

152   5.3 (GraphPad Software Inc., CA, USA). Feeding efficiency (proportion of fully fed

153   mosquitoes) was compared in matched experiments using the Wilcoxon matched-pairs

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154   signed-ranks test. Logistic regression was performed to compare infection status between *An.*

155   *arabiensis* and *An. stephensi* using individual mosquito data and a fixed effect for each

156   human participant to account for correlations between mosquito observations from the same

157   donor. Bland-Altmann plots were generated for differences in infectivity between mosquito

158   sources with Pitman's test of difference in variance.

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9

160 **Results**

161 **Most of the potential aquatic habitats were infested with *An. stephensi* developmental**

162 **stages**

163 Eighty-five water bodies within Awash Sebat Kilo town were assessed for *An. stephensi*

164 larvae and pupae. All of these water reservoirs were human-made (Figure 2; Supplemental

165 notes). *An. stephensi* larvae were detected in 75.3% (64/85) of sites (Table 1; Supplemental

166 notes); of which the final aquatic developmental stage (pupae) were detected in 37.5%

167 (24/64) of the waterbodies. Larvae were more commonly found in permanent (85.4%, 41/48)

168 compared to temporary containers (63.9%, 23/36; *P*=0.022). The most common water body

169 co-inhabitants were *Aedes aegypti* (39.1%, 25/64) and culicine mosquitoes (23.4%, 15/64). A

170 total of 49,393 immature *Anopheles* larvae and pupae were collected in 20 visits for rearing;

171 of which 45,316 (91.7%) emerged to adults. Morphological identification of 1,672 female

172 *Anopheles* confirmed that all were *An. stephensi*.

173 **Adult mosquitoes rest mainly in animal shelters and feed also on humans and are**

174 **infected with *Plasmodium***

175 A total of 89 adult female *Anopheles* mosquitoes, the majority of which were blood fed (72),

176 were collected in two monthly rounds (6 days each) of entomological surveillance (August

177 and September 2019) with a median of 10 *Anopheles* mosquitoes per productive trapping

178 night (range 1-22). The majority (80.9%, 72/89) were morphologically identified as *An.*

179 *stephensi;* the remainder were *An. gambiae* (n=16) and *An. pharoensis* (n=1). Most of the *An.*

180 *stephensi* mosquitoes were collected from animal shelters (91.7%, 66/72); the remainder

181 (8.3%, 6/72) were collected outdoors using HLC (Supplemental note). Almost half (43.8%,

182 7/16) of the *An. gambiae* were caught by CDC light traps, but no *An. stephensi* mosquitoes

183 were caught by this method. Of the adult caught *An. stephensi*, for two non-blood-fed

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184   mosquitoes the abdomen was positive for *P. vivax* (2.2%, 2/89) indicating oocyst level

185   infection and one blood-fed mosquito collected from an animal shelter was positive for *P.*

186   *falciparum* (1.1%, 1/89). From blood meal analysis the majority of adult *An. stephensi* had

187   fed on animals (52.8%, 38/72); such as goat (n=23), cow (n=7) and dog (n=5) with a non-

188   negligible number of them feeding on humans (12.5%, 9/72) (Supplemental notes). A quarter

189   of them (23.4%, 11/47) fed on multiple sources including humans.

190   ***An. stephensi* are highly susceptible to infection with Ethiopian *Plasmodium* isolates**

191   A total of 47 paired membrane feeding experiments were conducted using blood from

192   patients with microscopy confirmed *P. vivax* (n=36), *P. falciparum* (n=7) and mixed *P. vivax*

193   and *P. falciparum* (n=4) infections (Table 2). The majority of patients were female (73.8%,

194   31/42) with a median age of 27 years (IQR, 19-38). Gametocytes were detected by

195   microscopy in the majority of *P. vivax* mono-species infected patients (73.5%, 25/34) but

196   fewer in patients with *P. falciparum* (14.3%, 1/7) and mixed species infections (25.0%, 1/4;

197   only *P. vivax* gametocytes). A total of 4,088 female *An. stephensi* raised from field collected

198   larvae/pupae were fed alongside age-matched 6,130 colony derived *An. arabiensis*. The

199   proportion of blood fed mosquitoes was generally higher for *An. arabiensis* (median, 80.5%;

200   IQR, 72.5-85.0) compared to *An. stephensi* (median, 53.5%; IQR, 44.0-68.0; *P<0.001*;

201   Figure 3A).

202   For each blood feeding experiment, an average of 24 (range, 10-33) *An. stephensi* and 28

203   (range, 19-32) *An. arabiensis* mosquitoes were dissected for oocysts on day 7 post feeding.

