# Extensive genetic diversity among populations of the malaria mosquito *Anopheles moucheti* revealed by population genomics

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**Abstract**

**Background**: Recent intensive efforts to control malaria in African countries expose vector populations to additional adaptive challenges. The main malaria mosquitoes of the continent display an array of adaptive strategies to cope with such challenges. The development of genomic resources will empower genetic studies that are crucial to understand the evolutionary history and adaptive potential of these vectors.

**Methodology/Principal findings**: Here we constructed double-digest Restriction Associated DNA (ddRAD) libraries and generated 6461 Single Nucleotide Polymorphisms (SNPs) that we used to explore the population structure and demographic history of wild-caught *Anopheles moucheti* from Cameroon. The genome-wide distribution of allelic frequencies among sampled populations best fitted that of an old population at equilibrium, characterized by a weak genetic structure and extensive genetic diversity, presumably due to a large long term effective population size. In contrast to other important African malaria vectors, polymorphic chromosomal inversions play little role in the genome architecture and evolutionary adaptation of *An. moucheti*.

**Conclusions/Significance**: Our study provides the first investigation of the genetic structure and diversity in *An. moucheti* at the genomic scale. Despite a weak genetic structure and absence of adaptive divergence, the adaptive potential of this mosquito remains significant owing to a great diversity and standing genetic variation that can be used to face current vector control measures and other rapid anthropogenic and environmental changes.

## Background

*An. moucheti sensu lato* is a group of three related mosquito species (*An. moucheti moucheti*, *An. moucheti nigeriensis*, and *An. moucheti bervoetsi*) distributed across forested areas in Sub-Saharan Africa and distinguishable from each other by slight morphological differences [1]. The nominal species *An. moucheti moucheti* (hereafter *An. moucheti*) is the most widespread and one of the leading malaria vectors in the equatorial region [2–4]. The species breeds in slow moving streams and rivers close to villages where it contributes to high malaria transmission and often outcompete other main malaria vectors species. Despite this epidemiological significance, the evolutionary history and the evolvability of this mosquito remain understudied. Early investigations of the genetic structure based on allozymes and microsatellites detected no significant differentiation among *An. moucheti* populations over substantial geographic areas. This finding was particularly intriguing given the widespread distribution of *An. moucheti* in the evergreen forest areas of Central Africa. Moreover, African anopheline mosquito populations are increasingly exposed to intense selective pressure associated with the use of insecticides and insecticide-impregnated materials associated with recent expansive malaria control campaigns [5]. Such pressures are strong driving forces that often contribute to rapid diversification of vector populations at the scales of a few decades [6–8]. As a result, a more detailed characterization of the genomic architecture of *An. moucheti* will be crucial to: (1) understand the genetic bases allowing populations of this species to adapt to such a wide geographic area and (2)

56 predict the evolutionary potential of this vector in face of selective constraints
57 imposed by rapidly changing environments.
58
59 Thanks to recent progresses in sequencing technology, high-resolution sequence
60 information can be generated for virtually any living organism. However, while
61 significant progresses have been made in genomic studies of other African
62 anopheline mosquitoes [9], *An. moucheti* has lagged behind mainly because of the
63 absence of genomic resources. So far, this species crucially lacks a reference genome
64 assembly, a physical or linkage map and laboratory strains that are essential to
65 generate high-quality sequencing information and to enable robust interpretations
66 of natural polymorphisms.
67
68 To start filling this gap, we have conducted the first screening of genetic variation
69 across the genome of wild populations of *An. moucheti*. We have performed a high-
70 throughput sequencing of reduced representation libraries in 87 wild-caught
71 individuals from Cameroon and used a *de novo* assembly to identify thousands of
72 RAD loci scattered throughout the genome. Using high-quality Single Nucleotide
73 Polymorphisms (SNPs) identified within these loci, we have investigated the genetic
74 relatedness and population history of our samples. We found that populations of *An.*
75 *moucheti* are characterized by extensive gene flow and a great genetic diversity. This
76 vector appears particularly adapted to challenge the selective pressures imposed by
77 vector controls and rapid human-induced environmental modifications.
78

