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First detection of *Anopheles stephensi* Liston, 1901 (Diptera: culicidae) in Ethiopia using molecular and morphological approaches

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ABSTRACT

Malaria is a major public health concern in Ethiopia. With the increase in malaria cases in the Somali Region of Ethiopia, understanding the distribution and identifying the species of malaria vectors is vital to public health. Here we report the first detection of *Anopheles stephensi* in Ethiopia, a malaria vector typically found in the Middle East, the Indian subcontinent, and China, but recently found in Djibouti.

An entomological investigation was conducted during November to December 2016 in Kebri Dehar town of the Ethiopian Somali Regional State as ancillary work for *Anopheles* spp. surveillance. Mosquito larvae were collected from water reservoirs. Larvae were reared in the laboratory to the adult stage and identified morphologically. PCR and sequencing of cytochrome oxidase 1 (COI) and internal transcribed spacer 2 (ITS2) loci were performed. Basic Local Alignment Search Tool (BLAST) was used to compare sample sequences to sequences in the NCBI nucleotide database for species identification. To further analyze the relationship between the specimen we collected in Kebri Dehar and other *Anopheles* samples available in Genbank, phylogenetic analysis was performed using a maximum likelihood approach.

Molecular and morphological results confirm specimens were *An. stephensi*. The closest high scoring hit was for all specimens was for the *An. stephensi* sequence. Independent phylogenetic analyses of COI and ITS2 sequences revealed in both cases strong bootstrap (100) support for our sequence forming a clade with other *An. stephensi* sequences to the exclusion of any other species of *Anopheles*. In conclusion, *Anopheles stephensi* is present in Kebri Dehar town in Ethiopia. These findings highlight the need for additional research to examine the role of *An. stephensi* in malaria transmission in Ethiopia.

1. Background

Malaria is a serious public health threat in Ethiopia, where over 68% of the population is at risk for infection and an average of 2.5 million cases are reported each year (World Malaria Report, 2017). Malaria transmission exhibits a seasonal and unstable pattern in Ethiopia, with transmission varying with altitude and rainfall (Alemu et al., 2011). The highest levels of malaria transmission are observed in the north, west, and eastern lowlands of Ethiopia (World Malaria Report, 2017). In the eastern lowlands such as Afar and Ethiopian Somali Regional State (ESRS), malaria is endemic along the rivers where small-scale irrigation activities are practiced for agricultural purposes (Malaria programme review - Aide Memoire, 2011).

Over the past few years, malaria has become a growing public

Abbreviations: BLAST, Basic Local Alignment Search Tool; COI, cytochrome c oxidase subunit 1 gene; DNA, deoxyribonucleic Acid; ESRS, Ethiopian Somali Regional State; FMOH, Federal Ministry of Health; ITS2, internal transcribed spacer 2 region; NCBI, National Center of Biotechnology Information; PCR, polymerase chain reaction

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health threat in multiple zones and districts in Eastern Ethiopia (Bekele, 2016; Mohammed, 2015). Environmental changes are believed to be contributing with increased flooding being reported (Simane et al., 2016). With the migration of people in search of fertile land for crop production and livestock rearing along the river basin, there is concern that malaria transmission may continue to increase in and outside the region (Simane et al., 2016).

In addition to human movement, there are questions about the role of the mosquito vector population in the recent increase of malaria cases. Plans for malaria intervention and policy benefit from the knowledge of the specific malaria vector(s), in different regions of Ethiopia. Various species of *Anopheles* exhibit different feeding and breeding patterns which in turn dictate malaria transmission timing and seasonality (Gone et al., 2014; Massebo et al., 2015). Thus, determining the species and their role in malaria transmission across Ethiopia are crucial for planning of malaria control efforts.

