

Molecular Characterization Reveals Diverse and Unknown Malaria Vectors in the Western Kenyan Highlands

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Abstract. The success of mosquito-based malaria control is dependent upon susceptible bionomic traits in local malaria vectors. It is crucial to have accurate and reliable methods to determine mosquito species composition in areas subject to malaria. An unexpectedly diverse set of *Anopheles* species was collected in the western Kenyan highlands, including unidentified and potentially new species carrying the malaria parasite *Plasmodium falciparum*. This study identified 2,340 anopheline specimens using both ribosomal DNA internal transcribed spacer region 2 and mitochondrial DNA cytochrome oxidase subunit 1 loci. Seventeen distinct sequence groups were identified. Of these, only eight could be molecularly identified through comparison to published and voucher sequences. Of the unidentified species, four were found to carry *P. falciparum* by circumsporozoite enzyme-linked immunosorbent assay and polymerase chain reaction, the most abundant of which had infection rates comparable to a primary vector in the area, *Anopheles funestus*. High-quality adult specimens of these unidentified species could not be matched to museum voucher specimens or conclusively identified using multiple keys, suggesting that they may have not been previously described. These unidentified vectors were captured outdoors. Diverse and unknown species have been incriminated in malaria transmission in the western Kenya highlands using molecular identification of unusual morphological variants of field specimens. This study demonstrates the value of using molecular methods to compliment vector identifications and highlights the need for accurate characterization of mosquito species and their associated behaviors for effective malaria control.

INTRODUCTION

Malaria control methods focus largely on impacting the vector population to reduce transmission. Interventions such as use of indoor residual spray (IRS) and long-lasting insecticidal nets take advantage of species-specific vector behaviors that result in exposure to lethal insecticide.^{1–5} In many areas, multiple vector species contribute to malaria infection, which may prolong the transmission season through their different feeding behaviors and exploitation of the environment.^{3,6} There may also be more vector species in some areas than are presently known due to lack of regular in-depth descriptive surveys, the presence of cryptic or sibling species,^{7–9} or outdated keys to identify specimens, and inadequate descriptions used to morphologically identify species. Different species and even different members within species complexes can exhibit a variety of behaviors relevant to malaria epidemiology.^{10,11} For example, the *Anopheles gambiae* species complex is composed of at least eight distinct sibling species,⁸ some of which are generally nonhuman feeders, such as *An. quadriannulatus*,^{12,13} whereas others, such as *An. gambiae* s.s., *An. coluzzii*, and *An. arabiensis*, act as the primary malaria vectors in sub-Saharan Africa. *An. gambiae* s.s. is highly anthropophilic and endophagic, while in many areas *An. arabiensis* is considered to be more exophagic and exophilic.^{4,14–17} The outdoor feeding behavior of *An. arabiensis* and other vector species complicates control

interventions, such as IRS and insecticide-treated bednets, which are used indoors, and some species can shift their behavioral patterns over time in response to interventions.^{18–22} Morphological identification cannot distinguish between these cryptic species and can be inaccurate even for morphologically distinct species, resulting in skewed interpretation of species compositions in behavioral or monitoring studies. Because of the limitations of morphological identification tools, there is a need for detailed molecular taxonomy and phylogenetic analyses to accurately identify specific vectors and to associate these with specific behaviors.²³

The ribosomal DNA internal transcribed spacer region 2 (rDNA ITS2) and the mitochondrial DNA cytochrome oxidase subunit 1 (mtDNA CO1), two quickly evolving loci, have been used to differentiate members of species complexes, and to generate reference sequences and unique barcodes that identify and distinguish *Anopheles* species.^{9,10,24,25} Though an increasing number of *Anopheles* sequences at these two loci are available in databases, many common anophelines have not been part of molecular studies. There are approximately 200 *Anopheles* species represented by ITS2 and CO1 sequences in GenBank (National Center for Biotechnology Information [NCBI]), despite almost 500 recognized species in the genus.²⁶ Genotyping of local anophelines, even presumed nonvector species, will enable accurate linking of bionomic traits with species, thereby allowing for the appropriate assessment of efficacy or limitations of interventions being implemented.²⁷ Accurately identifying the bionomic vulnerabilities of specific populations or species that need to be targeted for malaria control also allows for more focused and potentially cost-effective intervention strategies.

