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*Published in:*  
Molecular Ecology

*DOI:*  
[10.1111/mec.13888](https://doi.org/10.1111/mec.13888)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2016

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Love, R. R., Steele, A., Coulibaly, M. B., Traore, S. F., Emrich, S. J., Besansky, N. J., & Fontaine, M. C. (2016). Chromosomal inversions and ecotypic differentiation in *Anopheles gambiae*: The perspective from whole-genome sequencing. *Molecular Ecology*, 25(23), 5889-5906. <https://doi.org/10.1111/mec.13888>

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# Chromosomal inversions and ecotypic differentiation in *Anopheles gambiae*: the perspective from whole-genome sequencing

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## Abstract

The molecular mechanisms and genetic architecture that facilitate adaptive radiation of lineages remain elusive. Polymorphic chromosomal inversions, due to their recombination-reducing effect, are proposed instruments of ecotypic differentiation. Here, we study an ecologically diversifying lineage of *Anopheles gambiae*, known as the Bamako chromosomal form based on its unique complement of three chromosomal inversions, to explore the impact of these inversions on ecotypic differentiation. We used pooled and individual genome sequencing of Bamako, typical (non-Bamako) *An. gambiae* and the sister species *Anopheles coluzzii* to investigate evolutionary relationships and genome-wide patterns of nucleotide diversity and differentiation among lineages. Despite extensive shared polymorphism and limited differentiation from the other taxa, Bamako clusters apart from the other taxa, and forms a maximally supported clade in neighbour-joining trees based on whole-genome data (including inversions) or solely on collinear regions. Nevertheless,  $F_{ST}$  outlier analysis reveals that the majority of differentiated regions between Bamako and typical *An. gambiae* are located inside chromosomal inversions, consistent with their role in the ecological isolation of Bamako. Exceptionally differentiated genomic regions were enriched for genes implicated in nervous system development and signalling. Candidate genes associated with a selective sweep unique to Bamako contain substitutions not observed in sympatric samples of the other taxa, and several insecticide resistance gene alleles shared between Bamako and other taxa segregate at sharply different frequencies in these samples. Bamako represents a useful window into the initial stages of ecological and genomic differentiation from sympatric populations in this important group of malaria vectors.

**Keywords:** adaptive radiation, *Anopheles gambiae*, chromosomal inversions, ecological divergence, genome scan, population genomics, selective sweeps

Received 3 February 2016; revision received 13 September 2016; accepted 3 October 2016

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## Introduction

Local adaptation to heterogeneous environments can produce genetically differentiated populations or

ecotypes and potentially lead to ecological speciation (Nosil 2012; Rundle & Nosil 2005; Schluter 2009). Although the genes and genetic mechanisms underlying local adaptation are elusive, empirical evidence and theoretical models suggest that initial genomic differentiation between ecotypes that are still exchanging genes should be confined to small chromosomal regions containing the loci directly responsible for phenotypes that are advantageous in one but not the other environment (Wu 2001; Nosil *et al.* 2009; Yeaman & Whitlock 2011; Andrew & Rieseberg 2013; Flaxman *et al.* 2014; Seehausen *et al.* 2014). Because migration, gene flow and recombination among diverging populations can break down locally adapted allelic combinations, genomic regions of reduced recombination – including chromosomal inversions – are likely to harbour loci that contribute to local adaptation and ecotypic differentiation (Rieseberg 2001; Butlin 2005; Kirkpatrick & Barton 2006; Hoffmann & Rieseberg 2008; Kirkpatrick 2010; Lowry & Willis 2010; Joron *et al.* 2011; Jackson *et al.* 2012; Yeaman 2013; Nishikawa *et al.* 2015; Roesti *et al.* 2015).

Structural rearrangements known as chromosomal inversions are now known to occur in most species, but they were first discovered in *Drosophila melanogaster* through their impact on recombination (Sturtevant 1917), which is suppressed between the break points of alternative arrangements in inversion heterozygotes. Their recombination-reducing effect may be the basis for the role of chromosomal inversions in local adaptation. As modelled by Kirkpatrick & Barton (2006), if a set of locally adapted alleles is captured between the break points of an inversion, the inversion can establish and spread in the population because the advantageous allelic combinations are maintained in the face of gene flow with migrant populations carrying other genetic backgrounds. Indeed, the adaptive significance of inversions is reflected in predictable shifts in polymorphic inversion frequencies in response to spatially or seasonally varying selection (Krimbas & Powell 1992; Hoffmann & Rieseberg 2008; Schaeffer 2008). Examples are clines in inversion frequencies related to climatic gradients that are replicated in different geographic locations, and in some cosmopolitan species, across continents (Knibb 1982; Krimbas & Powell 1992; Etges & Levitan 2004; Rako *et al.* 2006). Other examples are cyclical variations in inversion frequencies related to season (Dubinin & Tiniakov 1946; Dobzhansky 1948).