To date, forty-four species and subspecies of anopheline mosquitoes have been documented in Ethiopia (Systematic Catalog of Culicidae). The predominant malaria vector species in Ethiopia is *Anopheles arabiensis* (Massebo et al., 2013; Taye et al., 2006). Other common vectors include *An. funestus, An. pharoensis*, and *An. nili* (Krafsur, 1977; Lelisa et al., 2017; Taye et al., 2006). These species vary in breeding preferences and feeding behaviors. *An. arabiensis* is reported to feed both outdoors and indoors, is mostly zoophilic, and has exhibited both indoor and outdoor resting behavior (Gone et al., 2014; Kenea et al., 2016; Massebo et al., 2015). *An. arabiensis* larvae have been found in seasonal habits of both natural and manmade sources (Kenea et al., 2011). *An. funestus* has demonstrated both endophilic and exophilic behaviors and has shown to be zoophilic in certain settings (Gone et al., 2014) and breed in agricultural related puddles (Kibret et al., 2014). *An. pharoensis* has exhibited exophagic behavior (Taye et al., 2006) and larvae have been detected in swamps and sand mining pools (Kenea et al., 2011). Most studies on malaria vectors in Ethiopia have focused on the west, northern, and southern regions. However, entomological data on the *Anopheles* species composition and vector role in the eastern portion of Ethiopia are sparse. The limited mosquito surveys and molecular studies are due to remoteness and security challenges of the region. Accordingly, an entomological survey was undertaken with the aim of determining the bionomics of *Anopheles* mosquitoes in ESRS. Here we report the results of a study of *Anopheles* mosquitoes in ESRS in which we found *Anopheles stephensi*, a species primarily observed east of the Red Sea.

2. Methods

2.1. Description of study area

The current study was undertaken in Kebri Dehar, in the ESRS in eastern Ethiopia (Fig. 1). Kebri Dehar is located 1035 km from Addis Ababa. The topography of the study area is predominantly lowland plain with an average altitude of 493 m above sea level with a few foothills of higher altitude. The study area has a latitude and longitude of 6°44′25″N, 44° 16′38″E, respectively. The area has sparse shrubs and trees, including different species of *Acacia* and incense trees.

The climate of Kebri Dehar is characterized as tropical semiarid. The region's temperature ranges from 23 to 30 °C. The area has bimodal rainfall pattern. The first and main rainy season 'Gu' occurs from mid-April to the end of June. A secondary rainy season known as 'Deyr' occurs from early October to late December. Overall the annual precipitation averages 200 mm. Despite these patterns, the rainfall pattern is variable making the area prone to recurrent droughts that can last several months.

The inhabitants of the district are largely pastoralists, who raise cattle, camels, sheep and goats. Sorghum and maize are the main staple food crops in the area. Based on figures from the Ethiopian Central Statistical Agency in 2007, Kebri Dehar has an estimated population of 100,191 of whom 51,327 are men and 48,864 are women (National Statistics, 2007). Typically, each house contains a cement tank used as a water reservoir. The type of house in the study area is mainly rectangular with a corrugated iron roof and cement walls. The study area was selected as a sentinel site of malaria and is sprayed with carbamate insecticides during active malaria season in collaboration with the Federal Ministry of Health (FMOH) and the Regional Health Bureau.

2.2. Larval sampling and morphology

Mosquito larvae and pupae were collected from the water reservoirs between November to December 2016 (Fig. 2). We selected 10 larval sites based on the presence and density of larvae and pupae, and optimal time in the area. Dipping was done following WHO guidelines and standard operating procedures for entomological surveillance techniques (World Health Organization, 1992). The anopheline larvae collected were recorded according to their stage of development as either first to second instars (early) or third to fourth (late) instars. The larvae and pupae were transported to the field laboratory with jars and put in enamel trays. Larvae and pupae were reared at the field lab to adulthood in Kebri Dehar and they were maintained at 28 ± 20C and 70 ± 10% relative humidity. The pupae were sorted and transferred with pipettes from the enamel trays to beakers with small amounts of water. Each beaker was placed inside a cage for rearing. Pupae were provided with 10% sugar in the cage. After two to three days the pupae emerged to adults and the cages were put in safe place protected from contamination, ants, and other insects. The adults were collected using an aspirator, transferred to paper cups, and killed using chloroform for further species identification using morphological keys.

Emerged adults were identified to species level using dissecting microscope and magnifying lenses at 10x magnification. Mosquitoes were identified to species based on morphological characters of their palps, wings, abdomen and legs using standard identification keys (Gillies and Coetzee, 1987; Glick and Walter Reed Biosystemsatics Unit, 1992). Following the identification of species based on morphological characters, 529 adult mosquito specimens were shipped to the University of North Carolina at Charlotte for molecular analysis.

