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

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Abstract

Ongoing speciation in most African malaria vectors gives rise to cryptic populations, which differ remarkably in their behaviour, ecology and capacity to vector malaria parasites. Understanding the population structure and the drivers of genetic differentiation among mosquitoes is critical for effective disease control because heterogeneity within species contribute to variability in malaria cases and allow fractions of vector populations to escape control efforts. To examine the population structure and the potential impacts of recent large-scale control interventions, we have investigated the genomic patterns of differentiation in mosquitoes belonging to the *Anopheles nili* group, a large taxonomic group that diverged ~3-Myr ago. Using 4343 single nucleotide polymorphisms (SNPs), we detected strong population structure characterized by high F_{ST} values between multiple divergent populations adapted to different habitats within the Central African rainforest. Delineating the cryptic species within the *Anopheles nili* group is challenging due to incongruence between morphology, ribosomal DNA and SNP markers consistent with incomplete lineage sorting and/or interspecific gene flow. A very high proportion of loci are fixed (F_{ST} = 1) within the genome of putative species, which suggests that ecological and/or reproductive barriers are maintained by strong selection on a substantial number of genes.

Key words: *Anopheles nili*, divergent selection, high-F_{ST} regions, speciation.

1. Introduction

One of the principal goals of population genetics is to summarize the 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. The best known mosquito species of the genus *Anopheles* — which includes all vectors of *Plasmodium*, the parasite of human malaria — exhibit very complex range-wide population structure due to the combined effects of cryptic speciation, adaptive flexibility and ongoing gene flow (Harbach, 2013; Krzywinski and Besansky, 2003). For example, almost all major malaria vectors of the Afrotropical region 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 and Simard 2013; Coetzee and Koekemoer 2013; Dia et al. 2013; Lanzaro and 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; Weng et al. 2016), but can also have far-reaching practical consequences. Both spatial and temporal variability in malaria cases and the effectiveness of vector control measures are greatly impacted by heterogeneity within vector species (Molineaux and Gramiccia 1980; Van Bortel et al. 2001). For these reasons, research on the genetic structure among the major African malaria vector mosquitoes has intensified over the last few decades (Antonio-Nkondjio and Simard, 2013; Coetzee and Koekemoer, 2013; Dia et al., 2013; Lanzaro and Lee, 2013).

51 The recent scaling up of insecticide-treated nets usage and indoor insecticide
52 spraying to a lesser extent have led to a dramatic reduction in malaria morbidity across the
53 continent (WHO, 2016). However, other implications of these large-scale interventions
54 include increased insecticide resistance (reviewed by Hemingway et al. 2016; Ranson and
55 Lissenden 2016), range shift (e.g. Bøgh et al. 1998; Derua et al. 2012; Mwangangi et al.
56 2013) and profound evolutionary changes among vector populations. In contrast to
57 insecticide resistance and range shift, which has been extensively studied, the recent
58 adaptive changes among mosquito populations have just started to be addressed. These
59 changes — which involve local adaptation and genetic differentiation, introgressive
60 hybridization and selective sweeps across loci conferring resistance to xenobiotics — are
61 particularly evident in the most anthropophilic species (Barnes et al., 2017; Clarkson et al.,
62 2014; Kamdem et al. , 2017; Norris et al., 2015).

63 The ecology, taxonomic complexity, geographic distribution, role in transmission
64 and evolutionary potential of each major African malaria vector is unique. Consequently,
65 further research is needed to specifically resolve the fine-scale population structure and the
66 genomic targets of natural selection in all of the important taxa including currently
67 understudied species. The present work focused on a group of malaria vector species
68 representing a large taxonomic unit named *Anopheles nili* group. Despite the significant role
69 of some of its species in sustaining high malaria transmission, this group has received little
70 attention. To date, four species that occur in forested areas of Central and West Africa and
71 are distinguishable by slight morphological variations are known within the *An. nili* group:
72 *An. nili sensu stricto* (hereafter *An. nili*), *An. ovengensis*, *An. carnevalei*, and *An. somalicus*
73 (Awono-Ambene et al., 2004; Gillies and Coetzee, 1987; Gillies and De Meillon, 1968). These

74 species are characterized by reticulate evolution and complex phylogenies that have been
75 challenging to resolve so far (Awono-Ambene et al., 2004; Awono-Ambene et al., 2006;
76 Kengne et al., 2003; Ndo et al., 2010, 2013; Peery et al., 2011; Sharakhova et al., 2013).
77 Populations of *An. nili* and *An. ovengensis* are very anthropophilic and efficient vectors of
78 *Plasmodium* in rural areas where malaria prevalence is particularly high (Antonio-Nkondjio
79 et al., 2006).