Twenty-five years before the formal mathematical modelling of Kirkpatrick & Barton (2006), Coluzzi (1982) proposed a conceptually related verbal model for ecotype formation in anopheline mosquitoes, via chromosomal inversions which are abundant in the genus. Anophelines possess only three pairs of chromosomes, and inversions frequently span more than 5% of the

total chromosome complement, increasing the chance for an inversion to capture adaptively important variation. The high dispersal ability of anophelines, combined with the 'boom-bust' nature of their population dynamics, can produce situations in which populations at the margins of suitable habitat become temporarily isolated – spatially and/or ecologically – and locally adapted. Such population isolates could be stabilized by the association of adaptive variation with inversions (Coluzzi 1982).

The *Anopheles gambiae* complex is an African group of at least eight species that radiated within the last 2 million years (White *et al.* 2011; Fontaine *et al.* 2015). Best known for its medical importance, this group contains three of the principal vectors of human malaria in Africa (*Anopheles arabiensis*, *Anopheles coluzzii* and *Anopheles gambiae*), which also are the most widely distributed. Although morphologically indistinguishable at all developmental stages, even the most closely related pair of species (*An. coluzzii* and *An. gambiae*) differ in aquatic larval ecology (Gimonneau *et al.* 2012; Kamdem *et al.* 2012), a factor that may have been instrumental in the radiation of the *An. gambiae* complex as a whole (Coluzzi *et al.* 2002). In addition, both interspecific fixed chromosomal inversion differences and intraspecific inversion polymorphisms are common and nonrandomly distributed in the genome, within and among chromosomes (Coluzzi *et al.* 2002; Pombi *et al.* 2008). Particularly striking is the disproportionate involvement of chromosome 2R in the autosomal inversions described in the *An. gambiae* complex. Although this arm comprises only one-third of the autosomal complement, it bears 21 of 34 main fixed or polymorphic autosomal rearrangements recorded in the species complex (Coluzzi *et al.* 2002).

Heterogeneities within what was initially considered to be a single panmictic species were uncovered in Mali through intensive longitudinal cytogenetic surveys of inversions on chromosome 2 (Toure *et al.* 1998). Whereas significant and stable heterozygote deficits were observed in sympatric samples considered as one randomly mating population, their partitioning into three reproductive units ('chromosomal forms') restored Hardy-Weinberg equilibrium. Two of these forms, named Mopti and Savanna and later defined molecularly as M and S (Della Torre *et al.* 2001), have been subsequently elevated to species and are now known as *An. coluzzii* and *An. gambiae*, respectively (Coetzee *et al.* 2013). The third, Bamako, is indistinguishable from *An. gambiae* based on rDNA sequence (Favia *et al.* 2001), a locus that contains fixed sequence differences between all recognized species in the *An. gambiae* complex (Scott *et al.* 1993; Fettene & Temu 2003; Besansky *et al.* 2006; Coetzee *et al.* 2013). However, Bamako carries a unique

configuration of chromosomal inversions on chromosome 2R (Coluzzi *et al.* 1985). It is homokaryotypic (fixed) for inversions *j*, *c* and *u*, with only inversion *b* segregating; thus, it is considerably less polymorphic chromosomally than sympatric typical (i.e. non-Bamako) *An. gambiae* populations and *An. coluzzii* from Mali, in which inversions *b*, *c* and *u* segregate at high frequencies (Toure *et al.* 1998; Della Torre *et al.* 2005). Inversion 2Rj is absent or very rare in *An. coluzzii* and generally rare in most *An. gambiae* populations, only reaching higher frequencies in a few localities in West Africa (Toure *et al.* 1998; Della Torre *et al.* 2005). Bamako also has a far more restricted distribution than typical *An. gambiae* populations or *An. coluzzii*, being endemic to the upper Niger River in southern Mali and northern Guinea. Although adults of all three taxa freely co-occur in the same Malian localities, often resting together inside the same buildings, their larvae are associated with different habitat types; for this reason, breeding is not always synchronous. Larvae of *An. coluzzii* are associated with rice fields and other permanent or semi-permanent irrigated sites in this part of Africa, and therefore, this species can be abundant during the dry season (Gimonneau *et al.* 2012). Typical *An. gambiae* populations breed in ephemeral rain-dependent pools and puddles away from the river, but Bamako larvae are disproportionately found in more stable laterite rock pools beside the Niger River (Manoukis *et al.* 2008), which are only exposed as the river recedes at the end of the rains. With its peculiar karyotype, its association with a peripheral and ecologically marginal habitat, and cytogenetic evidence consistent with positive assortative mating (Toure *et al.* 1998; Coulibaly *et al.* 2007; Manoukis *et al.* 2008), Bamako appears to be in the earliest stages of ecological differentiation from sympatric typical *An. gambiae* populations.

