Genomic insights into adaptive divergence and speciation between malaria vectors of the *Anopheles nili* group

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10 **Abstract**

11 Ongoing speciation in most African malaria vectors gives rise to cryptic species that differ
12 remarkably in their behaviour, ecology and capacity to vector malaria parasites. Any vector
13 control measure can be undermined if cryptic populations exist and are mistargeted within
14 vector species. To examine population structure and the potential impacts of recent large-
15 scale control programs, we have investigated genomic patterns of differentiation in
16 mosquitoes belonging to a large taxonomic group that diverged ~3-Myr ago. Using 4343
17 single nucleotide polymorphisms (SNPs), we detected strong population structure
18 characterized by high F_{ST} values between multiple divergent populations. Delineating
19 cryptic species within the *Anopheles nili* group is challenging due to incongruence between
20 morphology, ribosomal DNA and SNP markers consistent with incomplete lineage sorting
21 and/or gene flow. Throughout the genome, a very high proportion of loci are fixed (F_{ST} = 1)
22 within putative species, which suggests that ecological and/or reproductive barriers are
23 maintained by strong selection on a substantial number of genes.

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25 **Key words:** *Anopheles nili*, divergent selection, high- *F*ST regions, speciation.

1. Introduction

One of the principal goals of population genetics is to summarize genetic similarities and differences between populations (Wright 1984). This task can be relatively straightforward for some taxa, but the genetic relationship among populations can also be difficult to summarize, especially for species whose evolutionary history is complex and reticulate. Most *Anopheles* mosquitoes have very complex range-wide population structure due to cryptic speciation and ongoing gene flow, which complicate species and population delimitations (Krzywinski & Besansky 2003; Harbach 2013). For example, almost all major African malaria vectors belong to large taxonomic groups encompassing multiple incipient species relatively isolated reproductively and geographically from one another (Reviewed by Sinka *et al.* 2010; Antonio-nkondjio & Simard 2013; Dia *et al.* 2013; Lanzaro & Lee 2013). These characteristics make them promising model systems to study speciation and the processes which contribute to reproductive barriers (e.g. Turner *et al.* 2005; Lawniczak *et al.* 2010; Neafsey *et al.* 2010; Fontaine *et al.* 2015), but can also have far-reaching practical consequences.

When the cryptic genetic structure underlying variations in behavior, distribution and vectorial capacity within mosquito species is not taken into account, vector populations can be mistargeted and major efforts at malaria control undermined (e.g. Molineaux & Gramiccia 1980; Van Bortel *et al.* 2001). The genetic structure of *Anopheles* species is most often based on macrogeographic or regional subdivisions (e.g. Touré *et al.* 1998; Wondji *et al.* 2005; Antonio-Nkondjio *et al.* 2007; Slotman *et al.* 2007; Ayala *et al.* 2011; Ndo *et al.* 2013; Pinto *et al.* 2013; Caputo *et al.* 2014; Kamdem *et al.* 2017), but can also involve more subtle differentiation between larvae and adults, or between adult populations found in or

around human dwellings (e.g. Riehle *et al.* 2011). This type of microgeographic genetic structure can have potentially significant impacts on malaria epidemiology and insecticide resistance because the main vector control measures target exclusively indoor mosquito populations (WHO 2016).

The recent scaling up of insecticide-treated nets usage and indoor insecticide spraying to a lesser extent have led to a dramatic reduction of malaria morbidity across the continent, but have also inevitably increased insecticide resistance among vectors (Hemingway *et al.* 2016; Ranson & Lissenden 2016). Although a direct causal link is not established, populations from urban areas and regions where pesticides are intensely used are strongly differentiated genetically implying a certain correlation between insecticide exposure and local adaptation (Kamdem *et al.* 2017). It has also been shown that this increased usage of xenobiotics can drive selective sweeps across genomes of the two major vectors *An. gambiae* and *An. funestus* (Clarkson *et al.* 2014; Barnes *et al.* 2017; Kamdem *et al.* 2017). The success of current malaria control measures relies among others on accurate knowledge of population structure and the environmental drivers that contribute or have contributed to genetic differentiation between mosquito populations. Extensive sampling combined with recent genomic tools and analytical approaches have helped resolve fine-scale population structure and genomic targets of selection in *An. gambiae* and *An. funestus* (White *et al.* 2011; Reidenbach *et al.* 2012; O'Loughlin *et al.* 2014; Barnes *et al.* 2017; Fouet *et al.* 2017; Kamdem *et al.* 2017), but important taxa remain understudied.