In this study, a set of anopheline species collected in the western Kenyan highlands, Nyanza Province (Figure 1), was

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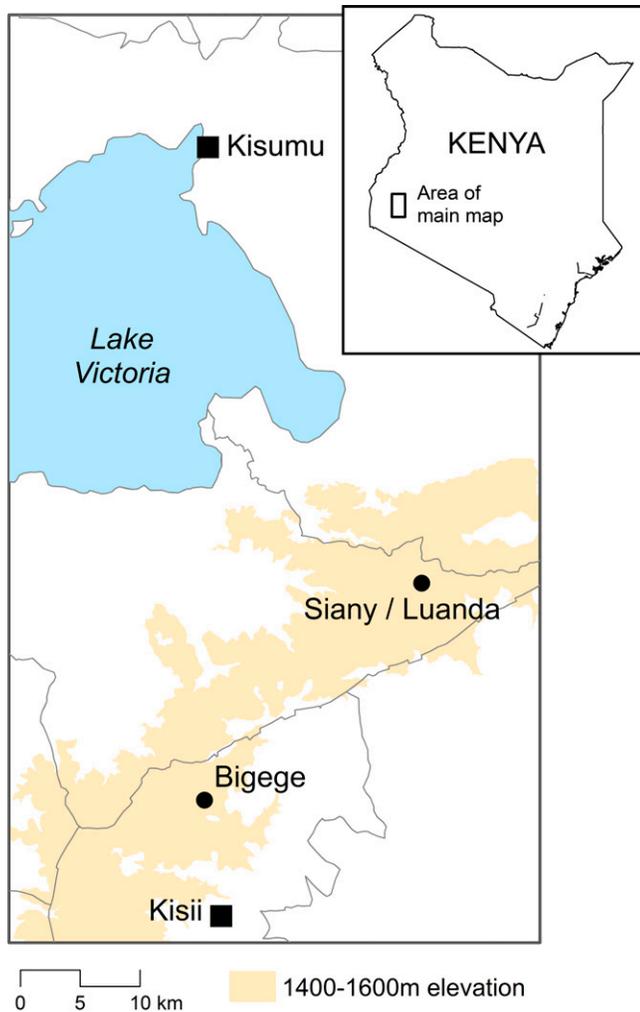


FIGURE 1. Map of the Kenya highlands. Malaria Transmission Consortium sites where entomological collections took place. Adult mosquitoes were collected in Bigege village in Kisii Central District and Luanda and Siany villages in Rachonyo South District (Luanda and Siany villages overlap), in Nyanza Province using Centers for Disease Control and Prevention miniature light traps placed indoors and outdoors, and a small number of immature stages were collected by larval dipping from May 2010 to December 2011.

analyzed using rDNA ITS2 and mtDNA CO1 loci for accurate molecular species identification. Published studies from Kisii district, Nyanza Province, of the Kenyan highlands have focused on the presumed primary malaria vectors in the region, namely species belonging to the *An. gambiae* and *An. funestus* complexes.^{28–33} There are no reports of other anophelines in the area acting as major vectors and only limited descriptive vector studies from the highlands of Rachuonyo South, Nyanza Province. The western Kenyan highlands is an area of unstable, sometimes epidemic malaria³⁴ where anopheline vector dynamics determine the spread and maintenance of malaria transmission.^{35–37} The aim of our study was to identify *Anopheles* species present in two neighboring districts in the western Kenyan highlands using both molecular and morphological tools. This study expands upon previously reported findings of unknown and potentially new vector species in Kisii district,³⁸ detailing the rDNA ITS2 and mtDNA CO1 regions,

and includes sequence analysis of two more recent sets of mosquito collections from an area in the neighboring district, Rachuonyo South.

MATERIALS AND METHODS

Study sites. Mosquitoes were collected in Bigege village, Kisii Central District, and Luanda and Siany villages, Rachuonyo South District, Nyanza Province, western Kenya (Figure 1). The global positioning system coordinates for these villages are the following: Bigege 0°35.576' S, 34°44.975' E; Luanda 0°25.909' S, 34°55.687' E; and Siany 0°25.619' S, 34°54.707' E. This is a rural area in the highlands (1,400–1,600 m above sea level) dominated by the Kisii and Luo ethnic groups, who are mainly subsistence farmers. The landscape is hilly with many small streams and rivers and households distributed on the sides of the valley. There are two seasonal peaks of malaria transmission reflecting a bimodal rainfall pattern, with the heaviest rainfall typically occurring between March and June with a smaller peak in October and November. Previous studies in Kisii have reported the primary vectors to be *An. gambiae* and *An. funestus*.³⁹

