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Molecular confirmation of *Anopheles stephensi* Mosquitoes in the Al Hudaydah Governorate, Yemen, 2021 and 2022

Appendix

The invasive malaria vector, *Anopheles stephensi* mosquito, in Africa was first detected in Djibouti in 2012, and was followed by Ethiopia, Somalia, Sudan, Nigeria, Ghana, Kenya, and Eritrea over the past decade (*1*–*7*, Afrane YA, unpub. data, https://pubmed.ncbi.nlm.nih.gov/38076990).

Site descriptions

Ad Dahi, located north of Hodeida city (15° 12′ 55” N / 43° 4′ 13” E). The city has a population size of ≈21,587 and the climate is tropical with high temperatures in the summer and moderate temperatures in the winter. Zabid, one of the southernmost districts of Al Hudaydah governorate, is located in the Tehama coastal plain near the Red Sea (14°12′02.6”N, 43°19′06.6”E) in a tropical climate and a population size of 34,686.

Molecular analysis

Two loci were selected for PCR-based species identifications: cytochrome oxidase subunit 1 (COI) and internal transcribed spacer 2 (ITS2). A *stephensi*-specific PCR endpoint assay was used to identify *An. stephensi* based on amplification (presence/absence) of a portion of the ITS2 locus. The primer sequences for PCR in the ITS2 endpoint assay were 5.8SB (5′-ATCACTCGGCTCGTGGATCG-3′) and 28SC (5′- GTCTCGCGACTGCAACTG-3′) (*7*). In addition, two PCR protocols for the *ITS2* locus and the *COI* gene were implemented to generate products for sequencing. PCR was conducted as detailed in Carter et al. (*3*). The primer sequences for the ITS2 PCR for sequencing were 5.8SB (5′-ATCACTCGGCTCGTGGATCG-3′) and 28SB (5′-ATGCTTAAATTTAGGGGGTAGTC-3′) (*7*). The primer sequences for *COI* PCR were LCO1490F (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198R (5′-

TAAACTTCAGGGTGACCAAAAAATCA-3′) (*8*). COI and ITS2 PCR products were sequenced using Sanger sequencing technology. Sequences were then trimmed using CodonCode Aligner (CodonCode Corporation, Centerville, MA). To avoid sequence ambiguity caused by the microsatellite loci located within ITS2 (*3*, *9*), we only considered the sequence upstream of a previous identified microsatellite. For the COI, a 317 bp region was used for phylogenetic analysis carried phytogeographically informative single nucleotide polymorphisms (SNPS) (*10*) (Carter et al. 2021). Trimmed sequences were submitted to the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST) to confirm successful amplification. The sequences were further aligned in CodonCode with previously published sequences retrieved from Genbank, and phylogenetic analyses were conducted using maximum-likelihood method with RAxML (*11*). The final trees were annotated using Figtree (Rambaut, http://tree.bio.ed.ac.uk/software/figtree/URL).

Availability of data and materials

The sequences generated in this study are available through NCBI Genbank Accession numbers PP410027, PP387837- PP387838.

Abbreviations

HoA: Horn of Africa; WHO: World Health Organization; PCR: polymerase chain reaction; ITS2: internal transcribed spacer 2; COI: cytochrome c oxidase I; NCBI: National Center for Biotechnology Information, BLAST: basic local alignment search tool; SNPs: single nucleotide polymorphisms

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