204   Overall, 72.2% (26/36) *P. vivax*, 14.3% (1/7) *P. falciparum* and 50.0% (2/4) mixed species

205   infected patients infected at least one *An. arabiensis* and one *An. stephensi* mosquito. A very

206   strong association was observed between the proportions of the two mosquito species

207   infected with *P. vivax* (ρ=0.82, *P<0.001*; Figure 3B) with a statistically significant higher

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208   proportion of infected mosquitoes for *An. stephensi* (median, 75.1%; IQR, 60.0-85.9)

209   compared to *An. arabiensis* (median, 58.4%; IQR, 40.0-85.6; *P<0.*042). Allowing for the

210   number of dissected mosquitoes for each set of paired feeding experiments results in higher

211   odds of infectivity to an individual mosquito for *An. stephensi* (Odds Ratio [OR], 1.99; 95%

212   CI, 1.52-2.59; *P<0.*001) (Figure 3C).

213   Oocyst intensity per infected midgut was higher for *An. stephensi* (median, 17; IQR, 6-33)

214   than *An. arabiensis* (median, 13; IQR, 4-30; *P<0.*001; Figure 4A). Oocyst intensity

215   associated positively with oocyst prevalence for both *An. stephensi* (ρ=0.553, *P<0.*001) and

216   *An. arabiensis* (ρ=0.576, *P<0.*001) mosquitoes (Figure 4B). To further determine

217   competence for transmission, random subsets of blood-fed mosquitoes from six paired feeds

218   were kept until day 12 post feeding for sporozoite quantitation in salivary glands. Sporozoites

219   were detected in both mosquito species and higher sporozoite loads were detected for

220   mosquitoes from batches where oocyst prevalence and intensity were higher (Supplemental

221   notes). Among paired feedings, after accounting for number of examined salivary glands, the

222   odds of detection of sporozoites was substantially higher in *An. stephensi* (OR, 4.6; 95% CI,

223   2.2-9.9; *P<0.*001) compared to *An. arabiensis*.

224   **Sequencing confirms Ethiopian *An. stephensi* mosquitoes are closely related to *An.***

225   ***stephensi* from Djibouti and Pakistan**

226   DNA extracted from 99 mosquitoes representing all larval habitats was used for

227   determination ITS2 and COI sequences. Of these, 76 were successfully amplified and

228   sequenced for ITS2 while 45 were successfully amplified and sequenced for COI. All of

229   sequences were confirmed to be *An. stephensi*. The ITS2 phylogeny was constructed from a

230   consensus alignment of 301bp, containing 124 variable sites; the COI phylogeny was

231   constructed from a consensus alignment of 465bp, containing 17 variable sites. The ITS2 tree

232 indicated that *An. stephensi* from Ethiopia form a well-supported monophyletic clade

233 (bootstrap value of 100%) with all other *An. stephensi* sequences from across the Arabian

234 Peninsula and South-East Asia (Figure 5). The COI tree was more resolutive, suggesting *An.*

235 *stephensi* from Ethiopia were most closely related to mosquitoes from Djibouti (64%) and

236 Pakistan (54%). Four haplotypes using COI and two genotypes using ITS2 were detected.

237

238   **Discussion**

239   In this study, we examined the abundance, behavior and vector competence of *An. stephensi*

240   in an Ethiopian town, Awash Sebat Kilo. *An. stephensi* was the dominant vector, larvae being

241   present in the majority of examined human-made water bodies. The detection of *Plasmodium*

242   developmental stages in adult *An. stephensi* demonstrates its receptivity to local parasites.

243   This was further demonstrated by mosquito feeding assays where *An. stephensi* more

244   frequently became infected and infectious when feeding on blood of *P. vivax* patients

245   compared to an insectary adapted colony of *An. arabiensis.* These data demonstrate the

246   widescale presence of a novel efficient vector in this urban area in Ethiopia.

247   Originally reported in India, *An. stephensi* has expanded westward from the Persian Gulf,

248   documented in farms and within the capital city of Kuwait in 1981 [28] and subsequently in

249   the Riyadh region of Saudi Arabia in 2007 [29]. More recently, it spread into the Horn of

250   Africa where it was reported in Djibouti city in 2013 [13] and Ethiopia in 2016 [14]. The

251   recent emergence in the Republic of Sudan [15] and more widespread sites in Ethiopia [19]

252   in 2019 suggests the species has the potential to become a widespread African malaria

253   vector. Our data demonstrate that *An. stephensi* is firmly established in an urban setting in

254   Ethiopia located on the main transportation corridor from Djibouti to Addis Ababa. The

255   detection of four haplotypes using COI and two genotypes using ITS2 suggests the

256   independent arrival of different populations or heterogeneity arising after the importation of

257   the mosquito species. Our findings further corroborate recent suggestions that *An. stephensi*

258   in Ethiopia is closely related to populations from Pakistan [14]. Regardless of its origin, it is

259   evident from our data that the mosquito is abundantly present; of the 85 water bodies

260   examined, 64 were infested with developmental stages of *An. stephensi* even in the driest

261   months of the year (May/June), further indicating how well-suited the mosquito is to local

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262   weather conditions and the availability of human-made water storage containers. The number

263   of larvae/pupae we detected (~50,000 in twenty rounds of sampling) and the development to

264   adulthood (>90%) is an alarming confirmation of adaptation in this setting.