Mosquito collections. Mosquitoes were collected from consenting households between May 2010 and December 2011 from a number of different entomological studies as part of the Malaria Transmission Consortium (MTC) (institutional review board approval Kenya Medical Research Institute (KEMRI) no. SSC 1399, 2007). This project sought to improve tools for evaluating malaria transmission and control in diverse transmission environments.³⁷ The MTC studies in the Kenya highlands have included entomological surveys that evaluate biting behavior, compare trapping techniques, and assess the impact of interventions in the highlands of Rachuonyo and Kisii districts, western Kenya.⁴⁰ Adult mosquitoes were collected using Centers for Disease Control and Prevention (CDC) miniature light traps set indoors and outdoors with either standard or ultraviolet bulbs. Indoor traps were either set next to a human under a bednet or baited with CO₂. In Bigege, limited larval collections were conducted in the vicinity of surveyed houses using the standard dipping method and a small number (N = 95) of these immature stages also underwent molecular analyses for species identification.⁴¹ Water bodies were mapped and sampling undertaken at the same time as adult collections in the peak transmission season. In addition to these collections, larval collections were made from fish ponds in multiple locations in Rachuonyo. The larvae were brought to a field insectary and raised to adults. Larval and pupal skins and adults were identified using standard morphological keys. Mosquitoes were identified in the field using standard morphological keys,^{7,42} scored visually as blood-fed or unfed, and dried on silica gel until molecular processing.

Infectivity. Female mosquitoes were analyzed for *Plasmodium* infection using circumsporozoite enzyme-linked immunosorbent assay (CS ELISA) for *Plasmodium falciparum*⁴³ at KEMRI/CDC laboratories in Kisumu, western Kenya. Positive samples were sent to the University of Notre Dame, Indiana, for subsequent polymerase chain reaction (PCR) confirmation of infection using a nested PCR assay for *P. falciparum* and *P. vivax*.⁴⁴

Blood meal analysis. Abdomens of mosquitoes found to be blood-fed were analyzed using a blood meal diagnostic

PCR assay based on vertebrate mitochondrial cytochrome b DNA sequences.⁴⁵ Blood meals that did not amplify in the diagnostic PCR were sequenced and blasted against the NCBI nr database to identify the source⁴⁶ of the blood meal.

Amplification by PCR and sequencing of the ITS2 and CO1 regions. Genomic DNA was isolated from female anophelines using a CTAB technique or simple 70% alcohol precipitation. The rDNA ITS2 was amplified from genomic DNA using the ITS2A (5'-TGTGAACTGCAGGACACAT-3') and ITS2B (5'-TATGCTTAAATTCAGGGGGT-3') primers.⁴⁷ The 25- μ L PCR mixture contained 2.5 μ L of 10 \times buffer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.2 mM MgCl₂, 0.5 units of Taq DNA polymerase, 0.75 μ L of 10 pmol/ μ L each of forward and reverse primers, and 1 μ L of DNA template prepared as above. The thermocycling conditions were as follows: 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes, with a final extension at 72°C for 5 minutes.

The mitochondrial DNA cytochrome oxidase subunit 1 (CO1) gene was amplified using LCO and HCO primers.⁴⁸ The primers used were LCO 1490 (5'-GGTCAACAAATCA TAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCA GGGTGACCAAAAATCA-3'). The 25- μ L PCR mixture contained 2.5 μ L of 10 \times buffer, 0.2 mM of each dNTP, 1.2 mM MgCl₂, 0.5 units of Taq DNA polymerase, 0.75 μ L of 10 pmol/ μ L each of forward and reverse primers, and 1 μ L of DNA template prepared as above. The thermocycling conditions were as follows: 94°C for 5 minutes, five cycles of denaturation at 94°C for 40 seconds, annealing at 45°C for 1 minute, and extension at 72°C for 1.5 minutes; then 30 cycles of denaturation at 94°C for 40 seconds, annealing at 51°C for 1 minute, and extension at 72°C for 1.5 minutes, with a final extension at 72°C for 5 minutes.

The amplified fragments were visualized by electrophoresis on a 1% agarose gel. The PCR product was purified using an enzyme cleanup; 2 units of exonuclease 1 (USB Corporation, Cleveland, OH), 1 unit of Shrimp Alkaline Phosphatase (USB Corporation), and 1.8 μ L of double distilled H₂O were added to 8 μ L of PCR product. This mixture was incubated at 37°C for 15 minutes, followed by 15 minutes at 80°C to inactivate the enzymes. The PCR products were sequenced directly (with one of the PCR primers) using Sanger sequencing on ABI 3730xl DNA Analyzer platform (PE Applied Biosystems, Warrington, England). A subset of specimens were independently confirmed using species-diagnostic PCRs for *An. funestus* and *An. gambiae*.^{9,24}

Sequence analysis. Raw ITS2 sequences were initially aligned using the Seqman pro assembler (Lasergene v 10.1.1, DNASTAR Inc., Madison, WI) with a minimum match of 90%. Assembled contigs were examined for repeats and further divided into subcontigs based on consistent single nucleotide polymorphisms (SNPs). A limit of 98% identity was used to assemble ITS2 sequences into final "species groups." Single sequence contigs, low quality, or contaminated sequences were not included in the analysis. The consensus sequences of these ITS2 contigs were compared (Basic Local Alignment Search Tool nucleotide [BLASTn]) to the NCBI nr database for confirmation of species identities. High sequence identity (99% or greater) to voucher specimen sequences in the database or a combination of sequence and morphological identification was used for final species confirmation. High sequence

similarity to non-voucher specimens was noted but not used for final species identity.