## Methods

### Mosquito sampling and sequencing

This study included two *An. moucheti* populations from the Cameroonian equatorial rainforest. A total of 98 mosquitoes (97 adults and 1 larva) were collected in August and November 2013 Olama and Nyabessan, respectively (Table 1). The two locations are separated by ~200 km (Fig. 1A) and are crossed respectively by the Nyong and the Ntem rivers that provide the breeding sites for *An. moucheti* larvae. Specimens were identified as *An. moucheti moucheti* using morphological identification keys and a diagnostic PCR [1,10,11]. We extracted genomic DNA of specimens using either the DNeasy Blood and Tissue kit (Qiagen) or the Zymo Research MinPrep kit. We used 10ul (~50ng) of genomic DNA to prepare double-digest Restriction-site Associated DNA libraries following a modified protocol of Peterson et al.,2012 [12]. *MluC1* and *NlaIII* restriction enzymes were used to digest DNA of individual mosquitoes yielding RAD-tags of different sizes to which short unique DNA sequences (barcodes) were ligated to enable the identification of reads belonging to each specimen. The digestion products were purified and pooled. DNA fragments with size ~400bp were selected and amplified via PCR. The distribution of fragment sizes was checked on a BioAnalyzer (Agilent Technologies, Inc., USA) before sequencing. The sequencing was performed on an Illumina HiSeq2000 platform (Illumina Inc., USA) (Genomic Core Facility, University of California, Riverside) to yield single-end reads of 101 bp.

### *De novo* assembly and SNP discovery

101 We used the bioinformatics pipeline Stacks v1.35 [13] to process Illumina short
102 reads. The program *process_radtags* was first used to sort the reads according to the
103 barcodes and to trim all reads to 96-bp in length by removing index and barcode
104 sequences from the ends of the reads. Reads with ambiguous barcodes, those that
105 did not contain the *NlaIII* recognition site and those with low-quality scores
106 (average Phred score < 33) were excluded. The program *ustacks* was then utilized
107 for a *de novo* assembly enabling the identification of consensus RAD loci in each
108 individual in our populations. We allowed a maximum of 2 nucleotide mismatches
109 between stacks (M parameter in *ustacks*) and we required a minimum of three reads
110 to create a stack (m parameter in *ustacks*). Using the *cstacks* program, a catalogue of
111 loci was built to synchronize variations across all individuals in our populations.
112 Finally, we utilized the *populations* program to calculate population genetic
113 parameters and output SNPs in different formats. To avoid bias associated with less
114 informative SNPs or possible false positive SNPs (due to sequencing or pipeline
115 errors), only RAD loci scored in at least 70-80% of individuals were retained for
116 further analyses.

117 **Population genomic analyses**

118 SNP files outputted by the *populations* program were used to assess the population
119 genetic structure with a Principal Component Analysis (PCA) and a Neighbor-joining
120 tree analysis using respectively the R packages *adegenet* and *ape* [14,15]. We also
121 explored patterns of ancestry and admixture in *An. moucheti* individuals in
122 ADMIXTURE v1.23 [16] with 10-fold cross-validation for k assumed ancestral
123 populations (k= 1 through 6). The optimal number of clusters was confirmed using