265   Uniquely, we directly determined the vector competence of wild-caught *An. stephensi* to

266   naturally circulating *Plasmodium* parasites from malaria patients via membrane feeding in

267   comparison to an established and membrane-adapted colony of *An. arabiensis* [31]. Our

268   mosquito feeding experiments predominantly included *P. vivax* clinical cases who are highly

269   infective [31, 32], and allow a sensitive comparison of mosquito species. Although the

270   membrane adapted colony of *An. arabiensis* had high feeding rates, mosquito infection rates

271   were statistically significantly higher for *An. stephensi* than for *An. arabiensis.* Our detection

272   of salivary gland sporozoites establishes that sporogonic development of local *P. vivax* can

273   be completed by *An. stephensi*. We recruited fewer clinical *P. falciparum* cases who, in line

274   with other findings, were less likely to infect mosquitoes compared to *P. vivax* patients [31].

275   Despite a modest number of observations, our findings demonstrate that also local *P.*

276   *falciparum* isolates are capable of infecting *An. stephensi. This ex vivo* evidence of

277   susceptibility to local *Plasmodium* isolates is further supported by the detection of adult

278   mosquitoes infected with *P. falciparum* and *P. vivax*. This is, to our knowledge, the first

279   direct evidence of infected *An. stephensi* in Ethiopia.

280   The spread of *An. stephensi* can be linked with movement of goods and people [13] and the

281   favorable conditions created by rapid social development and urbanization [33] that is

282   accompanied by increased availability of aquatic habitats in the form of water storage tanks.

283   Rapidly expanding urbanization often leads to informal settlements with poor housing and

284   sanitation [34]. Although housing conditions are improving in Africa, particularly in urban

285   settings, there are still major gaps such as poor estimates of combined water, sanitation and

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286   hygiene coverage [35]. Establishment and potential spread of *An. stephensi* in the Horn of

287   Africa poses considerable health risks of increased receptivity and local transmission of

288   malaria in the increasing urban African settings requiring realignment of malaria control

289   programs. Its frequent presence in human-made aquatic habitats [37] indicates that a simple

290   bifurcation between urban and rural settings may be misleading and is context dependent.

291   Additionally, urban populations are also at increased risk of *Aedes*-borne diseases, which are

292   increasing in incidence in Africa [38-41]. We regularly detected developmental stages of

293   both *An. stephensi* and *Aedes* in the same water body [42]. Outbreaks of chikungunya [43],

294   dengue [44], and yellow fever [45] were recently reported from Ethiopia within the same

295   geographic setting. The WHO recommends the use of integrated vector management [46] –

296   an adaptive and evidence-based approach to vector control which utilizes vector control

297   interventions from within and outside the health sector that may include larval source

298   management of both *An. stephensi* and *Aedes* vectors. Our findings support that larval source

299   management may be considered to prevent further spread of *An. stephensi* and *Aedes*-borne

300   disease outbreaks in African towns and cities and beyond their territories [47]. Further

301   investigation is required to understand how *An. stephensi* respond to the existing and novel

302   insecticides and vector control strategies.

303

Declarations

Ethics approval and consent to participate: The study protocol was reviewed and approved by the Institutional Ethical Review Board of the Aklilu Lemma Institute of Pathobiology of Addis Ababa University (Ref.No. ALIPB IRB/025/2011/2019), the Oromia Regional Health Bureau (Ref. No BEFO/MBTFH/1331), and AHRI/ALERT Ethics Review Committee (Ref.No.AF-10-015.1, PO07/19). All participants provided written informed consent; parent/legal guardians for participants younger than 18 years. Those collecting human landing collections also provided written informed consent and were monitored for 3 weeks following collections and treated if any malaria symptoms occurred.

Consent for publication: It was clearly indicated on the information sheet provided to the study participants that data generated from the study will be anonymously communicated to the wider scientific community in the form of peer-reviewed scientific publication.

Availability of data and materials: Data used to make the major conclusions of the study will be available together with this article and detailed data can be provided up on request.

Competing interests: All authors declare that they don't have competing interests.

Funding: The study was supported by the Bill and Melinda Gates Foundation grant (INDIE OPP1173572) to FGT, TB and CD. The Armauer Hansen Research Institute (AHRI) has supported WC, TA, EH, and EG through its Core funding from Sida and Norad. PMI VectorLink Ethiopia Project for financial support of the adult collection. CLJ and TW were supported by Wellcome Trust /Royal Society fellowships awarded to TW (101285/Z/13/Z): http://www.wellcome.ac.uk; https://royalsociety.org

Authors' contributions: Conceived the study: HT, PM, SC, MM, MB, SI, CD, EG, TB, FGT; participated in guiding the field activities: PM, DD, SC, MM, MB, SI, JHK, AW, CD, EG, TB, FGT; collected the developmental stages (larvae and pupae): TA, EE; reared adult

328   mosquitoes, collected blood samples, run feeding experiments, dissected mosquitoes: TA,

329   EE, WC, SWB, DAM, EH, SKT, TT, AG, TT, TE, GY, SK, GS, SAS; conducted laboratory

330   works: TA, HT, EE, LAM, WC, TW, SWB, KL, RH, CLJ, DAM, EH, SKT, TT, AG, TT,

331   TE, FGT; analyzed data: TA, LAM, LMK, KS, TSC, SI, CD, EG, TB, FGT; drafted the

332   manuscript: TA, HT, EE, LAM, TW, CLJ, CD, EG, TB, FGT; critically commented on the

333   manuscript: JHK, AW, TSC, SI, CD, EG, TB, FGT. All authors read and approved the final

334   manuscript.