The CO1 sequences were similarly assembled and compared with the NCBI nr database for confirmation of species identities. Sequence groups were merged (minimum identity of 94%) when CO1 BLAST results indicated that they belonged to the same species for a final minimum match of > 95%. Single sequence contigs were not included in this analysis.

Before phylogenetic analysis, ITS2 sequences were initially annotated in web interface for ITS2 delimitation accessible at the ITS2-DB (<http://its2.bioapps.biozentrum.uni-wuerzburg.de>). This database utilizes comprehensive Hidden Markov Model approach to define the boundaries (start and end positions) of the ITS2 region by comparing to a conserved structural motif at 5.8S/28S ribosomal RNA regions. The ITS2 sequences were then aligned in MAFT⁴⁹ using X-INS-i⁵⁰ strategy. This alignment method detects conserved secondary structures in noncoding RNA sequences and is based on the Four-way Consistency objective function to build a multiple alignment by combining Stem Candidate Aligner for RNAs (SCARNA) algorithm for the initial pairwise alignments.⁵⁰

Separate analyses for ITS2 sequences were done using Bayesian approach in MrBayes v3.1.2⁵¹ using a general time reversible substitution model. Each analysis was performed with two independent runs with four chains, and each run was carried out for 10,000,000 generations with a sample frequency of 1,000. The first 25% of trees were discarded as burn-in and the posterior probabilities were estimated from the remaining trees to infer branch support.

RESULTS

The ITS2 sequences representing 2,340 anopheline mosquitoes were aligned into 17 distinct groups with > 98% sequence identity within each group. These groups were arbitrarily designated *Anopheles* species A through Q. The consensus sequences of each group were compared (BLASTn) to the NCBI nr database to identify similarity to any sequence present in the database. *Anopheles* species B had a high (99.8%) sequence identity with ITS2 sequences of *An. arabiensis*, and those of *Anopheles* species D were identical to ITS2 sequences of *An. funestus*. The ITS2 consensus sequences from *Anopheles* species C, E, H, I, J, and L matched recently added non-voucher ITS2 sequences in the database. These included *An. coustani* (species C), *An. maculipalpis* (species E), *An. pretoriensis* (species H), *An. theileri* (species I), *An. rufipes* (species J), and *An. lesoni* (species L). The species' letter (B, C, D, E, H, I, J, and L) designations are henceforth referred to by their identified species' names. The consensus sequences from the other eight groups (A, F, G, K, M, N, O, P, and Q) did not share > 90% identity with any nr database sequence(s) (Table 1).

The CO1 sequences representing 336 mosquito specimens were aligned and the resulting distinct sequence groups were named as they correlated to the ITS2 groupings (again, A through Q). These included *An. arabiensis* (species B), *An. coustani* (species C), *An. funestus* (species D), *An. squamosus* (species M), and *An. christyi* (species P). *Anopheles* species O and Q sequences matched *An. coustani* sequences in the Barcode of Life Database (BOLD) database, though the ITS2 did not match *An. coustani*. The CO1 consensus sequences

TABLE 1
Summary of ITS2 and CO1 sequence groups, molecular and morphological identifications, and circumsporozoite ELISA and PCR positivity of anopheline specimens caught in the Kenyan highlands

