POLLUTANTS AND INSECTICIDES DRIVE LOCAL ADAPTATION IN AFRICAN MALARIA MOSQUITOES

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ABSTRACT

Urbanization presents unique environmental challenges to human commensal species. The Afrotropical *Anopheles gambiae* complex contains a number of synanthropic mosquito species that are major vectors of malaria. To examine ongoing cryptic diversification within the complex, we performed reduced representation sequencing on 941 mosquitoes collected across four ecogeographic zones in Cameroon. We find evidence for clear subdivision within *An. coluzzii* and *An. gambiae s.s.* – the two most significant malaria vectors in the region. Importantly, in both species rural and urban populations of mosquitoes were genetically differentiated. Genome scans of cryptic subgroups reveal pervasive signatures of selection centered on genes involved in xenobiotic resistance. Notably, a selective sweep containing eight detoxification enzymes is unique to urban mosquitoes that exploit polluted breeding sites. Overall, our study reveals that anthropogenic environmental modification is driving population differentiation and local adaptation in African malaria mosquitoes with potentially significant consequences for malaria epidemiology.

INTRODUCTION

Natural selection can drive local adaptation, increasing mean individual fitness and promoting biological diversification (Nosil, et al. 2005; Hereford 2009). Contemporary anthropogenic alteration of the landscape may be increasing pressure for local adaptation in diverse taxa (Gaston 2010). For example, the rise of urban centers over the past two centuries has presented unique challenges to human-commensal species, often necessitating the rapid evolution of resistance to pollutants and pesticides (Pelz, et al. 2005; Song, et al. 2011; Davies, et al. 2012). Successful local adaptation requires that selection overcome the homogenizing effect of gene flow from nearby populations (Kawecki and Ebert 2004). Theoretical simulations suggest that such divergence with gene flow can occur under a range of conditions (Berdahl, et al. 2015), although the most likely genomic distribution of the underlying adaptive variants remains unclear (Le Corre and Kremer 2012; Tiffin and Ross-Ibarra 2014). Studies of populations in the earliest stages of ecological divergence should help elucidate the conditions needed for local adaptation and relevant targets of natural selection (Feder, et al. 2013).

The Afrotropical *Anopheles gambiae* complex is a group of at least nine isomorphic mosquito species exhibiting varying degrees of geographic and reproductive isolation (White, et al. 2011; Coetzee, et al. 2013; Crawford, et al. 2014). Owing to the critical role its members play in sustaining malaria transmission, a wealth of genomic data exists on the complex including whole genome assemblies for most of the species (Holt, et al. 2002; Fontaine, et al.

58 2014). Because radiation of the complex began ~1.85 mya, interspecific genetic
59 comparisons will yield little insight into the establishment of divergence with gene
60 flow. However, both ecological and genetic evidence suggest that contemporary
61 local adaptation and diversification is occurring within *Anopheles gambiae s.s.*
62 (hereafter *An. gambiae*) and *Anopheles coluzzii*, two of the most widespread and
63 important vectors of malaria within the complex (Slotman, et al. 2007; Wang-
64 Sattler, et al. 2007; Lee, et al. 2013b; Caputo, et al. 2014). Up to now, shallow
65 sampling and/or or the use of low-resolution genetic markers limited the ability to
66 delineate new cryptic subgroups within either species.

67 We genotyped 941 mosquitoes collected from diverse environments in
68 Cameroon at >8,000 SNPs and find strong evidence for ongoing diversification
69 within both *An. gambiae* and *An. coluzzii*. In total, the two species harbor seven
70 cryptic subgroups distributed along a continuum of genomic differentiation. While
71 *An. gambiae* exhibits relatively high levels of panmixia, we did identify an ecotype
72 associated with intense suburban agriculture and a second subgroup that
73 appears partially reproductively isolated but exhibits no obvious
74 ecological/geographical distinctions. In contrast, *An. coluzzii* is separated into
75 multiple ecotypes exploiting different regional-scale habitats, including highly
76 urbanized landscapes. In most cryptic subgroups, selective sweeps contain an
77 excess of detoxification enzymes and insecticide resistance genes, suggesting
78 that human activity mediates spatially varying natural selection in both species.
79 The extensive population structure within both species represent an additional
80 challenge to vector control strategies. Moreover, ongoing local adaptation and

cryptic diversification of *Anopheles* species in human-dominated environments may contribute to increase malaria transmission.

RESULTS

Identification of An. gambiae s.l. *sibling species*

We performed extensive sampling of human-associated *Anopheles* across the main ecological zones in the central African country of Cameroon (Table S1) with the objective to collect diverse populations belonging to the four species of the *An. gambiae* complex that are present in the country (Simard et al. 2009). As recently shown (Riehle et al. 2011), certain cryptic subgroups can be overlooked when sampling is focused on the collection of only one type of population. Therefore, to maximize the chances that our samples best represent the genetic diversity within each species and to identify cryptic groups, we used several sampling methods (Service 1993) to collect both larvae and adult populations. In Addition, populations of *An. gambiae* and *An. coluzzii* segregate along urbanization gradients, which seem to be the most important driver of ecological divergence in the forest zone (Kamdem et al. 2012). To validate this hypothesis and to investigate the genomic targets of local adaptation in urban environments, we surveyed several neighborhoods representing the urban and suburban ecotypes in the two biggest cities of the forest area: Douala and Yaoundé (Figure S1).

To investigate the genetic relatedness among individuals and to detect any cryptic populations, we subjected all 941 mosquitoes that were morphologically identified as *An. gambiae s.l.* to population genomic analysis.

104 Individual mosquitoes were genotyped in parallel at a dense panel of markers
105 using double-digest restriction associated DNA sequencing (ddRADseq), which
106 enriches for a representative and reproducible fraction of the genome that can be
107 sequenced on the Illumina platform (Peterson, et al. 2012).

108 After aligning ddRADseq reads to the *An. gambiae* reference genome, we
109 used STACKS to remove loci present in <80% of individuals, leaving 8,476 SNPs
110 (~1 SNP every 30kb across the genome) for population structure inference
111 (Catchen, et al. 2011; Catchen, et al. 2013). First, we performed principal
112 component analysis (PCA) on genetic diversity across all 941 individuals (Figure
113 1A). The top three components explain 28.4% of the total variance and group
114 individuals into five main clusters. Likewise, a neighbor-joining (NJ) tree, based
115 on Euclidian distance of allele frequencies, shows five distinct clades of
116 mosquitoes (Figure 1B). We hypothesized that these groups at least partially
117 correspond to the four sibling species – *An. gambiae*, *An. coluzzii*, *An.*
118 *arabiensis*, and *An. melas* – known to occur in Cameroon. To confirm, we typed
119 a subset of 288 specimens using validated species ID PCRs and found that each
120 cluster comprised a single species (Scott, et al. 1993; Santolamazza, et al.
121 2004). In agreement with previous surveys (Wondji, et al. 2005; Simard, et al.
122 2009), our collections indicate that the brackish water breeding *An. melas* is
123 limited to coastal regions, while the arid-adapted *An. arabiensis* is restricted to
124 the savannah. In contrast, *An. gambiae* and *An. coluzzii* are distributed across
125 the four eco-geographic zones of Cameroon (Figure 1D). Lee and colleagues
126 (Lee, et al. 2013a) recently reported frequent bouts of hybridization between *An.*

127 *gambiae* and *An. coluzzii* in Cameroon. While both the PCA and NJ trees clearly
128 separate the two species, the PCA does show intermixing of some rare
129 individuals consistent with semi-permeable species boundaries.

130 In support of population structuring below the species level, Bayesian
131 clustering analysis with fastSTRUCTURE (Raj, et al. 2014) finds that 7
132 population clusters (*k*) best explain the genetic variance present in our sample
133 set (Figure 1C, Figure S2). Indeed, grouping of samples within *An. gambiae* and
134 *An. coluzzii* clades suggests that additional subdivision may exist within each
135 species (Figure 1A, 1B). Ancestry plots further support inference from the PCA
136 and NJ tree: at least two subgroups compose *An. coluzzii* and admixture is
137 present within *An. gambiae*, while *An. arabiensis* and *An. melas* are panmictic
138 (Figure 1C, Figure S2). Riehle et al. 2011 recently discovered a cryptic subgroup
139 by comparing indoor and outdoor fauna from the same village in Burkina Faso.
140 Visual inspections of our PCA, NJ and fastSTRUCTURE clustering results do not
141 indicate any genetic subdivision based on the collection methods or the
142 developmental stage. To explicitly test for the effects of the sampling methods
143 and the geographic origin of samples on the genetic variance among individuals,
144 we applied a hierarchical Analysis of Molecular Variance (AMOVA) (Excoffier et
145 al. 1992). This methods partition the genetic variance among individuals in order
146 to quantify the effects of several variables taken at different hierarchical levels on
147 the genetic diversity. We noted that the large majority of the genetic variation was
148 attributable to differences among individuals (86.3% ($p < 0.001$) in *An. coluzzii*
149 and 90.4% in *An. gambiae s.s.* ($p < 0.001$)). However, the geographic origin of

150 individuals retains a significant component of the genetic variation in the two
151 species. Respectively 13.2 % ($p < 0.001$) and 9.4% ($p < 0.001$) of the genetic
152 variation were partitioned across collection regions nested in sample type in *An.*
153 *gambiae s.s.* and *An. coluzzii*. The amount of variance due to the types of
154 sample was very low and not significant (less than 1%, $p = 0.29$ for *An. gambiae*
155 *s.s.* and $p = 0.20$ for *An. coluzzii*) implying that – as suggested by PCA, NJ and
156 fastSTRUCTURE analyses – no genetic structuring based on microhabitats or
157 temporal segregations is apparent in both species in Cameroon.
158
159 *Cryptic Population Structure within An. gambiae s.s.*
160 To further resolve the population structure within 357 *An. gambiae*
161 specimens, we performed population genetic analysis with a set of 9,345 filtered
162 SNPs. Using a combination of PCA, NJ trees, and ancestry assignment, we
163 consistently identify three distinct subgroups within *An. gambiae* (Figure 2, Figure
164 S2). The first and largest subgroup (termed *GAM1*) comprises the vast majority
165 of all *An. gambiae* specimens including individuals collected in all four eco-
166 geographic regions (Table S1). A total of 17 individuals make up a second small
167 subgroup (termed *GAM2*). Interestingly, individuals assigned to this cluster
168 include both larvae and adults collected in 3 different villages spread across 2
169 eco-geographic regions. In the absence of any obvious evidence of niche
170 differentiation between *GAM1* and *GAM2,* it is unclear what is driving and/or
171 maintaining divergence between the two sympatric subgroups. Specimens
172 collected from Nkolondom, a suburban neighborhood of Yaoundé where larval

173 sites associated with small-scale agricultural irrigation are common (Nwane, et
174 al. 2013; Tene, et al. 2013), form a genetically distinct third subgroup (termed
175 *Nkolondom*) that appears to be a locally-adapted ecotype.
176
177 *Cryptic Population Structure within An. coluzzii*
178 To examine population structure within 521 *An. coluzzii* specimens, we
179 utilized 9,822 SNPs that passed stringent filtration. All analyses show a clear split
180 between individuals from the northern savannah region and the southern three
181 forested regions of Cameroon (Coastal, Forest, Forest-Savannah) (Figure 3A-C).
182 In principle, the north-south structuring could be caused solely by differences in
183 chromosome 2 inversion frequencies, which form a cline from near absence in
184 the humid south to fixation in the arid north. However, we find SNPs from all five
185 chromosomal arms consistently separate northern and southern mosquitoes,
186 indicating a substantial genome-wide divergence between the two populations
187 (Figure S3).
188 Southern populations of *An. coluzzii* were collected from three different
189 areas: Douala (the largest city of Cameroon), Yaoundé (the second largest city)
190 and the rural coastal region. PCA, NJ trees, and fastSTRUCTURE show clear
191 clustering of southern samples by collection site (Figure 3D-F). Mosquitoes from
192 Douala, situated on the coastal border, contain a mixture of urban and coastal
193 polymorphisms as illustrated by their intermediate position along PC3 (Figure
194 3D). Despite considerable geographic segregation, clusters are not fully discrete,
195 likely owing to substantial migration between the three sites. Taken together, the