The nature, distribution and degree of genomic divergence between the Bamako ecotype and typical *An. gambiae* have been explored previously at increasing levels of genetic resolution, with puzzlingly inconsistent results. In 2006, analyses involving >10 000 AFLP bands uncovered no diagnostic differences between Bamako and typical *An. gambiae*, but revealed significant – albeit slight – genetic divergence in unknown genomic locations (Slotman *et al.* 2006). By 2010, a 400 000 SNP genotyping array (with median distance of ~300 bp between assayed SNPs) was available based on whole-genome sequencing of *An. gambiae* and *An. coluzzii* (Lawniczak *et al.* 2010), and results indicated that the greatest genomic differentiation between Bamako and typical *An. gambiae* was found in inversions on 2R, as expected under the hypothesis that inversions are instruments of ecotypic divergence, while no significant differentiation was found on the X chromosome (Neafsey *et al.* 2010).

However, in contradiction to these results, application of an even higher resolution whole-genome tiling microarray suggested that the majority of genomic divergence between Bamako and typical *An. gambiae* was located on the X chromosome, leading to the inference that genes mediating isolation are likely on the X (Lee *et al.* 2013a), as may be the case for the sister species *An. gambiae* and *An. coluzzii* (Aboagye-Antwi *et al.* 2015). Both the genotyping array and the whole-genome tiling microarray have technical and statistical limitations inherent in their design that may have biased or obscured measurements of genetic differentiation. To overcome these limitations and approach a more comprehensive understanding of the genetic architecture of ecotypic differentiation, we use whole-genome resequencing of individuals and population pools and assess the pattern of genomic differentiation between Bamako, typical *An. gambiae*, and for comparison, *An. coluzzii*. As expected, our data reveal extensive differentiation between *An. coluzzii* and either Bamako or typical *An. gambiae* in the centromere-proximal region of the X chromosome. However, we observed virtually no genomic differentiation between the latter two taxa on the X chromosome. The majority of differentiation was associated with chromosome 2 inversions, implicating these rearrangements in the ecotypification process.

## Materials and methods

### Sampling and sequencing strategy

We exploited prior indoor resting collections of adult *Anopheles gambiae* and *Anopheles coluzzii* from nearby villages in southern Mali, sampled in August–September of 2004 (Coulibaly *et al.* 2007). Cytogenetic karyotyping was performed using ovaries from females at the correct gonotrophic stage, and the corresponding carcass was molecularly identified to species (Coulibaly *et al.* 2007). Of the available population samples, a total of 87 adult female mosquitoes previously karyotyped cytogenetically and identified molecularly were used for this study: 39 *An. gambiae* Bamako, 16 typical *An. gambiae* and 32 *An. coluzzii* (see Tables S1 and S2, Supporting information, for sample provenances, karyotypes and chromosomal inversion frequencies). For each of the three population samples, we used pooled sequencing (Pool-seq) to identify SNPs and estimate allele frequencies (Futschik & Schlotterer 2010). In addition to the pools, we analysed individual whole-genome sequences from each taxon, determined as part of a previous sequencing effort (Fontaine *et al.* 2015; Neafsey *et al.* 2015): 10 Bamako, 12 typical *An. gambiae* and 12 *An. coluzzii* (Table S3, Supporting information).

### Library preparation, sequencing, data processing and filtering of pools

A single DNA pool was prepared for each of the three population samples: Bamako ( $N = 39$ ), typical *An. gambiae* ( $N = 16$ ) and *An. coluzzii* ( $N = 32$ ). Each pool (200 ng of total DNA) contained approximately equal contributions of genomic DNA from component mosquitoes. Pooled DNA was sheared into fragments with a Covaris S220 ultrasonicator (Covaris, Inc., Woburn, MA, USA). After end repair of the fragments, paired-end genomic libraries were constructed using the TruSeq DNA Nano Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA) and size-selected for an average fragment size of 550 bp using a BluePippin instrument (Sage Science™). Final average fragment library sizes were 539 bp for Bamako, 534 bp for typical *An. gambiae* and 536 bp for *An. coluzzii*, corresponding to average insert sizes of 353, 350 and 350 bp, respectively. Each library was barcoded, and the three libraries were multiplexed such that the relative contribution of each pool to the combined total volume was proportional to the number of individuals in the pool: 45% Bamako, 18% typical *An. gambiae* and 37% *An. coluzzii*. This was performed so that sequencing coverage (per individual) would be comparable across pools, given the different pool sizes. Multiplexed barcoded libraries were sequenced with paired-end 100-bp runs on two lanes of an Illumina HiSeq 2000 at BGI (University of California, Davis). Raw sequencing data from the pools are available as SRA BioProject PRJNA311062.