124 the Discriminant Analysis of Principal Component (DAPC) method which explores
125 the number of genetically distinct groups by running a k-means clustering
126 sequentially with increasing numbers and by comparing different clustering
127 solutions using Bayesian Information Criterion (BIC) [14]. We examined the
128 population genetic diversity, conformity to Hardy-Weinberg equilibrium and
129 demographic background using several statistics calculated with the *populations*
130 program. Precisely, to assess the global genetic diversity per population, we
131 calculated the overall nucleotide diversity (π) and the frequency of polymorphic
132 sites within population. To make inferences on the demographic history and to test
133 for departures from Hardy–Weinberg equilibrium, we utilized the Allele frequency
134 spectrum and the Wright's inbreeding coefficient ($F_{IS}$). To quantify the geographic
135 and genetic differentiation between allopatric populations, we estimated the
136 genome-wide average $F_{ST}$ [17] on 2000 randomly selected SNPs in Genodive v1.06
137 [18]. We also conducted an hierarchical Analysis of Molecular Variance (AMOVA)
138 [19] on the same SNP set to quantify the effects of the geographic origin on the
139 genetic variance among individuals. The statistical significance of $F_{ST}$ and AMOVA
140 was assessed with 10000 permutations. Finally, to examine the genomic
141 architecture divergence, we inspected the genome-wide distribution of locus-
142 specific estimates of $F_{ST}$.

143 **Identification of polymorphic chromosomal inversions**

144 The neutral recombination rate is notoriously reduced in genomic regions bearing
145 chromosomal inversions, which results in elevated linkage disequilibrium (LD) in
146 those regions relative to the rest of the genome. Thus, assessing genome-wide

147 patterns of LD can reveal clusters of highly correlated SNPs (LD blocks)
148 corresponding potentially to chromosomal inversions. The R package LDna [20]
149 allow the examination of the distinct LD network clusters within the genome of
150 nonmodel species without the need of a linkage map or reference genome. We
151 calculated LD, estimated as the $r^2$ correlation coefficient between all pairs of SNPs, in
152 PLINK v1.09 [21]. To avoid spurious LD due to the strong correlation between SNPs
153 located on the same RAD locus, we randomly selected only one SNP within each RAD
154 locus resulting in a dataset of 1056 variants containing less than 10% missing data.
155 LDna was then used to identify clusters of highly correlated SNPs that were
156 interpreted in light of the population demographic history, the population genetic
157 structure and previous cytological studies of *An. moucheti*.
158

159 **Results**

160 **De novo assembly**

161 In total, 518,218 unique 96-bp RAD loci were identified from *de novo* assembly of
162 reads in 98 individuals. We retained 946 loci that were present in all sampled
163 populations and in at least 75% of individuals in every population, and we identified
164 3027 high-quality biallelic SNPs from these loci.

165 **Population genetic structure**

166 First, we tested for the presence of cryptic genetic subdivision within *An. moucheti*
167 with PCA, NJ trees and the ADMIXTURE ancestry model. A NJ tree constructed from
168 a matrix of Euclidian distance using allele frequencies at 3027 genome-wide SNPs
169 showed a putative subdivision of *An. moucheti* populations in two genetic clusters
170 (Fig. S1). The first three axes of PCA also revealed a number of outlier individuals
171 separated from a main cluster (Fig. S1). However, when we ranked our sequenced
172 individuals based on the number of sequencing reads, we noticed that one of the
173 putative genetic clusters corresponded to a group of individuals having the lowest
174 sequencing coverage. We excluded all these individuals and reduced our dataset to
175 78 individuals. We conducted a *de novo* assembly and analyzed the relationship
176 between the 78 remaining individuals at 6461 genome-wide SNPs using PCA, NJ
177 trees and ADMIXTURE. Both the k-means clustering (DAPC) and the variation of the
178 cross-validation error as a function of the number of ancestral populations in
179 ADMIXTURE revealed that the polymorphism of *An. moucheti* resulted from only one
180 ancestral population (k = 1) (Fig. 1E,F). PCA and NJ depicted a homogeneous cluster
181 comprising all 78 individuals providing additional evidence of the lack of genetic or