196 data suggest a dynamic and ongoing process of local adaptation within southern
197 *An. coluzzii*. In contrast, no similar geographic clustering is observed in northern
198 populations (Figure S2, S4). Light variations can occur between genetic
199 clustering patterns suggested by different methods. We have only considered
200 subdivisions that were consistent across the three methods we employed. For
201 example, PCA showed that a few individuals from Yaoundé were relatively
202 detached from the main cluster, but this putative subdivision was not supported
203 by the NJ tree and fastSTRUCTURE analyses (Figure 3D-F). We have therefore
204 treated the Yaoundé subgroup as a single population. All populations described
205 as "urban" were collected from the most urbanized areas of the city, which are
206 characterized by a high density of built environments as described in Kamdem et
207 al. 2012.
208
209 *Relationships Between Species and Subgroups*
210 Population genomic analysis identified four different *An. gambiae* s.l.
211 species present within our samples. Within *An. gambiae* and *An. coluzzii* we
212 identified seven potential subgroups with apparently varying levels of isolation.
213 To further explore the relationships between different populations, we built an
214 unrooted NJ tree using pairwise levels of genomic divergence (F_{ST}) between all
215 species and subgroups (Figure 4, Table S2). As previously observed in a
216 phylogeny based on whole genome sequencing (Fontaine, et al. 2014), we find
217 that *An. melas* is highly divergent from all other species (F_{ST} ~ 0.8), while *An.*
218 *arabiensis* shows intermediate levels of divergence (F_{ST} ~ 0.4) from *An. gambiae*

219 and *An. coluzzii*. As expected, the sister species *An. gambiae* and *An. coluzzii*
220 are more closely related to each other (F_{ST} ~ 0.2) than any other species. When
221 examining differentiation between subgroups within *An. coluzzii*, we find that the
222 southern and northern subgroups are highly divergent (F_{ST} > 0.1), while
223 differentiation between local ecotypes within the south is much lower (F_{ST} <
224 0.04). The *An. gambiae* subgroups *GAM1* and *GAM2* are highly diverged (F_{ST} ~
225 0.1) from each other suggesting genuine barriers to gene flow despite sympatry,
226 while the suburban ecotype from Nkolondom shows a low level of divergence
227 from *GAM1* (F_{ST} ~ 0.05), characteristic of ongoing local adaptation. In sum, we
228 find a gradient of differentiation between species and subgroups ranging from
229 complete (or nearly complete) reproductive isolation down to the initial stages of
230 divergence with gene flow. To further examine the degree of isolation of
231 subgroups within species, we assessed the reductions in observed
232 heterozygosity with respect to that expected under Hardy–Weinberg Equilibrium
233 among *An. coluzzii* and *An. gambiae s.s.* populations, by computing the average
234 Wright's inbreeding coefficient, F_{IS}, across genome-wide SNPs. Values of F_{IS}
235 close to 1 indicate a deviation from Hardy–Weinberg Equilibrium and the
236 existence of cryptic subdivisions while F_{IS} close to 0 suggest that there are no
237 barriers to gene flow. In spite of the strong population genetic structure observed
238 within *An. coluzzii* and *An. gambiae*, we found surprisingly low genome-wide F_{IS}
239 values (less than 0.0003, $p < 0.005$) in both species, suggesting a lack of
240 assortative mating. Overall, in *An. gambiae* and *An. coluzzii*, ongoing local

241 adaptation and genetic differentiation are parallel to high levels of admixture and
242 extensive shared polymorphisms among individuals.
243
244 *Using genome scans to identify selective sweeps*
245 To find potential targets of selection within subgroups we performed scans
246 of nucleotide diversity (θ_w, θ_π) and allele frequency spectrum (*Tajima's D*) using
247 non-overlapping 150-kb windows across the genome. Scans of θ_w, θ_π, *and*
248 *Tajima's* D were conducted by importing aligned, but otherwise unfiltered, reads
249 directly into ANGSD, which uses genotype likelihoods to calculate summary
250 statistics (Korneliussen, et al. 2014). Natural selection can increase the
251 frequency of an adaptive variant within a population, leading to localized
252 reductions in genetic diversity as the haplotype containing the adaptive variant(s)
253 sweeps towards fixation (Maynard Smith and Haigh 1974; Tajima 1989).
254 Selective processes can also promote the coexistence of multiple alleles in the
255 gene pool of a population (balancing selection). Thus, genomic regions harboring
256 targets of recent selection should exhibit extreme values of diversity and allele
257 frequency spectra relative to genome-wide averages (Storz 2005).
258 We also performed genome scans using both a relative (F_{ST}) and absolute
259 (d_{xy}) measure of divergence calculated with STACKS and *ngsTools*, respectively.
260 If positive selection is acting on alternative haplotypes of the same locus in two
261 populations, values of F_{ST} and d_{xy} should increase at the target of selection.
262 Whereas spatially varying selection that acts on one population, but not the
263 other, should produce a spike in F_{ST} between populations and no change in d_{xy}.

264 Finally, parallel selection on the same haplotype in two populations should lead
265 to a decrease in both metrics (Cruickshank and Hahn 2014). For both diversity
266 and divergence scans we used a maximum of 40 mosquitoes per population,
267 prioritizing individuals with the highest coverage in populations where sample
268 size exceeded 40. In contrast to Tajima's *D* and F_{ST}, the genome-wide
269 distribution of d_{xy} and nucleotide diversity in 150-kb sliding windows yielded
270 relatively noisy patterns (Figure 5 and 6). As a result, we based the identification
271 of signatures of selection primarily on outliers of Tajima's *D* and F_{ST}. Estimates of
272 d_{xy} and nucleotide diversity were used only to confirm genomic locations that
273 were pinpointed as candidate selective sweep on the basis of values of Tajima's
274 *D* and F_{ST}. Precisely, genomic regions were considered as targets of selection if
275 they mapped to significant peaks or depressions of diversity and d_{xy}, and their
276 values of F_{ST} and Tajima's *D* were among the top 1% of the empirical distribution
277 in at least one population. Significantly negative values of Tajima's *D* relative to
278 the genome-wide average suggest an increase in low-frequency mutations due
279 to negative or positive selection whereas significantly positive values of Tajima's
280 *D* indicate a balancing selection.
281
282 *Targets of Selection within An. gambiae subgroups*
283 Our estimates of genome-wide diversity levels (Table S3) within *An.*
284 *gambiae* subgroups are comparable to previous estimates based on RAD
285 sequencing of East African *An. gambiae* s.l. populations (O'Loughlin, et al. 2014).
286 As expected, the large *GAM1* population harbors more genetic diversity than the

287 apparently rare *GAM2* population or the geographically restricted Nkolondom
288 ecotype (Table S3, Figure 5A-C). *Tajima's D* is consistently negative across the
289 entire genome of all three subgroups, indicating an excess of low-frequency
290 variants that are likely the result of recent population expansion (Figure 5A-C)
291 (Tajima 1989). Indeed, demographic models infer relatively recent bouts of
292 population expansion in all three subgroups (Table S4).

293 Genome scans of each subgroup reveal genomic regions that show
294 concordant dips in diversity and allele frequency spectrum consistent with recent
295 positive selection (highlighted in Figure 5 A-C). Based on the 1% cutoff of
296 Tajima's *D* (upper and lower bounds) and F_{ST}, we identified 4 candidate regions
297 exhibiting strong signatures of selection. It should be noted that due to the
298 reduced representation sequencing approach we used, our analysis is
299 necessarily conservative, highlighting only clear instances of selection, which are
300 likely both recent and strong (Tiffin and Ross-Ibarra 2014). An apparent selective
301 event on the left arm of chromosome 2 near the centromere is found in all
302 populations. This selective sweep is characterized by a prominent depression of
303 Tajima's *D* (~1.5Mb in width) and contains ~80 genes including the pyrethroid
304 knockdown resistance gene (*kdr*). Although it is difficult to precisely identify the
305 specific gene that has been influenced by selection in this region, the voltage-
306 gated sodium channel gene (*kdr*) involved in insecticide resistance in insects,
307 which is a pervasive target of selection in *Anopheles* mosquitoes (Clarkson et al.
308 2014; Norris et al. 2015) represents the strongest candidate in our populations.
309 Interestingly, the drop in Tajima's *D* in the putative *kdr* sweep is sharpest in the

310 Nkolondom population, which suggests that insecticide resistance is shaping the
311 genomic differentiation and local adaptation in *An. gambiae*. Indeed, Nkolondom
312 is a suburban neighborhood of Yaoundé where larvae can be readily collected
313 from irrigated garden plots that likely contain elevated levels of pesticides
314 directed at agricultural pests (Nwane, et al. 2013; Tene, et al. 2013). The *kdr*
315 allele of *para* confers knockdown resistance to pyrethroids and selective sweeps
316 in the same genomic location have been previously identified in many *An.*
317 *gambiae* s.l. populations (Donnelly, et al. 2009; Lynd, et al. 2010; Jones,
318 Liyanapathirana, et al. 2012; Clarkson, et al. 2014; Norris, et al. 2015). At the *kdr*
319 sweep, we observe contrasting patterns in local values of d_{xy} and F_{ST} (Figure 5D-
320 E). In both the *GAM1-GAM2* and *GAM1-Nkolondom* comparisons, d_{xy} dips while
321 local values of F_{ST} actually increase. While not definitive, the significant drop in
322 d_{xy} suggests that the same resistant haplotype is sweeping through each
323 population. Localized increases in F_{ST} could owe to differences in *kdr* allele
324 frequencies between populations; despite parallel selection, the sweep may be
325 closer to fixation in certain populations relative to others, perhaps due to
326 differences in selection intensity.

327 Another region exhibiting consistent signatures of selection in all
328 populations is found around the centromere on the X chromosome. Functional
329 analyses of gene ontology (GO) terms (Table S5) revealed a significant
330 representation of chitin binding proteins in this region (p = 1.91e-4). A strong
331 genetic divergence among the three subgroups of *An. gambiae* characterized by
332 significant F_{ST} and d_{xy} peaks is also observed at ~30 Mb and ~40 Mb on

333 chromosome 3R. Positive outliers of both Tajima's *D* and nucleotide diversity
334 suggest that this genetic divergence is due to balancing selection on multiple
335 alleles among populations. Functional analyses of GO terms indicate that the
336 region at ~40 Mb on 3R is enriched in cell membrane proteins, genes involved in
337 olfaction and epidermal growth factors (EGF) genes (Table S5). Finally, a striking
338 depression in Tajima's *D* supported by a marked dip in nucleotide diversity
339 occurs on chromosome 2L from ~33-35 Mb in the Nkolondom population
340 exclusively (Figure 5A-C). Despite the lack of genetic differentiation, this region –
341 enriched in six EGFs (Table S5) – is probably a recent selective sweep, which
342 could facilitate larval development of this subgroup in pesticide-laced agricultural
343 water.
344
345 *Targets of Selection within An. coluzzii subpopulations*
346 As above, we used diversity, allele frequency spectra and genetic
347 differentiation metrics to scan for targets of selection in the four subgroups of *An.*
348 *coluzzii*. Overall, genetic diversity is higher in the northern savannah population
349 than either of three southern populations, which all exhibit similar levels of
350 diversity (Table S3). Just as in *An. gambiae*, all subgroups have consistently
351 negative Tajima's *D* values confirming demographic models of population
352 expansion (Table S4). Based on the 1% threshold of Tajima's *D* and F_{ST}, we
353 found 5 putative selective sweeps in *An. coluzzii* populations including the *kdr*
354 region, the sweep on the X chromosome, and the two hot spots of balancing
355 selection detected on the chromosome 3R in *An. gambiae* (Figure 6A-D). The

356 fifth putative selective sweep characterized by a sharp drop in both diversity and
357 Tajima's *D* occurs on 3R from ~28.5-29.0 Mb, with the decline being more
358 significant in urban populations. Geographical limitation of the sweep to urban
359 mosquitoes strongly suggests it may contain variant(s) that confer adaptation to
360 extreme levels of anthropogenic disturbance. Indeed, this genomic region
361 harbors a cluster of both Glutathione S-transferase (*GSTE1-GSTE7*) and
362 cytochrome P450 (*CYP4C27*, *CYP4C35*, *CYP4C36*) genes, and functional
363 analyses of GO terms reveals an overrepresentation of terms containing
364 "Glutathione S-transferase" (Table S5, p = 5.14e-10). Both the *GSTE* and
365 cytochrome P450 gene families are known to confer metabolic resistance to
366 insecticides and pollutants in mosquitoes (Enayati, et al. 2005; David, et al. 2013;
367 Nkya, et al. 2013). In particular, *GSTE5* and *GSTE6* are intriguing candidate
368 targets of selection as each is up-regulated in highly insecticide resistant *An.*
369 *arabiensis* populations that recently colonized urban areas of Bobo-Dioulasso,
370 Burkina Faso (Jones et al. 2012).

