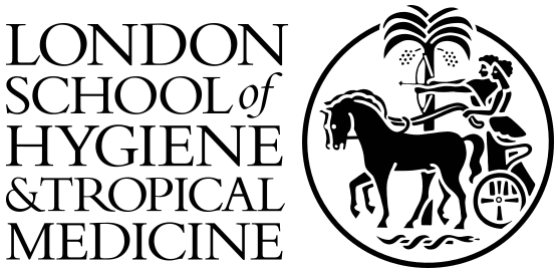

Anopheles stephensi as an emerging malaria vector in the Horn of Africa with high susceptibility to Ethiopian *Plasmodium vivax* and *Plasmodium falciparum* isolates

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Summary of the article: *An. stephensi*, a metropolitan malaria vector that recently expanded to the Horn of African, was highly susceptible to local *P. falciparum* and *P. vivax* isolates from Ethiopia and may increase malariogenic potential of rapidly expanding urban settings in Africa.

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

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

Abstract

Anopheles stephensi, an efficient Asian malaria vector, recently spread into the Horn of Africa and may increase malaria receptivity in African urban areas. We assessed occurrence, genetic complexity, blood meal source and infection status of *An. stephensi* in Awash Sebat Kilo town, Ethiopia. We used membrane feeding assays to assess competence of local *An. stephensi* to *P. vivax* and *P. falciparum* isolates from clinical patients. 75.3% of the examined waterbodies were infested with *An. stephensi* developmental stages that were genetically closely related to isolates from Djibouti and Pakistan. Both *P. vivax* and *P. falciparum* were detected in wild-caught adult *An. stephensi*. Local *An. stephensi* was more receptive to *P. vivax* compared to a colony of *An. arabiensis*. We conclude that *An. stephensi* is an established vector in this part of Ethiopia, highly permissive for local *P. vivax* and *P. falciparum* isolates and presents an important new challenge for malaria control.

Background

With expanded global malaria control efforts there have been two decades of substantial declines in malaria cases and deaths. These successes were mainly attributable to wide-scale deployment of vector control tools and availability of efficacious treatment [1]. Control programs in Africa traditionally focus on rural settings, which is where most infections occur [2] although malaria transmission is also a health concern in some urban settings [3, 4]. In 2015, 38% of Africans were living in urban settings; the number of Africans residing in urban areas is expected to double in the coming 25 years [5]. Urban settings can be sinks of malaria transmission primarily associated with importation of malaria from (rural) areas of intense transmission due to movement of people at the urban-rural interface [6]. With the adaptation of existing vectors to urban environments [7] and emerging vectors such as *Anopheles stephensi* in urban areas [8], malaria transmission in urban settings is becoming more likely. Urban areas can thereby form foci of active malaria transmission [9]. *An. stephensi* is an efficient vector for both *Plasmodium vivax* and *P. falciparum* in Asia and is the dominant malaria vector in India and the Persian Gulf [10]. *An. stephensi* predominantly breeds in urban settings with a preference for human-made water storage containers [11]. Recent reports indicate that *An. stephensi* is spreading in the Horn of Africa (Djibouti [13], Ethiopia [14] and the Republic of Sudan [15]). *An. stephensi* emergence has been epidemiologically linked to an unusual resurgence in local malaria cases in Djibouti city [16]. A recent technical consultative meeting convened at the World Health Organization (WHO) identified that there is potential for spread of *An. stephensi* across Africa and urged for more data on its distribution to allow monitoring of potential spread of *An. stephensi* from the currently affected areas and on the vector's susceptibility to local *Plasmodium* isolates [15].

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

Methods

Description of study site

This study was conducted in Awash Sebat Kilo town (916 meters above sea level; 8°58'59.99" N 40°10'0.01" E), on the main transportation corridor from Addis Ababa (220km southeast) to Djibouti (Figure 1). The town has an estimated total population of 24,700 [17]; the semi-arid climate is dominated by a major rainy season (July-August) and short intermittent rains (April/May) and an average temperature of 25.8^{o}C (17.3^{o}C-33.6^{o}C) [18]. The Awash River Valley is the most irrigated area in the country with extensive river-fed agriculture. Malaria transmission is perennial in the area surrounding the town with annual parasite incidence of 536 per 1000 population in 2019 (five-year trend summarized in supplemental notes). Entomological surveys conducted in 2018 detected the occurrence of *An. stephensi* in Awash Sebat Kilo town [19].

Characterization of aquatic habitat, resting, feeding and biting behavior

Aquatic sites were examined for the presence of *Anopheles* developmental stages by standard dipping (10x) for 5 consecutive days. Developmental stages were separated from culicines in the field and transported to Adama malaria center for rearing to adults. The resting, feeding and host-seeking behavior of *An. stephensi* was assessed using five conventional entomological sampling techniques: i) Centers for Disease Control (CDC) miniature light traps (Johns W. Hocks Company model 512) catches, ii) human landing catches (HLC), iii) pyrethrum spray sheet collection (PSC), iv) aspiration from animal shelters, and v) cattle-baited traps (Supplemental notes). Adult mosquitoes were identified morphologically using standard identification keys [20]. Fully fed mosquitoes identified as *An. stephensi* were kept in paper cups at Adama laboratory in ambient conditions and allowed to lay eggs on filter 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^{o}C) and relative humidity (70±10%) 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

head-thorax and abdomen separately [22], indicating sporozoite and oocyst-stage infections, respectively. Multiplex PCR that targets the mitochondrial cytochrome b gene and produces species-specific fragments of varying sizes was used to assess blood meal sources of individual mosquitoes [23]. For confirmation of morphologically identified *An. stephensi*, DNA was extracted from whole mosquito bodies using the DNeasy Blood and Tissue kit (Qiagen, UK). PCR was performed for each individual mosquito, targeting the nuclear internal transcribed spacer 2 region (ITS2) and the mitochondrial cytochrome oxidase subunit 1 gene (COI) [24]. Following PCR clean-up (Source BioScience Plc, Nottingham, UK), chain termination sequencing was performed to generate unambiguous consensus sequences for each sample (Supplemental notes). Sequences were assembled manually in BioEdit v7.2.5 [25] to create unambiguous consensus sequences for each sample. Consensus sequence alignments per gene were generated in ClustalW and used to perform nucleotide BLAST (NCBI) database queries [26]. *An. stephensi* ITS2 and COI sequences, from across the vector's geographic range, were downloaded from GenBank for phylogenetic analysis in MEGA X [27]. Additional outgroup ITS2 sequences were retrieved for *An. maculatus*, *An. maculipalpis*, *An. sawadwongporni* and *An. willmori.* Alternate maximum-likelihood (ML) phylogenies were constructed using the Jukes-Cantor (ITS2; final tree *ln*L=-916.913) or Tamura-Nei (COI; final tree *ln*L=-732.248) models, following appropriate nucleotide substitution model selection in MEGA X. Bootstrap support for clade topologies was estimated following the generation of 1000 pseudoreplicate datasets.