182 geographic structuring among populations. Unsurprisingly, the overall $F_{ST}$ was
183 remarkably low between populations from the two sampling locations Olama and
184 Nyabessan ($F_{ST}$ = 0.008, p < 0.005). Similarly, the distribution of $F_{ST}$ values across the
185 6461 SNPs showed a large dominance of very low $F_{ST}$ values (Fig. 2). The highest per
186 locus $F_{ST}$ was only 0.126, while 5006 of the 6461 loci revealed $F_{ST}$ near zero. The
187 modest geographic differentiation was also well illustrated by a hierarchical
188 AMOVA, which showed that the genetic variance was explained essentially by
189 within-individual variations (99.7%). Finally, we found very low overall Wright's
190 inbreeding coefficient ($F_{IS}$= 0.0014 in Nyabessan and $F_{IS}$ = 0.0025 in Olama) (Table
191 2) suggesting that allelic frequencies within both populations were in accordance
192 with proportions expected under the Hardy-Weinberg equilibrium.

193 **Genetic diversity and demographic history**

194 The estimates of the overall nucleotide diversity (π = 0.0020 and π = 0.0016,
195 respectively, in Olama and Nyabessan) (Table 2) were within the range of average
196 values found in other African *Anopheles* species using RAD-seq approaches [8,22–
197 24]. Notorious demographic expansions have been described in natural populations
198 of this insect clade [25], and the values of π observed in *An. moucheti* likely reflect
199 the level genetic diversity of a population with large effective size. The great genetic
200 diversity of *An. moucheti* was also illustrated by the percentage of polymorphic sites.
201 Of the 6461 variant sites, 89.60% were polymorphic in Olama and 34.82% in
202 Nyabessan (Table 2). The difference observed between the two locations can be
203 related to the sample size (n = 19 in Nyabessan and n = 59 in Olama) or to
204 demographic particularities that persists between the two geographic sites despite a

205 massive gene flow. To infer the demographic history of *An. moucheti*, we examined
206 the Allele Frequency Spectrum (AFS), summarized as the distribution of the major
207 allele in one population. This approach represented a substitute to model-based
208 methods that provide powerful examinations of the history of genetic diversity by
209 modeling the AFS at genome-wide SNP variants, but that couldn't be implemented
210 here due to the lack of reference genome assembly. The frequency distribution of
211 the major allele *p* (Fig. 3) indicates that the majority of loci that are polymorphic
212 between Olama and Nyabessan are fixed within each population as shown by the
213 predominance of SNPs at frequencies equal to 1. Ranges of allele frequencies are
214 similar in Olama and Nyabessan (between 0.47 and 1 in Olama and between 0.34
215 and 1 in Nyabessan). These frequency ranges are expected for old populations at
216 equilibrium capable of accumulating high amount of genetic diversity.

217 **Polymorphic chromosomal inversions**

218 LD analyses at 1056 highly filtered SNPs revealed a globally low LD in the *An.*
219 *moucheti* genome (average genome-wide $r^2$ = 0.0149) as expected in highly
220 polymorphic populations with large effective size. We used LDna to cluster the LD
221 values and to identify Single Outlier Clusters (SOC) that can be associated with
222 distinct or multiple evolutionary phenomena in the *An. moucheti* history. Results
223 showed the presence of two independent LD blocks in our samples (Fig. 4).
224 Cytogenetic analyses have identified three polymorphic chromosomal inversions in
225 Cameroon samples [26] and the two LD clusters could well correspond to linked
226 SNPs within inversions. Studies on *Anopheles baimaii* [20] have shown that when
227 SOCs are generated by polymorphic inversions, SNPs within the SOCs can clearly

228 separate the three expected karyotypes (inverted homozygotes, heterozygotes and
229 uninverted homozygotes). We conducted downstream analyses with a PCA using
230 respectively 30 and 34 SNPs identified within the two SOCs. As shown on Fig,
231 although individuals were spread along three PCA axes, no distinct cluster could be
232 identified for the first SOC. likewise, except three outlier individuals, the second SOC
233 formed a single genetic cluster (Fig. 4B,C). The two LD clusters were apparently not
234 associated with polymorphic inversions, but caution must be used when
235 interpreting this result as evidence of absence of inversions in our samples because
236 other LDna analyses conducted on *An. funestus* and *An. gambiae* which contain tens
237 of polymorphic inversions did not systematically identified the segregating
238 karyotypes of key adaptive inversions (results not shown). However, based on our
239 findings and previous cytogenetic studies, we can reasonably think that inversion
240 polymorphisms are less important in the genome architecture *An. moucheti*
241 compared with that of other important African malaria vectors such as *An. gambiae*
242 and *An. funestus* whose populations often segregate along clines of multiple
243 polymorphic inversions in nature.
244