Short reads from each pool were demultiplexed and trimmed following the pipeline described in Fontaine *et al.* (2015). Reads were mapped to the *An. gambiae* PEST AgamP4 reference assembly [www.vectorbase.org; (Giraldo-Calderon *et al.* 2015)] using *bwa-aln* 0.7.12-r1044 (Li & Durbin 2009) with parameters (-l, -k 2, -o 1, -l 32). Further processing of the mapped paired reads followed Fontaine *et al.* (2015). This included soft clipping of any reads where part of the read extended beyond the end of the sequence to which it was aligned (using Picard Tools 1.102 CleanSam.jar; <https://github.com/broadinstitute/picard>), sorting of reads based on alignment location (using Picard Tools' SortSam.jar), identification and marking of duplicate reads (using Picard Tools' MarkDuplicates.jar), and localized realignment near insertion-deletions [with the Indel Realignment tool of the Genome Analysis Toolkit, GATK v3.3; (McKenna *et al.* 2010; Depristo *et al.* 2011)]. Quality of the resulting mapped reads was assessed with QUALIMAP v2.1.1 (Garcia-Alcalde *et al.* 2012). Reads were compiled by position into pileup files using SAMTOOLS v.1.2 (Li *et al.* 2009), for each pool and collectively for all pools.

We considered only bases with a minimum Phred-scaled mapping quality of 20 and a base quality of 30.

For each pool, we set a minimum coverage threshold of 10 reads. To avoid repetitive genomic regions with high coverage, a maximum coverage threshold per pool was imposed, equal to the top 1% coverage (corresponding to 106 for Bamako, 45 for typical *An. gambiae* and 90 for *An. coluzzii*). For window-based analyses, any window with <60% of sites compliant with coverage requirements was excluded. For all analyses, we used a minimum minor allele count of 2. For all downstream analysis, only sites or windows that could be assigned to a chromosomal arm (2L, 2R, 3L, 3R or X) were retained.

### Data processing and filtering of individual whole-genome sequences

Reads from the 34 individual whole-genome sequences of Bamako, typical *An. gambiae* and *An. coluzzii* determined as part of a previous sequencing effort (Fontaine *et al.* 2015) were trimmed and aligned, following the workflow of Fontaine *et al.* (2015). Variants (SNPs) were called in a single cohort using the HaplotypeCaller walker of GATK v2.6-5. Variant calling was followed by variant quality score recalibration (VQSR) to differentiate true variants from false positives, as recommended for low-coverage samples (see [www.broadinstitute.org/gatk/guide/article?id=3225](http://www.broadinstitute.org/gatk/guide/article?id=3225)). This recalibration produced a top tranche of SNPs ( $n = 4\,697\,938$  SNPs) defined as having variant quality score log odds >2.007. Following VQSR, we retained biallelic SNPs with a minimum per-genotype depth of three and a minimum genotype quality of 15. We then constructed two data sets, one specific to Bamako individuals and used to estimate linkage disequilibrium (LD), and one for all 34 individuals from the three taxa. For the Bamako-only data set, sites with more than three missing genotypes (30%) were excluded. A total of 2 839 964 SNPs were retained after quality filtering. The second data set, after the exclusion of sites with more than 10 missing genotypes (29%) and the application of quality filters, consisted of 2 794 793 SNPs. For generating estimates of diversity, Tajima's  $D$  and differentiation, we retained only mosquito genomes sampled from the neighbouring West African countries of Mali and Burkina Faso [i.e. we excluded the more geographically distant samples from Central Africa (Cameroon)]. For analysis of population clustering, we added the *Anopheles christyi* reference genome (Neafsey *et al.* 2015), as *An. christyi* is the closest outgroup to the *An. gambiae* complex (Fontaine *et al.* 2015). Only sites that passed all previous filters and also had data for *An. christyi* (1 230 508 SNPs) were retained.

### Population clustering

We used the R package ADEGENET v2.0.0 (Jombart 2008; Jombart & Ahmed 2011) to store SNP genotype data from individual genome sequences. We then used the R package APE v3.3 (Paradis *et al.* 2004) to compute a genotype-based Euclidian distance matrix between individuals and to construct neighbour-joining (NJ) trees. Node support was assessed using 100 bootstrap replicates. In addition, we used ADMIXTURE 1.23 (Alexander *et al.* 2009) to conduct an unsupervised estimate of ancestry using  $K = 1-4$  with cross-validation.