371 As in *An. gambiae*, we also detected multiple regions that could be targets
372 of selection, but were less well supported because only one of the metrics (F_{ST} or
373 Tajima's *D*) was above the 99th percentile of the empirical distribution. We found
374 ~30 regions where significant F_{ST} peaks were not correlated to exceptional
375 values of Tajima's *D* and vice versa. This included at least 10 hotspots of F_{ST}
376 clustered within the 2L*a* inversion, which segregates between forest and
377 savannah populations (Figure 6H). Most notably, at ~25 Mb on 2L, a region
378 centered on the resistance to dieldrin (*rdl*) locus, large dips in Tajima's *D* are

379 evident in all southern groups. In the northern savannah population, a
380 pronounced dip in diversity occurs at this putative sweep, but Tajima's *D* stays
381 constant. This region contains ~40 genes, but just as with the *kdr* gene, the *rdl*
382 locus is arguably the prime candidate target of selection. This gene plays a key
383 role in insensitivity to insecticides (Ffrench-Constant et al. 2004), and studies
384 have confirmed the presence of footprints of selection around this locus in *An.*
385 *gambiae* s.l. populations (Lawniczak et al. 2010; Crawford et al. 2015).

386 The increased use of pesticides/insecticides in agriculture and vector
387 control imposes an unprecedented adaptive challenge to mosquito populations
388 (Bøgh et al. 1998; Moiroux et al. 2012; Mwangangi et al. 2013; Clarkson et al.
389 2014; Norris et al. 2015). As a result, both selection and adaptive introgression
390 are acting at the scale of a few decades around loci that provide selective
391 advantage against pesticides (Clarkson et al. 2014; Norris et al. 2015). Our
392 findings indicate that the genetic response to this challenge is also spreading
393 across multiple loci leaving sharp signatures of selection around clusters of
394 detoxification enzymes and major insecticide resistance genes in *An. gambiae*
395 and *An. coluzzii* populations. As expected, *An. coluzzii* populations that are
396 exposed to particularly high levels of insecticides/pollutants in human-dominated
397 environments (Kamdem et al. 2012; Fossog Tene et al. 2013; Tene Fossog et al.
398 2013) are more enriched in genomic regions bearing signatures of human-driven
399 selection. Both relative and absolute divergences between populations at the
400 three selective sweeps involved in xenobiotic resistance reflect the spatial
401 variation of selection along gradients of anthropogenic disturbance. In particular,

402 the *kdr* locus exhibits minimal divergence in all pairwise comparisons, suggesting
403 that the same resistance haplotype is under selection in each population (Figure
404 6E-H). In contrast, the region surrounding the *rdl* gene shows low F_{ST} and a
405 pronounced dip in d_{xy} between all southern populations, confirming that the same
406 haplotype is sweeping through these three populations. However, differentiation
407 between southern and northern populations at *rdl* may be obscured by the high
408 divergence between alternative arrangements of the 2L*a* inversion. Finally, the
409 urban-centric GSTE/CYP450 sweep on 3R shows a peak in F_{ST} between
410 Yaoundé and Coastal mosquitoes and minimal change in d_{xy} – a pattern
411 consistent with local adaptation. Comparisons between Douala and Coastal
412 populations show a more moderate increase in F_{ST}, presumably due to high rates
413 of mosquito migration between these nearby sites. The slight bump in F_{ST} is
414 coupled to a large dip in d_{xy} indicative of an ongoing, shared selective sweep
415 between the two cities.

416 To further explore the 3R GSTE/CYP450 sweep, we reconstructed
417 haplotypes for all 240 *An. coluzzii* southern chromosomes across the 28 SNPs
418 found within the sweep. In the Yaoundé population, a single haplotype is present
419 on 44 out of 80 (55%) chromosomes (all grey SNPs), while an additional 11
420 haplotypes are within one mutational step of this common haplotype (Figure 7A).
421 In Douala, the same haplotype is the most common, but present at a lower
422 frequency (31%) than in Yaoundé (Figure 7B). Strikingly, this haplotype is found
423 on only 6/80 (7.5%) coastal chromosomes (Figure 7C). The overall low
424 nucleotide variation and high frequency of a single haplotype in Yaoundé is

425 consistent with positive selection acting on a de novo variant(s) to generate the
426 3R GSTE/CYP450 sweep. Less intense selection pressure in Douala, and
427 particularly the Coast, would explain the markedly higher haplotype diversity in
428 these two populations relative to Yaoundé. It is also possible that Douala
429 mosquitoes experience similar selection pressures to Yaoundé mosquitoes, but
430 frequent migrant haplotypes from the nearby rural Coast populations decrease
431 the efficiency of local adaptation. Importantly, multiple population genomic
432 analyses of the same 28 SNPs (Figure 7D-F) mirror results of the haplotype
433 analysis, confirming that haplotype inference did not bias the results. In sum, we
434 hypothesize that divergence in xenobiotic levels between urban and rural larval
435 habitats is the main ecological force driving spatially variable selection at this
436 locus.
437

438 DISCUSSION

439 *Population genetic structure and cryptic subdivisions within the* An. gambiae
440 *complex*

441 The *Anopheles gambiae* complex, as a model of adaptive radiation with a
442 puzzling evolutionary history, has been recognized as a unique portal into the
443 genetic architecture of ecological speciation (Coluzzi et al. 2002; Ayala and
444 Coluzzi 2005). This system has however been refractory to traditional genetic
445 mapping methods, due mainly to the lack of observable phenotypes that
446 segregate between populations. Recently, patterns of genomic divergence have
447 started to be dissected thanks to the application of high-throughput sequencing
448 and genotyping methods, and significant insights have been gained into the
449 genomic targets of selection among populations at continental scale (Lawniczak
450 et al. 2010; Neafsey et al. 2010; White et al. 2011). Here we have applied a
451 population genomic approach to investigate the genomic architecture of selection
452 at the scale of one country. We showed that reduced representation sequencing
453 of 941 *An. gambiae* s.l. collected in or near human settlements in 33 sites
454 scattered across Cameroon facilitated rapid identification of known sibling
455 species and revealed multiple instances of novel cryptic diversification within *An.*
456 *gambiae* and *An. coluzzii*. This result is opposite to that found in East Africa,
457 where RADseq markers revealed no population genetic structure within species
458 of the *An. gambiae* complex (O'Loughlin et al. 2014). Historically, West African
459 populations have a greater tendency to be differentiated (Coluzzi et al. 1985;
460 Coluzzi et al. 2002), and the presence of cryptic subdivisions within *An. coluzzii*

461 has already been suspected in Cameroon (Wondji et al. 2005; Slotman et al.
462 2007). As revealed by genome-wide SNPs, these subdivisions result in at least
463 three genetically distinct clusters: a strongly differentiated population confined to
464 the arid savannah area, a coastal subgroup presumably adapted to tolerate high
465 concentrations of salt (Tene Fossog et al. 2015) and a cluster encompassing
466 urban populations that are known to thrive in breeding sites containing high
467 levels of organic waste and present a more complex insecticide resistance profile
468 (Antonio-Nkondjio et al. 2011; Fossog Tene et al. 2013; Tene Fossog et al. 2013;
469 Antonio-Nkondjio et al. 2015). The modelling of ecological niches of *An. gambiae*
470 s.l. in Cameroon predicts that favourable habitats of *An. coluzzii* populations are
471 fragmented and much more marginal landscapes in contrast to *An. gambiae*,
472 which occupies a broader environmental niche across the country (Simard et al.
473 2009). In line with this prediction, genome-wide SNPs indicate a weak genetic
474 differentiation and high genetic diversity in *An. gambiae* over large geographic
475 areas. Nevertheless, despite this broad geographic connectivity and extensive
476 gene flow among populations, ongoing local adaption results in the emergence of
477 geographic clusters in suburban areas.

478 It has been hypothesized that the actual number and the diversity of
479 malaria vector species across the African continent are largely underestimated
480 because of the limited power of morphological and genetic markers employed so
481 far (Stevenson et al. 2012). As a result, genome-wide studies and extensive
482 sampling are supposed to lead to the discovery of unknown species. Among the
483 941 *An. gambiae* s.l. we sequenced, we instead found a clear match between the

484 species identified by both morphological observations and PCR of the ribosomal
485 DNA and those suggested by thousands of SNPs scattered throughout the
486 genome. Furthermore, the intensive use of insecticide-treated bed nets has
487 triggered complex behavioural adaptations and changes in species distribution
488 that may ultimately lead to splits and the creation of cryptic populations within
489 species (Bøgh et al. 1998; Derua et al. 2012; Moiroux et al. 2012; Mwangangi et
490 al. 2013; Sokhna et al. 2013). For example, populations that are evolving to bite
491 outdoor or earlier at night to escape bed nets have an increased likelihood to
492 differentiated into distinct gene pools (Bøgh et al. 1998; Riehle et al. 2011;
493 Moiroux et al. 2012). Using a comprehensive sampling of larvae and adults at
494 different time points during diurnal and nocturnal activities, in or near human
495 settlements, we can conclude that no genetic clustering beyond the regional-
496 scale subdivisions we described is apparent over the scale of our study.
497
498 *Genomic signatures of selection*
499 Populations depicting increasing levels of genetic differentiation along a
500 speciation continuum are ideal to investigate the targets of selection at early
501 stages of ecological divergence (Savolainen et al. 2013). We have scanned
502 genomes of more or less divergent populations of *An. gambiae* and *An. coluzzii*
503 using several divergence and diversity metrics in order to identify outlier regions,
504 which likely contain factors involved in ecological divergence and/or reproductive
505 isolation. In principle, in weakly differentiated populations such as the subgroups
506 we described for which neutral and selective processes have yet to shape the

507 genomic architecture, signatures of selection are often clustered within a few
508 regions of the genome (Nosil and Feder 2012; Andrew and Rieseberg 2013).
509 Indeed, we found that footprints of natural selection in structured populations of
510 *An. gambiae* and *An. coluzzii* occur across a few loci enriched in genes whose
511 functions include insecticide resistance and detoxification, epidermal growth,
512 cuticle formation and olfaction. Although some targets of selection are likely
513 missing because of our experimental approach (Arnold et al. 2013; Tiffin and
514 Ross-Ibarra 2014), previous studies on ecological and phenotypic divergence in
515 *An. gambiae* s.l. suggest that footprints of selection we identified are particularly
516 relevant. First, the cuticle plays a major role at the interface between several
517 biological functions in insects and cuticular proteins are extremely diverse within
518 and among species (Vannini et al. 2014). Importantly, certain cuticular proteins
519 are associated with resistance to insecticides by contributing to a thicker cuticle
520 in *Anopheles* (Wood et al. 2010; Vannini et al. 2014). Olfaction also mediates a
521 wide range of both adult and larval behaviors in blood-feeding mosquitoes
522 (Bowen 1991; Takken and Verhulst 2013). A large family of olfactory receptors
523 has been characterized including candidate chemosensory genes directly
524 involved in the response to cues that are required for feeding, host preference,
525 and mate selection in *An. gambiae* s.l (Carey et al. 2010; Liu et al. 2010; Rinker
526 et al. 2013). Finally, malaria vectors of the *An. gambiae* complex are among the
527 most synanthropic insects in the world, which has led to the hypothesis that
528 human-driven selection is one of the main modulators of ecological divergence
529 between and within species (Coluzzi et al. 2002; Kamdem et al. 2012).

