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Article

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Isaiah Debrah [1,2,*,†], Kevin O. Ochwedo [2], Wilfred O. Otambo [2], Maxwell G. Machani [3], Edwin O. Magomere [2], Shirley A. Onyango [2], Daibin Zhong [4,*,†], Linda E. Amoah [5], Andrew K. Githeko [3], Yaw A. Afrane [6] and Guiyun Yan [4]

1 West Africa Centre for Cell Biology of Infectious Pathogen (WACCBIP), University of Ghana, Accra, Ghana
2 Sub-Saharan Africa International Centre of Excellence for Malaria Research, Homabay, Kenya; kevinochwedo@gmail.com (K.O.O.); oumaotambo@gmail.com (W.O.O.); emagomerre@gmail.com (E.O.M.); shirleyonyango@gmail.com (S.A.O.)
3 Centre for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya; machani.maxwe2011@gmail.com (M.G.M.); githeko@yahoo.com (A.K.G.)
4 Program in Public Health, College of Health Sciences, University of California, Irvine, CA, United States of America; guiyuny@uci.edu
5 Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana; lek.amoah@gmail.com
6 Department of Medical Microbiology, University of Ghana Medical School, College of Health Sciences, University of Ghana, Accra, Ghana; yaw_afrane@yahoo.com
* Correspondence: isaiah20009@gmail.com (I.D.); dzhong@hs.uci.edu (D.Z.)
† These authors contributed equally to this work.

Simple Summary: *An. funestus* is a major vector of human malaria responsible for high transmission in sub-Saharan Africa. In the malaria endemic region of western Kenya, it has adapted and colonized different ecological niches owing to its high resistance and changing breeding environments. The genetic basis of its ecological adaptations to various settings which could enrich our understanding of how the population is structured or segregated is poorly understood. This study sought to evaluate the population structure and genetic diversity of *Anopheles funestus* in different landscapes in western Kenya. To achieve this, the cytochrome oxidase subunits II gene (mtDNA-COII) was amplified and sequenced using Sanger sequencing. This study revealed population expansion with an excess of low-frequency variation due to evolutionary forces. This could serve as a guide for future genomic research involving this important vector to help design control strategies.

Abstract: The mitochondrial marker, cytochrome oxidase subunits II gene (mtDNA-COII) was employed to assess the genetic structure and diversity of *Anopheles funestus*, a very important malaria vector in Africa, that adapt and colonize different ecological niches in western Kenya. Mosquitoes were collected using mechanical aspirators in four sites (Bungoma, Port Victoria, Kombewa and Migori) in western Kenya. Following morphological identification, PCR was used to confirm species. The mtDNA-COII gene was amplified, sequenced and analyzed to determine genetic diversity and population structure. A total of 126 (Port Victoria-38, Migori-38, Bungoma-22 and Kombewa-28) sequences of mtDNA-COII were used for population genetic analysis. *Anopheles funestus* had high haplotype diversity (Hd= 0.97 to 0.98) but low nucleotide diversity (π =0.004 to 0.005). The neutrality test revealed significant ($p<0.05$) negative Tajima's *D* and Fs values indicating population expansion with an excess of low-frequency variation due to evolutionary forces across all sites. No genetic differentiation or structure (Fst = -0.01) and a high level of gene flow (Gamma St, Nm = 17.99 to 35.22) were observed among the populations across the sites. Population expansion suggests the high adaptability of this species to various ecological requirements hence sustaining its vectorial capacity and malaria transmission.