2.3. DNA extraction, amplification, and sequencing

DNA was extracted from the head and thorax of the mosquitos using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The polymerase chain reaction (PCR) was performed for each individual mosquito, targeting the mitochondrial cytochrome c oxidase subunit 1 gene (COI) and the nuclear internal transcribed spacer 2 (ITS2) region. The reagent components and concentrations for the PCR assays were 1x Promega HotStart Master Mix (Promega, Madison, WI) and 0.4 mM or 0.5 mM for each primer of COI and ITS2, respectively, plus 1 µl of isolated DNA template. For the COI assay, universal primers that amplifies a portion of the gene was utilized using previously published protocols (Folmer et al., 1994; Saeung et al., 2007), with expected amplicons between 658 to 710 bp. The PCR protocol was as follows: 95 °C 1 min; 30 cycles of 95 °C 30 s, 48 °C 30 s, 72 °C 1 min; 72 °C 10 min. For the ITS2 assay, a 650 bp region including the gene was PCR amplified using universal primers previously detailed in Djadid et al. (2006). PCR temperature protocol consisted of 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; followed by 72 °C for 5 min. All primers used are listed in Table 1.

PCR products were cleaned using ExoSAP. Amplicons were 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 analyzed using Codon Code Aligner Program V. 6.0.2 (CodonCode Corporation, Centerville, MA). Sequences from *Anopheles* from Ethiopia were submitted to the National Center for Biotechnology Information's (NCBI) Basic Local Alignment

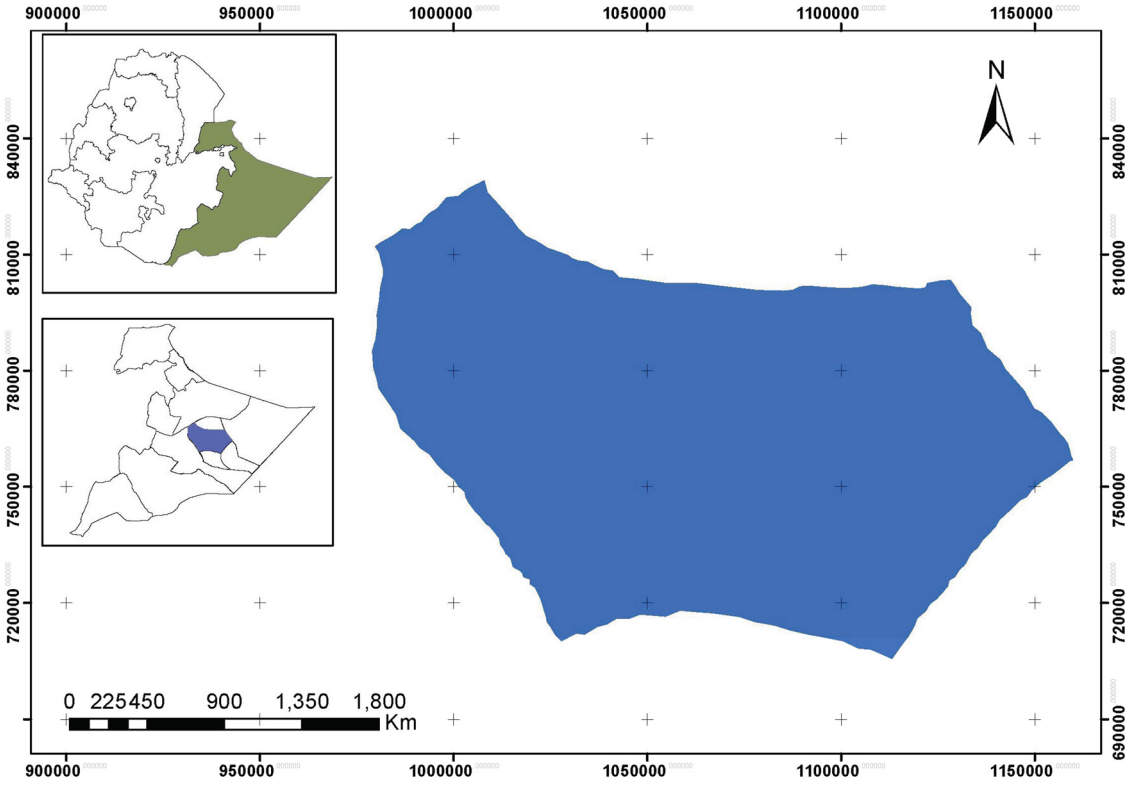

Fig. 1. Map of study site Kebri Dehar.