80 To delineate genomic patterns of differentiation, we sampled mosquito populations
81 throughout the range of species of the *An. nili* group in Cameroon and used reduced
82 representation sequencing to develop genome-wide SNP markers that we genotyped in 145
83 individuals. We discovered previously unknown subpopulations characterized by high
84 pairwise differentiation within *An. ovengensis* and *An. nili*. We further explored the genetic
85 differentiation across the genome and revealed the presence of a very high number of
86 outlier loci that are targets of selection among locally adapted subpopulations. These
87 findings provide significant baseline data on the genetic underpinnings of adaptive
88 divergence and pave the way for further genomic studies in this important group of
89 mosquitoes. Notably, a complete reference genome will allow in-depth studies to decipher
90 the functional and phenotypic characteristics of the numerous differentiated loci as well as
91 the contribution of recent selective events in ongoing adaptation.

92

93 **2. Materials and methods**

94 **(a) Mosquito species**

95 We surveyed 28 locations within the geographic ranges of species of the *An. nili*
96 group previously described in Cameroon (Figure 1) (Antonio-Nkondjio et al., 2009; Awono-

97 Ambene et al., 2004; Awono-Ambene et al., 2006; Ndo et al., 2010, 2013). The genetic
98 structure of *Anopheles* species is most often based on macrogeographic or regional
99 subdivisions of gene pools, but can also involve more subtle divergence between larvae and
100 adults, or between adult populations found in or around human dwellings (e.g. Riehle et al.,
101 2011). To effectively estimate the genetic diversity and identify potential cryptic
102 populations within species, we collected larvae and adult mosquitoes within and around
103 human dwellings using several sampling techniques (Service, 1993) in September-October
104 2013 (Table S1). To identify the four currently known members of the *An. nili.* group, we
105 used morphological keys and a diagnostic PCR, which discriminates species on the basis of
106 point mutations of the ribosomal DNA (Awono-Ambene et al., 2004; Gillies and Coetzee,
107 1987; Gillies and De Meillon, 1968; Kengne et al., 2003).

108 **(b) Library preparation, sequencing and SNP discovery**

109 We created double-digest Restriction site Associated DNA (ddRAD) libraries as
110 described in Kamdem et al. 2017 using a modified version of the protocol designed by
111 Peterson et al*.* 2012. Briefly, genomic DNA of mosquitoes was extracted using the DNeasy
112 Blood and Tissue kit (Qiagen) and the Zymo Research MinPrep kit for larvae and adult
113 samples respectively. Approximately 50ng (10µl) of DNA of each mosquito was digested
114 simultaneously with *MluC1* and *NlaIII* restriction enzymes. Digested products were ligated
115 to adapter and barcode sequences enabling identification of individuals. Samples were
116 pooled, purified, and 400-bp fragments selected. The resulting libraries were amplified via
117 PCR and purified, and fragment size distribution was checked using the BioAnalyzer. PCR
118 products were quantified, diluted and single-end sequenced to 100 base reads on Illumina
119 HiSeq2000.

120

121 **(b) SNP discovery and genotyping**

122 The *process_radtags* program of the Stacks v 1.35 pipeline (Catchen et al., 2013;
123 Catchen et al., 2011) was used to demultiplex and clean Illumina sequences. Reads that
124 passed quality filters were aligned to the *An. nili* Dinderesso draft genome assembly
125 (Giraldo-Calderon et al., 2015) made up of 51048 short contigs (~200-30512bp long) using
126 Gsnap (Wu and Nacu, 2010). To identity and call SNPs within consensus RAD loci, we
127 utilized the *ref_map.pl* program of Stacks. We set the minimum number of reads required to
128 form a stack to three and allowed two mismatches during catalogue creation. We generated
129 SNP files in different formats for further downstream analyses using the *populations*
130 program of Stacks and Plink v1.09 (Purcell et al., 2007).