Keywords: *Anopheles funestus*; western Kenya; mtDNA-COII; genetic diversity; gene flow

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Introduction

Malaria is a public health problem in Sub-Saharan Africa, spread mainly by the members of the *Anopheles funestus* group and *the Anopheles gambiae* species complex [1]. *An. funestus* group comprises five subgroups of which three of these subgroups contain at least thirteen (13) species; identified in various ecological niches across Africa [2]. *Anopheles funestus sensu stricto* (s.s.) (hereafter *An. funestus*) belongs to the *Funestus* subgroup which has seven members, namely: *An. funestus, An. aruni, An. vaneedeni, An. funestus-like, An. confuses, An. longipalpis type C* and *An. parensis* [2,3]. Of this group, *An. funestus* is a major vector responsible for high malaria transmission in sub-Saharan Africa. Three of the members of the *An. funestus* group; *An. funestus, An. rivulorum and An. leesoni* were found sympatrically in various ecological zones in Kenya, [4] Sudan [5] and Nigeria [6,7] suggesting that they have effective reproductive isolation mechanisms.

While most of the species of the *Anopheles funestus* group can be found only in certain geographical areas in Africa, *An. funestus* has a wide range of geographical distribution covering several various climatic types and is the most devastating efficient human malaria vector exhibiting consistent notorious anthropophilic (prefer human habitation), anthropophagic (biting humans), endophilic (indoor resting) and endophagic (indoor biting) behaviours [8,9]. Its capacity to transmit human malaria far outpaced *Anopheles gambiae* and *Anopheles arabiensis* in some endemic areas in Africa [8,10].

An. funestus can adapt and colonize different ecological niches owing to its high resistance and changing breeding environments. A previous study in Kenya revealed that *An. funestus* breeds in various habitats and co-breeds with *An. gambiae sensu.lato*, *Culex spp* and other vectors in the same habitats [11]. The vector survival rate, behaviour, ecology, vectorial capacity, and host-pathogen interactions are all affected by external environmental stress. This external stress such as temperature change, land-use changes, host migration, and insecticide use, plays a crucial role in mosquito population selection [12]. Based on microsatellite markers, Ogola et al [13] noted three genetically different clusters (FUN1, FUN2 and FUN3) of *An. funestus* in Kenya. The largest cluster (FUN1) is from samples from Rift Valley Regions and western regions and the other clusters (FUN2 and FUN3) are from the coastal region.

Mitochondrial DNA (mtDNA) sequences are genetic drift-sensitive and have high copy numbers, availability of conserved primer binding sequences, and ease of amplification hence they are widely employed in interpreting molecular taxonomy, phylogenetic relationships, population structure, and genetic diversity in malaria vectors [14]. Indeed, mtDNA markers have been utilized to study the genetic variances and evolutionary relationships of many mosquito species, as well as to correctly quantify gene flow and changes between populations [15,16]. Cytochrome oxidase subunits II gene (mtDNA-COII) is the mitochondrial marker that has unique properties of maternal inheritance are devoid of recombination, intraspecific polymorphism, highly variable compared to nuclear DNA and small effective population size, therefore they are good markers for studying genetic diversity, gene flow and population structure [17]. Mitochondrial genetic diversity and molecular phylogeny are becoming increasingly important in mosquito research [18].

The population structure and genetic diversity of *An. funestus* might influence its adaptation and efficiency of malaria transmission in western Kenya. Delineating the fine-scale population structure of vectors might be useful to investigate the genetic basis of speciation and local adaptation processes. Moreover, understanding gene flow among *An. funestus* populations could help to assess their movement in natural populations and therefore, how the populations are segregated. These could be of great importance in vector control strategies. This study was designed to investigate the genetic structure and diversity and gene flow of a major vector, *An. funestus* in a malaria-endemic region of western Kenya using mitochondrial marker mtDNA-COII.

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Materials and Methods

Mosquito Sampling

Anopheles funestus mosquitoes were collected from four malaria transmission areas (Port Victoria, Migori, Bungoma and Kombewa,) in four counties in western Kenya. Figure 1 shows the locations in the various counties where samples were collected. Adult mosquitoes were sampled inside indoor living rooms using pyrethrum spray catches and mechanical aspirators. All mosquitoes were morphologically identified as *An. funestus sensu lato* following morphological keys [19]. Each sample was stored in a 1.5 ml Eppendorf tube containing cotton wool and silica gel and was stored at -4^{o}C for subsequent molecular identification to confirm the species.