530 Consistent with this hypothesis, we have found that genomic regions harbouring
531 genes involved in resistance to insecticides and pollutants are the dominant
532 targets of selection in emerging subgroups adapted to urban areas. Although
533 more direct implications of genes within these selective sweeps will ultimately be
534 necessary to validate the role of human mediated selection in local adaptation,
535 our data provide a genomic perspective on the interactions between human
536 actions and the contemporary evolution of a mosquito species.
537
538 *Anthropogenic Mediated Selection*
539 Human activity has altered the evolutionary trajectory of diverse taxa. In
540 insects, spatially varying intensity of insecticide application can drive divergence
541 between populations, potentially leading to reproductive isolation. While a
542 plausible scenario, scant empirical evidence supports the hypothesis (Chen, et
543 al. 2012). Previous studies have documented significant reductions in the
544 population size of *An. gambiae* s.l. after introduction of long lasting insecticide
545 treated nets (LLINs), but did not determine the influence of exposure on
546 population structuring (Bayoh, et al. 2010; Athrey, et al. 2012). Among
547 Cameroonian populations of both *An. gambiae and An. coluzzii*, we find a
548 pervasive signature of selection at the *para* sodium channel gene. We infer that
549 this sweep confers globally beneficial resistance to LLINs, which are ubiquitous
550 in Cameroon and treated with pyrethroids that target *para* (Bowen 2013). In
551 contrast, selective sweeps centered on other insecticide resistance genes are
552 restricted to specific geographic locations/populations. For example, a sweep at

553 the *rdl* locus is limited to southern populations of *An. coluzzii*. Initial selection for
554 dieldrin resistance likely occurred during massive indoor residual spraying
555 campaigns conducted by the WHO in southern Cameroon during the 1950s.
556 Indeed, the spraying was so intense that it temporarily eliminated *An. gambiae*
557 s.l. from Yaoundé (and likely other locations in the forest region) (Livadas, et al.
558 1958). However, due to high human toxicity, dieldrin has been banned for use in
559 mosquito control since the mid-1970s. In the absence of insecticide exposure,
560 resistant *rdl* mosquitoes are significantly less fit than wild type mosquitoes
561 (Rowland 1991a, b; Platt, et al. 2015), making the continued persistence of
562 resistant alleles in southern *An. coluzzii* populations puzzling. One plausible
563 explanation is that other cyclodienes targeting *rdl*, such as fipronil and lindane,
564 are still commonly used in agriculture and may frequently runoff into *An. coluzzii*
565 larval habitats, imposing strong selection for resistant mosquitoes. A similar
566 phenomenon was recently proposed to explain the maintenance of resistance *rdl*
567 alleles in both *Culex* and *Aedes* mosquitoes (Tantely, et al. 2010).

568 Mosquitoes inhabiting Cameroon's two major cities, Yaoundé and Douala,
569 provide a clearer example of how xenobiotic exposure can directly influence
570 population structure. Both cities have seen exponential human population growth
571 over the past 50 years, creating a high concentration of hosts for anthropohilic
572 mosquitoes. Despite elevated levels of organic pollutants and insecticides in
573 urban relative to rural larval sites, surveys show substantial year-round
574 populations of *An. gambiae* and *An. coluzzii* in both cities (Antonio-Nkondjio, et
575 al. 2011; Kamdem, et al. 2012; Antonio-Nkondjio, et al. 2014). Bioassays of

576 insecticide resistance demonstrate that urban mosquitoes have significantly
577 higher levels of resistance to multiple insecticides compared to rural mosquitoes
578 (Nwane, et al. 2009; Antonio-Nkondjio, et al. 2011; Nwane, et al. 2013; Tene, et
579 al. 2013; Antonio-Nkondjio, et al. 2015). In support of human mediated local
580 adaptation, we find a selective sweep in urban *An. coluzzii* mosquitoes centered
581 on a cluster of GSTE/CYP450 detoxification genes. While the specific ecological
582 driver of the selective sweep is unknown, GSTE and P450 enzymes detoxify both
583 organic pollutants and insecticides (Suwanchaichinda and Brattsten 2001; David,
584 et al. 2010; Poupardin, et al. 2012). Indeed, the synergistic effects of the two
585 types of xenobiotics could be exerting intense selection pressure for pleiotropic
586 resistance in urban mosquitoes (Mueller, et al. 2008; David, et al. 2013; Nkya, et
587 al. 2013). Regardless of the underlying targets of selection, it is clear that
588 mosquitoes inhabiting highly disturbed urban and suburban landscapes are
589 genetically differentiated from rural populations. Further analysis of specific
590 sweeps using a combination of whole genome resequencing and emerging
591 functional genetics approaches (e.g. CRISPR/Cas9) should help resolve the
592 specific targets of local adaptation in urban mosquitoes, while also shedding light
593 on the evolutionary history of the enigmatic subgroup *GAM2*.
594
595 *Impacts on Vector Control*
596 Just five decades ago, there was not a single city in Sub-Saharan African
597 with a population over 1 million; today there are more than 40. Population shifts
598 to urban areas will only continue to increase with the United Nations estimating

599 that 60% of Africans will live in large cities by 2050 (United Nations 2014). When
600 urbanization commenced, it was widely assumed that malaria transmission would
601 be minimal because rural *Anopheles* vectors would not be able to complete
602 development in the polluted larval habitats present in cities (Donnelly, et al.
603 2005). However, increasingly common reports of endemic malaria transmission
604 in urban areas across Sub-Saharan Africa unequivocally demonstrate that
605 anophelines are exploiting the urban niche (Robert, et al. 2003; Keiser, et al.
606 2004; De Silva and Marshall 2012). Specifically, our study shows that *An.*
607 *gambiae* s.l. from the urban and suburban centers of southern Cameroon form
608 genetically distinct subgroups relative to rural populations. Local adaptation to
609 urban environments is accompanied by strong selective sweeps centered on
610 putative xenobiotic resistance genes, which are likely driven by a combination of
611 exposure to organic pollutants and insecticides in larval habitats. The rapid
612 adaptation of *Anopheles* to the urban landscape poses a growing health risk as
613 levels of resistance in these populations negate the effectiveness of almost all
614 commonly used insecticides. Moreover, repeated instances of beneficial alleles
615 introgressing between *An. gambiae* s.l. species make the emergence of highly
616 resistant subgroups even more troubling (Weill, et al. 2000; Clarkson, et al. 2014;
617 Crawford, et al. 2014; Fontaine, et al. 2014; Norris, et al. 2015). In essence,
618 urban populations can serve as a reservoir for resistance alleles, which have the
619 potential to rapidly move between species/populations as needed. Clearly,
620 sustainable malaria vector control, urban or otherwise, requires not only more
621 judicious use of insecticides, but also novel strategies not reliant on chemicals.

622 Towards this goal, various vector control methods that aim to replace or
623 suppress wild mosquito populations using genetic drive are currently under
624 development (e.g. (Windbichler, et al. 2011)). While promising, the complexities
625 of ongoing cryptic diversification within African *Anopheles* must be explicitly
626 planned for prior to the release of transgenic mosquitoes.
627

628 *MATERIALS AND METHODS*

629

630 *Mosquito collections*

631 In 2013, we collected *Anopheles* from 33 locations spread across the four
632 major ecogeographic regions of Cameroon (Table S1). Indoor resting adult
633 mosquitoes were collected by pyrethrum spray catch, while host-seeking adults
634 were obtained via indoor/outdoor human-baited landing catch. Larvae were
635 collected using standard dipping procedures (Service 1993). All researchers
636 were provided with malaria chemoprophylaxis throughout the collection period.
637 Individual mosquitoes belonging to the *An. gambiae* s.l. complex were identified
638 by morphology (Gillies and De Meillon 1968; Gillies and Coetzee 1987).

639

640 *ddRADseq Library Construction*

641 Genomic DNA was extracted from adults using the ZR-96 Quick-gDNA kit
642 (Zymo Research) and from larvae using the DNeasy Extraction kit (Qiagen). A
643 subset of individuals were assigned to sibling species using PCR-RFLP assays
644 that type fixed SNP differences in the rDNA (Fanello, et al. 2002). Preparation of
645 ddRAD libraries largely followed (Turissini, et al. 2014). Briefly, ~1/3rd of the DNA
646 extracted from an individual mosquito (10µl) was digested with *MluC1* and *NlaIII*
647 (New England Biolabs). Barcoded adapters (1 of 48) were ligated to overhangs
648 and 400 bp fragments were selected using 1.5% gels on a BluePippin (Sage
649 Science). One of six indices was added during PCR amplification. Each library

650 contained 288 individuals and was subjected to single end, 100 bp sequencing
651 across one or two flow cells lanes run on an Illumina HiSeq2500.
652 Raw sequence reads were demultiplexed and quality filtered using the
653 STACKS v 1.29 process_radtags pipeline (Catchen, et al. 2011; Catchen, et al.
654 2013). After removal of reads with ambiguous barcodes, incorrect restriction
655 sites, and low sequencing quality (mean Phred < 33), GSNAP was used to align
656 reads to the *An. gambiae* PEST reference genome (AgamP4.2) allowing up to
657 five mismatches per read. After discarding reads that perfectly aligned to more
658 than one genomic position, we used STACKS to identify unique RAD tags and
659 construct consensus assemblies for each. Individual SNP genotypes were called
660 using default setting in the maximum-likelihood statistical model implemented in
661 the STACKS genotypes pipeline.
662
663 *Population Genomic Analysis*
664 Population genetic structure was assessed using the SNP dataset output
665 by the *populations* program of STACKS. We used PLINK v 1.19 to retrieve
666 subsets of genome-wide SNPs as needed (Purcell, et al. 2007). PCA, neighbor-
667 joining tree analyses, and Bayesian information criterion (BIC) were implemented
668 using the packages *adegenet* and *ape* in R (Paradis et al. 2004; Jombart 2008; R
669 Development Core Team 2014). Ancestry analyses were conducted in
670 fastSTRUCTURE v 1.0 (Raj, et al. 2014) using the logistic method. The
671 choosek.py script was used to find the appropriate number of populations (k); in
672 cases where a range of k was suggested, the BIC-inferred number of clusters

673 was chosen. CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007) was used to
674 summarize assignment results across independent runs and DISTRUCT v1.1
675 (Rosenberg 2004) was used to visualize ancestry assignment of individual
676 mosquitoes. We used a subset of 1,000 randomly chosen SNPs to calculate
677 average pairwise F_{ST} between populations in GENODIVE v 2.0 using up to 40
678 individuals – prioritized by coverage – per population (Meirmans and Van
679 Tienderen 2004). Using this same subset of 1,000 SNPs, we conducted an
680 AMOVA to quantify the effect of the sampling method and the geographic origin
681 on the genetic variance among individuals in GENODIVE. We used 10,000
682 permutations to assess significance of F_{ST} values and AMOVA. We input
683 pairwise F_{ST} values into the program Fitch from the Phylip (Plotree and Plotgram
684 1989) suite to create the population-level NJ tree. F_{IS} values were computed with
685 the *populations* program in STACKS.
686
687 *Genome Scans for Selection*
688 We used ANGSD v 0.612 (Korneliussen, et al. 2014) to calculate
689 nucleotide diversity (θ_w and θ_π) and Tajima's *D* in 150-kb non-overlapping
690 windows. Unlike most genotyping algorithms, ANGSD does not perform hard
691 SNP calls, instead taking genotyping uncertainty into account when calculating
692 summary statistics. Similarly, absolute divergence (d_{xy}) was calculated using
693 *ngsTools* (Fumagalli, et al. 2014) based on genotype likelihoods generated by
694 ANGSD. Kernel smoothed values for 150-kb windows for all four metrics (θ_w, θ_π,
695 *D*, d_{xy}) were obtained with the R package *KernSmooth*. F_{ST} (based on AMOVA)

696 was calculated with the *populations* program in STACKS using only loci present
697 in 80% of individuals. A Kernel smoothing procedure implemented in STACKS
698 was used to obtain F_{ST} values across 150-kb windows. Because regions with
699 unusually high or low read depth can yield unreliable estimates of diversity and
700 divergence parameters due to the likelihood of repeats and local misassembly,
701 we checked that the average per-locus sequencing coverage was consistent
702 throughout the genome (Figure S5). To determine if selective sweeps were
703 enriched for specific functional annotation classes, we used the program DAVID
704 6.7 with default settings (Huang, et al. 2008). We physically delimitated the
705 selective sweep as the region corresponding to the base of the peak or the
706 depression of Tajima's *D*. Haplotypes across the GSTE/CYP450 sweep were
707 reconstructed by PHASE v 2.1.1 using the default recombination model
708 (Stephens, et al. 2001; Stephens and Scheet 2005).
709

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AUTHOR CONTRIBUTIONS

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

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1083

1084

1085 FIGURE LEGENDS

1086 **Figure 1. *Anopheles gambiae* complex sibling species are genetically**
1087 **distinct.** A) PCA B) NJ Tree, and C) fastSTRUCTURE analyses clearly separate
1088 the four *An. gambiae* complex species that occur in Cameroon into discrete
1089 genetic clusters. Additional subdivision below the species level is apparent within
1090 *An. coluzzii* and *An. gambiae.* D) Species composition varies strongly between
1091 eco-geographic regions. Sampling sites are denoted by black dots.