245 **Discussion**

246 We have analyzed genome-wide polymorphism and characterized some of the
247 baseline population genomic parameters in *An. moucheti*, an important malaria
248 vector in the African rainforest. We found very little differentiation among our
249 samples, with most of the genetic variation distributed within individuals. Although
250 a more substantial sampling will be necessary to fully appreciate the population
251 genetic structure of this species, our sampling likely reflects the current dynamic of
252 *An. moucheti* populations in Cameroon. It is worth mentioning that we have
253 surveyed a total of 28 locations across the country, some of which were known from
254 several past surveys to harbor *An. moucheti* populations [1–4,27,28], but we
255 confirmed the presence of the species in only 2 villages. Extant populations of *An.*
256 *moucheti* are distributed in patches of favorable habitats along river networks
257 where larval populations breed. Our results indicate that despite this apparent
258 fragmentation, connectivity and gene flow are high among population aggregates.
259 The weak population genetic structure of *An. moucheti* observed with genome-wide
260 markers corroborated results obtained with microsatellites and allozymes [2,3]. A
261 survey of eight microsatellite loci revealed that the highest $F_{ST}$ among Cameroonian
262 populations was as low as 0.003. Nevertheless, a substantial differentiation was
263 found between samples from different countries consistent with an isolation-by-
264 distance model [3]. It is clear that a deep sequencing of continental populations is
265 necessary to further clarify the status of these putative subpopulations that seem to
266 be endemic to different countries. But, samples collected at lower spatial scales like
267 ours are also relevant as they can allow robust inferences about ongoing selective

268 processes. We have found that signatures of selection are very rare in the genome of
269 *An. moucheti* populations from the Cameroonian rainforest. Populations remain
270 largely undifferentiated throughout the genome, with $F_{ST}$ values near zero across the
271 vast majority of segregating sites. Interestingly, polymorphic chromosomal
272 inversions that are the prime source of adaptive polymorphisms in many other
273 important malaria vectors seem to be less vital in the evolution of *An. moucheti*. The
274 characterization of chromosomal inversions with cytogenetic methods can be
275 laborious, particularly with regard to the identification of new inversions [26,29].
276 We have implemented a recently designed method that used Next Generation
277 Sequencing and LD estimates to identify paracentric inversions. Although the
278 method holds great promise in identifying inversions in genomes of nonmodel
279 species like *An. moucheti*, care should be taken when interpreting the results. So far,
280 only three paracentric polymorphic inversions have been discovered in *An. moucheti*
281 thanks to cytogenetics [26]. The ecological, behavioral or functional roles of these
282 inversion polymorphisms remain unknown. In keeping with cytogenetic
283 observations, LD analyses revealed the presence of only a few LD clusters that could
284 not be unambiguously associated with inversions. On the other hand, the low overall
285 LD observed across the genome reflected the significant genetic polymorphism that
286 seems to prevail in *An. moucheti* populations. This polymorphism translates into
287 exceptional levels of overall genetic diversity and very high percentage of
288 polymorphic sites that are in the range of values found in other mosquito species
289 undergoing a significant demographic expansion [8,24,25]. One of our main goals
290 was to know if the genetic diversity of *An. moucheti* can provide cues about its