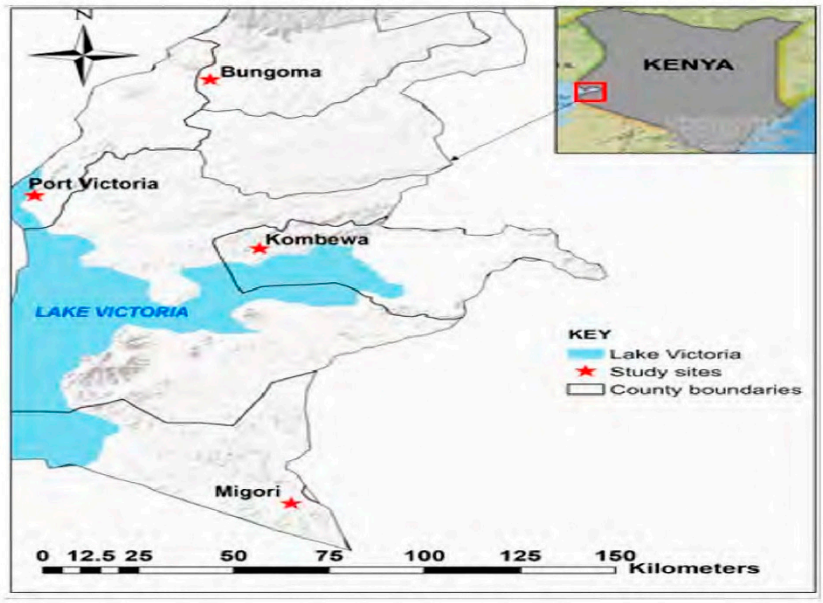

Figure 1. Map of study areas in western Kenya. This map was prepared with ESRI ArcGIS Pro 2.8 using field survey results and publicly available datasets. The Open Database License, which is used to make the data available, was used to compile the material on the map from OpenStreetMap and the OpenStreetMap 115 Foundation.

DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from the whole mosquito using the Chelex®-100 method [20]. Funestus-specific PCR was conducted to confirm species using the species-specific primers (ITS2A/FUN) in the internal transcribed spacer region (ITS2) on the ribosomal DNA as described by [21]. The mtDNA-COII gene was amplified using forward (5'-TCT AAT ATG GCA GAT TAG TGC A-3') and reverse (5'-ACT TGC TTT CAG TCA TCT AAT G-3') primers. A 23µl PCR mixture containing 3 µl genomic DNA, 0.5 µl each of forward and reverse primers, 11.5 µl master mix with PerfeCTa® ToughMix® (5x) and 7.5 of PCR water. The PCR conditions include initial denaturation at 95^{0}C for 3 minutes, denaturation of 95^{0}C for 15 seconds, annealing at 41^{0}C for 30 seconds, extension at 72^{0}C for 1 minute and 30 seconds for 35 cycles and final extension at 72^{0}C for 7 minutes. The amplicons' quality was assessed using agarose gel electrophoresis in 1.5% w/v agarose gel stained with 2 µl smart glow and visualized using the SmartDoc imaging system (Accuris ™ instruments). All the amplicons were bidirectionally sequenced with the same primers used for the PCR using 3730 BigDye® Terminator v3.1 Sequencing Standard kit on ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

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Data Analysis

De novo assembly of paired raw reads was performed using Geneious prime software version 2022.0.1 [22] and CLC Genomics Workbench [23]. Low-quality reads with low base calling accuracy below 99% (Phred 20) were excluded from the analysis. ClustalW algorithm, a platform in BioEdit version 7.2.5 [24], was used in generating multiple sequence alignment. DnaSP Version 6.12.03 [25] was used to compute genetic diversity indices [haplotype diversity (Hd), number of haplotypes (h), nucleotide diversity (π), number of segregating sites (S) and mean number of pairwise difference (k)] and Neutrality tests (Tajima's *D*, Fu and Li's D, Fu and Li's F, and Fu's Fs statistics). The analysis of molecular variance (AMOVA) was performed in Arlequin version 3.5.2 [26] to partition genetic variations among groups (Port Victoria, Migori, Bungoma and Kombewa,) and within groups. Population Analysis with Reticulate Trees (Popart) version 1.7 [27] was used to infer haplotype networks.