131 **(d) Population genomics analyses**

132 We analyzed the genetic structure of *An. nili* sensu lato (s.l.) populations using a
133 Principal Component Analysis (PCA) and an unrooted Neighbor-Joining tree (NJ). We also
134 examined ancestry proportions and admixtures between populations in Admixture v1.23
135 (Alexander et al., 2009) and Structure v2.3.4 (Pritchard et al., 2000). We used the package
136 *adegenet* (Jombart, 2008) to implement the PCA in R (R Development Core Team 2016).
137 The NJ tree was generated from SNP allele frequencies via a matrix of Euclidian distance
138 using the R package *ape* (Paradis et al., 2004). We ran Admixture with 10-fold cross-
139 validation for values of k from 1 through 8. Similarly, we analyzed patterns of ancestry from
140 k ancestral populations in Structure, testing five replicates of k = 1-8. We used 200000
141 iterations and discarded the first 50000 iterations as burn-in for each Structure run.
142 Clumpp v1.1.2 (Jakobsson and Rosenberg, 2007) was used to summarize assignment

143 results across independent runs. To identify the optimal number of genetic clusters in our
144 sample, we applied simultaneously the lowest cross-validation error in Admixture, the ad-
145 hoc statistic deltaK (Earl and VonHoldt, 2012; Evanno et al., 2005) and the Discriminant
146 Analysis of Principal Component (DAPC) method implemented in *adegenet.* To examine the
147 level of genomic divergence among populations, we assessed genetic differentiation (F_{ST})
148 across SNPs using the *populations* program of the Stacks pipeline. Mean F_{ST} values were also
149 used to quantify pairwise divergence between populations. To infer the demographic
150 history of different populations, we used the diffusion approximation method implemented
151 in the package ∂a∂i v 1.6.3 (Gutenkunst et al., 2009). Single-population models were fitted
152 to allele frequency spectra, and the best model was selected using the lowest likelihood and
153 Akaike Information Criterion as well as visual inspections of residuals.
154

155 **3. Results**

156 **(a) SNP genotyping**

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

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

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

185 As suggested by the long internal branches, which connect subpopulations on the NJ
186 tree, there is strong differentiation between and within morphological species
187 characterized by globally high F_{ST} values (Table 1). Relatively lower F_{ST} values observed
188 between certain clusters may be due to greater inter-population migration and intermixing

189 or more recent divergence. The F_{ST} values do not reflect the morphological delimitation of
190 species, and the level of genetic differentiation is even higher between some subpopulations
191 within the same morphological species. Overall, patterns of genetic structure and
192 differentiation reveal a group of populations whose phylogenies and species status are
193 likely confounded by hybridization and/or incomplete lineage sorting. We argue that the
194 current taxonomy based on morphology and ribosomal DNA does not capture the optimal
195 reproductive units among populations of this group of mosquitoes.

196 **(c) Genomic signatures of divergent selection and demographic history**

197 We analyzed patterns of genetic differentiation across SNP loci throughout the
198 genome. Pairwise comparisons are based on filtered variants that satisfy all criteria to be
199 present in both populations, which explain the discrepancy in the number of SNPs observed
200 between specific paired comparisons (Figure 5). The distribution of locus-specific F_{ST}
201 values between the five subpopulations revealed a bimodal shape characterized by two
202 peaks around 0 and 1. The great majority of SNPs have low to moderate divergence, but a
203 substantial number of variants are extremely differentiated between populations. The
204 maximum F_{ST} among SNPs is 1 and the proportion of loci with F_{ST} = 1 varies from 6.52%
205 between the populations we termed *An. nili* group 1 and *An. ovengensis* group 1 to 44.74%
206 between the subgroups called *An. nili* group 2 and *An. nili* group 3 (Figure 5). This pattern of
207 genome-wide divergence suggests that a very high number of sites with abrupt
208 differentiation — which likely contain genes that contribute to divergent selection and/or
209 reproductive isolation — coexist with regions of weak divergence that can be freely
210 exchanged between species. As is the case with the overall genetic differentiation,
211 morphology is not a reliable predictor of locus-specific divergence. Precisely, the lowest

212 percentage of fixed SNPs is found between *An. ovengensis* from Nyabessan and *An. nili*
213 collected from Mbébé and Ebebda (Figure 1, Figure 5). In contrast, the greatest proportion
214 of fixed loci occurs between locally adapted subgroups within the same morphological
215 species, *An. nili*. The draft reference genome made up of short contigs didn't allow us to test
216 hypotheses about the genomic distribution of differentiated loci. For example, it remains
217 unknown whether the numerous SNPs that are fixed among populations are spread
218 throughout the entire genome or clustered in genomic regions of low recombination
219 including chromosomal inversions and chromosome centers (Nosil and Feder, 2012; Roesti
220 et al., 2012).