Fig. 2. Collection of larvae from breeding habitats (water reservoirs) in Kebri Dehar town.

Search Tool (BLAST) (Altschul et al., 1990) against the nucleotide collection in Genbank to find other *Anopheles* sequences that formed high scoring similarity pairs (HSP) at the NCBI web server using these values (i.e. max hsp 500, expect threshold 10, word size 28).

2.4. Phylogenetic analysis

Phylogenetic analysis was performed with the sequence data collected from the *Anopheles* specimens isolated in Ethiopia and closely related within the *Anopheles* genus found in Genbank's nucleotide

Table 1
Primers used for PCR amplification.

Primer Name	Sequence	Locus	Reference	Annealing Temperature
LCO1490F	GGTCAACAAATCATAAAGATATTGG	COI	Folmer et al. (1994)	48
HCO2198R	TAAACTTCAGGGTGACCAAAAAATCA	COI	Folmer et al. (1994)	48
5.8S	ATCACTCGGCTCGTGGATCG	ITS2	Djadid et al. (2006)	50
28S	ATGCTTAAATTTAGGGGGTAGTC	ITS2	Djadid et al. (2006)	50

database (https://www.ncbi.nlm.nih.gov/nuccore) for both the COI and ITS2 sequences separately. Alignments were created with MAFFT version 7 (Katoh and Standley, 2013) and were trimmed using MEGA version 7 (Kumar et al., 2016). Phylogenetic relationships between the Ethiopian samples we created and samples accessed via Genbank sequences were inferred using a maximum likelihood approach with RAxML (Stamatakis, 2014). We applied the GTRGAMMA option that uses GTR model of nucleotide substitution with gamma model of rate of heterogeneity. A total 100 runs were completed with a strategy to identify the heuristically-best-scoring tree under the maximum likelihood criterion and rapid bootstrap analysis in one run. Outgroups were chosen based on availability of data in Genbank and reported relatedness from previous studies (Alam et al., 2008; Harbach and Kitching, 2016). *Anopheles implexus* and *Anopheles sawadwongporni* were assigned as the outgroup for COI analysis and ITS2 respectively. RAxML output was viewed in FigTree (Rambaut, 2007), rooted on the outgroup, and a final phylogenetic tree image was created.

3. Results

Both molecular and morphological data confirmed the larvae were *Anopheles stephensi* based on the following analyses:

3.1. Morphological analysis

In total, 535 larval samples were collected and reared to adulthood. Each adult mosquito emerged from the pupa was identified to species level using morphological characters under a dissecting microscope, two days of post emergence. The following characteristics were observed: the palps were smooth with three distinct pale bands and pale spots were also present in the maxillary palps, the palpomere four is white at the base and apex, and the palpomere five was completely pale. The legs femora and tibiae were speckled with pale scales. The abdominal terga II-VIII and sterna V-VIII were covered with pale scales. The wing anal vein had 3 dark spots and its scutal fossa was covered with scattered pale scales.

3.2. Genetic analysis

Thirty-six samples were selected at random for genetic analysis. Each specimen had identical COI and ITS2 sequences. BLAST of Genbank nucleotide collection under default settings with COI and ITS2 from the Kebri Dehar samples as query sequences matched to *An. stephensi* sequences with 99% identity and 100% identity for COI and ITS2 sequences, respectively. Sequences from Kebri Dehar were deposited in Genbank (MH650999 for ITS2, and MH651000 for COI).

Phylogenetic analyses were performed for COI and ITS2 separately to confirm the species identified through BLAST search. COI phylogenetic analysis included 129 top high-scoring segment pair (HSP) sequences retrieved from Genbank via Nucleotide BLAST (Altschul et al., 1990) analysis from background specimens that had sufficient geographic metadata (Supplemental File 1). Sequences were collected from the following species: *An. annularis*, *An. arabiensis*, *An. argyritarsis*, *An. azaniae*, *An. coluzzii*, *An. culicifacies*, *An. dthali*, *An. gambiae*, *An. implexus*, *An. intermedius*, *An. jeyporiensis*, *An. lepidotus*, *An. minimus*, *An. minimus C*, *An. nr. dravidicus* YML2012, *An. pampanai*, *An. sawyeri*, *An. splendidus*, *An. stephensi*, and *An. varuna*. *An. stephensi* sequences were collected from Pakistan, India, Djibouti, Sri Lanka, Iran, and Saudi Arabia. The final alignment was trimmed to 618 base pairs (Supplemental File 2). In total, 175 variable sites were identified across all taxa. Within the 44 *An. stephensi* sequences, 65 variables sites were identified. Phylogenetic analysis of the alignment revealed strong bootstrap support for the sequence from the Ethiopian sample forming a monophyletic clade with all other *An. stephensi* sequences. The bootstrap value for the key clade was 100% (see arrow in Fig. 3).