The best-fitting nucleotide substitution model was estimated with the Akaike Information Criterion (AIC) and the Bayesian information criterion (BIC) implemented in MEGA version 11.0.13 [28]. The Bayesian phylogenetic analysis was performed by MrBayes v3.2.7 [29] using Markov chain Monte Carlo (MCMC) methods. The MCMC was run for 2,000,000 generations with sampling tree topologies every 1,000 generations, after excluding the initial 25% as 'burn-in'. To root the tree, *Anopheles gambiae*, *Aedes albopictus*, and *Culex pipiens pallens* were the "outgroup" (accession # MG930872, MG930866, KX383916, and KT851543). The previously reported mtDNA-COII sequences of *An. funestus* from Kenya and other African countries were also included in the phylogenetic tree. The 50% majority rule consensus tree was constructed with Bayesian posterior probabilities of the nodal supports. The output tree was visualized and edited with an online tool Interactive Tree of Life (iTOL) v5 [30].

Results

Genetic Diversity of An. funestus in Western Kenya

A total of 126 (Port Victoria-38, Migori-38, Bungoma-22, and Kombewa-28) amplicon sequences of mtDNA-COII were used for population genetic analysis. A total of 64 haplotypes were identified, suggesting that there is high haplotype diversity in the populations (Hd= 0.97 to 0.98) but low nucleotide diversity (Π=0.004) based on mtDNA-COII sequences (Table 1). Moreover, the statistical test of neutrality revealed significant ($p<0.05$) negative Tajima's *D* and Fs values indicating a deviation from a standard neutral model and population expansion with an excess of low-frequency alleles due to evolutionary forces (Table 1). There was no significant difference in observed mutations across the four populations ($X2$=181.744, df 189, P=0.635).

Table 1. Nucleotide diversity indices based on mtDNA-COII of *An. funestus* from four sites in western Kenya.

Sites	N	Mutated Sites	Π ($\times10^{-2}$)	h	Hd	Tajima's		Fu's		Fu and Li's		Fu and Li's	
						D	P	Statistics	P	*D*	P	*F*	P
Port Vic.	38	32	0.005	30	0.98	-1.86	< 0.05	-31.51	0.000	-1.99	> 0.10	-2.31	> 0.05
Migori	38	26	0.004	25	0.97	-1.76	> 0.05	-21.85	0.000	-2.75	< 0.05	-2.86	< 0.05
Bungoma	22	19	0.004	18	0.98	-1.64	> 0.05	-16.42	0.000	-1.89	> 0.10	-2.12	> 0.05
Kombewa	28	17	0.004	23	0.98	-1.33	> 0.10	-25.04	0.000	-1.06	> 0.10	-1.34	> 0.10
All sites	126	41	0.004	64	0.97	-1.81	< 0.05	-32.91	0.000	-1.42	> 0.10	-1.89	> 0.05

N: Sampled population, Π: Nucleotide diversity, h: Number of Haplotypes, Hd: aplotype diversity, Statistical significance (P<0.05).

Population Structure and Gene Flow

AMOVA results showed that there was no genetic differentiation across all four populations (Fst = -0.01). Specifically, our result revealed that there was no genetic differentiation between Port

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Victoria and Migori (Fst= -0.010), Bungoma and Kombewa (Fst= -0.016), Port Victoria and Bungoma (Fst = -0.020), Migori and Bungoma (Fst= -0.008), Port Victoria and Kombewa (Fst = -0.009), Migori and Kombewa (Fst= -0.003) (Table 2). The lack of population structure was supported by a high level of gene flow across the four populations (Gamma St, Nm=15.40). The highest gene flow was between Port Victoria and Bungoma (Gamma St. Nm= 35.22). This was followed by Port Victoria and Migori (Gamma St, Nm= 30.25). The lowest gene flow was between Migori and Kombewa (Gamma St. Nm=17.99) (Table 2).