Statistical analysis

Analyses were performed in STATA version 13 (StataCorp., TX, USA) and GraphPad Prism 5.3 (GraphPad Software Inc., CA, USA). Feeding efficiency (proportion of fully fed mosquitoes) was compared in matched experiments using the Wilcoxon matched-pairs

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.
159

Results

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

Eighty-five water bodies within Awash Sebat Kilo town were assessed for *An. stephensi* larvae and pupae. All of these water reservoirs were human-made (Figure 2; Supplemental notes). *An. stephensi* larvae were detected in 75.3% (64/85) of sites (Table 1; Supplemental notes); of which the final aquatic developmental stage (pupae) were detected in 37.5% (24/64) of the waterbodies. Larvae were more commonly found in permanent (85.4%, 41/48) compared to temporary containers (63.9%, 23/36; P=0.022). The most common water body co-inhabitants were *Aedes aegypti* (39.1%, 25/64) and culicine mosquitoes (23.4%, 15/64). A total of 49,393 immature *Anopheles* larvae and pupae were collected in 20 visits for rearing; of which 45,316 (91.7%) emerged to adults. Morphological identification of 1,672 female *Anopheles* confirmed that all were *An. stephensi*.

Adult mosquitoes rest mainly in animal shelters and feed also on humans and are infected with *Plasmodium*

A total of 89 adult female *Anopheles* mosquitoes, the majority of which were blood fed (72), were collected in two monthly rounds (6 days each) of entomological surveillance (August and September 2019) with a median of 10 *Anopheles* mosquitoes per productive trapping night (range 1-22). The majority (80.9%, 72/89) were morphologically identified as *An. stephensi;* the remainder were *An. gambiae* (n=16) and *An. pharoensis* (n=1). Most of the *An. stephensi* mosquitoes were collected from animal shelters (91.7%, 66/72); the remainder (8.3%, 6/72) were collected outdoors using HLC (Supplemental note). Almost half (43.8%, 7/16) of the *An. gambiae* were caught by CDC light traps, but no *An. stephensi* mosquitoes were caught by this method. Of the adult caught *An. stephensi*, for two non-blood-fed

mosquitoes the abdomen was positive for *P. vivax* (2.2%, 2/89) indicating oocyst level infection and one blood-fed mosquito collected from an animal shelter was positive for *P. falciparum* (1.1%, 1/89). From blood meal analysis the majority of adult *An. stephensi* had fed on animals (52.8%, 38/72); such as goat (n=23), cow (n=7) and dog (n=5) with a non-negligible number of them feeding on humans (12.5%, 9/72) (Supplemental notes). A quarter of them (23.4%, 11/47) fed on multiple sources including humans.

An. stephensi are highly susceptible to infection with Ethiopian _Plasmodium_ isolates

A total of 47 paired membrane feeding experiments were conducted using blood from patients with microscopy confirmed *P. vivax* (n=36), *P. falciparum* (n=7) and mixed *P. vivax* and *P. falciparum* (n=4) infections (Table 2). The majority of patients were female (73.8%, 31/42) with a median age of 27 years (IQR, 19-38). Gametocytes were detected by microscopy in the majority of *P. vivax* mono-species infected patients (73.5%, 25/34) but fewer in patients with *P. falciparum* (14.3%, 1/7) and mixed species infections (25.0%, 1/4; only *P. vivax* gametocytes). A total of 4,088 female *An. stephensi* raised from field collected larvae/pupae were fed alongside age-matched 6,130 colony derived *An. arabiensis*. The proportion of blood fed mosquitoes was generally higher for *An. arabiensis* (median, 80.5%; IQR, 72.5-85.0) compared to *An. stephensi* (median, 53.5%; IQR, 44.0-68.0; $P<0.001$; Figure 3A).

For each blood feeding experiment, an average of 24 (range, 10-33) *An. stephensi* and 28 (range, 19-32) *An. arabiensis* mosquitoes were dissected for oocysts on day 7 post feeding. Overall, 72.2% (26/36) *P. vivax*, 14.3% (1/7) *P. falciparum* and 50.0% (2/4) mixed species infected patients infected at least one *An. arabiensis* and one *An. stephensi* mosquito. A very strong association was observed between the proportions of the two mosquito species infected with *P. vivax* ($\rho=0.82$, $P<0.001$; Figure 3B) with a statistically significant higher

proportion of infected mosquitoes for *An. stephensi* (median, 75.1%; IQR, 60.0-85.9) compared to *An. arabiensis* (median, 58.4%; IQR, 40.0-85.6; $P<0.042$). Allowing for the number of dissected mosquitoes for each set of paired feeding experiments results in higher odds of infectivity to an individual mosquito for *An. stephensi* (Odds Ratio [OR], 1.99; 95% CI, 1.52-2.59; $P<0.001$) (Figure 3C).

Oocyst intensity per infected midgut was higher for *An. stephensi* (median, 17; IQR, 6-33) than *An. arabiensis* (median, 13; IQR, 4-30; $P<0.001$; Figure 4A). Oocyst intensity associated positively with oocyst prevalence for both *An. stephensi* ($\rho=0.553$, $P<0.001$) and *An. arabiensis* ($\rho=0.576$, $P<0.001$) mosquitoes (Figure 4B). To further determine competence for transmission, random subsets of blood-fed mosquitoes from six paired feeds were kept until day 12 post feeding for sporozoite quantitation in salivary glands. Sporozoites were detected in both mosquito species and higher sporozoite loads were detected for mosquitoes from batches where oocyst prevalence and intensity were higher (Supplemental notes). Among paired feedings, after accounting for number of examined salivary glands, the odds of detection of sporozoites was substantially higher in *An. stephensi* (OR, 4.6; 95% CI, 2.2-9.9; $P<0.001$) compared to *An. arabiensis*.