The present work focused on a set of African malaria vectors representing a large taxonomic unit named "*An. nili* group". Species that compose this group are characterized by reticulate evolution and complex phylogenies that have been challenging to resolve

(Kengne *et al.* 2003; Awono-Ambene *et al.* 2004, 2006, Ndo *et al.* 2010, 2013; Peery *et al.* 2011; Sharakhova *et al.* 2013). To date, four species distinguishable by slight morphological variations are known within the group and occur in forested areas of Central and West Africa: *An. nili sensu stricto* (hereafter *An. nili*), *An. ovengensis*, *An. carnevalei*, and *An. somalicus*. Populations of *An. nili* and *An. ovengensis* are very anthropophilic and efficient vectors of *Plasmodium* in rural areas where malaria prevalence is particularly high (Antonio-Nkondjio *et al.* 2006).

To delineate genomic patterns of differentiation, we sampled mosquito populations throughout the range of species of the *An. nili* group in Cameroon and used reduced representation sequencing to develop genome-wide SNP markers that we genotyped in 145 individuals. We discovered new strongly differentiated subpopulations within *An. ovengensis* and *An. nili*. We analyzed the genetic differentiation at 4343 SNPs and revealed the presence of a very high number of outlier loci that are targets of selection. Further studies using a more complete reference genome will help answer important questions concerning the functional and phenotypic characteristics of differentiated loci as well as the contribution of recent selective events.

2. Materials and methods

(a) Mosquito species

We surveyed 28 locations within the geographic ranges of species of the *An. nili* group previously described in Cameroon (Figure 1) (Awono-Ambene *et al.* 2004, 2006; Antonio-Nkondjio *et al.* 2009; Ndo *et al.* 2010, 2013). We collected larvae and adult mosquitoes in and around human dwellings using several sampling techniques (Service 1993) (Table S1). The four currently known members of the *An. nili.* group were identified using morphological keys and a diagnostic PCR, which discriminates species on the basis of point mutations of the ribosomal DNA (Gillies & De Meillon 1968; Gillies & Coetzee 1987; Kengne *et al.* 2003; Awono-Ambene *et al.* 2004).

(b) Library preparation, sequencing and SNP discovery

We created double-digest RAD libraries using a modified version of the protocol described by Peterson *et al.* 2012. Genomic DNA of mosquitoes was extracted using the DNeasy Blood and Tissue kit (Qiagen) and the Zymo Research MinPrep kit for larvae and adult samples respectively. Approximately 50 ng (10µl) of DNA of each mosquito was digested simultaneously with *MluC1* and *NlaIII* restriction enzymes. Digested products were ligated to adapter and barcode sequences enabling identification of individuals. Samples were pooled, purified, and 400-bp fragments selected. The resulting libraries were amplified via PCR and purified, and fragment size distribution was checked on BioAnalyzer (Agilent Technologies, Inc., USA). PCR products were quantified, diluted and sequenced on Illumina HiSeq2000 (Illumina Inc., USA).

(b) SNP discovery and genotyping

The *process_radtags* program of the Stacks v 1.35 pipeline (Catchen *et al.* 2011, 2013) was used to demultiplex and clean raw reads. Reads that passed quality filters were aligned to the *An. nili* Dinderesso draft genome assembly made up of 51048 short contigs (~200-30512bp long) using Gsnap (Wu & Nacu 2010). The *ref_map.pl* program of Stacks was used to call SNPs within consensus RAD loci. We set the minimum number of reads required to form a stack to three and allowed two mismatches during catalogue creation. We generated SNP files in different formats for further downstream analyses using the *populations* program of Stacks and Plink v1.09 (Purcell *et al.* 2007).