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477

478   **Table 1. Characteristics of the aquatic habitats surveyed**

Characteristics		Habitats	Larvae detected	Pupae detected
Localities within the town (Kebeles)	Sebat Killo	60	73.3% (44/60)	43.2% (19/44)
	Lemlefan	17	70.6% (12/17)	0% (0/12)
	Alalamo	8	100.0% (8/8)	62.5% (5/8)
Water body type	Permanent	48	85.4% (41/48)	41.5% (17/41)
	Temporary	37	62.2% (23/37)	30.4% (7/23)
Shade status	Fully shaded	22	63.6% (14/22)	42.9% (6/14)
	Partially shaded	24	99.7% (22/24)	31.8% (7/22)
	Not shaded	39	71.8% (28/39)	39.3% (11/28)
Usage	in use	71	76.1% (54/71)	37.0% (20/54)
	not in use	14	71.4% (10/14)	40.0% (4/10)
Container material	Fiber jar/tyre	23	43.5% (10/23)	40.0% (4/10)
	Metal (steel tanks/drum/barrel)	17	94.1% (16/17)	31.3% (5/16)
	Cemented/Ceramic	45	84.4% (38/45)	39.5% (15/38)
Cleanliness	Clean water	56	80.4% (45/56)	37.8% (17/45)
	Turbid water	28	67.9% (19/28)	36.8% (7/19)

479

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25

481 **Table 2. Membrane feeding assays: characteristics of malaria patients and mosquito**

482 **feeding outcomes.**

	P. vivax	*P. falciparum*	**Mixed species**
N	36	7	4
Parasites/µL, median (IQR)	7783 (3603-13440)	2431 (867-8756)	4516 (1589-10563)
Gametocyte positivity, % (n/N)	73.5 (25/34)	14.3 (1/7)	25.0 (1/4)
Infectious feeds, % (n/N)	72.2 (26/36)	14.3 (1/7)	50.0 (2/4)
Infected *An. stephensi*, % (n/N)	52.5 (446/849)	2.2 (4/180)	34.6 (36/104)
Infected *An arabiensis*, % (n/N)	45.2 (452/1000)	9.0 (18/200)	36.9 (45/122)
Oocyst per infected *An. arabiensis* midgut, median (IQR)	15 (5-35)	NA	3 (2-5)
Oocyst per infected *An. stephensi* midgut, median (IQR)	20 (7-35)	NA	3 (2-8)

483 Parasite and gametocyte densities were determined by microscopy; IQR, Interquartile range;

484 NA, not available.

485

486 **Figure legends:**

487 **Figure 1. Map of study site, *An. stephensi* aquatic habitats and adult mosquito resting**

488 **sites.** Indicated are the map of Ethiopia with regional boundaries and study site (starred) (A)

489 and an aerial view of Awash town (B) with larvae/pupae (red dots) and adult mosquito

490 collection sites (blue dots).

491 **Figure 2. *An. stephensi* larval habitats**. Images are of waterbodies that were infested with

492 developmental stages of *An. stephensi,* namely water reservoirs made of bricks or cemented

493 tanks (A – B), custom-made metal containers (C and D), barrels (E – F) or plastic containers

494 (G – H). The median volume of the aquatic containers was $4m^3$ (IQR, 1.0 – 15.6) and ranged

495 from $0.06m^3$ to $360m^3$. The majority of the containers were uncovered and were in use for

496 household (32) and construction purposes (34). The material from which the different types

497 of reservoirs were made of included cement (n=45), plastic (n=9), fiber (n=14) and steel

498 (n=17).

499 **Figure 3. Comparison of feeding efficiency and infection rates for *An. stephensi* and *An.***

500 ***arabiensis* in paired feeding experiments.** The percentage of fully fed mosquitoes for *An.*

501 *arabiensis* (red circles) and *An. stephensi* (green circles) (A). The percentage infected

502 mosquitoes for the two mosquito sources (*An. stephensi* on the Y-axis and *An. arabiensis* on

503 the X-axis) (B). The Bland-Altman plot (difference plots) for mosquito infection rates in

504 different mosquito species (C). Symbols indicate the difference in infection rate in *An.*

505 *stephensi* versus *An. arabiensis* (Y-axis) in relation to average infection rate in these two

506 species (X-axis). Positive values (57.1%; 16/28) indicate a higher infection rate in *An.*

507 *stephensi*; dotted lines indicate the 95% limits of agreement. There was no evidence that

508 correlation coefficient between the paired differences and means differed significantly from

509 zero (Pitman's Test of difference in variance, r=0.026, *P*=0.864).