Table 2. *Anopheles funestus* population structure in western Kenya region.

Populations	No. of Shared Mutations (%)	Dxy	Hs	Ks	Kxy	Gst	Gamma St		Fst		
							Value	Nm	Value	Nm	*P*-Value
Port Vic. *vs* Migori	20/40 (50)	0.004	0.97	3.400	3.365	0.001	0.008	30.25	-0.010	-24.45	0.855
Bungoma *vs* Kombewa	12/25 (48)	0.004	0.98	2.878	2.836	-0.006	0.013	19.43	-0.016	-16.04	0.892
Port Vic *vs* Bungoma	18/34 (52.9)	0.004	0.98	3.389	3.222	-0.004	0.007	35.22	-0.020	-12.80	0.982
Migori *vs* Bungoma	14/32 (43.8)	0.004	0.97	3.048	2.994	0.001	0.013	19.02	-0.008	-31.71	0.649
Port Vic *vs* Kombewa	15/36 (41.7)	0.004	0.98	3.324	3.234	-0.003	0.011	23.43	-0.009	-28.29	0.838
Migori *vs* Kombewa	15/30 (50)	0.004	0.97	3.015	2.985	0.0003	0.014	17.99	-0.003	-82.77	0.468

Dxy: Average number of nucleotide substitution per site between populations, Ks: the number of synonymous substitutions per synonymous site, Kxy: Average number of nucleotide differences between populations, Nm: Number of migrants, Gst: a measure of population differentiation, Hs: mean diversity within each population.

Phylogenetic Relationships and Network Analyses

The best-fit model for nucleotide substitution was identified by MEGA 11 as a Tamura 3-parameter with gamma-distributed rate heterogeneity (T92 + G) according to the Bayesian information criterion for mtDNA-COII haplotypes. The phylogenetic tree was inferred by Bayesian analyses with the standard deviation of split frequency values (<0.01). The phylogenetic estimates from the MCMC analyses strongly supported the monophyletic group (posterior probability = 1) (Figure 2). There is no clear clustering of haplotypes observed and all the 64 identified haplotypes in western Kenya shared a common ancestor with *An. funestus-like* (accession # MT917161) from Malawi, with the closest haplotype (Hap 40) coming from the Migori and Port Victoria study sites, which border Tanzania and Uganda, respectively. *Anopheles funestus* samples from Port Victoria and Bungoma (Hap 2) shared a recent common descendant with *An. funestus* (accession # MT917175) from Uganda.