### Estimation of population genetic parameters

Using a genome scan approach to compare patterns of polymorphism and divergence within and between taxa, we estimated standard population genetic parameters of within-population variation (average pairwise nucleotide diversity,  $\pi$ ), deviation of the allele frequency spectrum from neutral expectation (Tajima's  $D$ ) and pairwise population differentiation ( $F_{ST}$ ) and divergence ( $D_{xy}$ ). From the Pool-seq data, parameter estimates were obtained using POPOOLATION (Kofler *et al.* 2011a) and POPOOLATION2 (Kofler *et al.* 2011b), as appropriate, based on nonoverlapping windows of 1 kb. The window-based approach to parameter estimation was adopted in the light of the unavoidably small sample sizes of diploid individuals included in our pools (<40 individuals), as recommended by Schlotterer *et al.* (2014). Tajima's  $D$  (Tajima 1989) within the pools was calculated using the correction of Achaz (2008), and because one of its components (the number of segregating sites) strongly depends on pool size, which differed between our taxa, we standardized the pooled data by randomly downsampling to a read coverage matching the smallest pool (typical *An. gambiae*) using Picard Tools' Downsampler.jar, following Nolte *et al.* (2013). The average number of pairwise differences,  $D_{xy}$  (Nei 1987; equation 10.20), was calculated between reference sequences from each pool using POPOOLATION 1.2.2. Reference sequences were generated with a custom Python script (accessible at [github.com/rrlove/Bamako](https://github.com/rrlove/Bamako)) that incorporates alleles with a probability proportional to their frequency in the population; PROGRESSIVEMAUVE (Darling *et al.* 2010) was used to generate the input file required by POPOOLATION. From the individual sequences, parameters were estimated from larger (200 kb) nonoverlapping windows using VCFTOOLS 0.1.11 and 0.1.12a (Danecek *et al.* 2011). All downstream analysis of population genetic parameter estimates was performed in R (R Development Core Team 2016). Population genetic parameters were plotted in R using the packages GGPLOT2 (Wickham 2009), GRIDEXTRA (Auguie 2016) and SCALES (Wickham 2014).

### Linkage disequilibrium

From individual sequences and within pools, we analysed patterns of linkage disequilibrium (LD). From pools, LD was calculated using LDx (Feder *et al.* 2012) and custom R scripts. For each chromosome arm, we calculated the correlation coefficient  $r^2$  between pairs of SNPs spaced 200–300 bp apart in sliding windows of 1000 SNPs with step size of 100 SNPs. Because differences in pool size strongly affect LD estimates, we first down-sampled to a coverage matching the smallest (typical *An. gambiae*) pool, as described above. Variants used for LD calculations were called from the down-sampled data using the UnifiedGenotyper walker of GATK (due to the pooled nature of the data), and filtered to retain only variant sites that met the following criteria: minimum read depth of 10; maximum read depth fixed to the 99th percentile of coverage for the pool; PHRED-scaled base quality score of 20; minimum allele frequency of 0.1; insert sizes as reported above. LD calculated from pools was plotted using the R package ZOO (Zeileis & Grothendieck 2005).

From individual Bamako sequences, LD ( $r^2$ ) was estimated on chromosome 2R with PLINK 1.90b (Purcell *et al.* 2007) and plotted with the R packages RESHAPE2 (Wickham 2007) and LDHEATMAP (Shin *et al.* 2006). The heatmap was coloured using RCOLORBREW (Neuwirth 2011).

### Functional enrichment analysis in outlier genomic regions

We used the Functional Annotation Clustering tool of DAVID 6.8 (Beta) (Huang *et al.* 2009) to determine whether certain pathways and functional annotation terms are statistically overrepresented in Bamako genomic regions with elevated differentiation from typical *An. gambiae*. The annotation databases used by this software (i.e. DAVID Knowledgebase) are current (updated May 2016). Input gene lists were compared to the background reference list (*An. gambiae* PEST AgamP4.3 gene set, 20 October 2015). Annotation clusters (groups of related terms that share gene members) whose overall enrichment score exceeds 1.3 ( $P < 0.05$ ) are considered significantly enriched. The overall enrichment score for a cluster is the geometric mean (in  $-\log$  scale) of individual scores assigned to each annotation term in the cluster. Individual scores (known as EASE scores) are  $P$ -values based on a modified Fisher's exact test (Huang *et al.* 2009).

## Results

Sequencing of three population pools resulted in 682 456 384 paired-end reads; after adapter and

quality trimming, we retained 579 119 542 paired-end reads (84.9%), with an average length of 95.5 bases. Of the retained reads, 434 130 146 (75.0%) mapped to the reference genome. These reads, after filtering for base and mapping quality, produced total mean coverage of 117X across all three pools, and mean coverage per pool (individual) of 51.1X (1.3X), 20.5X (1.3X) and 45.6X (1.4X), for Bamako, typical *Anopheles gambiae* and *Anopheles coluzzii*, respectively (Table 1 and Fig. S1, Supporting information). At least 80% of the chromosomally assigned reference genome was accessible for analysis after implementation of quality filters in Bamako, typical *An. gambiae* and *An. coluzzii*: 88.1% (203 059 1-kb windows), 80.5% (185 544 1-kb windows) and 88.7% (204 484 1-kb windows), respectively.