To better investigate the relationship with the Ethiopian *An. stephensi* sequenced isolate with sequenced isolates from other countries, phylogenetic analysis was performed with only *An. stephensi* COI sequences that contained accurate location details using *An. maculatus* as the outgroup (Fig. 4, Supplemental File 3). Our results show the Ethiopian isolate is sister to the clade that contains

isolates from Pakistan, India, Iran, Sri Lanka, and Djibouti. The bootstrap value of the key clade is 100 percent (see arrow in Fig. 4). The analysis also indicated that the Ethiopian *An. stephensi* is most closely related to a Pakistan sequenced isolate.

Similar results were observed for the ITS2 phylogenetic analysis. ITS2 phylogenetic analysis included 35 sequences from NCBI (Supplemental File 4) that formed HSPs with the sequence from the Ethiopian sample forming in the BLAST search and had sufficient geographic metadata. The following species were included in the analysis: *An. stephensi*, *An. willmori*, *An. maculipalpus*, *An. maculatus*, and *An. sawadwongporni*. The final alignment was trimmed to 350 base pairs. In total, 112 variable sites were identified across taxa. All *An. stephensi* sequences were identical except for one (HQ703001) which had 40 variables sites compared the other *An. stephensi* sequences. The ITS2 alignment included multiple gaps and a microsatellite region. As with COI, the Ethiopian ITS2 sequence shared a clade with other *An. stephensi* isolates to the exclusion of other species with a bootstrap value of 100 percent for the key clade (see arrow in Fig. 5).

4. Discussion

Here we illustrate for the first time the presence of *Anopheles stephensi* in Ethiopia with both molecular and morphological evidence. Both COI and ITS2 phylogenetic analysis confirm the monophyly of Ethiopian *An. stephensi* specimens with other *An. stephensi* specimens to the exclusion of other species of *Anopheles*. The differentiation of *An. stephensi* isolates from *Anopheles gambiae* complex isolates, including *An. arabiensis*, is consistent with previous phylogenetic studies (Hao et al., 2017; Jiang et al., 2014). While COI showed more sequence diversity across the *Anopheles* species, ITS2 is more conserved. The morphological data also support the identification of *An. stephensi*. Morphology of the specimen resembled the features for the *An. stephensi* reported in Sri Lanka (Gayan Dharmasiri et al., 2017).

The finding of *An. stephensi* in Ethiopia is unexpected. Previous sources describe *An. stephensi*'s range being restricted to east of the Red Sea (Gaffigan et al., 2018; Sinka et al., 2011). One recent exception is the detection of *An. stephensi* in Djibouti in 2013 during a malaria outbreak (Faulde et al., 2014). The authors of the Djibouti report speculate that the source of the outbreak was host migration from Ethiopia. While host movement likely plays a role in cross transmission of malaria, it will be very important to investigate how vector movement may also contribute to transmission across political borders. In our *An. stephensi* species phylogenetic analysis (Fig. 4), the Djibouti sequenced isolate is in a distinct clade to the clade containing the isolate from Ethiopia suggesting different introductions to the Horn of Africa. Interestingly, the sister isolate for *Anopheles stephensi* in Ethiopia was from Pakistan. Additional genetic analysis of samples from the Horn of Africa, Pakistan, and other regions within *An. stephensi*'s range can provide needed information on the origin and movement of *An. stephensi*.