(d) Population genomics analyses

We analyzed the genetic structure of *An. nili* sensu lato (s.l.) populations using Principal Component Analysis (PCA) and Neighbor-Joining trees (NJ). We also examined ancestry proportions and admixtures between populations in Admixture v1.23 (Alexander *et al.* 2009) and Structure v2.3.4 (Pritchard *et al.* 2000). We used the R package *adegenet* (Jombart 2008) to implement the PCA. Neighbor-Joining trees were generated from SNP allele frequencies via a matrix of Euclidian distance using the R package *ape* (Paradis *et al.* 2004). We ran Admixture with 10-fold cross-validation for values of k from 1 through 8. We analyzed patterns of ancestry from k ancestral populations in Structure, testing five replicates of k = 1-8. We used 200000 iterations and discarded the first 50000 iterations as burn-in for each Structure run. Clumpp v1.1.2 (Jakobsson & Rosenberg 2007) was used to summarize assignment results across independent runs. To identify the optimal number of genetic clusters in our sample, we applied simultaneously the lowest cross-validation error

in Admixture, the ad-hoc statistic deltaK (Evanno *et al.* 2005; Earl & VonHoldt 2012) and the Discriminant Analysis of Principal Component (DAPC) method implemented in *adegenet.* To examine the extent of genome divergence, we estimated locus-specific genetic differentiation (F_{ST}) using the *populations* program of the Stacks pipeline. Mean F_{ST} values were also used to assess pairwise divergence among populations. To infer the demographic history of populations, we used the diffusion approximation method implemented in the ∂a∂i package (Gutenkunst *et al.* 2009). Single-population models were fitted to allele frequency spectra and the best model was selected with the lowest likelihood and Akaike Information Criterion as well as visual inspections of residuals.

3. Results

(a) SNP genotyping

We collected mosquitoes from four locations out of 28 sampling sites (Figure 1, Table S1) and sequenced 145 individuals belonging, according to morphological identifications and diagnostic PCRs, to two species (*An. nili* (n = 24) and *An. ovengensis* (n = 121)). We assembled 197724 RAD loci that mapped to unique positions throughout the reference genome. We applied stringent filtering rules and retained 408 loci present in all populations and in at least 50% of individuals in each population. Within these loci, we identified 4343 high-quality biallelic markers that were used to analyze population structure and genetic differentiation.

(b) Morphologically-defined species do not correspond to genetic clusters

156 PCA and NJ trees show that the genetic variation across 4343 SNPs is best explained
157 by more than two clusters, implying subdivisions within *An. nili* and *An. ovengensis* (Figure
158 2). Three subgroups are apparent within *An. nili* while two distinct clusters segregate in *An.*
159 *ovengensis*. These five subpopulations are associated with the different sampling sites
160 suggesting local adaptation of divergent populations. Importantly, Structure and Admixture
161 analyses reveals that, at k = 2, one population identified by morphology and diagnostic
162 PCRs as *An. nili* has almost the same ancestry pattern as the largest *An. ovengensis* cluster
163 (Figure 3). Such discrepancies between morphology-based and molecular taxonomies can
164 be due to a variety of processes including phenotypic plasticity, introgressive hybrydization
165 or incomplete lineage sorting (i.e., when independent loci have different genealogies by
166 chance) (Arnold 1997; Combosch & Vollmer 2015; Fontaine *et al.* 2015; Weng *et al.* 2016).
167 At k = 2 and k = 3, some populations also depict half ancestry from each morphological
168 species suggestive of gene flow. We found a conflicting number of genetic clusters in our
169 samples reflecting the complex history of subdivisions and admixtures among populations
170 (Figure 4). The Evanno et al. method, which highlights early divergence between *An. nili* and
171 *An. ovengensis* indicates two probable ancestors. DAPC and the Admixture cross-validation
172 error, which are more sensitive to recent hierarchical population subdivisions, show five or
173 more distinct clusters as revealed by PCA and NJ trees (Figure 4).

174 As suggested by the long internal branches connecting subpopulations on NJ trees,
175 there is strong differentiation between and within morphological species characterized by
176 globally high F_{ST} values (Table 1). Relatively lower F_{ST} observed between certain clusters
177 can be due to greater inter-population migration and intermixing or more recent
178 divergence. Overall, both genetic structure and differentiation reveal a set of populations

whose ancestries and species status are likely confounded by hybridization and/or incomplete lineage sorting. The current taxonomy based on morphology and ribosomal DNA does not effectively describe the optimal reproductive units among populations of this group of mosquitoes.

(c) Genomic signatures of divergent selection and demographic history

Estimates of genetic differentiation varied substantially across 4343 SNP loci (Figure 5). The distribution of locus-specific F_{ST} between the five subpopulations revealed a bimodal shape characterized by two peaks around 0 and 1. The great majority of SNPs have low to moderate divergence, but a substantial number of variants are extremely differentiated between populations. The maximum F_{ST} among SNPs is 1 and the number of loci with $F_{ST} = 1$ varies from 406 between the populations we termed *An. nili* goup 1 and *An. nili* group 2 to 838 between the subgroups called *An. nili* group 3 and *An. ovengensis* group 2. This pattern of genome-wide divergence suggests that a very high number of sites with abrupt differentiation, which likely contain genes that contribute to divergent selection and/or reproductive isolation, coexist with regions of weak divergence that can be freely exchanged between species.