291 extensive spatial distribution and can help to predict its environmental resilience. In
292 principle, evolutionary responses of species to human-induced or natural changes
293 rely largely on available heritable variation, which reflects the evolutionary
294 potential and adaptability to novel environments [30]. Therefore, the screening of
295 genome-wide variation is supposed to be a sensible approach that may provide a
296 generalized measure of evolutionary potential in species like *An. moucheti* for which
297 direct ecological, evolutionary or functional tests are impossible [31]. Our
298 population genomic analyses have depicted *An. moucheti* as a species with a great
299 genetic diversity and consequently a sustainable long-term adaptive resilience.
300 Implications of our findings in malaria epidemiology and control can be very
301 significant. First, *An. moucheti* is essentially endophilic and is particularly sensitive
302 to the principal measures currently employed to control malaria in Sub-Saharan
303 Africa such as the massive use of Insecticide Treated Nets (ITNs) and Indoor
304 Residual insecticide Spraying (IRS). For example, estimates of population effective
305 size in one village in Equatorial Guinea indicated that both mass distribution of ITNs
306 and IRS campaigns resulted in a decline of approximately 55% of *An. moucheti* [32].
307 However, the great genetic diversity and the massive gene flow we observed within
308 populations could easily enable *An. moucheti* to challenge population declines and
309 recover from shallow bottlenecks. Moreover, most insecticide resistance
310 mechanisms found in insects exploit standing genetic variation that allow to rapidly
311 respond to the evolutionary challenge by increasing the frequency of existing
312 variations rather than that of *de novo* mutations [33]. As a result, despite the current
313 sensitivity of *An. moucheti* to common insecticides, the significant amount of

314 standing genetic variation provides the species with a great potential to challenge
315 insecticides and other types of human-induced stress.
316
317 Recent advances in sequencing allow sensitive genomic data to be generated for
318 virtually any species [30]. However, the most important information we can obtain
319 from population resequencing approaches often depends on the availability and the
320 quality of genomic resources such as a well-annotated reference genome.
321 Nevertheless, the reduced genome sequencing strategy (RAD-seq) offers a cost-
322 effective strategy that can been used to effectively study the genetic variation in a
323 broad range of species from yeast to plants, insects, etc., in the absence of reference
324 genome. We have extended this approach to the study of the genetic structure of an
325 understudied epidemiologically important mosquito species. We have provided
326 both significant baseline population genomic data and the methodological validation
327 of one approach that should motivate further studies on this species and other
328 understudied anopheline mosquitoes lacking genomic resources.
329

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425

## Author contributions

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

## Competing interests

The authors declare that they have no competing interests.

432 **Tables**

433 **Table 1**: Information on *An. moucheti* samples included in this study.

| Sampling locations | Geographic coordinates | Sampling methods | | | Total |
|---|---|---|---|---|---|
| | | HLC-OUT | HLC-IN | LC | |
| Nyabessan | 2°24'00"N, 10°24'00"E | 21 | 15 | 1 | 37 |
| Olama | 3°26'00"N, 11°17'00"E | 30 | 31 | 0 | 61 |
| Total | | | | | 98 |