Sequencing confirms Ethiopian *An. stephensi* mosquitoes are closely related to *An. stephensi* from Djibouti and Pakistan

DNA extracted from 99 mosquitoes representing all larval habitats was used for determination ITS2 and COI sequences. Of these, 76 were successfully amplified and sequenced for ITS2 while 45 were successfully amplified and sequenced for COI. All of sequences were confirmed to be *An. stephensi*. The ITS2 phylogeny was constructed from a consensus alignment of 301bp, containing 124 variable sites; the COI phylogeny was 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

Discussion

In this study, we examined the abundance, behavior and vector competence of *An. stephensi* in an Ethiopian town, Awash Sebat Kilo. *An. stephensi* was the dominant vector, larvae being present in the majority of examined human-made water bodies. The detection of *Plasmodium* developmental stages in adult *An. stephensi* demonstrates its receptivity to local parasites. This was further demonstrated by mosquito feeding assays where *An. stephensi* more frequently became infected and infectious when feeding on blood of *P. vivax* patients compared to an insectary adapted colony of *An. arabiensis.* These data demonstrate the widescale presence of a novel efficient vector in this urban area in Ethiopia.

Originally reported in India, *An. stephensi* has expanded westward from the Persian Gulf, documented in farms and within the capital city of Kuwait in 1981 [28] and subsequently in the Riyadh region of Saudi Arabia in 2007 [29]. More recently, it spread into the Horn of Africa where it was reported in Djibouti city in 2013 [13] and Ethiopia in 2016 [14]. The recent emergence in the Republic of Sudan [15] and more widespread sites in Ethiopia [19] in 2019 suggests the species has the potential to become a widespread African malaria vector. Our data demonstrate that *An. stephensi* is firmly established in an urban setting in Ethiopia located on the main transportation corridor from Djibouti to Addis Ababa. The detection of four haplotypes using COI and two genotypes using ITS2 suggests the independent arrival of different populations or heterogeneity arising after the importation of the mosquito species. Our findings further corroborate recent suggestions that *An. stephensi* in Ethiopia is closely related to populations from Pakistan [14]. Regardless of its origin, it is evident from our data that the mosquito is abundantly present; of the 85 water bodies examined, 64 were infested with developmental stages of *An. stephensi* even in the driest months of the year (May/June), further indicating how well-suited the mosquito is to local

weather conditions and the availability of human-made water storage containers. The number of larvae/pupae we detected (~50,000 in twenty rounds of sampling) and the development to adulthood (>90%) is an alarming confirmation of adaptation in this setting.

Uniquely, we directly determined the vector competence of wild-caught *An. stephensi* to naturally circulating *Plasmodium* parasites from malaria patients via membrane feeding in comparison to an established and membrane-adapted colony of *An. arabiensis* [31]. Our mosquito feeding experiments predominantly included *P. vivax* clinical cases who are highly infective [31, 32], and allow a sensitive comparison of mosquito species. Although the membrane adapted colony of *An. arabiensis* had high feeding rates, mosquito infection rates were statistically significantly higher for *An. stephensi* than for *An. arabiensis*. Our detection of salivary gland sporozoites establishes that sporogonic development of local *P. vivax* can be completed by *An. stephensi*. We recruited fewer clinical *P. falciparum* cases who, in line with other findings, were less likely to infect mosquitoes compared to *P. vivax* patients [31]. Despite a modest number of observations, our findings demonstrate that also local *P. falciparum* isolates are capable of infecting *An. stephensi*. This *ex vivo* evidence of susceptibility to local *Plasmodium* isolates is further supported by the detection of adult mosquitoes infected with *P. falciparum* and *P. vivax*. This is, to our knowledge, the first direct evidence of infected *An. stephensi* in Ethiopia.

The spread of *An. stephensi* can be linked with movement of goods and people [13] and the favorable conditions created by rapid social development and urbanization [33] that is accompanied by increased availability of aquatic habitats in the form of water storage tanks. Rapidly expanding urbanization often leads to informal settlements with poor housing and sanitation [34]. Although housing conditions are improving in Africa, particularly in urban settings, there are still major gaps such as poor estimates of combined water, sanitation and

hygiene coverage [35]. Establishment and potential spread of *An. stephensi* in the Horn of Africa poses considerable health risks of increased receptivity and local transmission of malaria in the increasing urban African settings requiring realignment of malaria control programs. Its frequent presence in human-made aquatic habitats [37] indicates that a simple bifurcation between urban and rural settings may be misleading and is context dependent. Additionally, urban populations are also at increased risk of *Aedes*-borne diseases, which are increasing in incidence in Africa [38-41]. We regularly detected developmental stages of both *An. stephensi* and *Aedes* in the same water body [42]. Outbreaks of chikungunya [43], dengue [44], and yellow fever [45] were recently reported from Ethiopia within the same geographic setting. The WHO recommends the use of integrated vector management [46] – an adaptive and evidence-based approach to vector control which utilizes vector control interventions from within and outside the health sector that may include larval source management of both *An. stephensi* and *Aedes* vectors. Our findings support that larval source management may be considered to prevent further spread of *An. stephensi* and *Aedes*-borne disease outbreaks in African towns and cities and beyond their territories [47]. Further investigation is required to understand how *An. stephensi* respond to the existing and novel insecticides and vector control strategies.

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.

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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

mosquitoes, collected blood samples, run feeding experiments, dissected mosquitoes: TA, EE, WC, SWB, DAM, EH, SKT, TT, AG, TT, TE, GY, SK, GS, SAS; conducted laboratory works: TA, HT, EE, LAM, WC, TW, SWB, KL, RH, CLJ, DAM, EH, SKT, TT, AG, TT, TE, FGT; analyzed data: TA, LAM, LMK, KS, TSC, SI, CD, EG, TB, FGT; drafted the manuscript: TA, HT, EE, LAM, TW, CLJ, CD, EG, TB, FGT; critically commented on the manuscript: JHK, AW, TSC, SI, CD, EG, TB, FGT. All authors read and approved the final manuscript.