27

510   **Figure 4. Comparison of oocyst intensity and prevalence for *An. stephensi* and *An.***

511   ***arabiensis* in paired feeding experiments.** Oocyst intensity (number of oocysts per

512   dissected midgut) for individual mosquitoes of each of the two species (A). The violin plot

513   presents the estimated kernel density, the median is indicated with horizontal lines, the

514   interquartile range by the box and upper and lower-adjacent values by the spikes. In panel B,

515   oocyst prevalence (proportion of midguts with detectable oocyst) (Y-axis) is indicated in

516   association with Log_{10} transformed oocyst intensity (X-axis) for *An. stephensi* (green dots)

517   and *An. arabiensis* (orange dots). Data are presented for 24 feeding experiments where 723

518   *An. arabiensis* and 643 *An. stephensi* were dissected.

519   **Figure 5. Maximum-likelihood phylogenies of ITS2 (left) and COI (right).** Maximum-

520   likelihood topologies were constructed using representative reference sequences with

521   published geographical data downloaded from GenBank. Within the Ethiopian population,

522   due to the presence of a hyper-variable microsatellite region, ITS2 sequences (A) were

523   trimmed to create a consensus alignment of 289bp; one polymorphic site separated samples

524   into two genotypes (indicated with filled asterisk together with the previously reported

525   genotype, MH650999, Carter, et al. [14] in unfilled asterisk). COI sequences (B) were

526   assembled into a consensus alignment of 687bp; a total of four variable sites were identified,

527   corresponding to four haplotypes (indicated with filled asterisk together with the previously

528   reported genotype, MH651000, Carter, et al. [14], unfilled asterisk). Nucleotide sequences

529   for ITS2 and COI were deposited in GenBank under the following accession numbers:

530   Ethiopia Genotype1, MN826065; Ethiopia Genotype2, MN826066; Ethiopia Haplotype1,

531   MN826067; Ethiopia Haplotype2, MN826068; Ethiopia Haplotype3, MN826069; and
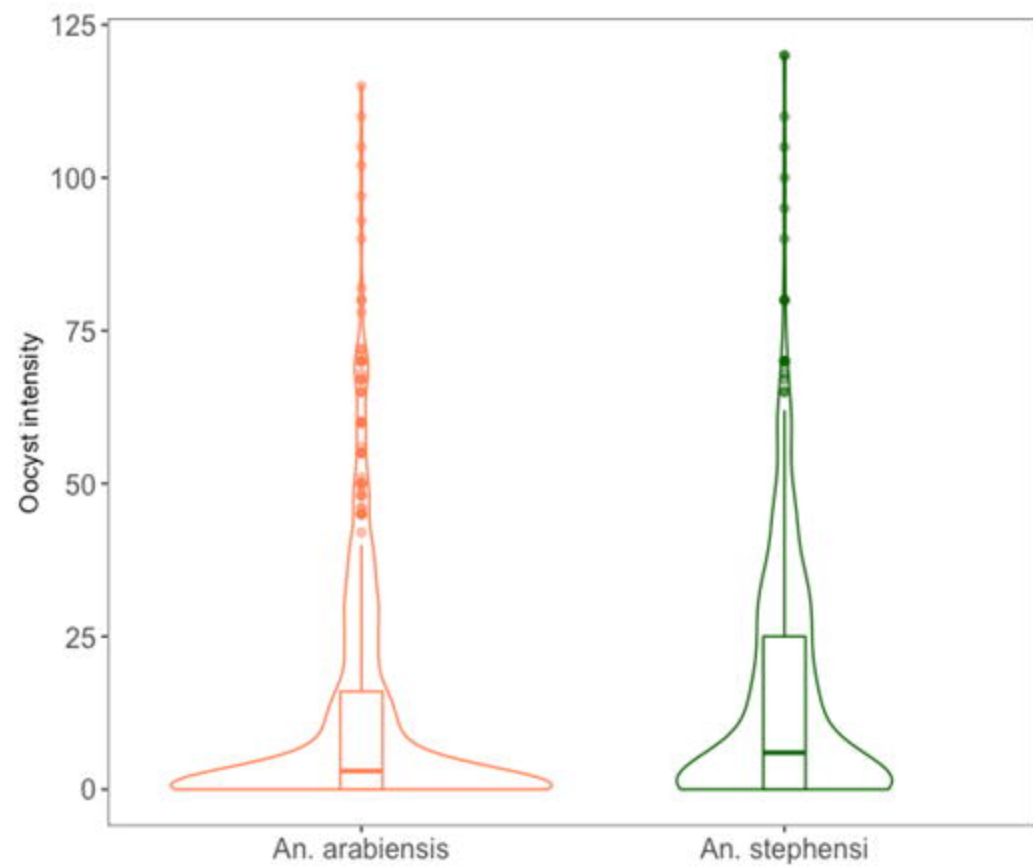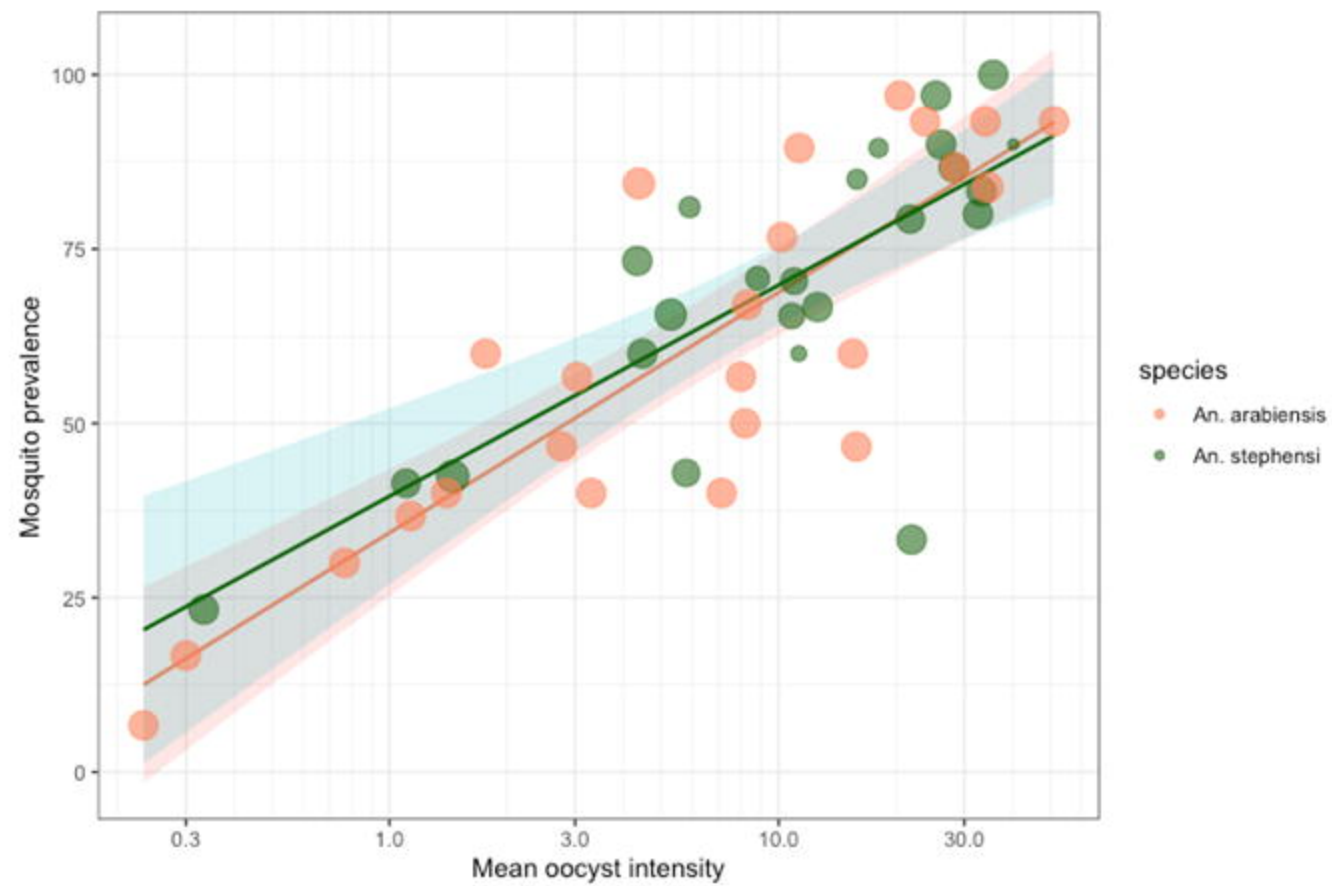
532   Ethiopia Haplotype4, MN826070.