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

434

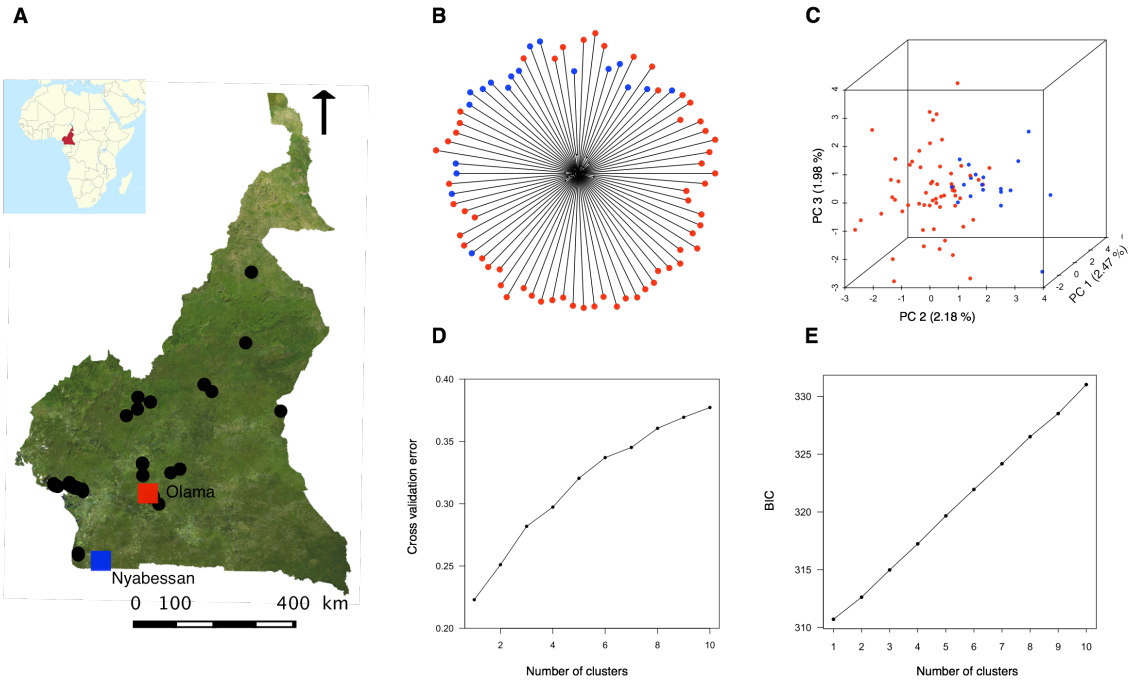
435 **Table 2**: Population genomic parameters based on 6461 variant sites reflecting the

436 genetic diversity and conformity to Hardy-Weingberg equilibrium.

| | n | Sites | Observed heterozygosity | $F_{IS}$ | π | % polymorphic sites |
|---|---|---|---|---|---|---|
| **Variant positions** | | | | | | |
| **Olama** | 54.12 | 6 461 | 0.0445 | 0.0631 | 0.0505 | 89.60 |
| **Nyabessan** | 16.78 | 6 461 | 0.0334 | 0.0372 | 0.0402 | 34.82 |
| **All positions** | | | | | | |
| **Olama** | 54.47 | 165 975 | 0.0017 | 0.0025 | 0.0020 | 3.49 |
| **Nyabessan** | 16.86 | 165 975 | 0.0013 | 0.0014 | 0.0016 | 1.36 |

## Figures

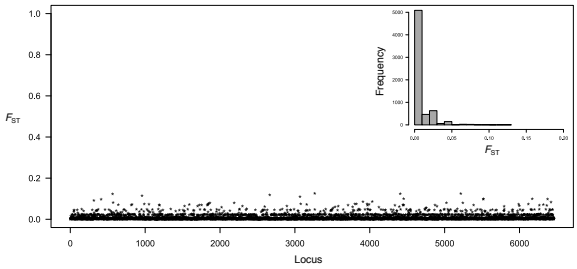
**Figure 1**: Relationship between *An. moucheti* individuals from Olama and Nyabessan. (A) Map of the study site showing both the locations surveyed (small black dots) and the two villages (large red and blue squares) where *An. moucheti* samples were collected. (B) and (C) Absence of genetic structure within populations illustrated by neighbor-joining and PCA. The percentage of variance explained by each PCA axis is indicated. (D) and (E) Plots of the ADMIXTURE cross-validation error and the Bayesian Information Criterion (BIC) (DAPC) as a function of the number of genetic clusters indicating that k = 1. The lowest BIC and CV error indicate the suggested number of clusters.