Sequence species group	Field-based morphological identification	Morphological identification	ITS2 no. sequenced	ITS2 sequence homology	CO1 no. sequenced	CO1 sequence homology	Tentative species ID	Final species ID	ELISA <i>P.f.</i> spz +ve/no. tested	PCR +ve/no. tested
Species A (multiple species)	<i>An. demeilloni</i>	<i>An. demeilloni</i> group	529	–	78	–	Subgenus <i>Cellia</i> , Myzomyia series	Unknown	11/318	5 <i>P.f.</i> 1 <i>P.</i> /343
Species B	<i>An. gambiae</i> s.l.	<i>An. gambiae</i> s.l.	272	<i>An. arabiensis</i>	126	<i>An. arabiensis</i>	<i>An. arabiensis</i>	<i>An. arabiensis</i>	0/102	0/105
Species C (multiple species)	<i>An. coustani</i>	<i>An. coustani</i>	216	<i>An. coustani</i>	25	<i>An. coustani</i>	<i>An. coustani</i>	<i>An. coustani</i>	0/169	0/174
Species D (multiple species)	<i>An. funestus</i> s.l.	<i>An. funestus</i> s.l.	724	<i>An. funestus</i>	21	<i>An. funestus</i>	<i>An. funestus</i>	<i>An. funestus</i>	10/549	16 <i>P.f.</i> 7 <i>P.</i> /687
Species E (multiple species)	<i>An. maculipalpis</i>	<i>An. maculipalpis</i>	138	<i>An. maculipalpis</i>	16	–	Subgenus <i>Cellia</i> , Neocellia series, likely <i>An. maculipalpis</i>	<i>An. maculipalpis</i>	0/97	0/108
Species F	<i>An. gambiae</i> s.l.	–	51	–	10	–	Subgenus <i>Cellia</i> , Myzomyia series, near <i>An. theileri</i>	Unknown	2/37	2 <i>P.f.</i> /38
Species G (multiple species)	–	–	38	–	5	–	Subgenus <i>Cellia</i> , Myzomyia series, near <i>An. theileri</i>	Unknown	1/23	1 <i>P.f.</i> /23
Species H	<i>An. pretoriensis</i>	–	38	<i>An. pretoriensis</i>	9	–	Subgenus <i>Cellia</i> , Neocellia series, possibly <i>An. pretoriensis</i>	<i>An. pretoriensis</i>	0/28	0/28
Species I	<i>An. gambiae</i> s.l. (multiple species)	–	198	<i>An. theileri</i>	28	–	Subgenus <i>Cellia</i> , Myzomyia series, possibly <i>An. theileri</i>	Unknown	4/164	1 <i>P.</i> /168
Species J (multiple species)	–	–	30	<i>An. rufipes</i>	4	–	Subgenus <i>Cellia</i> , Neocellia series, possibly <i>An. rufipes</i>	<i>An. rufipes</i>	0/20	0/24
Species K (multiple species)	–	–	29	–	3	–	Subgenus <i>Cellia</i> , Neocellia series, possibly <i>An. rufipes</i>	Unknown	0/25	0/25
Species L	<i>An. funestus</i> s.l.	<i>An. funestus</i> s.l.	62	<i>An. leesoni</i>	2	–	Subgenus <i>Cellia</i> , Myzomyia series, likely <i>An. leesoni</i>	<i>An. leesoni</i>	0/59	1 <i>P.</i> /60
Species M (multiple species)	–	<i>An. squamosus</i>	4	–	2	<i>An. squamosus</i>	<i>An. squamosus</i>	<i>An. squamosus</i>	0/2	0/2
Species N	–	–	3	–	2	–	Subgenus <i>Cellia</i> , Myzomyia series, near <i>An. theileri</i>	Unknown	0/1	0/1
Species O	<i>An. coustani</i>	–	5	–	2	<i>An. coustani</i>	Subgenus <i>Anopheles</i> , possibly an <i>An. coustani</i> sibling	Unknown	0/3	0/3
Species P (multiple species)	–	–	3	–	3	<i>An. christyi</i>	Subgenus <i>Cellia</i> , <i>Cellia</i> series, possibly <i>An. christyi</i>	Unknown	0/3	0/3
Species Q	–	–	2	–	2	<i>An. coustani</i>	Subgenus <i>Anopheles</i> , possibly an <i>An. coustani</i> sibling	Unknown	0/2	0/2

COI = cytochrome oxidase subunit I; ELISA = enzyme-linked immunosorbent assay; ITS2 = internal transcribed spacer region 2; mtDNA = mitochondrial DNA; PCR = polymerase chain reaction; rDNA = ribosomal DNA. Sequence groups were determined based on sequence identity of > 98% based on ribosomal DNA ITS2 sequences. The composition of the groups was confirmed with mitochondrial CO1 sequences. In total, 2,340 and 336 specimens were sequenced for rDNA ITS2 and mtDNA CO1, respectively. Detailed morphological identifications were carried out on a subsample of specimens by at least three entomologists in reference laboratories. *P.f.* refers to *Plasmodium falciparum*. PCR confirmation of *P.f.* circumsporozoite ELISA positives. *P.* refers to PCR positives for *Plasmodium* that could not be confirmed to species using a second species-specific *Plasmodium* PCR. Positive specimens are bolded. The total number of each sequence group tested by ELISA and PCR are listed in the last two columns. Voucher sequences are underlined.

from *Anopheles* species groups A, E, F, G, H, I, J, K, and N did not share > 90% identity with any database sequence(s) (Table 1), indicating that these CO1 regions have not been previously sequenced.