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References

1. O'Meara WP, Mangeni JN, Steketee R, Greenwood B. Changes in the burden of malaria in sub-Saharan Africa. The Lancet Infectious Diseases **2010**; 10(8): 545-55.
2. Fillinger U, Lindsay SW. Larval source management for malaria control in Africa: myths and reality. Malar J **2011**; 10(1): 353.
3. Wilson ML, Krogstad DJ, Arinaitwe E, et al. Urban Malaria: Understanding its Epidemiology, Ecology, and Transmission across Seven Diverse ICEMR Network Sites. Am J Trop Med Hyg **2015**; 93(3 Suppl): 110-23.
4. Dash AP, Valecha N, Anvikar AR, Kumar A. Malaria in India: challenges and opportunities. J Biosci **2008**; 33(4): 583-92.
5. The World Bank. Productive, Livable Cities Will Open Africa's Doors to the World. Available at: https://www.worldbank.org/en/region/afr/publication/africa-cities-opening-doors-world. Accessed 08 November.
6. Molina Gomez K, Caicedo MA, Gaitan A, et al. Characterizing the malaria rural-to-urban transmission interface: The importance of reactive case detection. PLoS Negl Trop Dis **2017**; 11(7): e0005780.
7. Azrag RS, Mohammed BH. Anopheles arabiensis in Sudan: a noticeable tolerance to urban polluted larval habitats associated with resistance to Temephos. Malar J **2018**; 17(1): 204.
8. Seyfarth M, Khaireh BA, Abdi AA, Bouh SM, Faulde MK. Five years following first detection of Anopheles stephensi (Diptera: Culicidae) in Djibouti, Horn of Africa: populations established-malaria emerging. Parasitol Res **2019**; 118(3): 725-32.

9. Chaparro PE, Molina K, Alzate A, Padilla J, Arevalo-Herrera M, Herrera S. Urban malaria transmission in a non-endemic area in the Andean region of Colombia. Mem Inst Oswaldo Cruz **2017**; 112(12): 797-804.

10. Sinka ME, Bangs MJ, Manguin S, et al. The dominant *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. Parasites & vectors **2011**; 4: 89-.

11. Surendran S, Sivabalakrishnan K, Sivasingham A, et al. Anthropogenic Factors Driving Recent Range Expansion of the Malaria Vector Anopheles stephensi. Front. Public Health **2019**; 7: 53.

12. Surendran SN, Sivabalakrishnan K, Sivasingham A, et al. Anthropogenic Factors Driving Recent Range Expansion of the Malaria Vector Anopheles stephensi. Frontiers in Public Health **2019**; 7(53).

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

14. Carter TE, Yared S, Gebresilassie A, et al. First detection of Anopheles stephensi Liston, 1901 (Diptera: culicidae) in Ethiopia using molecular and morphological approaches. Acta Trop **2018**; 188: 180-6.

15. World Health Organization. Vector alert: Anopheles stephensi invasion and spread Available at: https://www.who.int/publications-detail/vector-alert-anopheles-stephensi-invasion-and-spread. Accessed 08 November.

16. Seyfarth M, Khaireh BA, Abdi AA, Bouh SM, Faulde MK. Five years following first detection of *Anopheles stephensi* (Diptera: Culicidae) in Djibouti, Horn of Africa: populations established—malaria emerging Parasitology Research **2019**.

17. City Population. The population of the regions of Ethiopia. Available at: http://www.citypopulation.de/Ethiopia.html. Accessed 08 November.

18. Climate data. Climate: Ethiopia. Available at: https://en.climate-data.org/africa/ethiopia-249/. Accessed 08 November.

19. Balkew M, Mumba P, Dengela D, et al. Geographical distribution of Anopheles stephensi in eastern Ethiopia. bioRxiv **2019**: 802587.

20. Gillies MT, Coetee M. The supplement to the anophelinae of Africa south of the sahara(Afrotropical Region). South African Institute for Medical Research **1987**; (55).

21. Snounou G, Viriyakosol S, Zhu XP, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol **1993**; 61(2): 315-20.

22. Schriefer ME, Sacci JB, Jr., Wirtz RA, Azad AF. Detection of polymerase chain reaction-amplified malarial DNA in infected blood and individual mosquitoes. Exp Parasitol **1991**; 73(3): 311-6.

23. Kent RJ, Norris DE. Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochrome B. Am J Trop Med Hyg **2005**; 73(2): 336-42.

24. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol **1994**; 3(5): 294-9.

25. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic acids symposium series: [London]: Information Retrieval Ltd., c1979-c2000., 1999:95-8.

414 26. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
415 tool. J Mol Biol **1990**; 215(3): 403-10.
416 27. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary
417 Genetics Analysis across Computing Platforms. Mol Biol Evol **2018**; 35(6): 1547-9.
418 28. Salit A, Zakaria M, Balba M, Zaghloul T. The mosquito fauna of Kuwait.
419 JOURNAL-UNIVERSITY OF KUWAIT SCIENCE **1994**; 21: 77-.
420 29. Al-Ghamdi K, Alikhan M, Mahyoub J, Afifi Z. Studies on identification and
421 population dynamics of anopheline mosquitoes from Jeddah province of Saudi
422 Arabia. Biosci Biotechnol Res Commun **2008**; 1: 19-24.
423 30. Subbarao SK, Vasantha K, Adak T, Sharma VP, Curtis CF. Egg-float ridge number in
424 Anopheles stephensi: ecological variation and genetic analysis. Med Vet Entomol
425 **1987**; 1(3): 265-71.
426 31. Tadesse FG, Slater HC, Chali W, et al. The Relative Contribution of Symptomatic
427 and Asymptomatic Plasmodium vivax and Plasmodium falciparum Infections to the
428 Infectious Reservoir in a Low-Endemic Setting in Ethiopia. Clin Infect Dis **2018**;
429 66(12): 1883-91.
430 32. Kiattibutr K, Roobsoong W, Sriwichai P, et al. Infectivity of symptomatic and
431 asymptomatic Plasmodium vivax infections to a Southeast Asian vector, Anopheles
432 dirus. Int J Parasitol **2017**; 47(2-3): 163-70.
433 33. Alahmed AM. Mosquito fauna (Diptera: Culicidae) of the Eastern Region of Saudi
434 Arabia and their seasonal abundance. Journal of King Saud University-Science **2012**;
435 24(1): 55-62.