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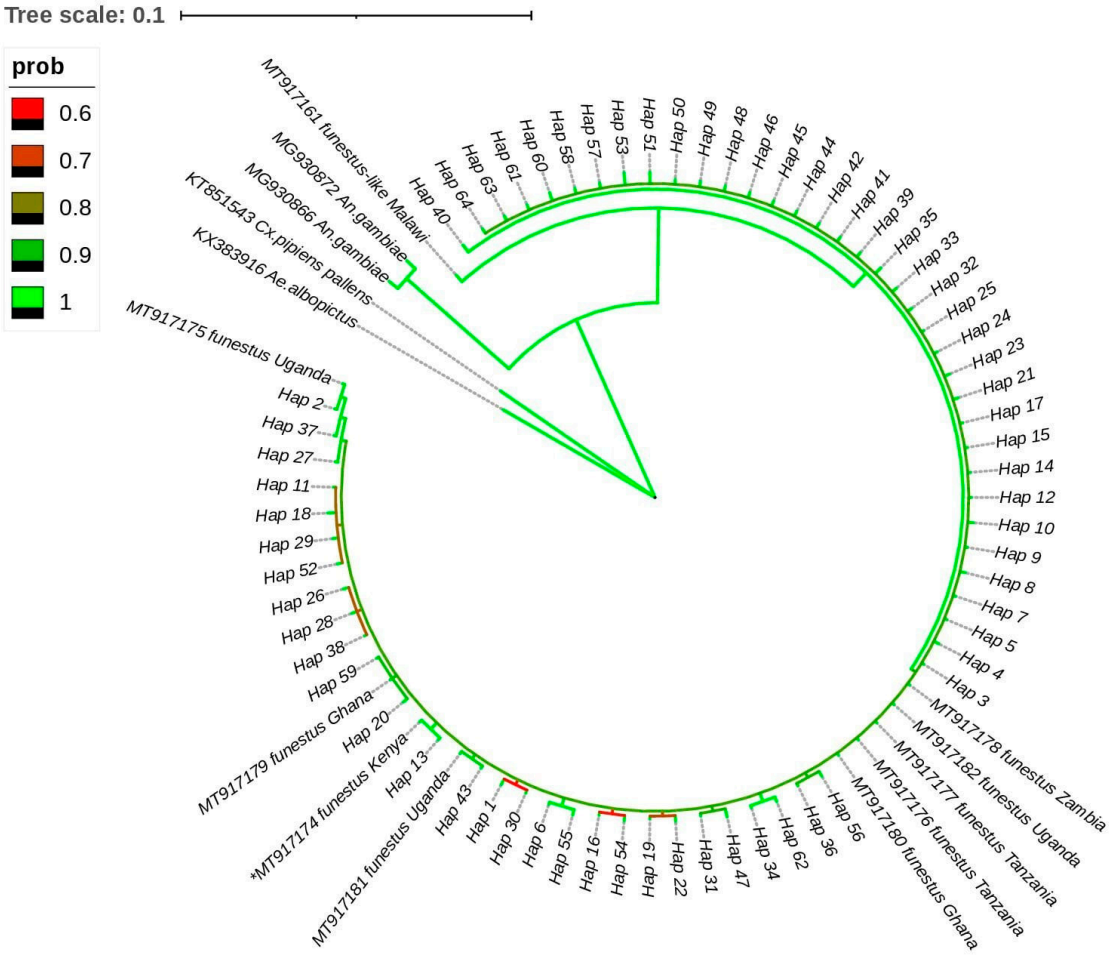

Figure 2. Bayesian phylogenetic tree inferred from cytochrome oxidase subunit II gene (COII). The 50% majority rule consensus tree was constructed using Markov chain Monte Carlo (MCMC) methods and posterior probabilities of the nodal supports are indicated by colour lines where the green corresponds to 100% support value).

Haplotype networks were constructed using the Median-Joining method in PopART software. Out of the 64 distinct haplotypes identified in western Kenya, 30 (46.9%) were found from the Port Victoria study site (Table 1). Twenty (31.3%) of the 64 haplotypes were shared across the study sites. The median-joining haplotype network of the mtDNA-COII gene revealed the genealogy of each of the observed haplotypes with haplotypes S1-S20 being shared across the study sites (Figure 3). In the four study sites, the most common haplotype was S1 (14/64, 21.9%), followed by S3 (7/64, 10.9%), S2 (7/64, 10.9%), S4 (6/64, 9.4%), S6 (6/64, 9.4%), and S8 (4/64, 6.3%). The distribution of the S1 haplotype among the 64 observed haplotypes was as follows: 8%, 6%, 5%, and 3% in Port Victoria, Migori, Bungoma, and Kombewa, respectively. The haplotype (S1) could be an ancestral haplotype (recent common ancestor) among *An. funestus* in western Kenya. Port Victoria and Bungoma populations had the most shared mutations (52.9%) followed by Migori and Port Victoria as well as with Kombewa each at 50%. Port Victoria and Kombewa populations had the least shared number of mutated sites (42%). Nucleotide sequences of the 64 identified haplotypes were submitted to Gene Bank and assigned accession numbers ON931353-ON931416.