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

226

227 **4. Discussion**

228 **(a) Genetic differentiation**

229 Advances in sequencing and analytical approaches have opened new avenues for the
230 study of genomes of disease vectors. We have focused on malaria mosquitoes of the *An. nili*
231 group, whose taxonomy and population structure have been challenging to resolve with
232 low-resolution markers. We analysed the genetic structure using genome-wide SNPs and
233 found strong differentiation and local adaption among populations belonging to the two
234 morphologically defined species *An. nili* and *An. ovengensis*. The exact number of

235 subpopulations remains contentious, with the suggested number of divergent clusters
236 varying from two to five within both species. Significant population structure at eight
237 microsatellite loci has been described among *An. nili* populations from Cameroon, with F_{ST}
238 values as high as 0.48 between samples from the rainforest area (Ndo et al., 2013). By
239 contrast, *An. ovengensis* was discovered recently and the genetic structure of this vector
240 remains understudied. This species was initially considered as a sibling of *An. nili* (Awono-
241 Ambene et al., 2004; Awono-Ambene et al., 2006; Kengne et al., 2003), but more recent
242 studies have started to challenge the assumed relatedness between the two species due to
243 the high divergence of their polytene chromosomes (Sharakhova et al., 2013). Our findings
244 call for careful review of the current taxonomy within this group of species, which is a
245 necessary first step for accurately delineating the role played by the different
246 subpopulations in malaria transmission.

247 Our samples were collected from locations characterized by a more or less degraded
248 forest in the rainforest area of Cameroon. In these habitats, larvae of *An. nili* s.l. exploit
249 relatively similar breeding sites consisting of slow-moving rivers (Antonio-Nkondjio et al.,
250 2009). The ecological drivers of genetic differentiation remain unknown, and will be
251 difficult to infer from our data given the apparent similarity of habitats among populations.
252 Further study is needed to clearly address the environmental variables that may be
253 correlated with ongoing adaptive divergence at adult and larval stages. One of the most
254 expected outcomes of current large-scale malaria control measures that are underway in
255 Sub-Saharan African countries concerns the effects of increased insecticide exposure on the
256 genetic diversity and population demography of vectors. A substantial population decline
257 that may considerably affect the adaptive potential of vector species has been occasionally

258 reported following the distribution of insecticide-treated bed nets and/or indoor residual
259 house spraying (e.g. Athrey et al., 2012). The inferred demographic history of the different
260 subpopulations within the *An. nili* group does not reveal signatures of bottlenecks that can
261 be potentially correlated with increased usage of insecticides and insecticide-treated nets.
262 This result is consistent with the demography of several other important malaria vectors of
263 the Afrotropical region, including *An. gambiae*, *An. coluzzii*, *An. funestus* and *An. moucheti*,
264 which depict a substantial population increase suggesting that recent intense insecticide
265 exposure has yet to leave deep or detectable impacts on patterns of genetic variation among
266 mosquito populations (Fouet et al., 2017; Kamdem et al., 2017; O'Loughlin et al., 2014).

267 **(b) Genomic architecture of geographic and reproductive isolation**

268 Understanding the genomic architecture of reproductive isolation may reveal crucial
269 information on the sequence of events that occur between the initial stages of divergence
270 among populations and the onset of strong reproductive barriers (e.g. Turner et al., 2005;
271 Harr 2006; Nadeau et al., 2012; Ellegren et al., 2012; Carneiro et al., 2014; Burri et al.,
272 2015). One influential concept of speciation coined the "genic view of species" proposes
273 that boundaries between species are properties of individual genes or genome regions and
274 not of whole organisms or lineages (Barton and Hewitt, 1985; Harrison and Larson, 2014;
275 Harrison, 1990; Key, 1968; Nosil and Feder, 2012; Rieseberg et al., 1999; Wu, 2001). We
276 have discovered a dramatically high number of SNPs that are strongly differentiated
277 between populations and often fixed within subgroups of *An. nili* s.l. Interpreting this
278 intriguing pattern of genomic differentiation is not straightforward due to the complex
279 interactions between numerous forces — including positive or negative selection,
280 recombination, introgressive hybridization and incomplete lineage sorting — that can affect