436 34. Ferreira Goncalves MJ, Alecrim WD. Non-planed urbanization as a contributing
437 factor for malaria incidence in Manaus-Amazonas, Brazil. Revista de salud publica
438 (Bogota, Colombia) **2004**; 6(2): 156-66.
439 35. Roche R, Bain R, Cumming O. A long way to go - Estimates of combined water,
440 sanitation and hygiene coverage for 25 sub-Saharan African countries. PLoS One
441 **2017**; 12(2): e0171783.
442 36. Takken W, Lindsay S. Increased Threat of Urban Malaria from Anopheles stephensi
443 Mosquitoes, Africa. Emerg Infect Dis **2019**; 25(7): 1431-3.
444 37. Surendran SN, Sivabalakrishnan K, Gajapathy K, et al. Genotype and biotype of
445 invasive Anopheles stephensi in Mannar Island of Sri Lanka. Parasit Vectors **2018**;
446 11(1): 3.
447 38. Lilay A, Asamene N, Bekele A, et al. Reemergence of yellow fever in Ethiopia after
448 50 years, 2013: epidemiological and entomological investigations. BMC Infectious
449 Diseases **2017**; 17(1): 343.
450 39. Ferede G, Tiruneh M, Abate E, et al. A serologic study of dengue in northwest
451 Ethiopia: Suggesting preventive and control measures. PLoS neglected tropical
452 diseases **2018**; 12(5): e0006430.
453 40. Gutu MA, Bekele A, Seid Y, et al. Another dengue fever outbreak in eastern Ethiopia
454 – an emerging public health threat (Under Review).
455 41. Daniel E, Eshetu N, Techalew S, et al. Sero-positivity of dengue virus and its
456 associated factors among febrile patients in Arbaminch districts, Southern Ethiopia
457 (Under Review).

458 42. Thomas S, Ravishankaran S, Justin JA, et al. Overhead tank is the potential breeding
459 habitat of Anopheles stephensi in an urban transmission setting of Chennai, India.
460 Malar J **2016**; 15(1): 274.
461 43. Mengesha Tsegaye M, Tayachew A, Belay D, Alemu A, Beyene B. A35 The first
462 laboratory confirmation of chikungunya outbreak in Ethiopia. Virus Evolution **2019**;
463 5(Supplement_1): vez002. 34.
464 44. Degife LH, Worku Y, Belay D, Bekele A, Hailemariam Z. Factors associated with
465 dengue fever outbreak in Dire Dawa administration city, October, 2015, Ethiopia -
466 case control study. BMC Public Health **2019**; 19(1): 650.
467 45. Lilay A, Asamene N, Bekele A, et al. Reemergence of yellow fever in Ethiopia after
468 50 years, 2013: epidemiological and entomological investigations. BMC Infect Dis
469 **2017**; 17(1): 343.
470 46. World Health Organization. A toolkit for integrated vector management in sub-
471 Saharan Africa. Available at:
472 https://www.who.int/neglected_diseases/resources/9789241549653/en/. Accessed 16
473 December.
474 47. Donnelly MJ, McCall PJ, Lengeler C, et al. Malaria and urbanization in sub-Saharan
475 Africa. Malar J **2005**; 4(1): 12.
476
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

480

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

Parasite and gametocyte densities were determined by microscopy; IQR, Interquartile range; NA, not available.

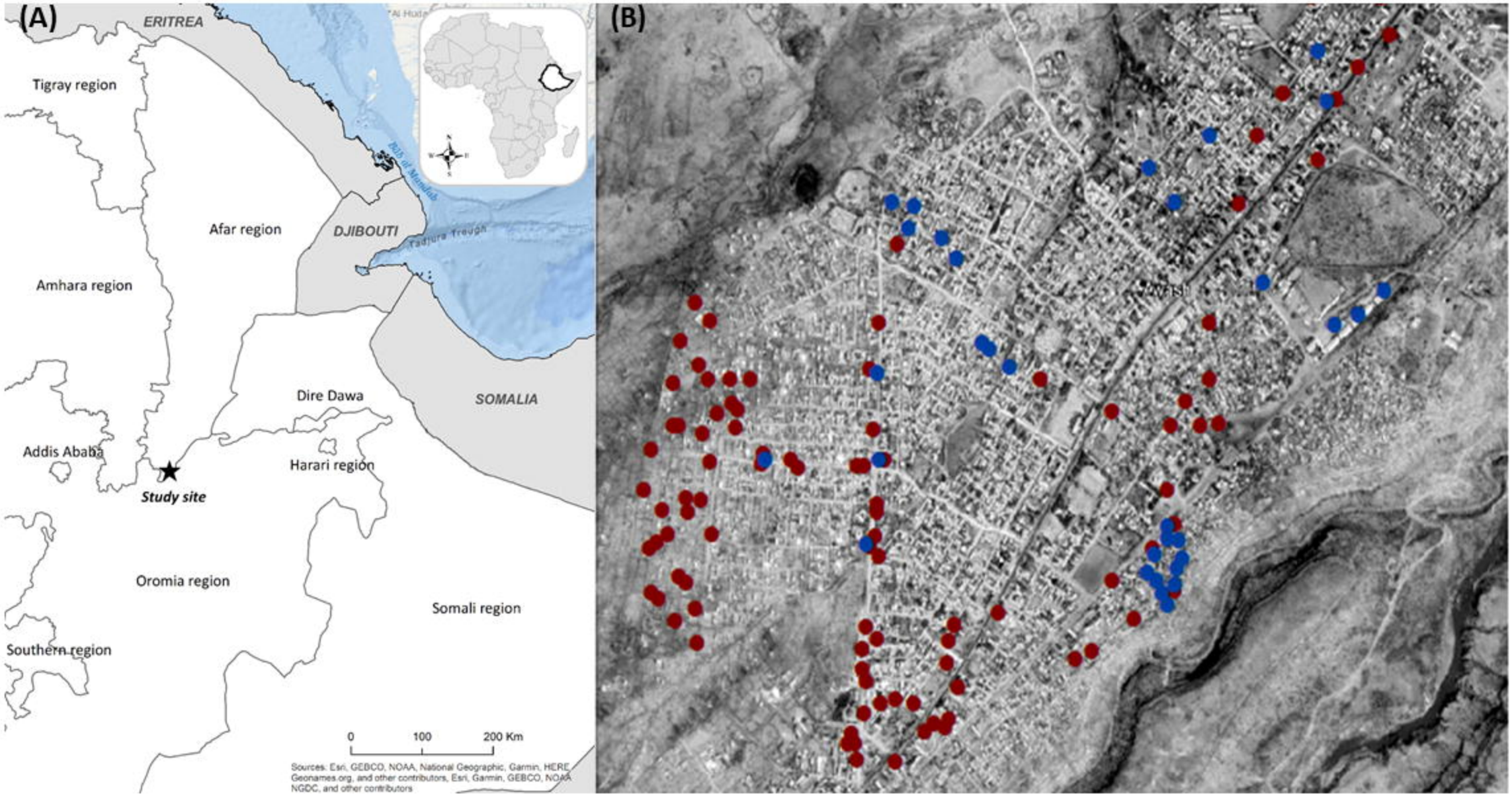
Figure legends:

Figure 1. Map of study site, *An. stephensi* aquatic habitats and adult mosquito resting sites. Indicated are the map of Ethiopia with regional boundaries and study site (starred) (A) and an aerial view of Awash town (B) with larvae/pupae (red dots) and adult mosquito collection sites (blue dots).