Consensus ITS2 sequences were structurally examined and aligned to construct a phenogram (Figure 2). This phenogram is meant only to demonstrate sequence divergence based on ITS2 sequences rather than represent the exact phylogenetic relationships of these groups. Insertions seen in group-specific sequences might increase the divergence between similar sequences. The distinct clustering and sequence divergence between molecular sequence groups indicate that many very distinct species of anophelines are present in these collections from a small geographic area. Notably, *Plasmodium*-positive *Anopheles* species A, I, F, and G cluster together, as do *An. maculipalpis*, *An. pretoriensis*, and *An. rufipes*, and *Anopheles* species K, P, and *An. squamosus*. Both the ITS2 and CO1 sequence of species O, Q, and *An. coustani* are very different than the other species analyzed and reference sequences. These are *An. coustani* and a potential *An. coustani* sibling species, which belong to subgenus *Anopheles*, whereas the rest of the species are members of subgenus *Cellia*. The grouping of *An. maculipalpis*, *An. pretoriensis*, and *An. rufipes* is consistent with other ITS2 phylogenies.⁵² *Anopheles* species B (*An. arabiensis*) and *Anopheles* species D (*An. funestus*) cluster closely at the top of the tree.

As ITS2 sequence groups were eventually used to characterize the number of species, a stringent threshold of 98% identity was used to determine the maximum number of possible clusters and final number of species. Because of a typically higher rate of sequence divergence, a lower threshold of 94% sequence identity, after a higher initial threshold, was used for the assembly of CO1 groups, resulting in lower sequence similarity in the final species groupings.^{53,54} The

CO1 group sequences that matched the same anopheline species reference sequence were collapsed into one group. This was reflected in the lower CO1 sequence similarity within a final species group when compared with that within the same species ITS2 sequence. To validate the proper assembly of these sequences, contigs were manually inspected for SNPs and repeat regions that would inflate divergence and decrease identity scores. These sequences are available in GenBank with accession numbers KJ522813–KJ544843.

Comparison of ITS2 and CO1 sequence groups. The final description of 17 ITS2 sequence groups from the entire set of mosquitoes was validated by their 1:1 association with 15 separate CO1 sequence groups, except for two groups, *Anopheles* species O and Q, which matched the *An. coustani* CO1 sequence, but diverged at the ITS2 locus. This may be due to the presence of sibling species or introgression, which would need to be determined through further collections.

The molecular species identification was compared with morphological identification and many specimens could not be morphologically identified to terminal species in multiple keys. There was a low degree of concordance between molecular identification and field-based morphological identification of most groups (Table 1). For example, only half (51%) of the specimens morphologically identified as *An. funestus* s.l. belonged to that group when compared with well-characterized ITS2 reference sequences.^{9,55} Those not belonging to the *An. funestus* group fell into 12 other sequence groups. Results were similar for *An. gambiae* s.l. In most cases, the morphological identifications were mixed and not predictive of sequence groupings.

Preliminary morphological identification suggested that some of the specimens were not of the more commonly reported species in the area, and putatively identified two of the species groups—*Anopheles* species E was putatively

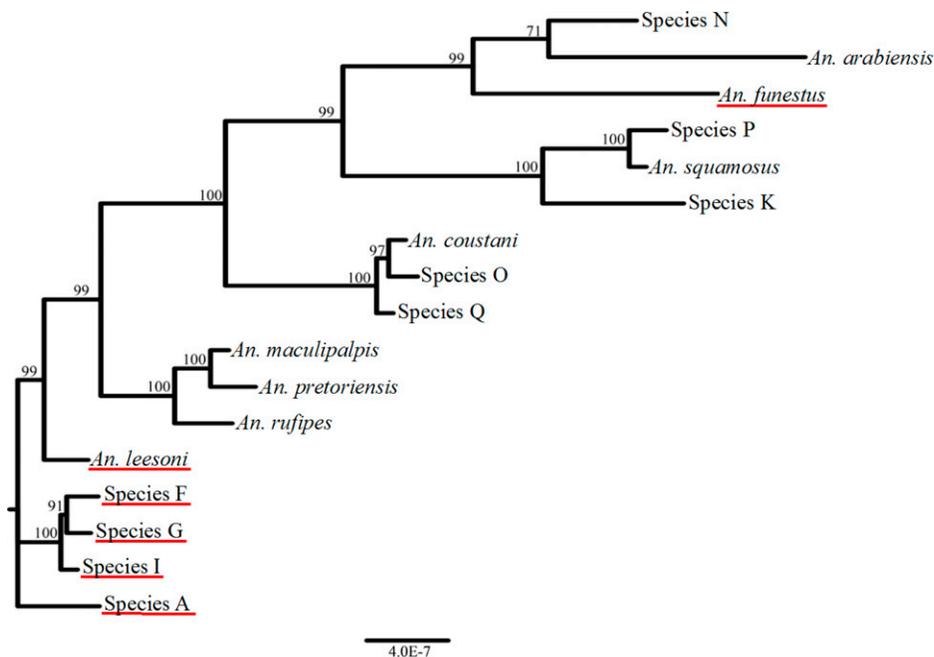


FIGURE 2. Majority rule consensus tree from Bayesian analysis of internal transcribed spacer region 2 (ITS2) sequences of anopheline specimens collected in the Kenyan highlands. Bayesian posterior probabilities are shown above branches. The ITS2 sequence groups are named by their formal species name or by unknown Species A–Q. Red underscoring indicates groups that were found positive for *Plasmodium* infection. Note that *Plasmodium* positive unknown species A, F, G, and I group together.