Previous resequencing of individual samples produced an average coverage depth of 8.9X across the three taxa, 9.5X for Bamako + typical *An. gambiae*, and 7.5X for *An. coluzzii* [see Table S5, Supporting information of Fontaine *et al.* (2015) for further detail].

### Population clustering

Using SNPs called from individual whole-genome sequences representing our ingroup (Bamako, typical *An. gambiae* and *An. coluzzii*) and the outgroup *An. christyi*, we constructed NJ distance trees (Fig. 1). When the NJ trees were constructed from the genome-wide SNP set (Fig. 1A), all Bamako individuals form a maximally supported cluster exclusive of the other two taxa. As expected, all internode distances – both within and between taxa – are very short, reflecting the low levels of differentiation in this group of taxa. To explore whether exclusive clustering of Bamako mosquitoes is driven solely by inverted regions on chromosome 2R that formally define this taxon, we next constructed trees based on SNPs from collinear parts of the genome. The Bamako mosquitoes again clustered together exclusive of the other taxa with maximal bootstrap support

(Fig. 1B), suggesting an important degree of differentiation even outside inverted genomic regions.

As an independent assessment of population clustering, we used ADMIXTURE to conduct an unsupervised estimate of ancestry. Although cross-validation error was lowest for  $K = 1$  (Table S4, Supporting information), consistent with the very recent and modest differentiation of the taxa studied,  $K = 3$  correctly identified Bamako as a population group (ancestral component), as shown in Fig. 1C.

### Genomewide patterns of variation

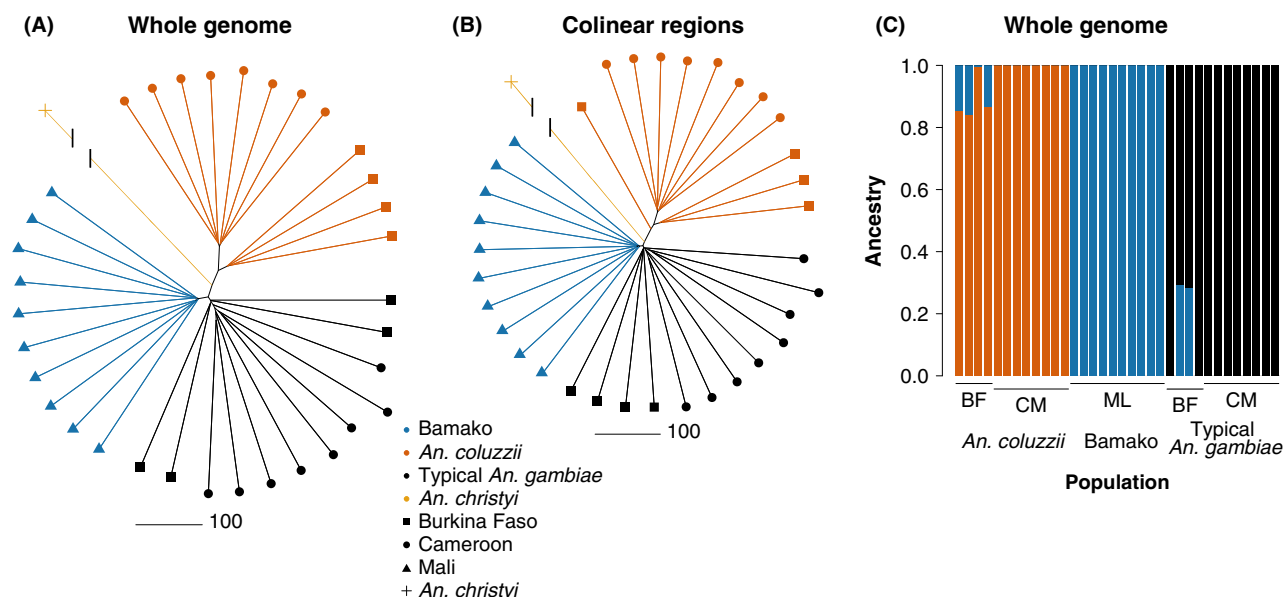
We conducted genome scans and generated summaries of genetic diversity in Bamako, typical *An. gambiae* and *An. coluzzii* (Fig. 2, Table 1; Figs S2 and S3, Table S5, Supporting information). Overall, nucleotide diversity ( $\pi$ ) is lowest on the X chromosome in all three pools (Fig. S3b-e, Supporting information). In addition, all three taxa show sharp drops in nucleotide diversity near the centromeric regions where the recombination rate declines (Pombi *et al.* 2006), particularly on the X chromosome. This pattern is expected (Begun & Aquadro 1992) and was observed previously for species in the *An. gambiae* complex (Fontaine *et al.* 2015), although it was not observed in a recent population genomic study of *Drosophila mauritiana* (Nolte *et al.* 2013). In our data, this pattern may be exacerbated by lower coverage and missing data in centromere-proximal regions (Fig. S1, Supporting information). Overall, the patterns of diversity along chromosome arms are very similar among taxa, with the notable exception of inversions 2Rc and 2Ru, which show strongly decreased diversity in Bamako (Figs 2 and S2, Table S5, Supporting information).