1092

1093 **Figure 2. *Anopheles gambiae* is divided into three cryptic subpopulations.**
1094 A) PCA, B) NJ Tree, and C) fastSTRUCTURE analyses reveal subdivisions
1095 within *An. gambiae.* We term the most abundant group *GAM1*, while a second
1096 small, but widely distributed group, is termed *GAM2*. Finally, most individuals
1097 from the village of Nkolondom are genetically distinct from other *An. gambiae*
1098 suggestive of local adaptation.

1099

1100 **Figure 3. *Anopheles coluzzii* is divided into four subgroups.** A) PCA, NJ
1101 Tree, and C) fastSTRUCTURE reveal major population structuring between *An.*
1102 *coluzzii* from the northern Savannah eco-geographic region and *An. coluzzii* from
1103 the southern three forested regions. Within the south, D) PCA, E) NJ Tree, and
1104 F) fastSTRUCTURE analyses separate mosquitoes based on geographic origin,
1105 although clustering is not fully discrete indicating a dynamic interplay between
1106 local adaptation and migration.

1107

1108 **Figure 4. Phylogenetic relationships between populations show recent**
1109 **radiation within *An. gambiae* and *An. coluzzii* clades**. In an unrooted, F_{ST}-
1110 based NJ tree, *An. melas* is most distant from all other species, while *An.*
1111 *gambiae* and *An. coluzzii* are sister species. Southern populations of *An. coluzzii*
1112 are more closely related to each other than to the northern savannah population.
1113 In contrast to geographic distance, the Douala subpopulation is genetically closer
1114 to Yaoundé rather than Coastal mosquitoes. Within *An. gambiae*, a relatively
1115 deep split is present between *GAM2* and *GAM1*, while *Nkolondom* appears to
1116 have recently diverged from *GAM1*.
1117
1118 **Figure 5. Genome scans reveal footprints of global and local adaptation in**
1119 ***An. gambiae* subpopulations.** A-C) Diversity and Tajima's *D* are plotted for
1120 each of the three subpopulations. Brown asterisks denote windows above the
1121 99th percentile or below the 1st percentile of empirical distribution of Tajima's *D*.
1122 D-E) Both absolute (d_{xy}) and relative (F_{ST}) divergence between populations are
1123 plotted across 150-kb windows. Red asterisks indicate windows above the 99th
1124 percentile of empirical distribution of F_{ST}. In all populations, concordant dips in
1125 diversity and Tajima's *D* are evident near the pericentromeric region of 2L where
1126 the *para* sodium channel gene is located. Three other selective sweeps located
1127 on the X and 3R chromosomes are highlighted (grey boxes).
1128
1129 **Figure 6. Strong positive selection acts on xenobiotic resistance loci in**
1130 **subpopulations of *An. coluzzii.*** A-H) Grey boxes highlight five selective

1131 sweeps characterized by extreme values of F_{ST} and Tajima's *D*. As in *An.*
1132 *gambiae*, sharp declines in diversity and allele frequency spectrum at the *para*
1133 sodium channel gene are present in all populations. A sweep encompassing a
1134 cluster of detoxification genes on 3R is limited to urban mosquitoes. The region
1135 centered on the resistance to dieldrin (*rdl*) gene also shows signs of local
1136 selection in southern subpopulations. No evidence for locally elevated
1137 divergence is observed at the *para* or *rdl* loci suggesting a shared sweep
1138 amongst populations. In contrast, urban-rural mosquitoes show extreme levels of
1139 divergence at the detoxification-enriched sweep on 3R.
1140
1141 **Figure 7. Spatially varying selection between urban and coastal**
1142 **populations.** For each of the three southern *An. coluzzii* subpopulations, 80
1143 reconstructed haplotypes are visualized by color-coding 28 bi-allelic SNPs in the
1144 3R GSTE/CYP450 sweep either grey or white. A single invariant haplotype -- all
1145 grey SNPs -- is common in (A) Yaoundé, less so in (B) Douala, and very rare in
1146 (C) coastal populations. D-E) Similarly, in PCA and NJ Tree analysis of the same
1147 28 SNPs, coastal individuals (navy blue) are diffuse across genotypic space,
1148 while Yaoundé mosquitoes (purple) are tightly clustered. As expected, Douala
1149 (pink) exhibits an intermediate degree of variation. F) STRUCTURE analysis
1150 based solely on the 28 SNPs within the sweep shows clear distinctions between
1151 the three populations.
1152
1153

1154 SUPPLEMENTAL FIGURE LEGENDS

1155 **Figure S1.** Detailed map of sampling sites in Cameroon.

1156

1157 **Figure S2.** Bayesian information criterion was used to determine the most likely
1158 number of clusters/populations for A) all 941 samples, B) all *An. coluzzii*, C) *An.*
1159 *coluzzii* from the northern savannah, D) *An. arabiensis*, E) the 309 individuals
1160 used for genome scans F) all *An. gambiae*, G) southern *An. coluzzii*, and H) *An.*
1161 *melas.* BIC scores for 1 to 50 clusters are plotted. The lower the BIC score the
1162 better the model fits the observed genetic diversity.

1163

1164 **Figure S3.** Southern (pale pink) and northern populations (deep pink) of *An.*
1165 *coluzzii* are readily separated in PCA (top) and NJ trees (bottom) using SNPs
1166 exclusively from any of the five chromosomal arms.

1167

1168 **Figure S4.** No population substructure is detectable within northern *An. coluzzii*
1169 using PCA and NJ tree analysis.

1170

1171 **Figure S5.** Mean sequencing coverage per individual is plotted in 300kb non-
1172 overlapping windows across the genome. Only individuals used in the genome
1173 scans are included in the coverage calculation.

Figure 1.

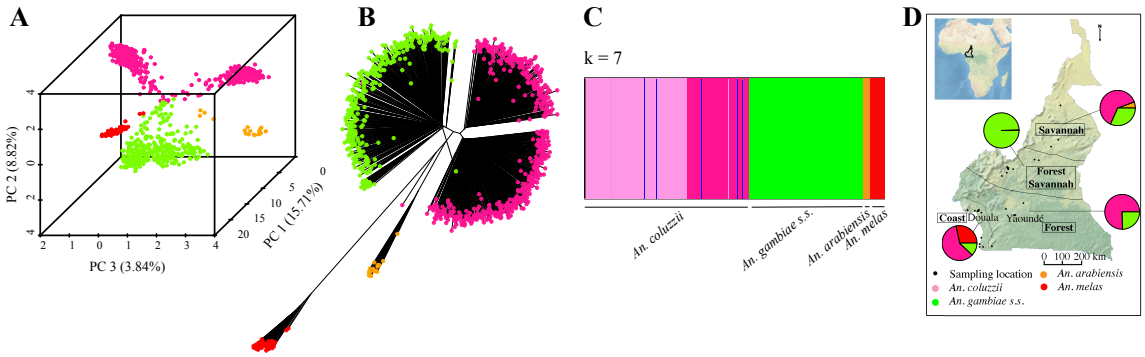

A
PC 2 (8.82%)
PC 3 (3.84%)
PC 1 (15.71%)
B
C
k = 7
An. coluzzii
An. gambiae s.s.
An. arabiensis
An. melas
D
Savannah
Forest Savannah
Coast
Douala
Yaoundé
Forest
0 100 200 km
Sampling location
An. coluzzii
An. gambiae s.s.
An. arabiensis
An. melas

Figure 2.

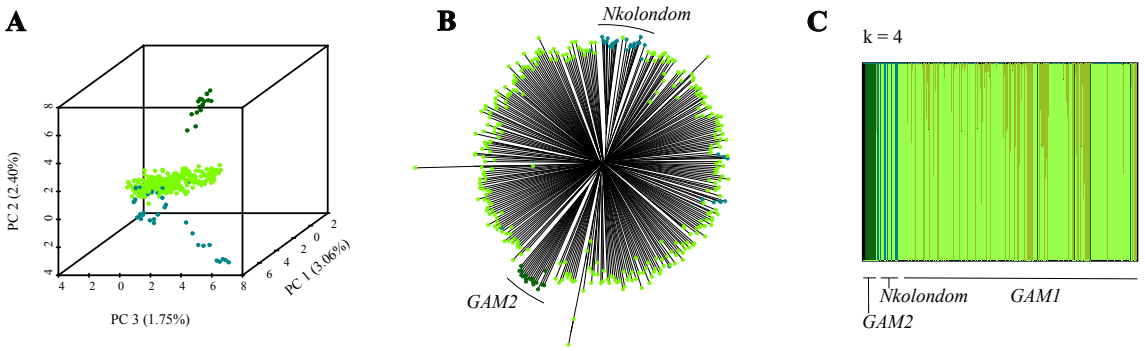

A
PC 2 (2.40%)
PC 3 (1.75%)
PC 1 (3.06%)
B
Nkolondom
GAM2
C
k = 4
Nkolondom
GAM1
GAM2

Figure 3.

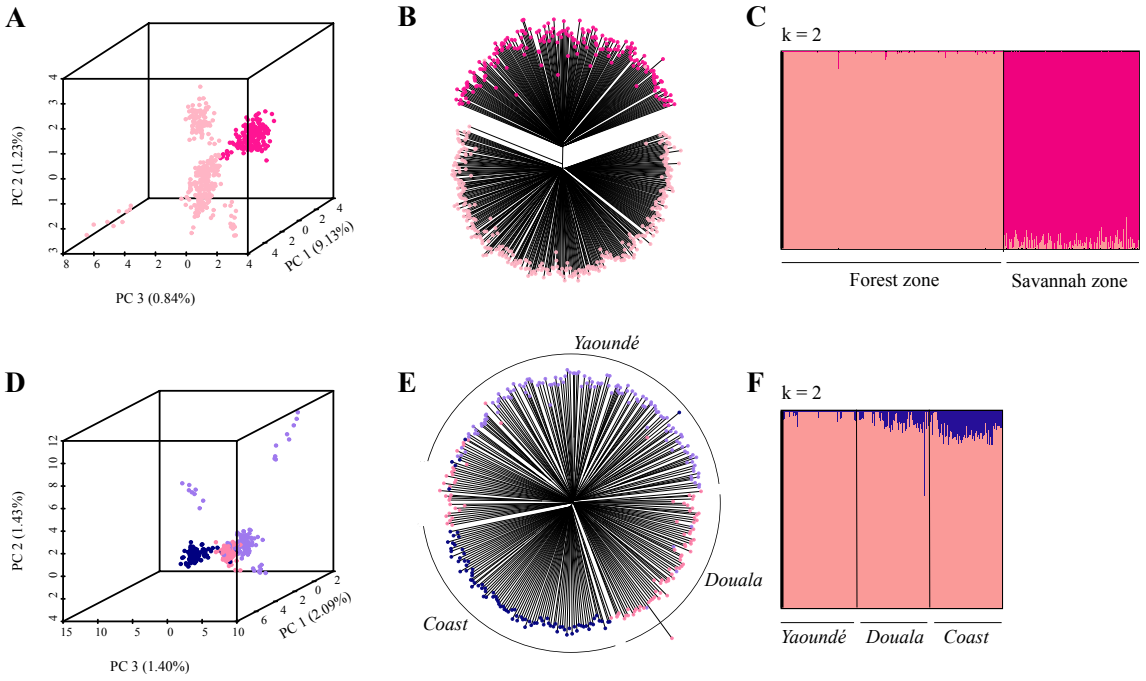

A
PC 2 (1.23%)
PC 3 (0.84%)
PC 1 (9.13%)
B
C
k = 2
Forest zone
Savannah zone
D
PC 2 (1.43%)
PC 3 (1.40%)
PC 1 (2.09%)
E
Yaoundé
Douala
Coast
F
k = 2
Yaoundé
Douala
Coast

Figure 4.