We establish some working hypotheses about the arrival of the Ethiopian *An. stephensi* in Ethiopia. Some competing scenarios are possible: 1) *An. stephensi* was recently introduced into Ethiopia. The COI data currently shows that the Ethiopian isolate is most closely related to a Pakistani isolate. Further analysis of mosquitoes from South Asia, the Middle East, and the Arabian Peninsula can provide more granular information about the timing and source of *An. stephensi* in the Horn of Africa. 2) An alternative hypothesis is that *An. stephensi* has been present in Ethiopia for a long time but undetected. There are some morphological similarities between *An. stephensi* and *An. arabiensis,* which is considered the dominant vector in most of Ethiopia. Therefore, it is

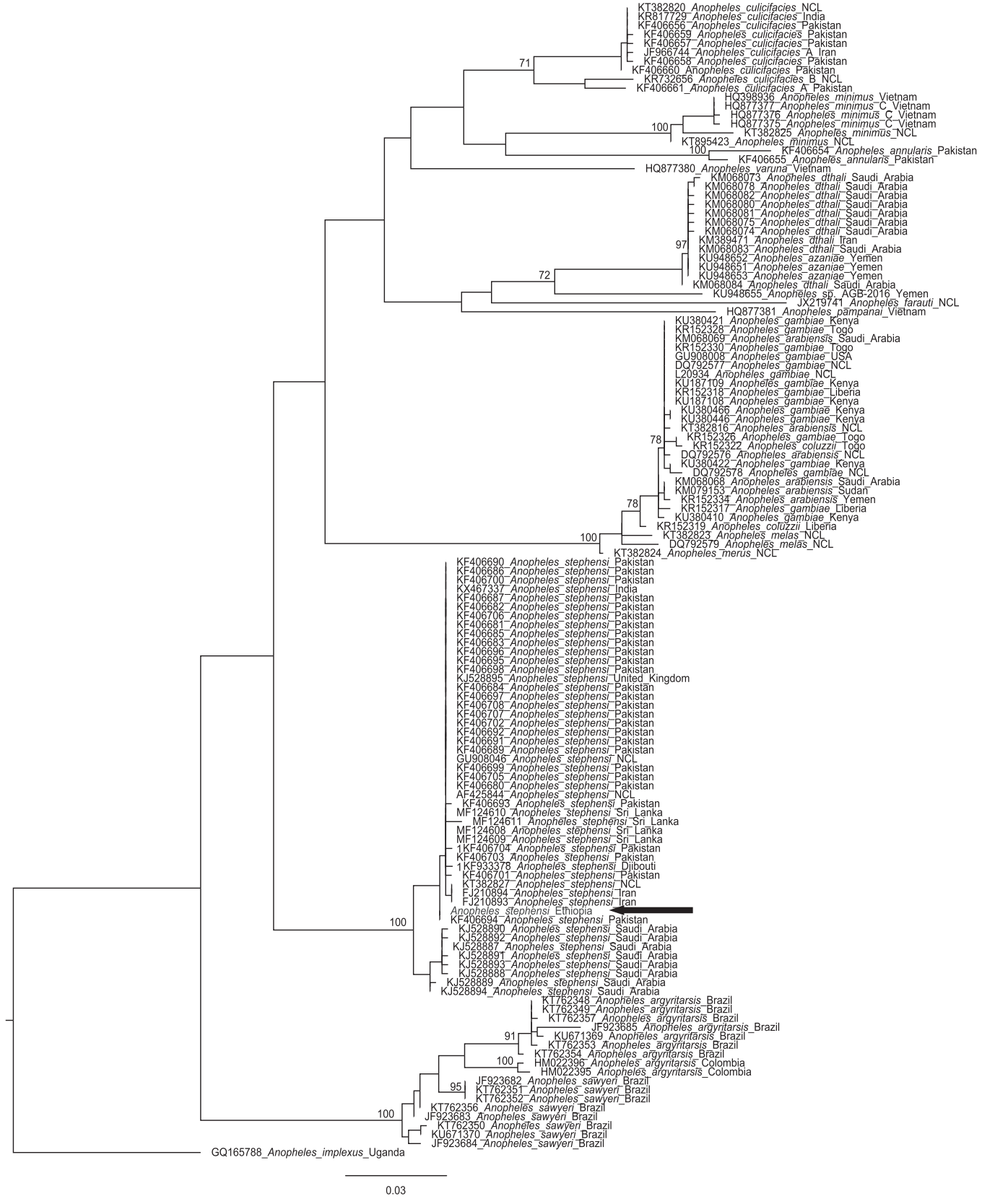

Fig. 3. Phylogeny of COI sequence from *Anopheles* isolates. Bootstrap values > 70 for notable species clades shown at nodes. Nodes without numbers had a value < 70. Ethiopian sequenced isolated denoted with arrow. Final ML Optimization Likelihood: -3549.017267. Sequences with no country listed or country incorrectly listed in Genbank are labeled as NCL.