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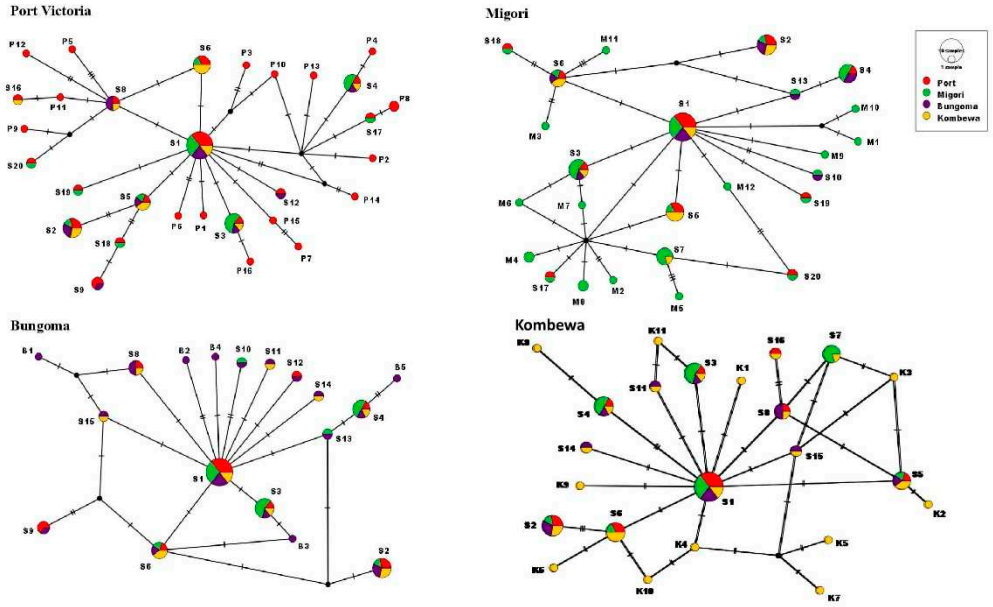

Figure 3. Haplotype distribution in the four study sites. S represents shared haplotypes across the four study sites, P haplotypes in Port Victoria only, M haplotypes in Migori, K in Kombewa and B for Bungoma. Hatch marks represent the number of mutated sites resulting in particular haplotypes whereas the size of the circle corresponds to haplotype frequency or numbers of the sample under that specific haplotype.

Discussion

This study revealed the population genetic structure of *An. funestus* population across four counties in western Kenya using a mitochondrial marker, mtDNA-COII. In the context of malaria control strategies including genetic alteration of vector species, information on genetic diversity and population structure of vectors is critical [31]. In recent years, changes in the ecology of vector populations have been documented [11]. High tolerance to a variety of ecological niches, insecticide resistance, and vast geographic distribution make *An. funestus* a highly adaptable dominant vector species [32].

This study revealed the signature of the population expansion, with weak population structure and high levels of gene flow among the population of *An. funestus* from areas with varied *P. falciparum* transmission intensities in western Kenya. Generally, *An. funestus* exhibited low nucleotide diversity with Port Victoria exhibiting a high level of genetic diversity as compared to other regions. In western Kenya, the high genetic diversity of *An. funestus* population compared to coastal regions based on microsatellite markers was reported a decade ago [33]. The observed high number of haplotypes in Kombewa, Migori, and Port Victoria among this primary vector is consistent with high levels of *Plasmodial* transmission in these study sites as compared to the rest of other malaria vectors [34,35]. Port Victoria which is also proximal to Lake Victoria had a significantly highest number of haplotypes circulating in the area. Port Victoria is the main Lake Victoria transport corridor between Kenya, the Islands, and Uganda. It was earlier reported that small wooden boats played a significant role in the transportation of mosquitoes between the mainland and the Islands [36]. This might have resulted in high gene flow hence low nucleotide and high haplotype diversity in that site. Port Victoria has also been documented to have a high malaria prevalence with vector control interventions ongoing [37]. The high gene flow observed in the populations could be due to migration and a lack of geographical barriers [15]. Mountain ranges, rivers, forests, and other physical barriers, in combination with climatic or biological obstacles like flight range and breeding grounds, may obstruct gene flow between *Anopheles* populations. However, none of these factors served as a barrier to gene flow in the *An. funestus* population in western Kenya. The Rift Valley is known to serve as a barrier to gene flow

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[4] nonetheless our findings showed that there are no physical barriers to hamper gene flow among mosquito populations in western Kenya. Different breeding sites, mosquito migration, environmental changes, and human activities act to shape the genetic diversity of *An. funestus* populations [38].