Figure 2. *An. stephensi* larval habitats. Images are of waterbodies that were infested with developmental stages of *An. stephensi*, namely water reservoirs made of bricks or cemented tanks (A – B), custom-made metal containers (C and D), barrels (E – F) or plastic containers (G – H). The median volume of the aquatic containers was $4m^3$ (IQR, 1.0 – 15.6) and ranged from $0.06m^3$ to $360m^3$. The majority of the containers were uncovered and were in use for household (32) and construction purposes (34). The material from which the different types of reservoirs were made of included cement (n=45), plastic (n=9), fiber (n=14) and steel (n=17).

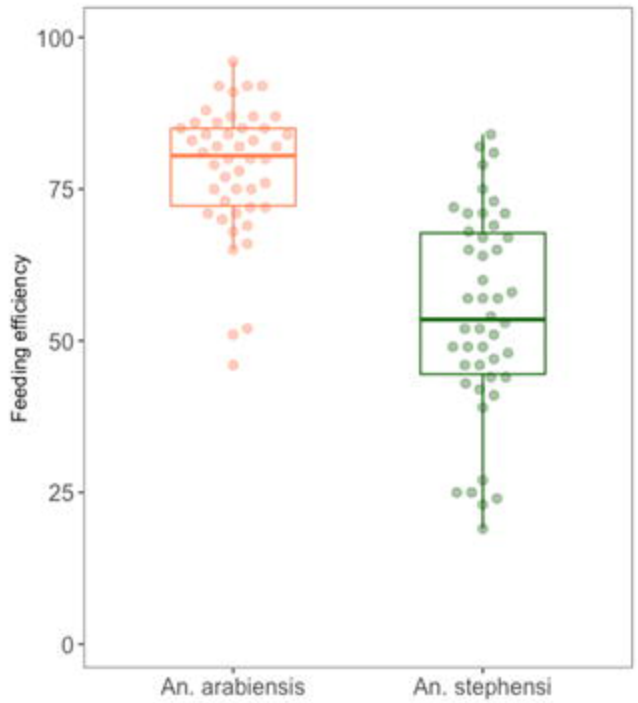
Figure 3. Comparison of feeding efficiency and infection rates for *An. stephensi* and *An. arabiensis* in paired feeding experiments. The percentage of fully fed mosquitoes for *An. arabiensis* (red circles) and *An. stephensi* (green circles) (A). The percentage infected mosquitoes for the two mosquito sources (*An. stephensi* on the Y-axis and *An. arabiensis* on the X-axis) (B). The Bland-Altman plot (difference plots) for mosquito infection rates in different mosquito species (C). Symbols indicate the difference in infection rate in *An. stephensi* versus *An. arabiensis* (Y-axis) in relation to average infection rate in these two species (X-axis). Positive values (57.1%; 16/28) indicate a higher infection rate in *An. stephensi*; dotted lines indicate the 95% limits of agreement. There was no evidence that correlation coefficient between the paired differences and means differed significantly from zero (Pitman's Test of difference in variance, r=0.026, *P*=0.864).

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

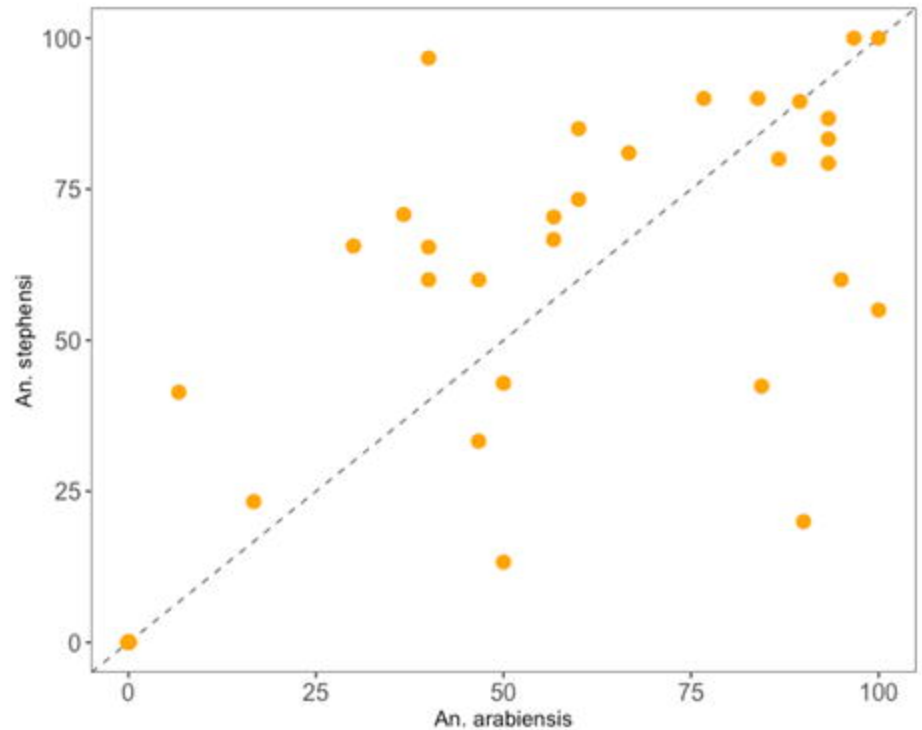

(A)
ERITREA
Tigray region
Afar region
DJIBOUTI
Amhara region
Dire Dawa
SOMALIA
Addis Ababa
Harari region
Study site
Oromia region
Somali region
Southern region
0
100
200 Km
Sources: Esri, GEBCO, NOAA, National Geographic, Garmin, HERE, Geonames.org, and other contributors, Esri, Garmin, GEBCO, NOAA NGDC, and other contributors
(B)