281 the level of divergence among SNPs (Begun and Aquadro, 1992; Cutter and Payseur, 2013;
282 Harrison and Larson, 2016; Nachman and Payseur, 2012; Roesti et al., 2012). Some of these
283 variants exhibiting high divergence among populations certainly contain markers of
284 ecological and/or reproductive isolation. However, as far as reproductive barriers are
285 concerned, recent studies have indicated a complex relationship between genetic
286 differentiation and gene flow at the genome level (e.g. Gompert et al., 2012; Hamilton et al.,
287 2013a, b; Larson et al., 2013, 2014; Parchman et al., 2013; Taylor et al., 2014). Highly
288 divergent genomic regions do not necessarily coincide with regions of reduced gene flow.
289 Several alternative interpretations exist for the numerous high-F_{ST} regions we detected in
290 all pairwise comparisons (Cruickshank and Hahn, 2014; Delmore et al., 2015; Nachman and
291 Payseur, 2012; Noor and Bennett, 2009). Nevertheless, careful examination of these outliers
292 of differentiation may reveal significant insights into the wide range of genes and traits that
293 contribute to ecological divergence and/or reproductive isolation between subgroups of *An.*
294 *nili* s.l. A complete genome assembly will be necessary to better delineate specific regions of
295 the genome under natural selection, and thereby clarify the genomic basis of phenotypic
296 fitness differences between divergent populations. This will also help understand the extent
297 to which recent selection associated with human interventions contribute to local
298 adaptation and genetic differentiation as observed in *An. gambiae* and *An. coluzzii* (Kamdem
299 et al., 2017).

300 Signals consistent with gene flow between *An. nili* and *An. ovengensis* are apparent
301 in our data despite significant time since divergence (~3M-yr) (Ndo et al., 2013). Some
302 individuals display almost half ancestry from each morphological species. The
303 disagreement between morphology/PCR and molecular taxonomies observed in Structure

304 and Admixture analyses also suggests that incongruent genealogies may be widespread
305 along chromosomes due to hybridization. However, hybridization can be difficult to detect
306 because other factors such as incomplete lineage sorting or technical artefacts can leave
307 signatures that are similar to those of interspecific gene flow (Liu et al., 2014; Patterson et
308 al., 2012). A complete reference genome is also needed to analyze the detailed distribution
309 of genealogies across small genomic windows and to disentangle the relative contribution
310 of processes that generate the putative admixtures and species confusion observed among
311 divergent populations (Fontaine et al., 2015; Martin et al., 2013; Weng et al., 2016).
312

313 **5. Conclusions and implications**

314 Delineating the fine-scale population structure of mosquito populations is crucial for
315 understanding their epidemiological significance and their potential response to vector
316 control measures. Moreover, recent malaria control efforts are affecting interspecific gene
317 flow, genetic differentiation, population demography and natural selection in mosquitoes
318 (Athrey et al., 2012; Barnes et al., 2017; Clarkson et al., 2014; Kamdem et al., 2017; Norris et
319 al., 2015). Deciphering the signatures of all these processes across mosquito genomes is
320 important to minimize their negative impacts on vector control. Our findings shed some
321 light on the complex evolutionary history and provide a framework for future
322 investigations into the genetic basis of ecological and reproductive barriers among species
323 of the *An. nili* group.
324

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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: CF CK BJW. Performed the experiments: CF CK SG BJW. Analysed the data: CF CK. Wrote the paper: CF CK BJW.

621 **Tables**

622

623 **Table 1**: Pairwise F_{ST} between divergent subpopulations of *An. Nili* s.l.

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	-

624

625 **Table 2:** Demographic models of different subgroups of *An. nili* s.l.