533
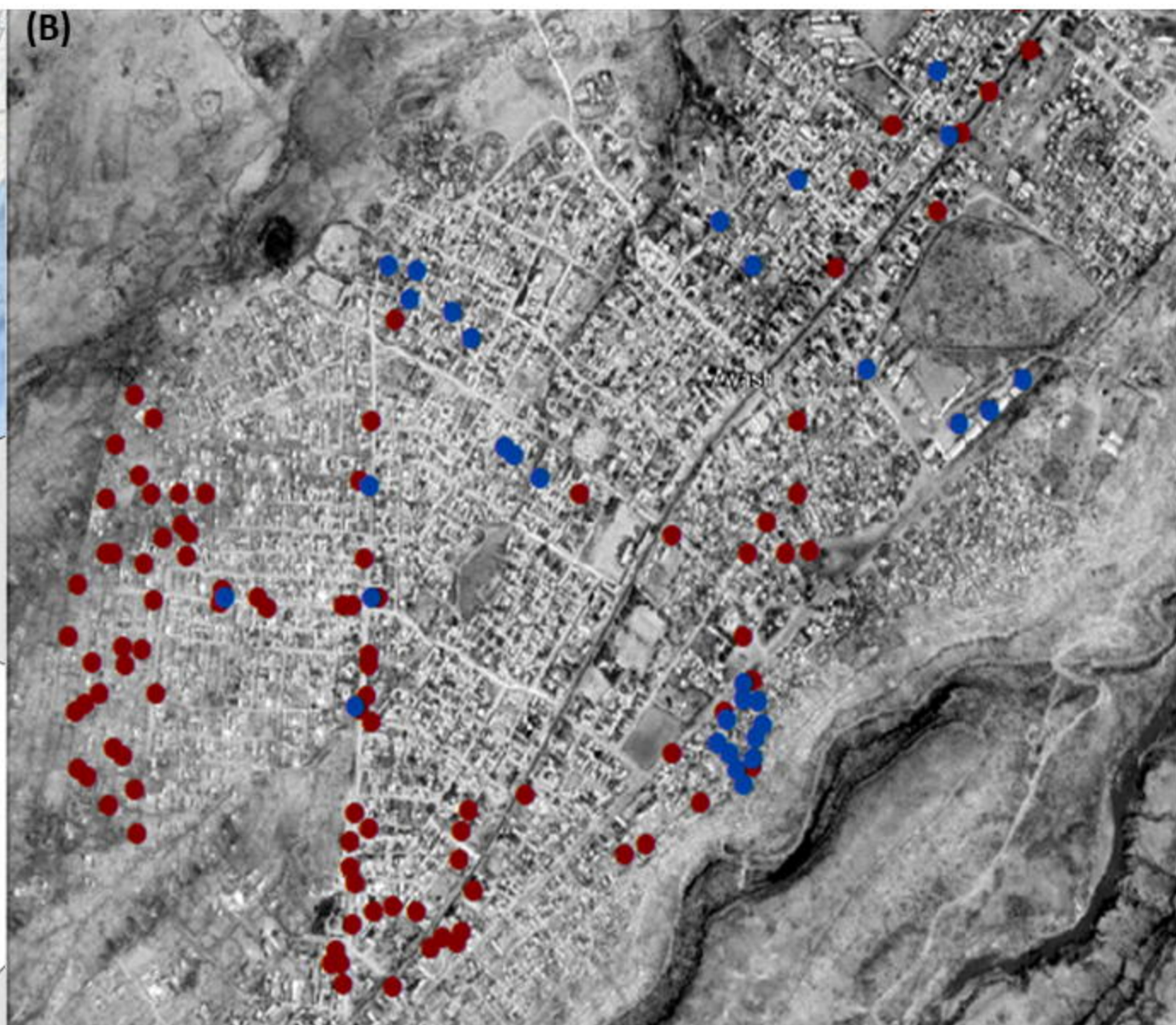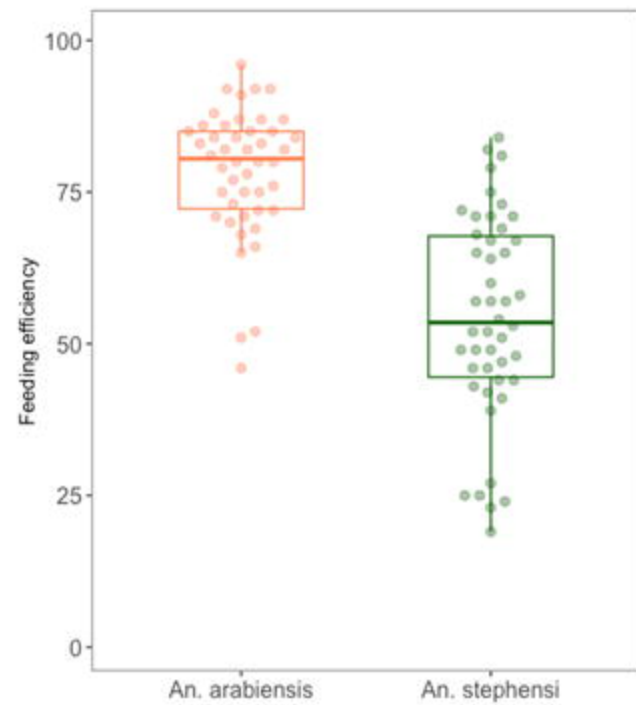