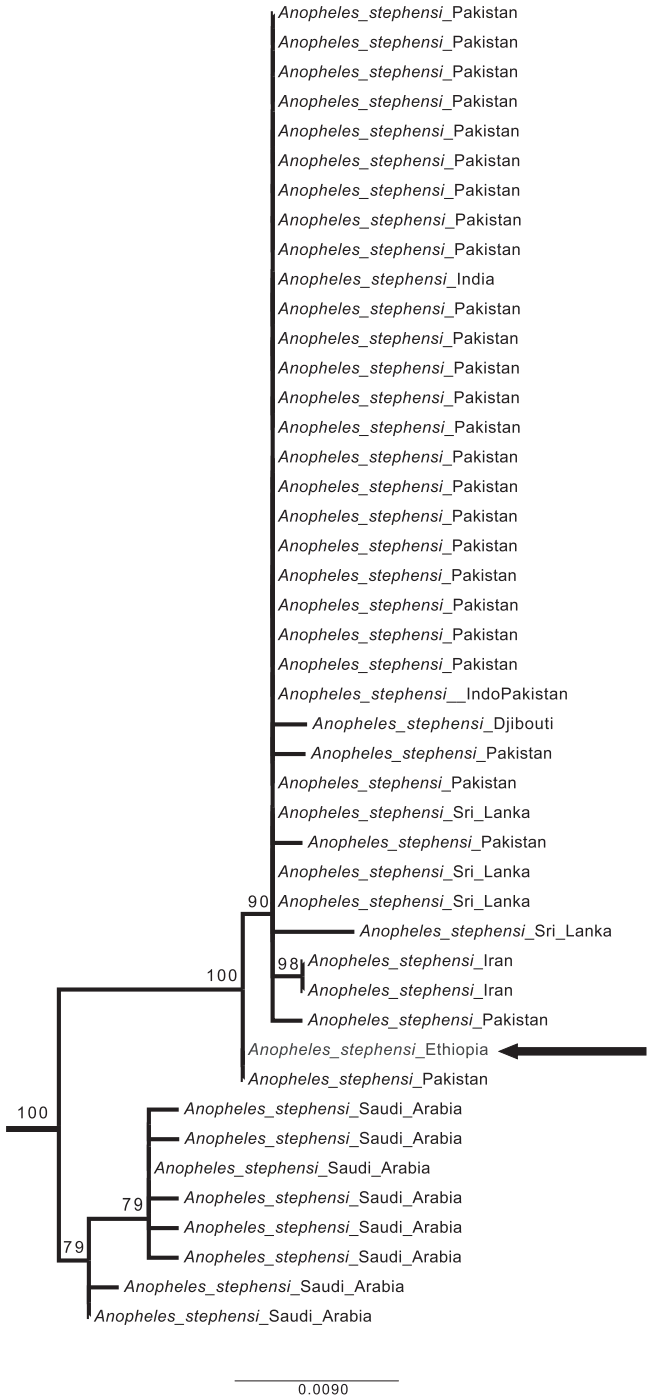

Fig. 4. Phylogeny of COI sequence from *Anopheles stephensi* isolates. Bootstrap values > 70 shown at nodes. Nodes without numbers had a value < 70. Final ML Optimization Likelihood: -1252.592081. Ethiopian sequenced isolated denoted with arrow.