identified as *An. maculipalpis* and *Anopheles* species I as possibly *An. harperi*. *Anopheles* species A, which was the second most abundant species in the collections, shared morphological features with members of the *An. demeilloni* group, for which there are no published ITS2 or CO1 sequences. The inconclusive nature of the morphological observations was primarily due to limitations in using available morphological keys in identifying some species, though several field specimens were also damaged. High-quality specimens representing the unknown groups could not be identified when compared with museum voucher specimens or identified to species using currently available keys or species descriptions. Only adult females were compared with morphological keys and museum voucher specimens.

Some molecular species identifications that were supported by morphological identifications and could be paired with high sequence identity to non-voucher sequences in the database were factored into the final species identification presented in Table 1. For example, *Anopheles* species M and E had morphological features supporting their ID to one species and had CO1 and ITS2 sequences matching with a high similarity to published sequences, it was concluded these were *An. maculipalpis* and *An. squamosus*, respectively. Since many of the sequences in NCBI and BOLD are incorrectly morphologically identified, species with matches in the databases to only non-voucher specimens, and not confirmed by any other method, could not be resolved to species in our final identification (Table 1). This is particularly true for sequence matches only within the BOLD database, such as *Anopheles* species O, P, and Q when the sequence alignment and source of the match could not be confirmed. The lack of species resolution of some sequence groups is based on the absence of any similar ITS2 or CO1 datasets.³⁸

Several species were confirmed using both molecular and morphological tools, such as *Anopheles* species L, which morphologically matched with the *An. funestus* group, had high ITS2 sequence homology to a non-voucher sequence, and several specimens were tested using a species-diagnostic PCR for members of that group,^{9,24,56} indicating that the specimens were *An. lesoni*. Similarly, *An. maculipalpis*, *An. squamosus*, and *An. coustani* were confirmed through multiple indicators for their identification.

Sporozoite analysis. Of 1,601 mosquitoes tested for the presence of *P. falciparum* sporozoites using CS ELISA, 28 were found positive. Eleven belonged to the *Anopheles* species A sequence group, 10 to *An. funestus*, two to *Anopheles* species F, one to *Anopheles* species G, and four to *Anopheles* species I (Table 1). All 1,601 specimens tested by ELISA, including those found positive, were tested by PCR. In addition, a further 193 specimens of those species shown to be positive for sporozoite by ELISA, that is, specimens with sequences matching *Anopheles* species A, *An. funestus*, *Anopheles* species F, *Anopheles* species G, and *Anopheles* species I with positive CS ELISA from the same area were confirmed to be positive for *Plasmodium* infection by PCR.⁴⁴ A total of five specimens of *Anopheles* species A, 16 of *An. funestus*, two of *Anopheles* species F, and one of *Anopheles* species G were positive for *P. falciparum* DNA by PCR (Table 1). These PCR positives were confirmed by sequencing. In addition, one *Anopheles* species A, seven *An. funestus*, one *Anopheles* species I, and one *An. lesoni* were positive for *Plasmodium* DNA but

could not be confirmed to be *Plasmodium* species in a secondary species-specific PCR.⁴⁴ *Anopheles* species A, F, G, and I were species that could not be definitively identified using morphology or molecular tools to known species. No mosquitoes of the third most abundant sequence group, *An. arabiensis*, a presumed primary vector in the area, were found to be positive for *Plasmodium* infections in these collections.

Blood meal analysis. Of 42 blood-engorged mosquitoes, 28 analyzed from the light trap collections were identified to specific blood meal hosts by PCR. Specimens that fell into the *Anopheles* species A group were found to contain human blood mixed with cow blood in two blood meals, as well as eight blood meals from cow, one from a dog, and one from a donkey. One specimen of *An. pretoriensis* had a mixed human and cow blood meal. Of four blood-fed *Anopheles* species I, one had a human blood meal and three had cow blood meals. These blood meal identifications confirm only that these species had fed on human and other blood hosts. *An. maculipalpis*, *An.* species F, and *Anopheles* species G had two, three, and three cow blood meals, respectively. No blood meals were found in females of the known vector species *An. arabiensis* and *An. funestus* available for analysis.