To identify deviations in the allele frequency spectrum from neutral expectation, we calculated Tajima's  $D$  in windows across the genome (Fig. 2, Table 1). In general, all three taxa show negative Tajima's  $D$  values genomewide (Table 1), indicating an excess of rare

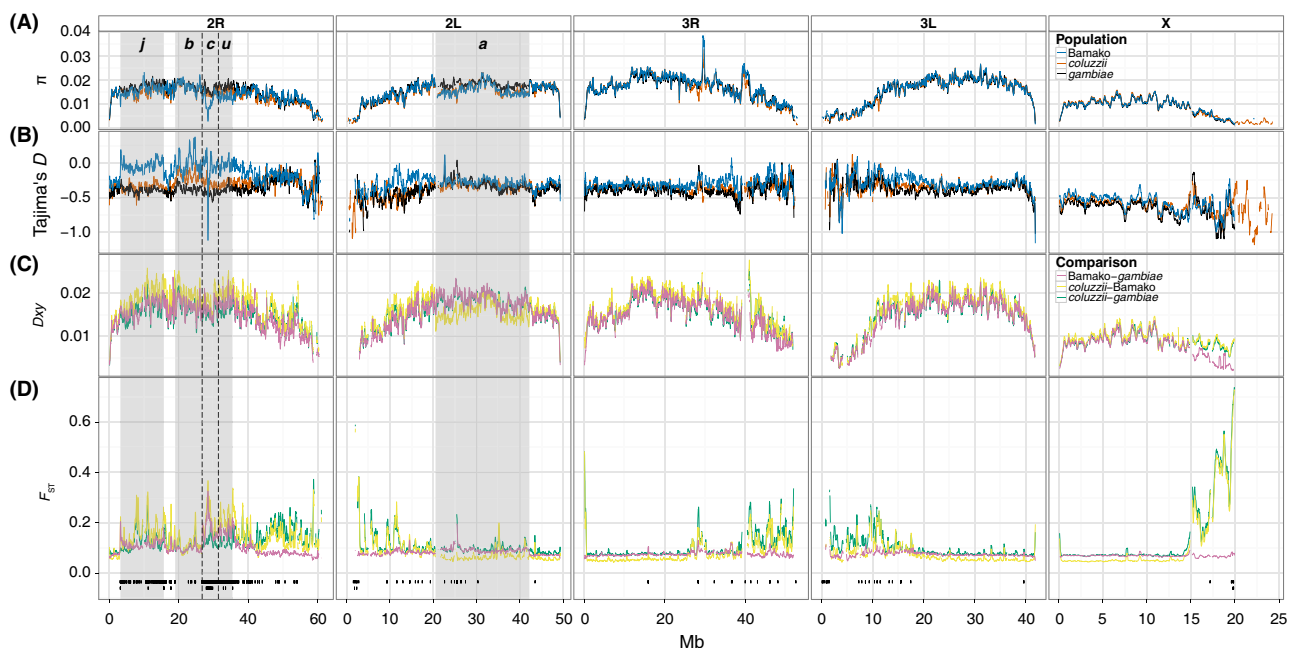
**Table 1** Mean (median) [5–95 percentiles] for nucleotide diversity and Tajima's  $D^*$  from pools of *Anopheles gambiae* s.l. from southern Mali

	Bamako	<i>Anopheles gambiae</i>	<i>Anopheles coluzzii</i>
Individuals per pool	39	16	32
Coverage	51.1 (53.0)	20.5 (20.0)	45.6 (48.0)
$\pi$ , Autosomes	0.015 (0.014) [0.004 to 0.030]	0.016 (0.015) [0.004 to 0.030]	0.015 (0.014) [0.004 to 0.029]
$\pi$ , X	0.009 (0.008) [0.001 to 0.020]	0.009 (0.008) [0.001 to 0.020]	0.008 (0.007) [0.001 to 0.020]
$D$ , Autosomes	−0.270 (−0.268) [−0.985 to 0.438]	−0.414 (−0.408) [−1.040 to 0.188]	−0.377 (−0.367) [−1.089 to 0.290]
$D$ , X	−0.590 (−0.588) [−1.427 to 0.210]	−0.659 (−0.665) [−1.434 to 0.106]	−0.628 (−0.632) [−1.479 to 0.202]

\*Tajima's  $D$  calculations based on down-sampled data sets to achieve uniform coverage.