A
B
C
D
E
F
G
H
BASF

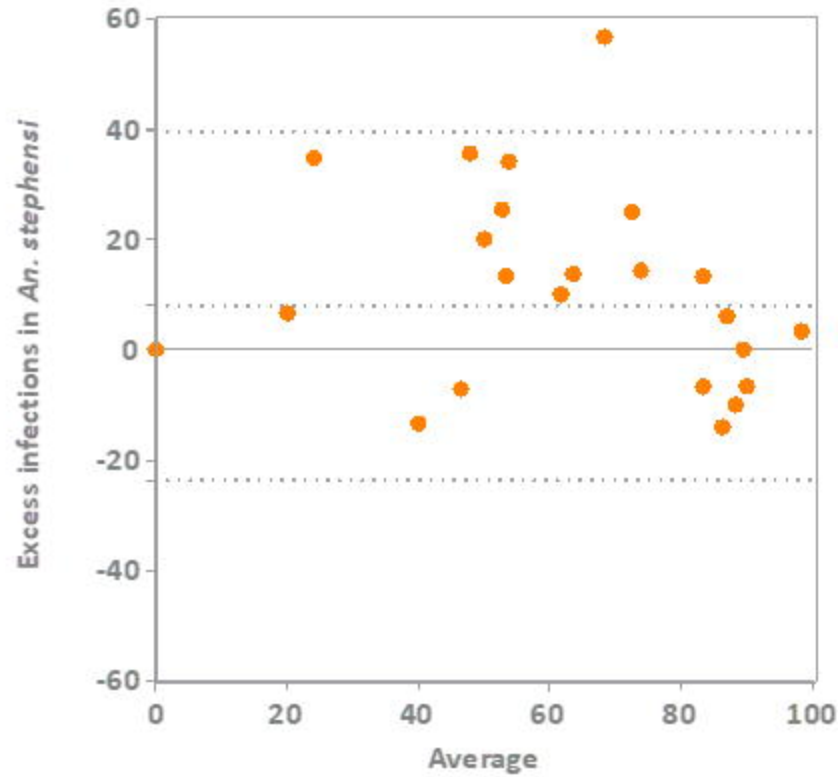
(A)


100
75
50
25
0
Feeding efficiency
An. arabiensis
An. stephensi

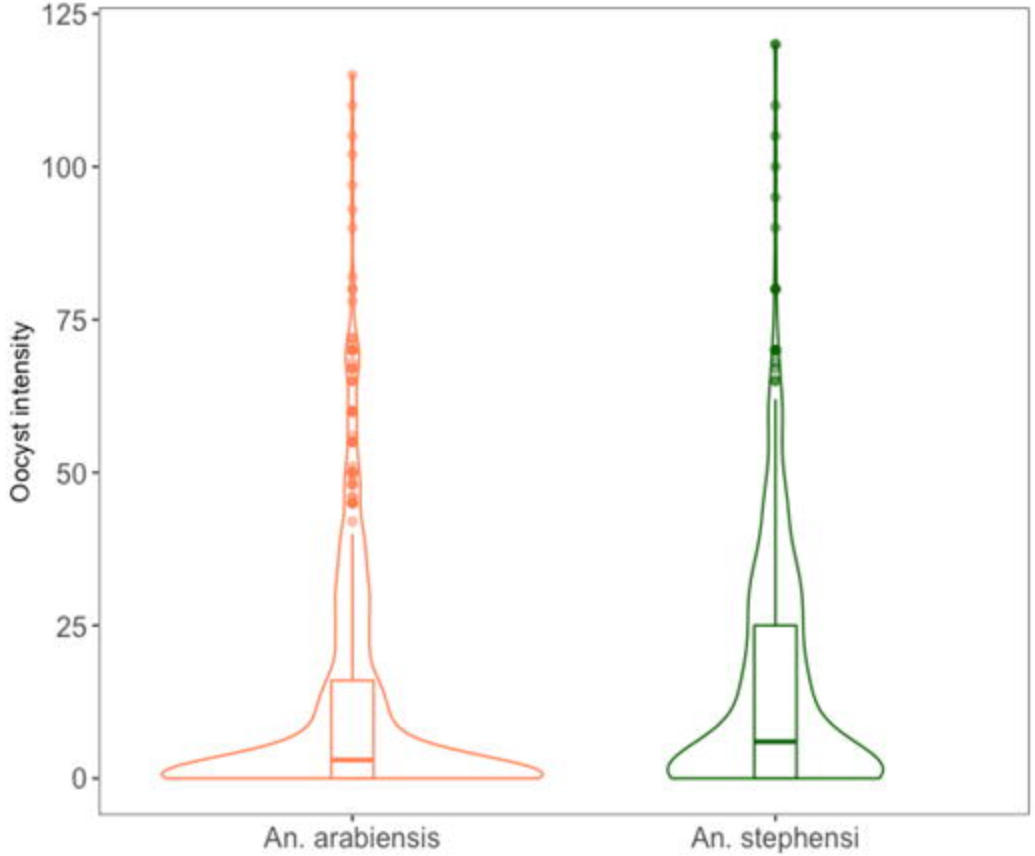
(B)