Models of population demography indicate that all subgroups have experienced an increase in effective size in more or less recent past (Table 2). Nevertheless, confidence intervals of population parameters are high in some populations, and our results should be interpreted with the necessary precautions. The population growth is less significant in *An. nili* group 1.

4. Discussion

(a) Genetic differentiation

Advances in sequencing and analytical approaches have opened new avenues for the study of genomes of disease vectors. We have focused on malaria mosquitoes of the *An. nili* group, whose taxonomy and population structure have been challenging to resolve with low-resolution markers. We analysed genetic structure using genome-wide SNPs and found strong differentiation and local adaption among populations belonging to the two morphological species *An. nili* and *An. ovengensis*. The exact number of subpopulations remains contentious, with the suggested number of divergent clusters varying from two to five within both species. Significant population structure at eight microsatellite loci has been described among *An. nili* populations from Cameroon, with F_{ST} values as high as 0.48 between samples from the forest area (Ndo *et al.* 2013). By contrast, *An. ovengensis* was discovered recently and the population structure of this vector remains understudied. This species was initially considered as a sibling of *An. nili* (Kengne *et al.* 2003; Awono-Ambene *et al.* 2004, 2006), but more recent studies have started to challenge the assumed relatedness between the two species due to the high divergence of their polytene chromosomes (Sharakhova *et al.* 2013). Our findings call for careful review of the current taxonomy within this group of species, which is a necessary first step for accurate delineation of the role played by the different subpopulations in malaria transmission.

Our samples were collected from locations characterized by more or less degraded forest in the equatorial region of Cameroon. In those habitats, larvae of *An. nili* s.l. exploit relatively similar breeding sites consisting of slow-moving rivers (Antonio-Nkondjio *et al.* 2009). Given the similarity of habitats among populations, it is hard to pinpoint the

environmental variables that may be correlated with adaptive divergence. One of the most expected outcomes of current large-scale malaria control measures that are underway in Sub-Saharan African countries concerns the effects of increased insecticide exposure on genetic diversity and population structure of vectors. The inferred demographic history of the different subpopulations of the *An. nili* group suggests that there are currently no bottlenecks that can be potentially linked with increased usage of insecticides and insecticide-treated nets (e.g. Athrey *et al.* 2012). Nevertheless, further study is needed to understand whether recent selection associated with human intervention is among the drivers of local adaptation as observed in *An. gambiae* (Kamdem *et al.* 2017).

(b) Genomic architecture of reproductive isolation

The genomic architecture of reproductive isolation can reveal crucial information on the mode of speciation and has been the focus of much research over the last few decades (e.g. Turner *et al.* 2005; Harr 2006; Nadeau *et al.* 2012; Ellegren *et al.* 2012; Carneiro *et al.* 2014; Burri *et al.* 2015). One influential concept of speciation coined the "genic view of species" proposes that boundaries between species are properties of individual genes or genome regions and not of whole organisms or lineages (Key 1968; Barton & Hewitt 1985; Harrison 1990; Rieseberg *et al.* 1999; Wu 2001; Nosil & Feder 2012; Harrison & Larson 2014). We have found that a high number of SNPs are strongly differentiated between populations and often fixed within subgroups. These variants can presumably be assimilated with regions of the genome bearing signals of reproductive isolation. However, recent studies analyzing interactions between pairs of related taxa at their hybrid zone indicate a complex relationship between genetic differentiation and gene flow at the

248 genome level (e.g. Gompert *et al.* 2012; Hamilton *et al.* 2013a; b, Larson *et al.* 2013, 2014;
249 Parchman *et al.* 2013; Taylor *et al.* 2014). Highly divergent genomic regions do not
250 necessarily coincide with regions of reduced gene flow. Several alternative interpretations
251 exist for the numerous high-F_{ST} regions we detected in all pairwise comparisons (Noor &
252 Bennett 2009; Nachman & Payseur 2012; Cruickshank & Hahn 2014; Delmore *et al.* 2015),
253 but careful examination of these outlier of differentiation will reveal significant insights into
254 the markers of ecological divergence and/or reproductive isolation. More complete genome
255 assemblies will be necessary to better delineate specific regions of the genome under
256 natural selection, and thereby clarify the genomic basis of phenotypic fitness differences
257 between divergent populations.