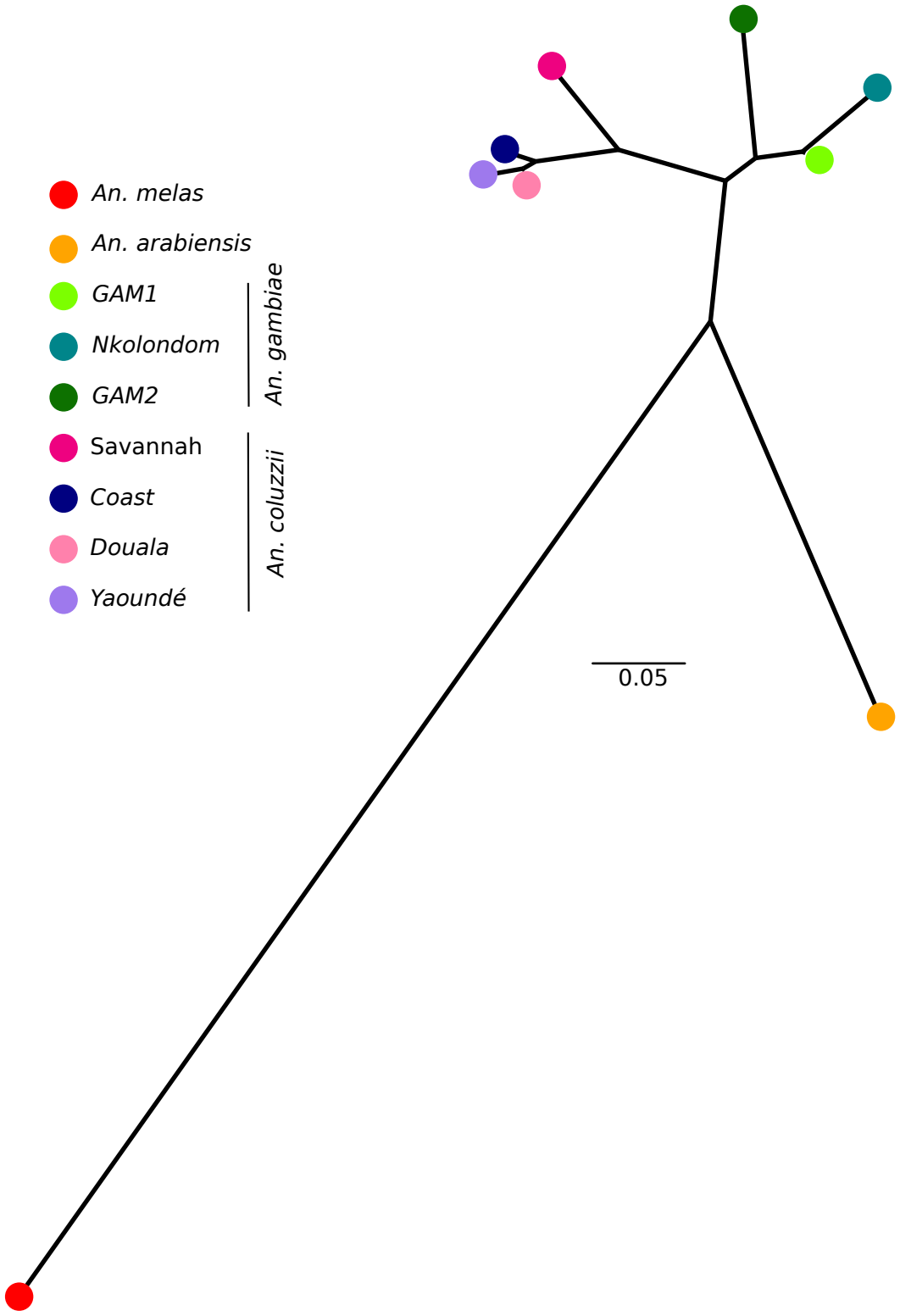

An. melas
An. arabiensis
GAM1
Nkolondom
GAM2
Savannah
Coast
Douala
Yaoundé
An. gambiae
An. coluzzii
0.05

Figure 5.

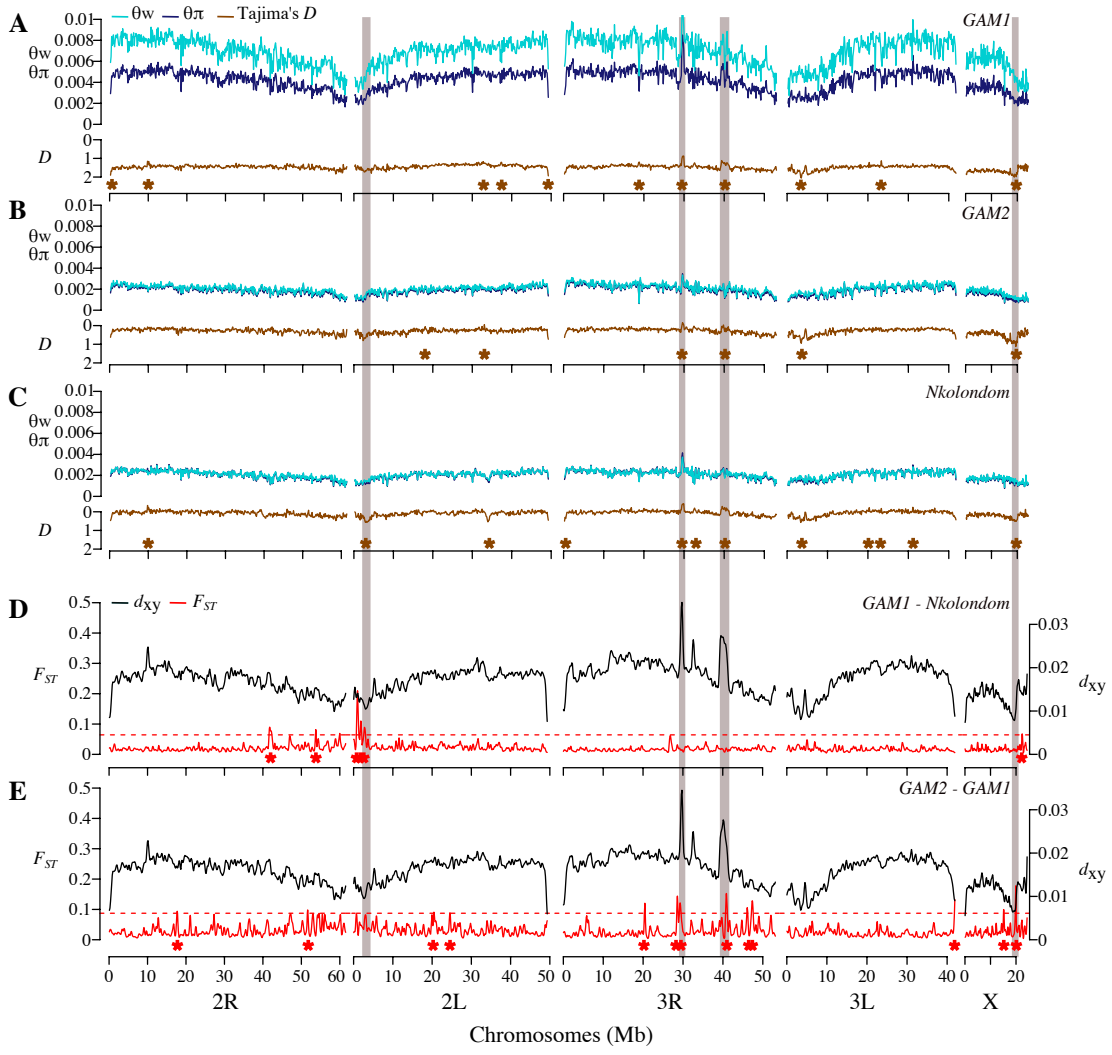

A
θw θπ Tajima's D
GAM1
B
GAM2
C
Nkolondom
D
dxy FST
GAM1 - Nkolondom
E
GAM2 - GAM1
2R
2L
3R
3L
X
Chromosomes (Mb)

Figure 6.

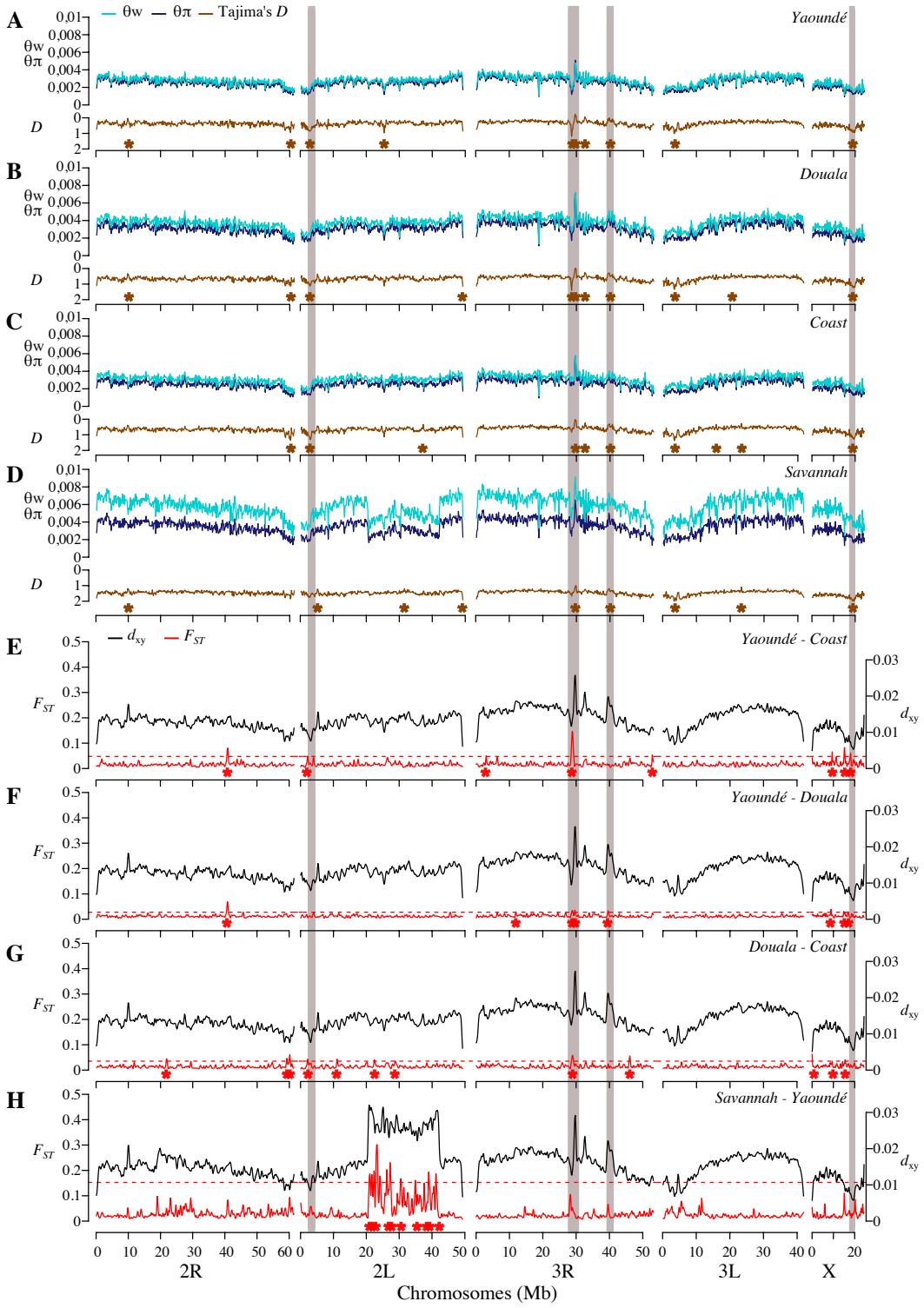

A
B
C
D
E
F
G
H
θw
θπ
Tajima's D
Yaoundé
Douala
Coast
Savannah
d_{xy}
F_{ST}
Yaoundé - Coast
Yaoundé - Douala
Douala - Coast
Savannah - Yaoundé
2R
2L
3R
3L
X
Chromosomes (Mb)

Figure 7.