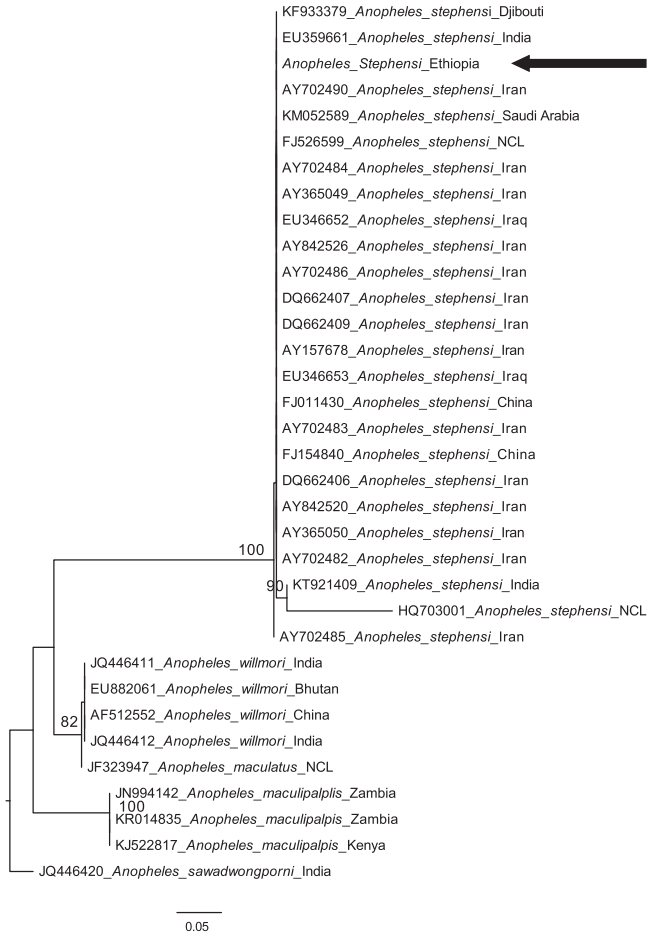

Fig. 5. Phylogeny of ITS2 sequence from *Anopheles stephensi* isolates. Bootstrap values > 70 shown at nodes. Nodes without numbers had a value < 70. Final ML Optimization Likelihood: -1104.708501. Ethiopian sequenced isolated denoted with arrow.

possible that *An. stephensi* has been overlooked or misidentified over the years and came from the other side of the Red Sea a long time ago. Our findings highlight the need for wider survey of the malaria vectors in Ethiopia. Here we show that sequence-based methods are particularly useful for differentiating the species that may be morphologically similar.

Finding *An. stephensi* in Ethiopia has important public health implications. *An. stephensi*, like *An. arabiensis*, has demonstrated both indoor and outdoor behaviors but is most often endophagic (Manouchehri et al., 1976; Tirados et al., 2006). If the same endophagic behavior patterns are observed in Ethiopia, different malaria control strategies may need to be implemented. Additionally, more studies are needed to determine the vector compentency of *An. stephensi* in Ethiopia for *Plasmodium* spp. Wild-caught mosquitoes will be surveyed to confirm the transmission of *Plasmodium* in the region and to identify which parasite species is being transmitted. Ethiopia is one of the few countries in Africa where *P. vivax* and *P. falciparum* are both actively transmitted (Carter et al., 2018; Tadesse et al., 2018; World Health Organization, 1992). In the Somali Region, *P. falciparum* is believed to the be the primary species. *An. stephensi*'s ability to carry both *Plasmodium falciparum* and *Plasmodium vivax*, as observed in other areas (e.g. India and Djibouti) (Balabaskaran Nina et al., 2017; Faulde et al., 2014; Thomas et al., 2017), makes it a public health concern for this region. In addition to studies of behavior, range, and vector competence for *An. stephensi*, studies on *An. stephensi*'s sensitivity or resistance to insecticides will provide important information on the type of vector control interventions to implement.

To gain better insight into the geographic range of *An. stephensi*, the next step is to conduct mosquito surveys in multiple locations throughout Ethiopia. Much of the effort should center on the eastern portion where information on malaria vectors in general is scarce. Both rural and urban surveys are needed, particularly to investigate the role that livestock presence plays in *An. stephensi* abundance. All target sites should include both larvae and adult collections. Sampling ten additional study sites with two sample collection time points will provide preliminary information on the temporal, epidemiological, geographic, and climatic variation of the distribution of *Anopheles* populations in

Ethiopia. This information will be used to design a long-term surveillance study.

In conclusion, this study describes the first confirmed report of *An. stephensi* in Ethiopia. The identification of *An. stephensi* in Ethiopia has important implications for understanding malaria transmission in Ethiopia. The results of this study contribute the broader understanding of malaria vector composition in the ESRS and in Ethiopia as a whole.

Competing interests

The authors declare that they have no competing interest exist 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.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2018.09.001.

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