258 Signals consistent with gene flow between *An. nili* and *An. ovengensis* are apparent
259 in our data despite significant time since divergence (~3M-yr) (Ndo *et al.* 2013). Some
260 individuals depict almost half ancestry from each morphological species. The disagreement
261 observed between morphology/PCR and molecular taxonomies also suggests that
262 incongruent genealogies may be widespread along chromosomes due to hybridization
263 and/or incomplete lineage sorting. However, hybridization can be difficult to detect
264 because other factors such as incomplete lineage sorting or technical artefacts can leave
265 signatures that are similar to those of interspecific gene flow (Patterson *et al.* 2012; Liu *et*
266 *al.* 2014). A complete reference genome is also necessary to analyze the detailed
267 distribution of genealogies across genomic windows and to disentangle the relative
268 contribution of processes that generate the putative admixtures and species confusion
269 observed among divergent populations (Fontaine *et al.* 2015; Weng *et al.* 2016).
270

5. Conclusions and implications

Delineating the fine-scale population structure of mosquito populations is crucial to understand their epidemiological significance and their potential response to vector control measures. Moreover, recent malaria control efforts are affecting interspecific gene flow, genetic differentiation, population demography and natural selection in mosquitoes (Athrey *et al.* 2012; Clarkson *et al.* 2014; Norris *et al.* 2015; Barnes *et al.* 2017; Kamdem *et al.* 2017). Deciphering the signatures of all these processes across mosquito genome is important to minimize their negative impacts on vector control. Our findings shed some light on the complex evolutionary history of members of the *An. nili* group and provide a framework for future investigations into the genetic basis of ecological and reproductive barriers.

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Data Archiving Statement

Data for this study are available at: to be completed after manuscript is accepted for publication

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Author contributions

Conceived and designed the experiments: CK CF BJW. Performed the experiments: CK CF SG BJW. Analyzed the data: CK CF. Wrote the paper: CK CF BJW.

Tables

Table 1: Pairwise F_{ST} between divergent subpopulations.

F_{ST}	*An. nili* group 1	*An. nili* group 2	*An. nili* group 3	*An. ovengensis* group 1	*An. ovengensis* group 2
***An. nili* group 1**	-				
***An. nili* group 2**	0.374	-			
***An. nili* group 3**	0.506	0.552	-		
***An. ovengensis* group 1**	0.135	0.275	0.364	-	
***An. ovengensis* group 2**	0.432	0.458	0.492	0.349	-

Table 2: Demographic models of different subgroups.