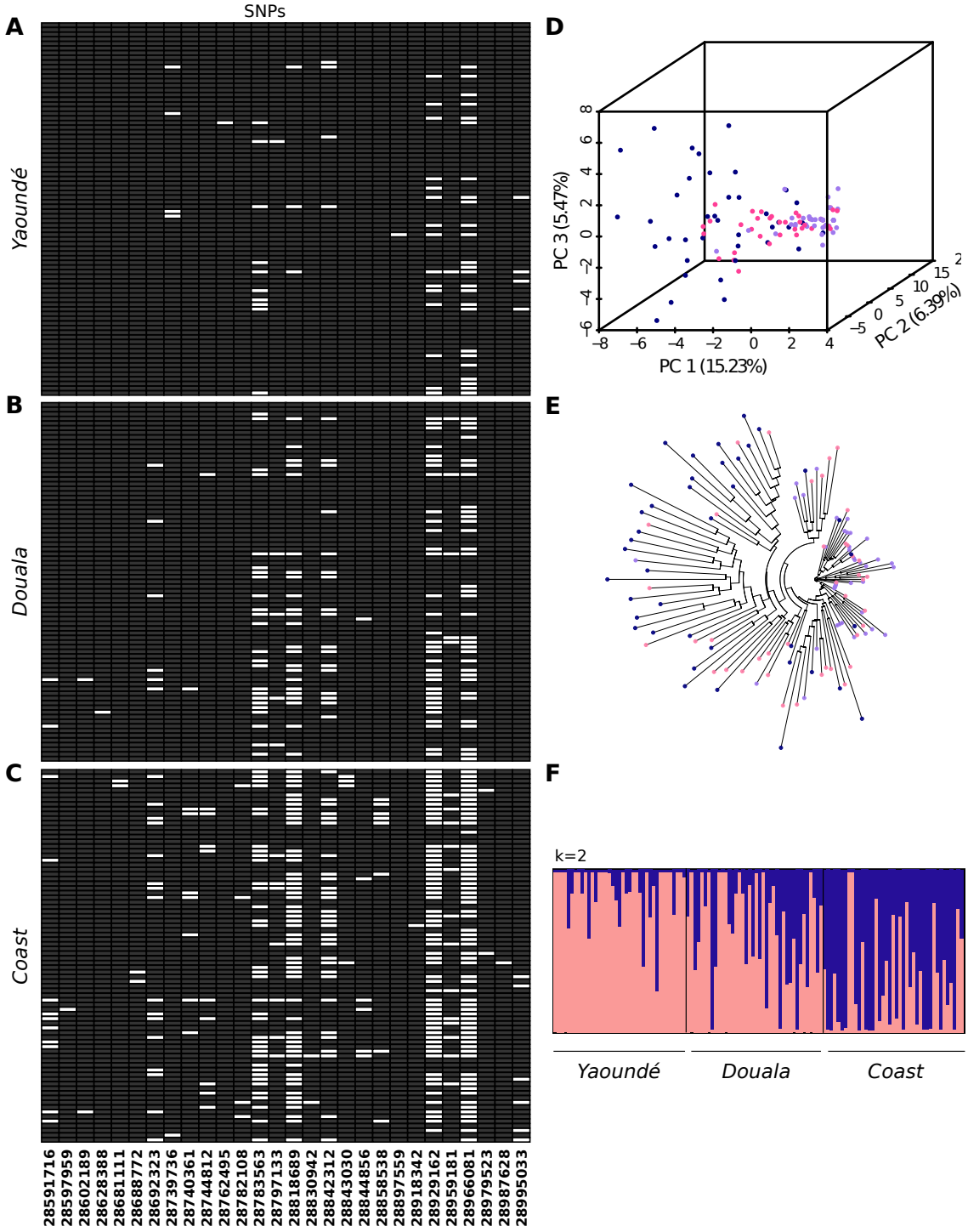

SNPs
A
B
C
D
E
F
Yaoundé
Douala
Coast
28591716
28597959
28602189
28628388
28681111
28688772
28692323
28739736
28740361
28744812
28762495
28782108
28783563
28797133
28818689
28830942
28842312
28843030
28844856
28858538
28897559
28918342
28929162
28959181
28966081
28979523
28987628
28995033
PC 1 (15.23%)
PC 2 (6.39%)
PC 3 (5.47%)
k=2
Yaoundé
Douala
Coast

Figure S1.

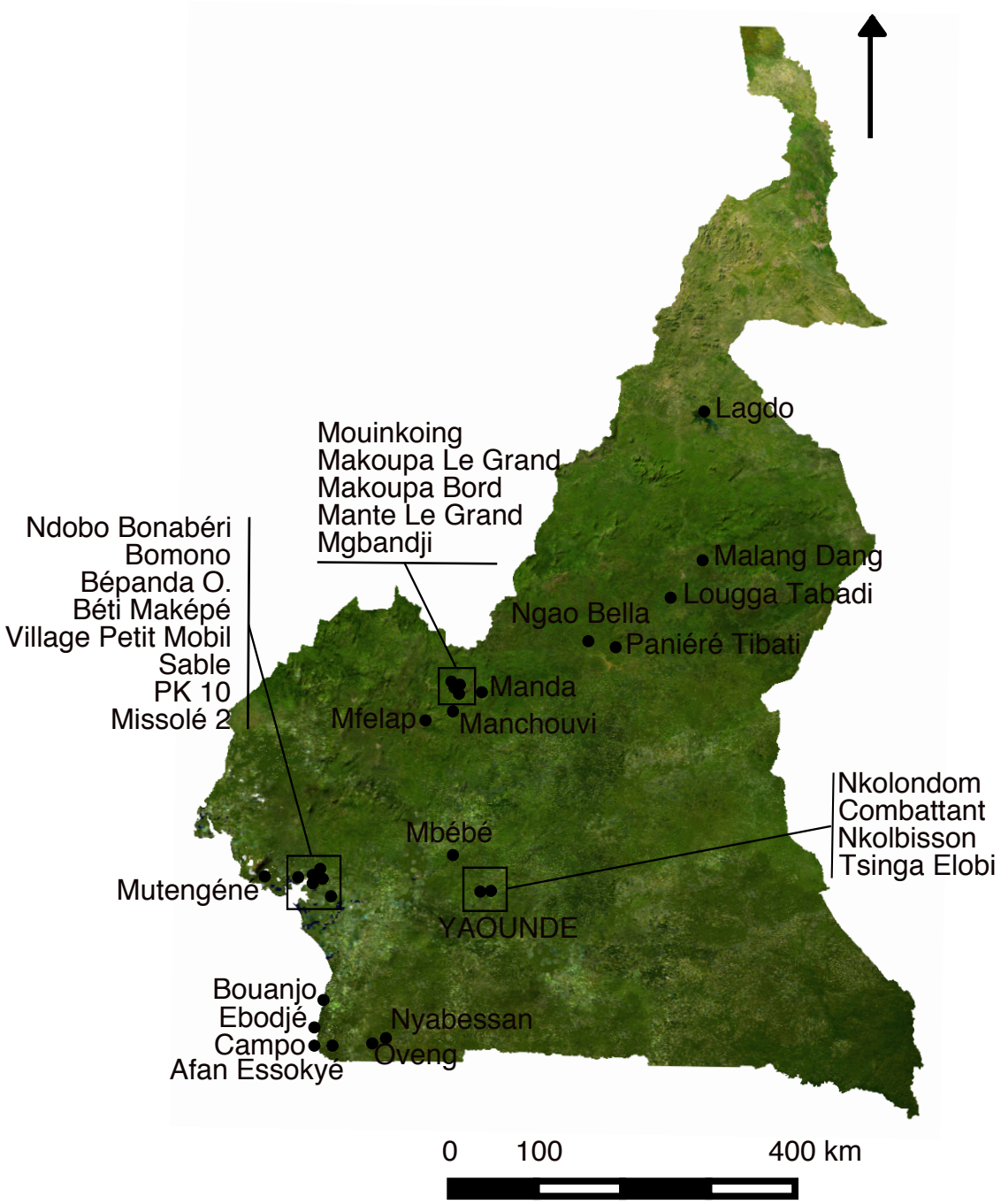

Lagdo
Mouinkoing
Makoupa Le Grand
Makoupa Bord
Mante Le Grand
Mgbandji
Ndobo Bonabéri
Bomono
Bépanda O.
Béti Maképé
Village Petit Mobil
Sable
PK 10
Missolé 2
Malang Dang
Lougga Tabadi
Ngao Bella
Paniéré Tibati
Manda
Mfelap
Manchouvi
Nkolondom
Combattant
Nkolbisson
Tsinga Elobi
Mbébé
Mutengéné
YAOUNDE
Bouanjo
Ebodjé
Campo
Afan Essokyé
Nyabessan
Oveng
0
100
400 km

Figure S2.

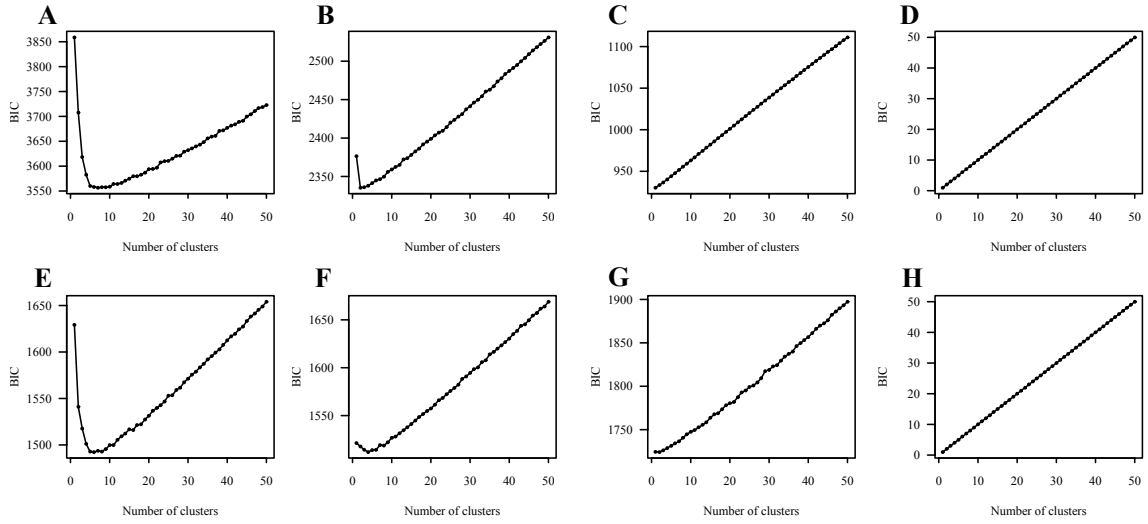


Figure S3.

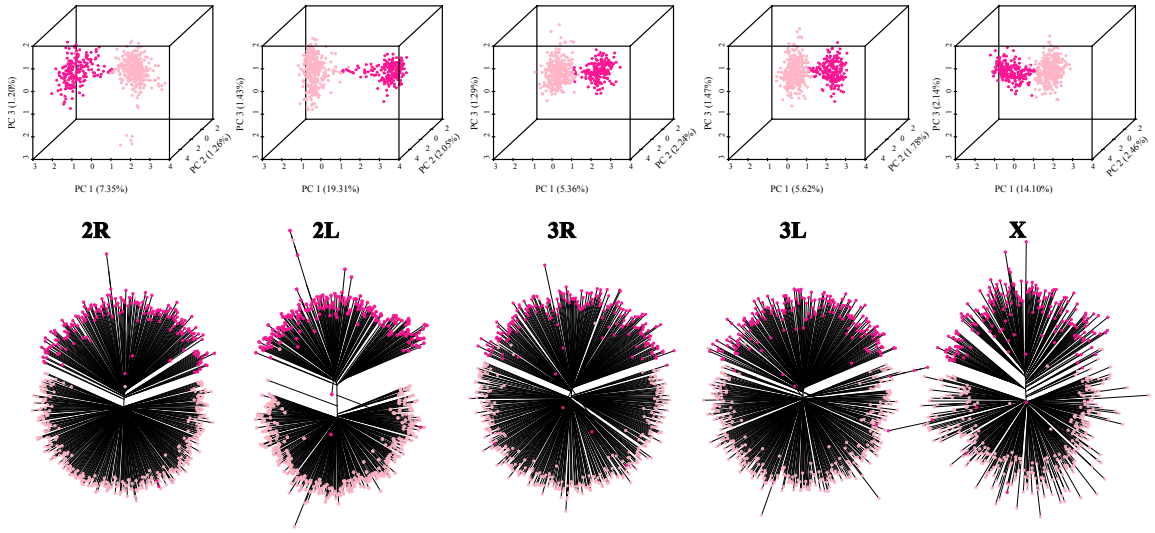

PC 1 (7.35%)
PC 2 (1.26%)
PC 3 (1.20%)
PC 1 (19.31%)
PC 2 (2.05%)
PC 3 (1.43%)
PC 1 (5.36%)
PC 2 (2.28%)
PC 3 (1.29%)
PC 1 (5.62%)
PC 2 (1.78%)
PC 3 (1.47%)
PC 1 (14.10%)
PC 2 (2.46%)
PC 3 (2.14%)
2R
2L
3R
3L
X

Figure S4.