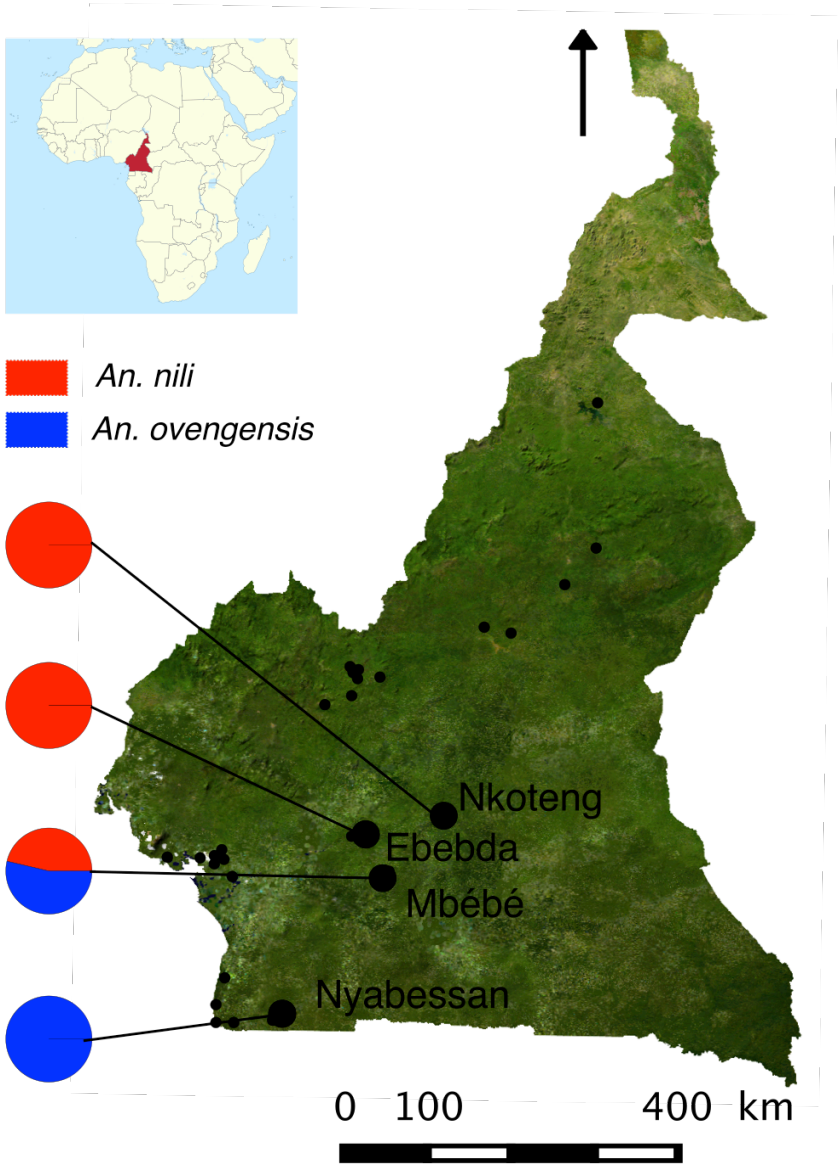
Population	**Best Model**	**Log Likelihood**	**Final Population Size[a] (95% CI)**	**Time[b] (95% CI)**
An. nili group 1	*Growth*	-18.42	6.41 (5.326 - 20.71)	3.70 (1.11 - 13.31)
An. nili group 2	*Two-epoch*	-19.97	17.87 (9.33 - 35.50)	11.27 (4.93 - 19.64)
An. ovengensis group 1	*Growth*	-112.18	13.04 (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.

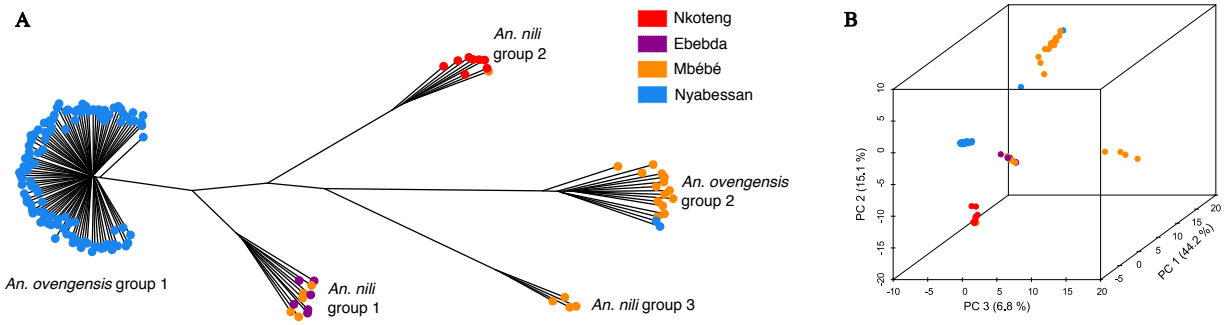
[b] Expressed in units 2Ne generations from start of growth to present.