100
75
50
25
0
An. stephensi
0
25
50
75
100
An. arabiensis

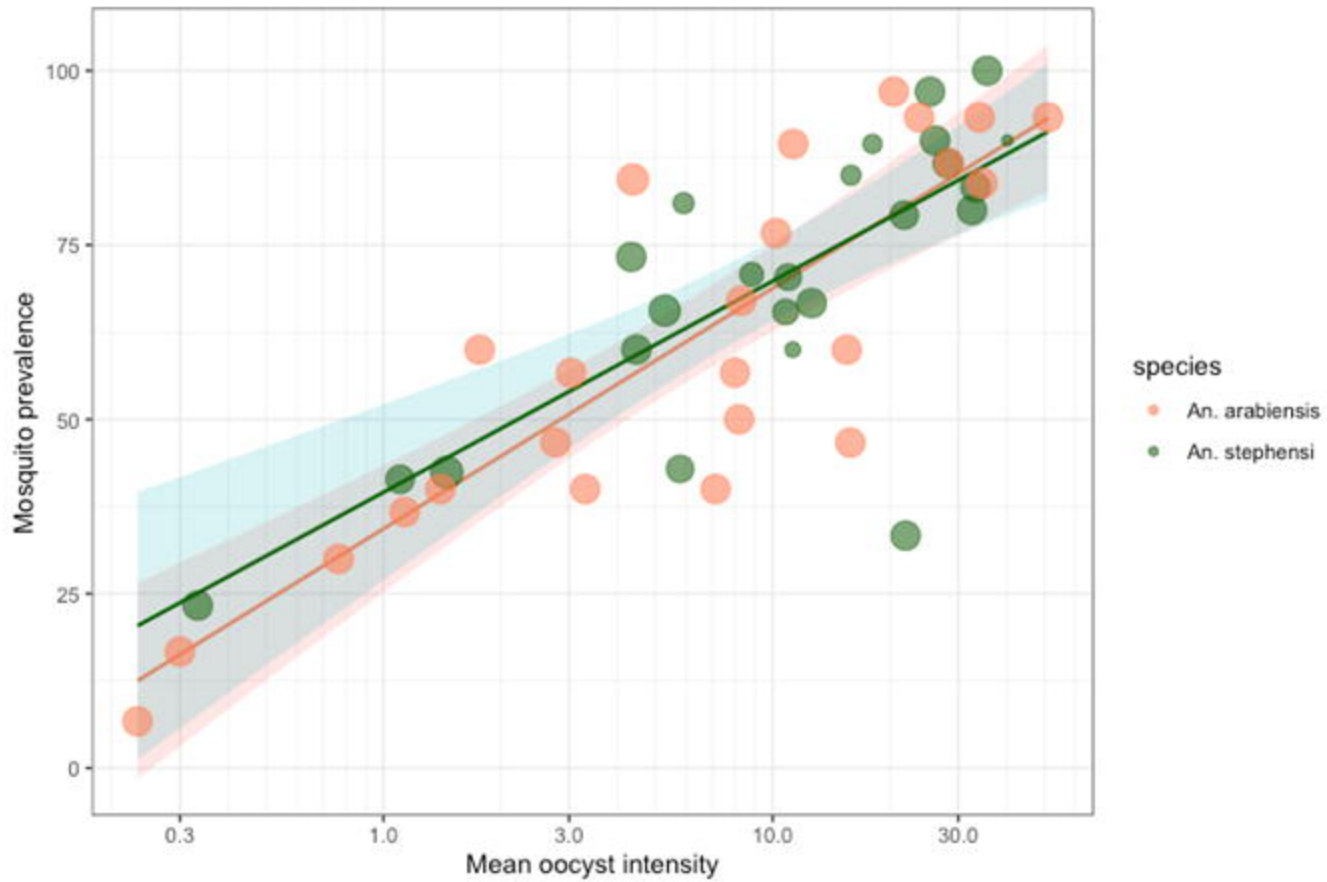
(C)

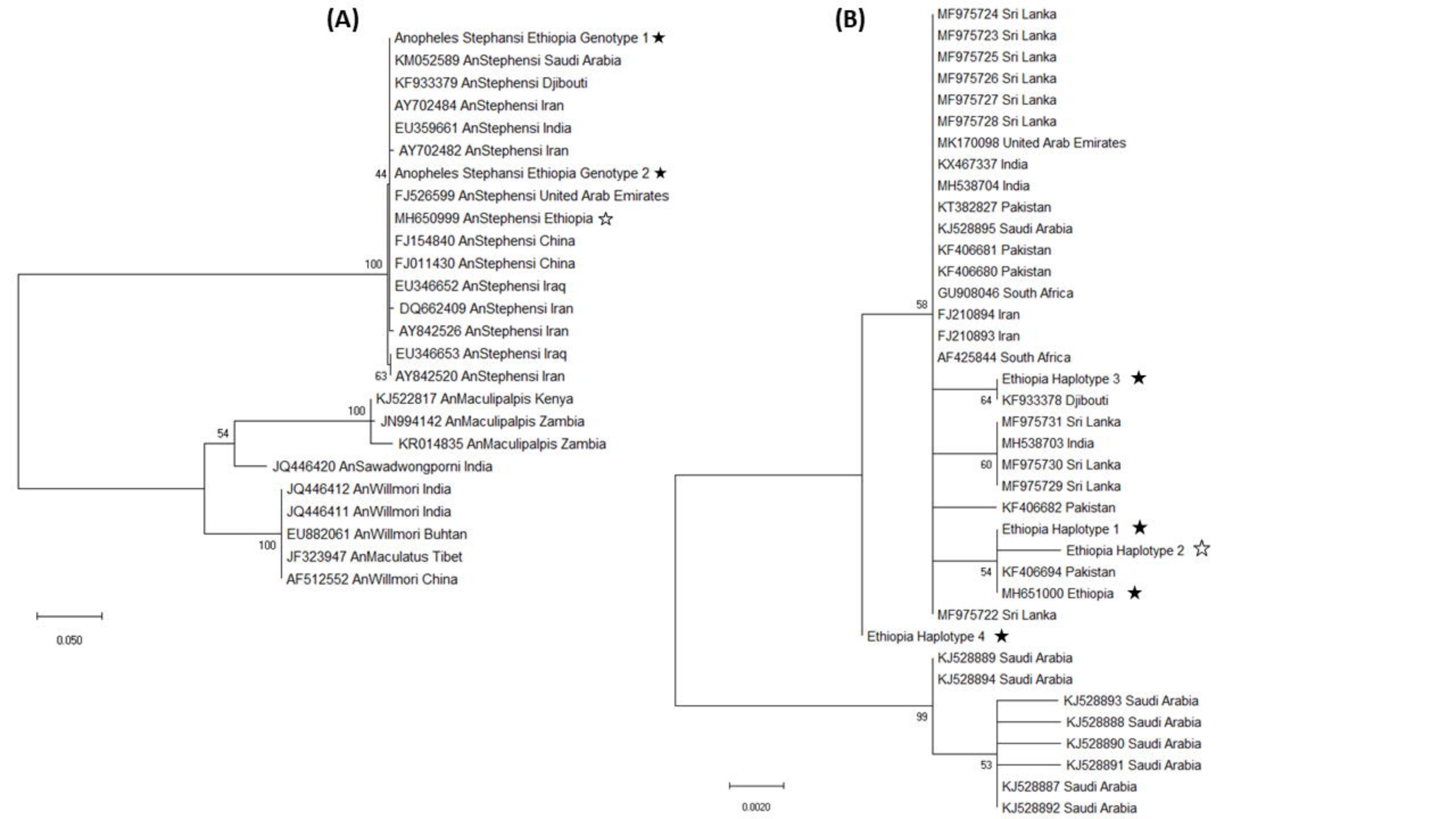

60
40
20
0
-20
-40
-60
Excess infections in An. stephensi
0
20
40
60
80
100
Average

(A)


125
100
75
50
25
0
Oocyst intensity
An. arabiensis
An. stephensi

(B)


100
75
50
25
0
Mosquito prevalence
0.3
1.0
3.0
10.0
30.0
Mean oocyst intensity
species
An. arabiensis
An. stephensi


(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
54
KR014835 AnMaculipalpis Zambia
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
99
KJ528893 Saudi Arabia
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53
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