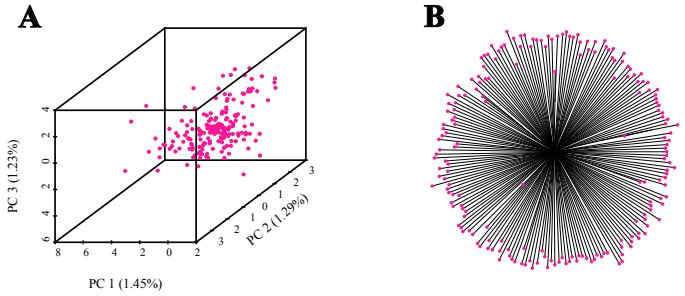
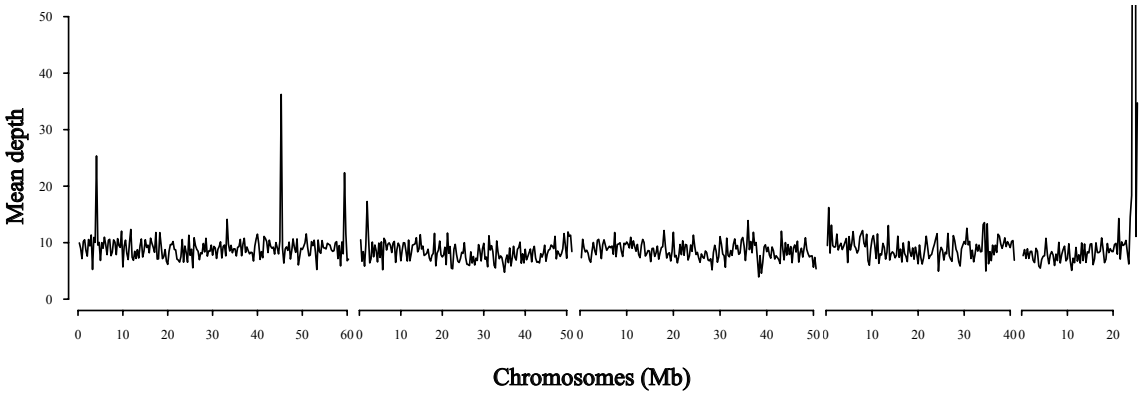


Figure S5.



1174

1175 SUPPLEMENTAL INFORMATION

1176 Consistently negative Tajima's *D* across all subgroups may reflect recent
1177 population expansions. To further address this hypothesis we modeled the
1178 demographic history of each population using a diffusion-based approach
1179 implemented in the software package ∂a∂i v 1.6.3 (Gutenkunst et al. 2009). We
1180 fit four alternative demographic models (*neutral, growth, two-epoch, bottle-*
1181 *growth*), without migration or recombination, to the folded allele frequency
1182 spectrum of each cryptic subgroup of *An. gambiae s.s.* and *An. coluzzii*. The best
1183 model was selected based on the highest composite log likelihood, the lowest
1184 Akaike Information Criterion (AIC), and visual inspection of residuals. As the
1185 choice of model can be challenging in recently diverged populations, we
1186 prioritized the simplest model when we found it difficult to discriminate between
1187 conflicting models. To obtain uncertainty estimates for the demographic
1188 parameters we used the built-in bootstrap function implemented in ∂a∂i to derive
1189 95% bootstrap confidence intervals.

1190 Results indicate that GAM1, GAM2, and Savannah populations have
1191 experienced recent size increases. However, for the southern populations of
1192 *Yaoundé, Coast, Douala, and Nkolondom* the best demographic model is a
1193 *bottle-growth* (Table S4). While most classical studies report *An. gambiae s.l.*
1194 populations that are in expansion (Donnelly et al. 2001), a more recent study
1195 employing RAD markers revealed that some East African populations have more
1196 complex demographic histories, often involving several changes in effective
1197 population size (N_e) as we observed in southern forest populations of both *An.*

1198 *coluzzii* and *An. gambiae*. It has also been shown that *Anopheles* mosquitoes
1199 can experience drastic declines in N_e due to insecticidal campaigns (Athrey et al.
1200 2012). Such events affect demographic parameters and could be a plausible
1201 explanation for the difficulty we encountered in distinguishing between *bottle-*
1202 *growth* and *two-epoch* models in some populations.
1203

1204 SUPPLEMENTAL REFERENCES

1205

1206 Athrey G, Hodges TK, Reddy MR, Overgaard HJ, Matias A, Ridl FC,
1207 Kleinschmidt I, Caccone A, Slotman M a. 2012. The effective population size
1208 of malaria mosquitoes: large impact of vector control. PLoS Genet.
1209 8:e1003097.

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1211 expansion in the evolutionary history of the malaria vectors Anopheles
1212 arabiensis and Anopheles gambiae. Mol. Biol. Evol. 18:1353–1364.

1213 Gutenkunst RN, Hernandez RD, Williamson SH, Bustamante CD. 2009. Inferring
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1215 SNP frequency data. PLoS Genet. 5:e1000695.

1216

1217

Table S1. Locations of *An. gambiae s.l.* mosquitoes sequenced in Cameroon

Ecogeographic regions	Sampling locations	Geographic coordinates	Sampling methods				Total	*Anopheles gambiae sensu lato* populations identified								
			HLC-OUT	HLC-IN	LC	SPRAY		YDE	DLA	CST	SAV	GAM2	NKO	GAM1	A	ME
Savannah	Lagdo	9°02'56"N, 13°39'22"E	52	27	21	43	143				116			9	18	
	Lougga Tabadi	7°06'36"N, 13°12'36"E	2				2							2		
	Malang Dang	7°26'06"N, 13°33'14"E				11	11				2			9		
	Ngao Bella	6°30'04"N, 12°25'34"E	28	17		69	114				65	14		35		
	Paniéré Tibati	6°28'08"N, 12°37'44"E	5		22	16	43				13	1		29		
	Total		87	44	43	139	313				196	15		84	18	
Forest-Savannah Transition	Makoupa Bord	6°06'06"N, 11°11'29"E	2			16	18							18		
	Makoupa Le Grand	6°02'28"N, 11°10'39"E			4	10	14							14		
	Manchoutvi	5°52'48"N, 11°06'36"E			70		70					2		68		
	Manda	5°43'32"N, 10°52'06"E	1	2		33	36							36		
	Mante Le Grand	6°03'44"N, 11°12'18"E				8	8				1			7		
	Mfelap	5°43'30"N, 10°52'00"E				8	8							8		
	Mgbandji	6°05'49"N, 11°08'26"E			3	5	8							8		
	Mouinkoing	6°02'56"N, 11°24'36"E				3	3							3		
	Total		3	2	77	83	165				1	2		162		
Forest (urban)																
	Bepanda Omnisport (Doula)	4°03'18"N, 9°43'16"E			7		7		7							
	Beti Maképé (Douala)	4°03'54"N, 9°45'40"E			25		25		6					19		
	Bomono Gare (Douala)	4°04'55"N, 9°35'35"E			13		13		9					4		
	PK10 (Douala)	4°02'49"N, 9°46'47"E			20		20		16					4		
	Missolé 2 (Douala)	3°59'17"N, 9°54'22"E			8		8		8							
	Ndobo Bonabéri (Douala)	4°04'39"N, 9°40'12"E			1		1		1							
	Sable (Douala)	4°04'52"N, 9°43'34"E	28		5		33		33							
	Village Petit Mobil (Douala)	4°00'16"N, 9°45'18"E	1		16		17		17							
	Nkolbisson (Yaoundé)	3°52'29"N, 11°26'58"E			9		9	9								
	Nkolondom (Yaoundé)	3°58'20"N, 11°30'56"E			48		48	12					33	3		
	Tsinga Elobi (Yaoundé)	3°52'49"N, 11°30'23"E			46		46	46								
	Combattant (Yaoundé)	3°52'36"N, 11°30'46"E	18		48		66	65					1			
	Total		47	0	246	0	293	132	97				34	30		
Forest (rural)																
	Mbébé	4°10'00"N, 11°04'00"E				5	5							5		
	Nyabessan Centre	2°24'00"N, 10°24'00"E	4	1			5							5		
	Oveng	2°44'00"N, 11°27'00"E	1				1							1		
	Total		5	1	0	5	11							11		
Coast																
	Afan Essokyé	2°22'01"N, 9°58'59"E	1			13	14			3				11		
	Bouanjo	2°48'00"N, 9°54'00"E			5		5			5						
	Campo	2°22'01"N, 9°49'01"E	48		16	27	91			79				1		11
	Ebodjé	2°30'00"N, 9°49'05"E	35	4		3	42			5				3		34
	Mutengéné	4°06'53"N, 9°14'51"E			7		7		1	2				4		
	Total		84	4	28	43	159		1	94				19		45
Total			226	51	394	270	941	132	98	94	197	17	34	306	18	45

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

Table S2. Pairwise comparison of genetic distance (F_{ST}) among cryptic subgroups and sibling species of *An. gambiae s.l.*

F_{ST}	***Yaoundé***	***Douala***	***Coast***	***Savannah***	***GAM2***	***Nkolondom***	***GAM1***	***An. arabiensis***
Yaoundé	-							
Douala	0.016	-						
Coast	0.035	0.023	-					
Savannah	0.127	0.109	0.103	-				
GAM2	0.244	0.199	0.230	0.168	-			
Nkolondom	0.197	0.201	0.200	0.247	0.161	-		
GAM1	0.183	0.153	0.178	0.188	0.090	0.050	-	
An. arabiensis	0.451	0.406	0.498	0.383	0.478	0.372	0.327	-
An. melas	0.851	0.836	0.830	0.792	0.841	0.818	0.781	0.872

Table S3. Average nucleotide diversity in seven cryptic subgroups of *An. coluzzii* and *An. gambiae s.s.*

Population	θπ (bp^{-1})	θw (bp^{-1})
Yaoundé	0.0025	0.0028
Douala	0.0031	0.0037
Coast	0.0025	0.0031
Savannah	0.0035	0.0057
GAM2	0.0019	0.0020
Nkolondom	0.0020	0.0021
GAM1	0.0042	0.0069

Table S4. Parameters of demographic models inferred from folded Site Frequency Spectrum (SFS) of autosomal SNPs in seven cryptic subpopulations of *An. gambiae*

Species	Population	Best Model	Log Likelihood	Final Pop Size[a] (95% CI)	Bottleneck Size[b] (95% CI)	Time[c] (95% CI)
Anopheles coluzzii	*Yaoundé*	*Bottle-growth*	-83.45	1.18 (0.92 – 1.69)	24.85 (11.94 - 125.77)	0.58 (0.43 - 1.01)
	Douala	*Bottle-growth*	-84.50	2.20 (1.52 - 3.77)	5.66 (2.80 - 142.42)	0.59 (0.41 - 1.77)
	Coast	*Bottle-growth*	-82.95	1.72 (1.28 - 2.33)	23.83 (10.95 - 128.52)	0.67 (0.50 - 1.10)
Anopheles gambiae s.s.	*Savannah*	*Two-epoch*	-102.65	6.73 (6.35 - 7.21)		0.62 (0.54 - 0.72)
	GAM2	*Two-epoch*	-37.48	3.60 (1.74 - 7.70)		3.60 (0.56 - 9.30)
	Nkolodom	*Bottle-growth*	-74.92	1.08 (0.82 - 1.12)	24.95 (47.91 - 99.84)	0.63 (0.47 - 0.91)
	GAM1	*Two-epoch*	-103.33	6.75 (6.33 - 7.24)		0.68 (0.60 - 0.78)

[a] Ratio of contemporary to ancient population size.
[b] Ratio of population size after instantaneous change to ancient population size.
[c] Time in the past at which instantaneous change happened and growth began (in units of 2*Na generations).

Table S5. Functional analysis of gene ontology terms in candidate regions.
Supplementary online information