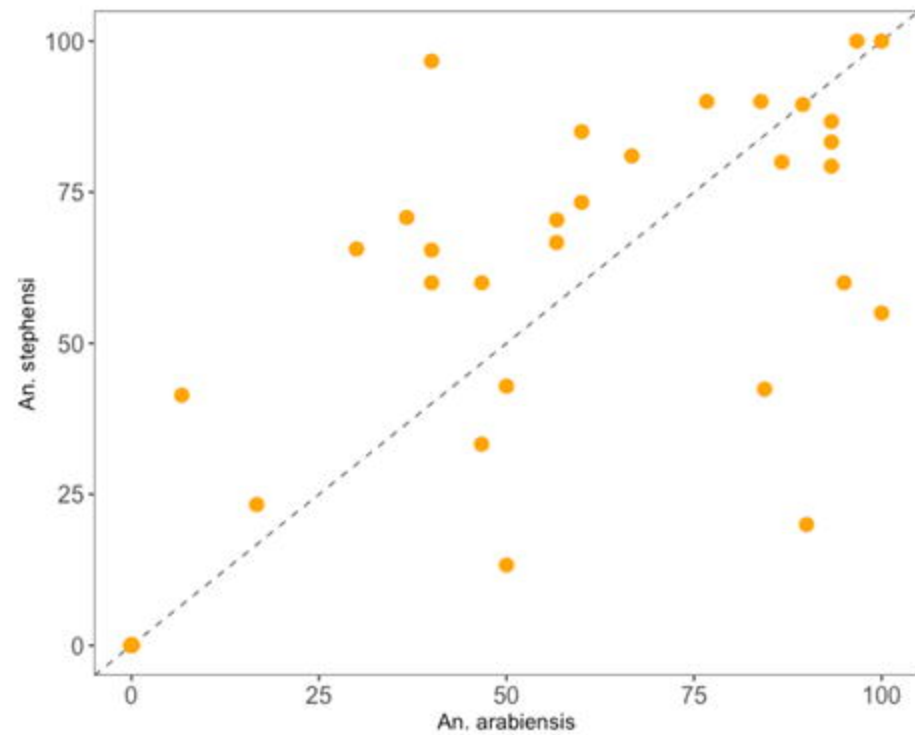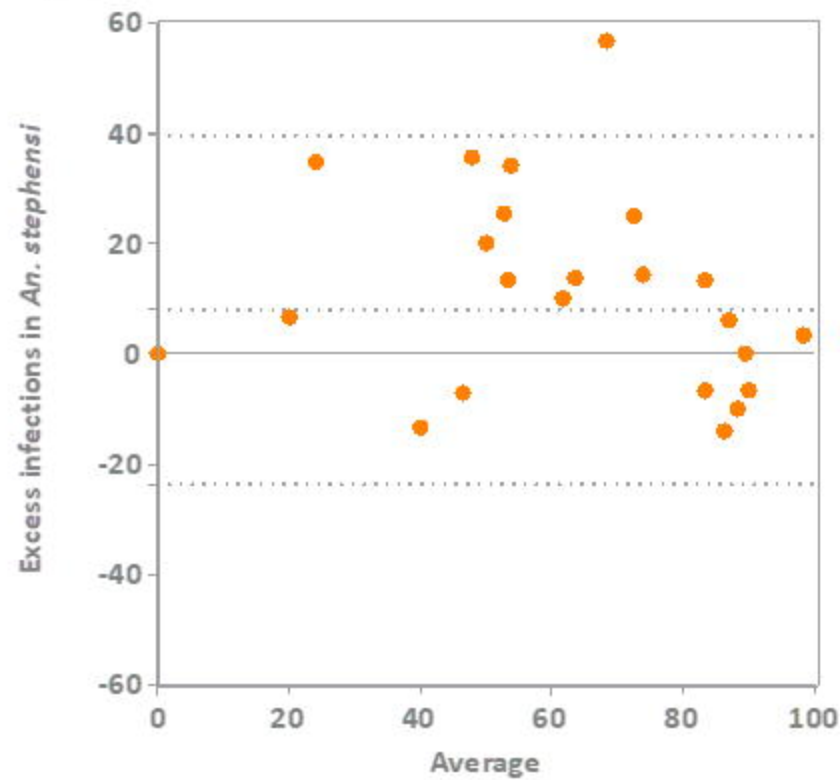
(A)