Figures

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



634 **Figure 2**: Population genetic structure inferred from 4343 SNPs using PCA (A) and a
635 neighbor-joining tree (B). The percentage of variance explained is indicated on each PCA
636 axis.
637



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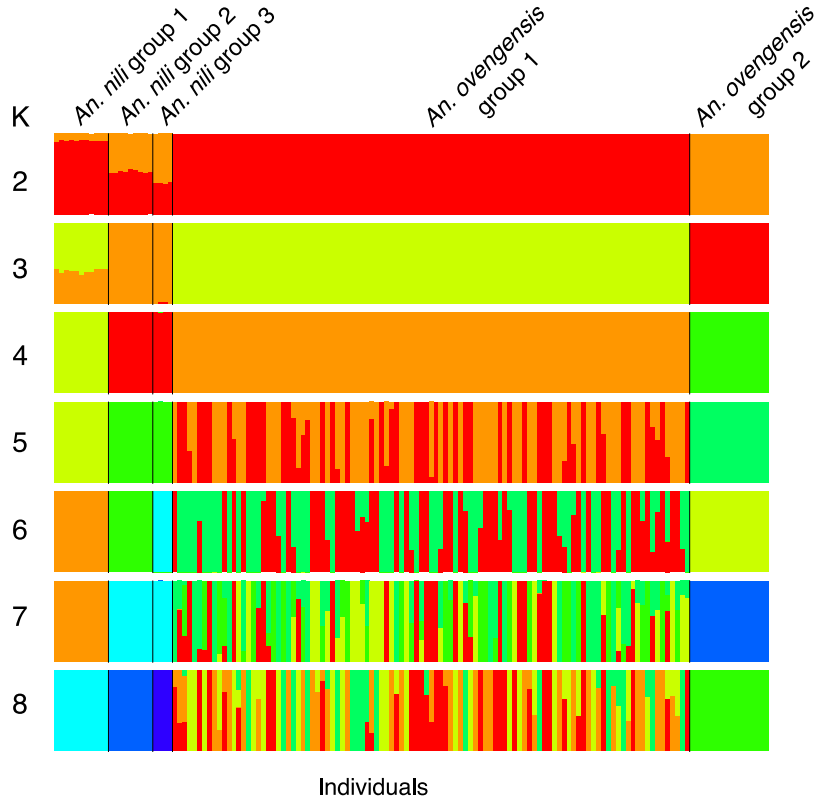
641 **Figure 3:** Ancestry proportions inferred in ADMIXTURE with k = 2 – 8.
642