450 **Figure 2**: Frequency distribution of $F_{ST}$ between Olama and Nyabessan across 6461

451 SNP loci and plot of these $F_{ST}$ values along arbitrary positions in the genome.

452



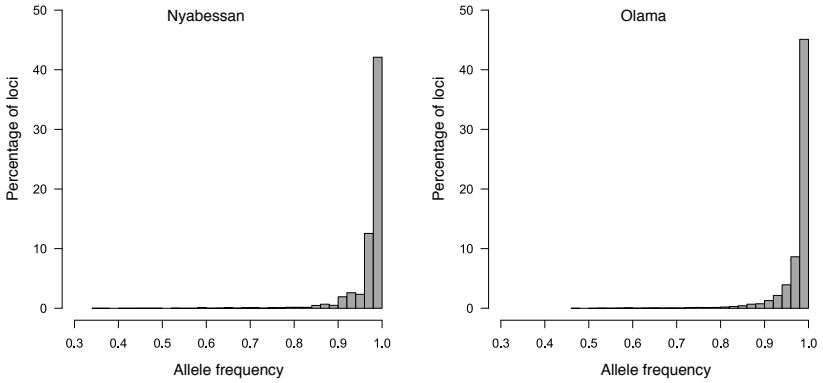

453

454 **Figure 3**: Allele Frequency Spectrum for 6461 SNP loci in Nyabessan and Olama

455 populations. The x-axis presents the frequency of the major allele and the y-axis the

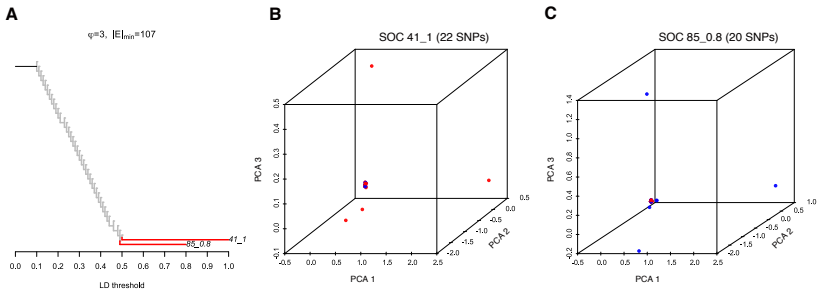
456 frequency distribution of loci in each class of the major allele frequency.

457




458

459 **Figure 4**: LDna analyses on 1056 SNPs showing the presence of two Single Outlier
460 Clusters (SOCs) of linkage disequilibrium in *An. moucheti*. (A) Graph presenting the
461 results obtained with values of the two parameters: $\varphi$ (which controls when clusters
462 are defined as outliers) and $|E|_{\min}$, the minimum number of edges required for a LD
463 cluster to be considered as an outlier, indicated on top. LD thresholds are shown on
464 the x-axis. (B) and (C) PCA indicating the population genetic structure inferred from
465 SNPs within the two SOCs (red: Olama; blue: Nyabessan).
466

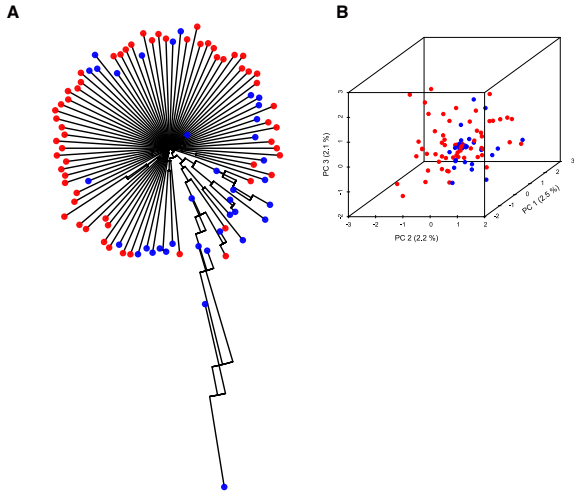


467

468 **Supplemental Material**

469 **Figure S1**: Selection of individuals included in final analyses based on the average
470 per individual sequencing coverage. (A) and (B) Neighbor-joining tree and PCA
471 indicating spurious population structure due to individuals with low sequencing
472 coverage in Olama (red) and Nyabessan (blue).

473

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