Anopheles Stephansi Ethiopia Genotype 1 ★
KM052589 AnStephensi Saudi Arabia
KF933379 AnStephensi Djibouti
AY702484 AnStephensi Iran
EU359661 AnStephensi India
AY702482 AnStephensi Iran
44 | Anopheles Stephansi Ethiopia Genotype 2 ★
FJ526599 AnStephensi United Arab Emirates
MH650999 AnStephensi Ethiopia ☆
FJ154840 AnStephensi China
100 | FJ011430 AnStephensi China
EU346652 AnStephensi Iraq
DQ662409 AnStephensi Iran
AY842526 AnStephensi Iran
EU346653 AnStephensi Iraq
63 | AY842520 AnStephensi Iran
KJ522817 AnMaculipalpis Kenya
100 | JN994142 AnMaculipalpis Zambia
KR014835 AnMaculipalpis Zambia
54 | JQ446420 AnSawadwongporni India
JQ446412 AnWillmori India
JQ446411 AnWillmori India
EU882061 AnWillmori Buhtan
100 | JF323947 AnMaculatus Tibet
AF512552 AnWillmori China

0.050

(B)

MF975724 Sri Lanka
MF975723 Sri Lanka
MF975725 Sri Lanka
MF975726 Sri Lanka
MF975727 Sri Lanka
MF975728 Sri Lanka
MK170098 United Arab Emirates
KX467337 India
MH538704 India
KT382827 Pakistan
KJ528895 Saudi Arabia
KF406681 Pakistan
KF406680 Pakistan
GU908046 South Africa
58 | FJ210894 Iran
FJ210893 Iran
AF425844 South Africa
Ethiopia Haplotype 3 ★
64 | KF933378 Djibouti
MF975731 Sri Lanka
MH538703 India
60 | MF975730 Sri Lanka
MF975729 Sri Lanka
KF406682 Pakistan
Ethiopia Haplotype 1 ★
Ethiopia Haplotype 2 ☆
54 | KF406694 Pakistan
MH651000 Ethiopia ★
MF975722 Sri Lanka
Ethiopia Haplotype 4 ★
KJ528889 Saudi Arabia
KJ528894 Saudi Arabia
KJ528893 Saudi Arabia
99 | KJ528888 Saudi Arabia
KJ528890 Saudi Arabia
53 | KJ528891 Saudi Arabia
KJ528887 Saudi Arabia
KJ528892 Saudi Arabia

0.0020