Given the low Fst values and high Gamma St Nm values, there is a strong indication of a high level of inbreeding across the population. As a result, most of the haplotypes in this study are shared among the western Kenya region contributing to a weak or lack of population structure. However, with the excess frequency of rare alleles, a genetic signature for population expansion can persist for a long period, masking any genuine ecological population or genetic structure that may exist [39]. The observed no genetic differentiation, negative selection, and population expansion suggest that there was a free exchange of genes among *An. funestus* populations in western Kenya. The presence of a negative signature of selection on this gene is an indicator of purifying selection acting on the gene to preserve the genetic structure by eliminating deleterious mutations [40].

With evidence of purifying selection and population expansion, it is possible evolutionary forces shaping *An. funestus* population in western Kenya could be the usage of LLINs, IRS and insecticide use for agricultural activities, especially those affecting larval breeding sites [41,42]. The high number of haplotypes per study site, new species and the population expansion observed in this study suggest the high adaptability of *An. funestus* to various ecological requirements hence sustaining its vectorial capacity and malaria transmission.

Molecular identification confirmed that *An. funestus* is the main vector of the *An. funestus* group in western Kenya which is consistent with previous findings [11,43]. The other members of the *An. funestus* group identified include *Anopheles funestus-like, Anopheles longipalpis* and *Anopheles parensis* in Kenya. *Anopheles funestus-like* was first discovered in Malawi, Southern Africa in 2009 [44]. *Anopheles funestus-like* and *An. funestus* are members of the *Funestus* subgroup which exist sympatrically [44,45]. Experiments have shown that they are separate species and no evidence of hybridization was observed between them [44]. The role of *Anopheles funestus-like* in malaria transmission is unknown. Most of the sibling species of *An. funestus* group's role in malaria transmission is not fully understood although speculations are rife that they may be playing a role as secondary vectors. The recent identification of new species suggests that mosquitoes are invading new ecological niches and may be involved in malaria transmission. It is critical to correctly identify sibling species to design successful vector management measures. Failure to detect sibling species, for example, can make it difficult to tell the difference between a vector and a non-vector. When two or more sibling species are not distinguished, assessing the impact of control measures can be extremely imprecise. The discovery of sibling species gives vector and vector-borne control a whole new level. With the unravelling of new species and the presence of selection pressure, continuous entomological surveillance is needed to ascertain the distribution and role of new species in the malaria ecosystem and their response to various vector control tools.

Conclusions

This study had shown that the *An. funestus* population in western Kenya is under selection pressure leading to demographic expansion and the spread of variants through inbreeding among varied transmission sites in western Kenya. Population expansion suggests there is high adaptability of this species to various ecological requirements hence sustaining its vectorial capacity and malaria transmission.

Author Contributions: Conceptualization, I.D. and Y.A.A.; methodology, I.D. and S.A.O; software, I.D., K.O.O. and D.Z.; validation, I.D. and D.Z.; formal analysis, I.D., K.O.O. and D.Z.; investigation, I.D., M.G.M., K.O.O. and E.O.M.; resources, G.Y.; data curation, I.D. and D.Z.; writing—original draft preparation, I.D.; writing—review and editing, I.D., K.O.O., W.O.O., S.A.O., D.Z. and L.E.A.; visualization, I.D.; supervision, A.K.G., Y.A.A., L.E.A. and G.Y.; project administration, I.D. and A.K.G.; funding acquisition, G.Y. and Y.A.A. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Verbal consent was sought from heads of households before mosquitoes were collected inside the living rooms.

Data Availability Statement: This study's datasets are available in online repositories. The accession numbers for the haplotype sequence data submitted to the gene bank are ON931353-ON931416.

Conflicts of Interest: The authors have no conflict of interest to disclose.

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