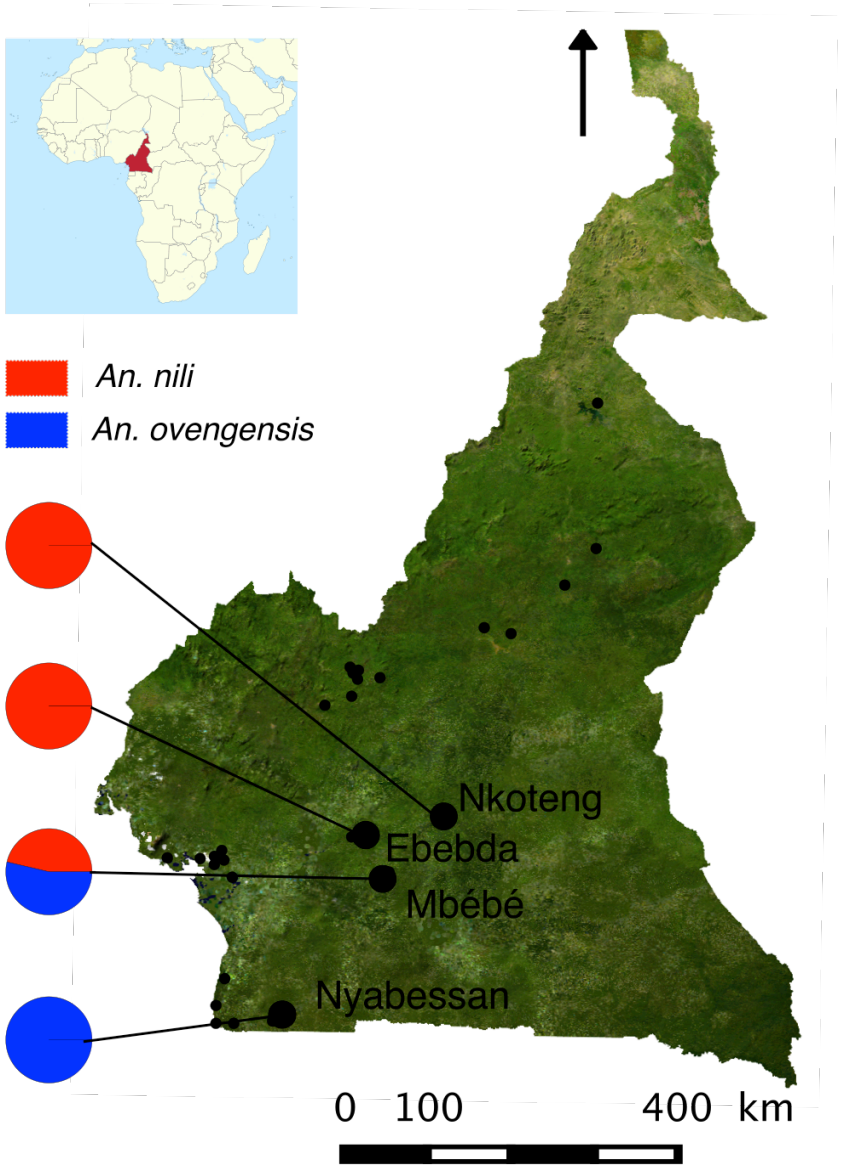
Population	Best Model	Log Likelihood	Final Population Size[a] (95% CI)	Time[b] (95% CI)
An. nili group 1	*Growth*	-18.42	6.41 (5.326 - 20.71)	3.70 (1.11 - 13.31)
An. nili group 2	*Two-epoch*	-19.97	17.87 (9.33 - 35.50)	11.27 (4.93 - 19.64)
An. ovengensis group 1	*Growth*	-112.18	13.04 (12.15 - 17.26)	0.70 (0.58 - 1.08)
An. Ovengensis group 2	*Growth*	-22.98	19.95 (14.45 - 45.70)	5.11 (2.33 - 15.13)

[a] Relative to ancestral population size.

[b] Expressed in units 2Ne generations from start of growth to present.

520 **Figures**

521 **Figure 1**: Map showing the sampling locations and the relative frequencies of *An. nili* and
522 *An. ovengensis*. Small and large black dots indicate respectively the 28 locations surveyed
523 and the four sampling sites where mosquitoes were collected.
524



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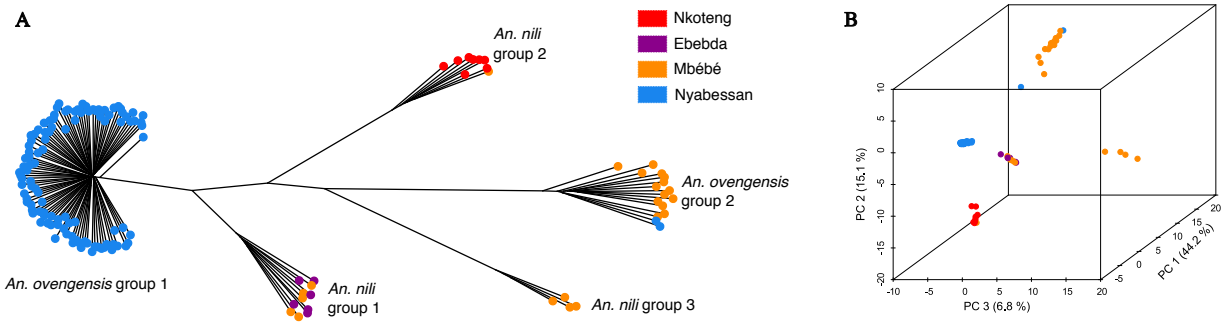
527 **Figure 2**: Population genetic structure inferred from 4343 SNPs using PCA (A) and a
528 neighbor-joining tree (B). The percentage of variance explained is indicated on each PCA
529 axis.
530



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534 **Figure 3:** Ancestry proportions inferred in ADMIXTURE with k = 2 – 8.
535