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644
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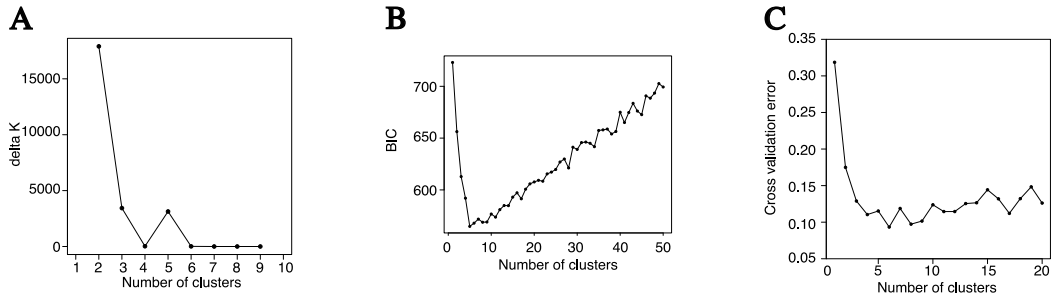
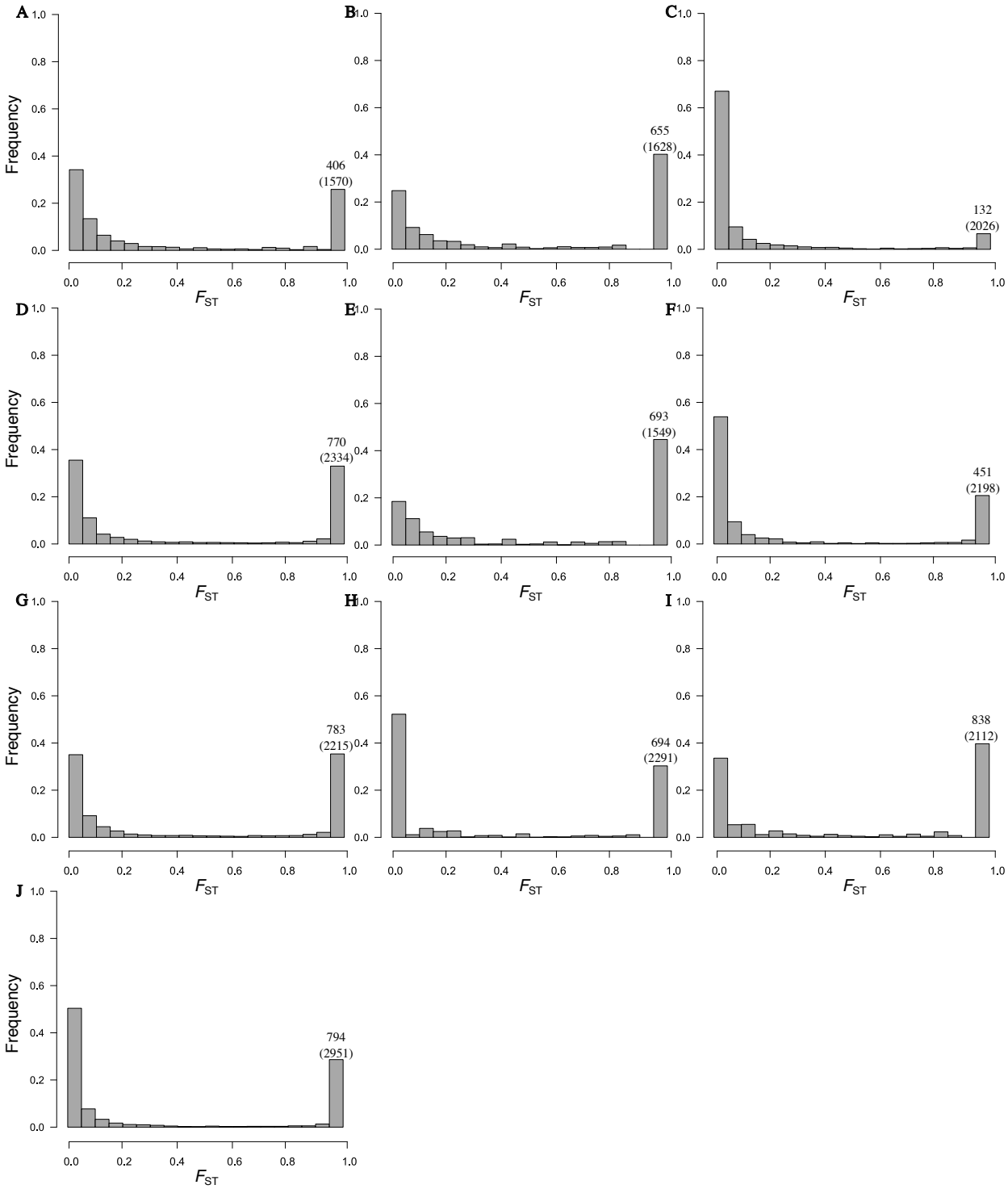
Figure 4: Identification of the optimal number of genetic clusters using the delta k method of Evanno et al (A), DAPC (B) and 10-fold cross-validation in ADMIXTURE (C). The lowest BIC and CV error and the highest delta k indicate the most probable number of clusters.



654 **Figure 5:** Distribution of F_{ST} values throughout the genome between *An. nili* group 1 and *An.*
655 *nili* group 2 (A); *An. nili* group 1 and *An. nili* group 3 (B); *An. nili* group 1 and *An. ovengensis*
656 group 1 (C); *An. nili* group 1 and *An. ovengensis* group 2 (D); *An. nili* group 2 and *An. nili*
657 group 3 (E); *An. nili* group 2 and *An. ovengensis* group 1 (F); *An. nili* group 2 and *An.*
658 *ovengensis* group 2 (G); *An. nili* group 3 and *An. ovengensis* group 1 (H); *An. nili* group 3 and
659 *An. ovengensis* group 2 (I); *An. ovengensis* group 1 and *An. ovengensis* group 2 (J). The
660 number of SNPs with F_{ST} = 1 is indicated in each pairwise comparison as well as the total
661 number of SNPs in parenthesis.
662


A
B
C
D
E
F
G
H
I
J
Frequency
F_{ST}
406
(1570)
655
(1628)
132
(2026)
770
(2334)
693
(1549)
451
(2198)
783
(2215)
694
(2291)
838
(2112)
794
(2951)

665 **Supplemental information**

666

667 **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

668