DISCUSSION

Downstream associations of bionomic characteristics, vectorial capacity, and entomological inoculation rates based solely on morphologically identified mosquitoes may differ substantially from those obtained from the more precise types of molecular identifications presented here. Molecular analysis of divergence at both rDNA ITS2 and mtDNA CO1 loci of over 2,300 mosquitoes collected over an 18-month period in three villages in the Kenyan highlands of Nyanza Province indicated the presence of *P. falciparum* sporozoite-infected mosquitoes that are not members of the commonly known vector species in Kenya. More than half of the specimens, 63%, were assigned to known vector taxa and the remaining 37% fell into groups that could not be conclusively identified using morphology or multiple molecular markers. The latter contained most of the sporozoite-positive specimens and females that had fed on humans. Of those specimens for which there are no published ITS2 and CO1 genetic sequences, CS ELISA positive specimens of *Anopheles* species A, F, and G were confirmed positive for parasite DNA by PCR and sequencing. The specimens of *Anopheles* species I that were ELISA positive for *P. falciparum* sporozoites could not be confirmed with PCR.⁵⁷

The sequences were assembled into 17 (ITS2) and 15 (CO1) groups. These sequence groups had a 1:1 correspondence, that is, all specimens in an ITS2 sequence group had a matching set of CO1 sequences, except for one group (*Anopheles* species O) that had a different ITS2 sequences but a matching CO1 sequence. A large number of sequences were not present in the NCBI database. This result alone does not necessarily represent a novel (i.e., unnamed) species, but rather that the ITS2 or CO1 sequences may belong to formally described species which have not been sequenced previously.

The comparison of specimens within the sequence groups to voucher sequences in public databases, to each other, and

detailed morphological criteria has enabled the identification of reference sequences for several Kenyan species: ITS2 for *An. squamosus* and *An. christyi*, and CO1 for *An. rufipes*, *An. pretoriensis*, and *An. maculipalpis*. Several specimens that shared some features, but not all, with those of species of the *An. gambiae* complex were later shown to be *An. arabiensis* because of high sequence similarity (100%) to published sequences. A match of a single non-voucher sequence to either ITS2 or CO1 was not considered in the final species identification because many unpublished or non-voucher sequences that are deposited in sequence databases are misidentified. This issue underlies the need for sequences of voucher specimens of both vector and nonvector species to be represented in the database.

Anopheles species A, F, G, and I are described here as species that have not previously been identified as malaria vectors in the study area, and which may or may not be species of *Anopheles* new to science. These four *Plasmodium* positive species also cluster together in the ITS2 phenogram, indicating that they could be closely related or sibling species (Figure 2). Specimens with ITS2 sequences matching those of *Anopheles* species A have been found in multiple sites in Zambia (N. Lobo, unpublished data), indicating that this species may be distributed more widely in Africa and could be playing a role in malaria transmission elsewhere. This study represents only an important first step in identifying any potentially new species. Detailed descriptions of adult female mosquitoes need to be further validated with careful inspection of a greater number of high-quality adult female and male specimens and other life stages. The specific behaviors of *Anopheles* species A, one of the most abundant species in our study sites with a *P. falciparum* infection rate comparable to that previously published for *An. funestus* in western Kenya,^{58,59} are currently being characterized.

This study emphasizes the importance of combining molecular tools with morphological identifications, particularly in areas with diverse species. Available adult female anopheline morphological keys and species descriptions may be suitable for identifying common and well-known species but do not include more recently described species, some life stages or sexes are missing from keys, descriptions can be incomplete and open to misinterpretation, and captured mosquitoes may be damaged and missing key morphological features. Members of the *An. gambiae* and *An. funestus* complexes are the most studied mosquitoes in Africa. Studies that focus on primarily identifying these mosquito species, often based on the assumptions that they are the most common anophelines or the only vectors, might result in misidentification or discarding of unexpected or unknown vectors. Even within these well-studied species complexes, new species are being discovered using molecular techniques, such as a new species in the *An. funestus* complex that was found indoors and could not be identified using the common species diagnostic PCR.⁵⁵ *Anopheles* species A, for example, thought by the authors to belong to the *An. demeilloni* group, might be mistaken for *An. funestus*. An initial or periodic molecular characterization of species composition, using sequencing or available PCR diagnostics, could be used to compliment morphological approaches for more cost-effective monitoring of vector species. This would also enable the identification of field specimens that are often too damaged to identify mor-

phologically and enable quick processing of large numbers of specimens.

The presence of new or unknown vectors with uncharacterized behaviors will impact the efficiency of interventions and hints at the complex nature of the malaria transmission paradigm. The two most common interventions, indoor residual spraying and use of insecticide-treated bednets, target indoor behaviors exhibited by members of the *An. gambiae* and *An. funestus* complexes that may not be characteristic of other species involved in malaria transmission. Detailed descriptions of vector species, their behaviors, vectorial capacity, insecticide resistance, and other characteristics relevant to transmission are vital to our understanding of local transmission dynamics and for the deployment of effective interventions.⁶⁰

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