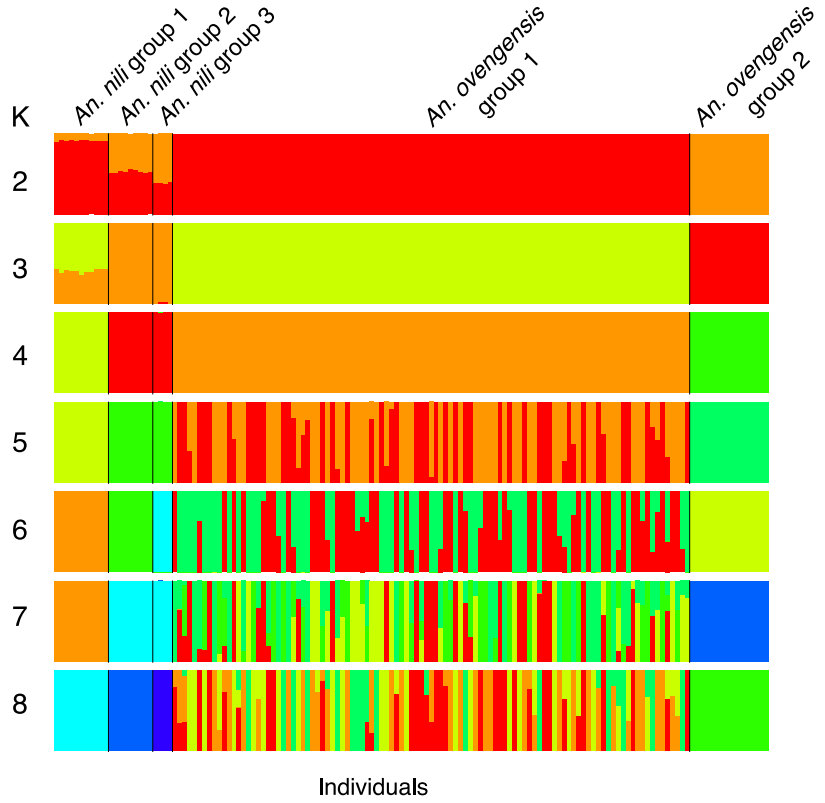

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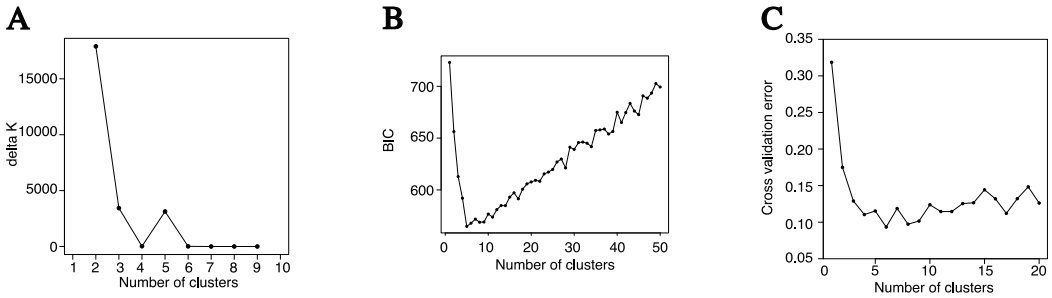
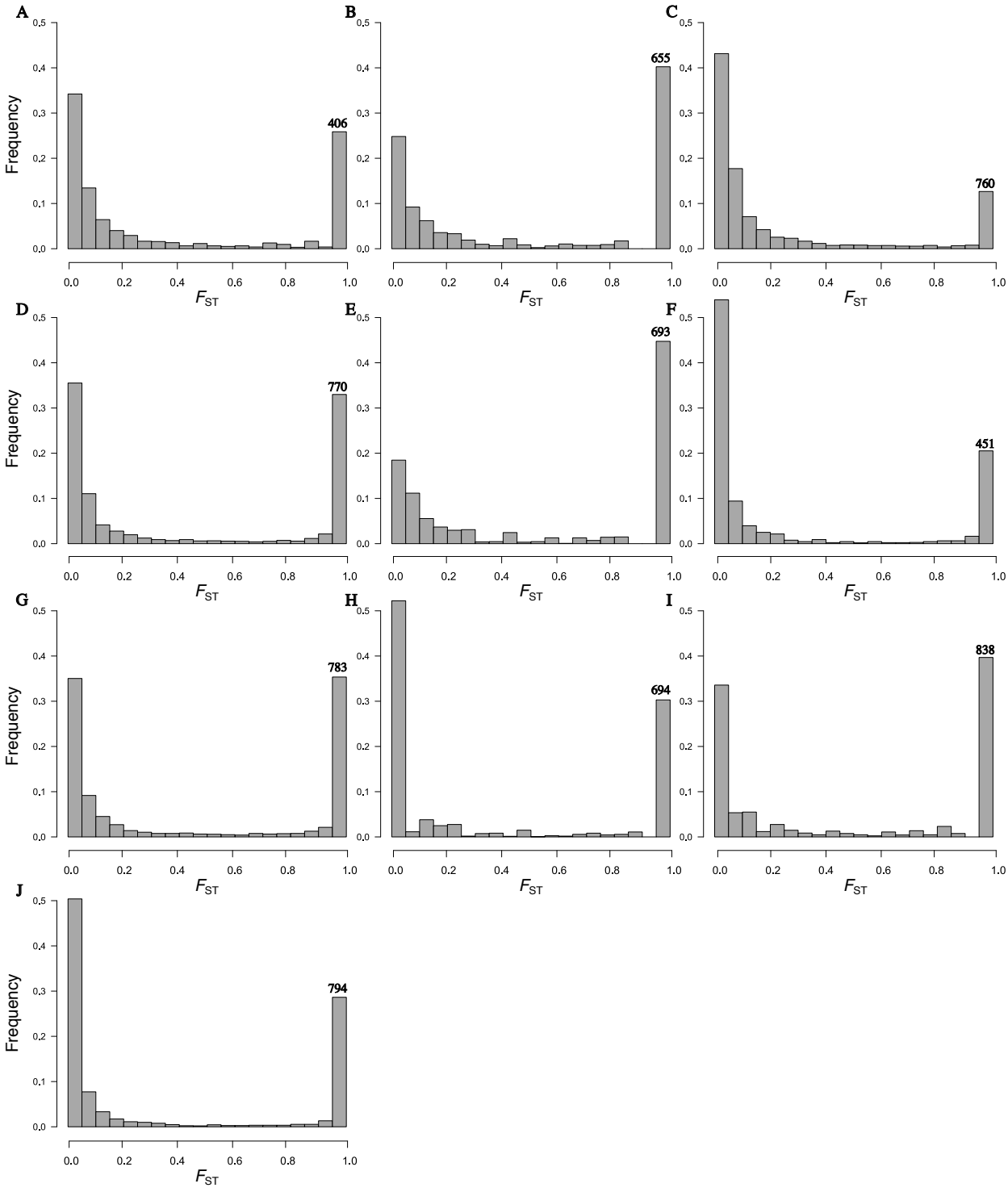
Figure 4: Identification of the optimal number of genetic clusters using the delta k method of Evanno et al (A), DAPC (B) and 10-fold cross-validation in ADMIXTURE (C). The lowest BIC and CV error and the highest delta k indicate the most probable number of clusters.



547 **Figure 5:** Distribution of F_{ST} values across 4343 SNPs between *An. nili* group 1 and *An. nili*
548 group 2 (A); *An. nili* group 1 and *An. nili* group 3 (B); *An. nili* group 1 and *An. ovengensis*
549 group 1 (C); *An. nili* group 1 and *An. ovengensis* group 2 (D); *An. nili* group 2 and *An. nili*
550 group 3 (E); *An. nili* group 2 and *An. ovengensis* group 1 (F); *An. nili* group 2 and *An.*
551 *ovengensis* group 2 (G); *An. nili* group 3 and *An. ovengensis* group 1 (H); *An. nili* group 3 and
552 *An. ovengensis* group 2 (I); *An. ovengensis* group 1 and *An. ovengensis* group 2 (J). The
553 number of SNPs with $F_{ST} = 1$ is indicated in each pairwise comparison.
554



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Supplemental information

Table S1: Information on mosquito samples included in this study.

Sampling locations	Geographic coordinates	Sampling methods			Total
		HLC-OUT	HLC-IN	LC	
Ebebda	4°20'00"N, 11°17'00"E			6	6
Nkoteng	4°31'00"N, 12°02'00"E			8	8
Nyabessan	2°24'00"N, 10°24'00"E	63	44		107
Mbébé	4°10'00"N, 11°04'00"E	13	3	8	24
Total		76	47	22	145

HLC-OUT, human landing catches performed outdoor; HLC-IN, human landing catches performed indoor; LC, larval collection