**Fig. 1** Population clustering of 34 individual samples of Bamako, typical *Anopheles gambiae* and *Anopheles coluzzii*. Neighbour-joining trees based on whole-genome data (A) or collinear (uninverted) genomic regions (B), rooted with the outgroup *Anopheles christyi*. In both trees, Bamako clades are maximally supported by bootstrapping. Length of the branch leading to *An. christyi* is not to scale. (C) Unsupervised ancestry estimation from ADMIXTURE based on  $K = 3$  (BF, Burkina Faso; CM, Cameroon; ML, Mali).



**Fig. 2** Whole-genome scans of diversity and pairwise divergence for Bamako, typical *Anopheles gambiae* and *Anopheles coluzzii* population pools. Shaded boxes span genomic regions corresponding to known chromosome 2 inversions. All parameters are displayed in 200-kb windows slid 20 kb. (A) Mean nucleotide diversity,  $\pi$ ; (B) mean Tajima's  $D$ ; (C) mean pairwise absolute divergence,  $D_{xy}$ ; (D) mean pairwise differentiation,  $F_{ST}$ . Below the line graph in (D), positions of 1-kb outlier  $F_{ST}$  windows (top row) and 1-kb outlier windows of putative positive selection (bottom row) are displayed as black vertical bars.

variants, as observed in previous studies (e.g. Donnelly *et al.* 2001, 2002; Cohuet *et al.* 2008; Crawford & Lazzaro 2010; O'Loughlin *et al.* 2014). The lower level of

variation observed on the X vs. autosomes is paralleled by slightly more negative Tajima's  $D$  values on the X chromosome in all taxa (Table 1). Across the rearranged



regions of 2R, Bamako generally exhibits less strongly negative values for Tajima's  $D$  relative to the other taxa. The exception to this trend is a strong negative spike in  $D$  inside the 2Rc inversion in the Bamako pool (Fig. 2), which was also captured in the genome scan from individually sequenced Bamako mosquitoes (Fig. S2, Supporting information).

As expected owing to reduced recombination near centromeres, linkage disequilibrium (LD) is elevated in the centromere-proximal regions in all taxa. Patterns of LD across chromosome arms are comparable among taxa except on chromosome 2. On this chromosome, Bamako is distinguished by elevated LD inside inversions on 2R and strongly elevated LD inside inversion 2Rc; LD in Bamako is also moderately elevated on 2L, albeit outside the 2La inversion in a ~6-Mb proximal region (Fig. 3). Inside the 2La inversion (fixed or at very high frequency in all three taxa in southern Mali; Table S2, Supporting information), LD does not appear to be elevated.

#### Genomewide patterns of differentiation and divergence

We investigated how allelic frequencies in Bamako differ from the other two taxa (Fig. 2, Table 2; Figs S2 and S4, Table S6, Supporting information). Mean absolute pairwise divergence ( $D_{xy}$ ) is virtually identical between all pairs of taxa, ~0.015. Mean genomewide differentiation ( $F_{ST}$ ) is 0.084 between Bamako and typical *An. gambiae*, and slightly higher between *An. coluzzii* and either Bamako or typical *An. gambiae* (0.108 and 0.120, respectively). Overall, differentiation is slight among all three taxa, reflecting extensive shared ancestral polymorphism and likely some contemporary gene flow (Table 2). Of note, the strongly elevated levels of  $F_{ST}$  observed between *An. coluzzii* and either Bamako or typical *An. gambiae* near the centromeres [prompting the metaphor 'speciation islands' (Turner *et al.* 2005)] are not echoed by  $D_{xy}$  (Fig. 2). Indeed, overall  $D_{xy}$  levels appear relatively constant across the chromosome arms, with one exception: in contrast to  $F_{ST}$ ,  $D_{xy}$  is moderately depressed near all centromeres (and possibly also near telomeres), as expected (Cruickshank & Hahn 2014). The depression in  $D_{xy}$  near the centromeres is strongest between Bamako and typical *An. gambiae* – an effect most dramatic on the X chromosome. In the inverted regions on chromosome 2, especially on 2R, corresponding  $F_{ST}$  values between Bamako and typical *An. gambiae* are elevated.

#### Outlier loci between Bamako and typical *Anopheles gambiae*

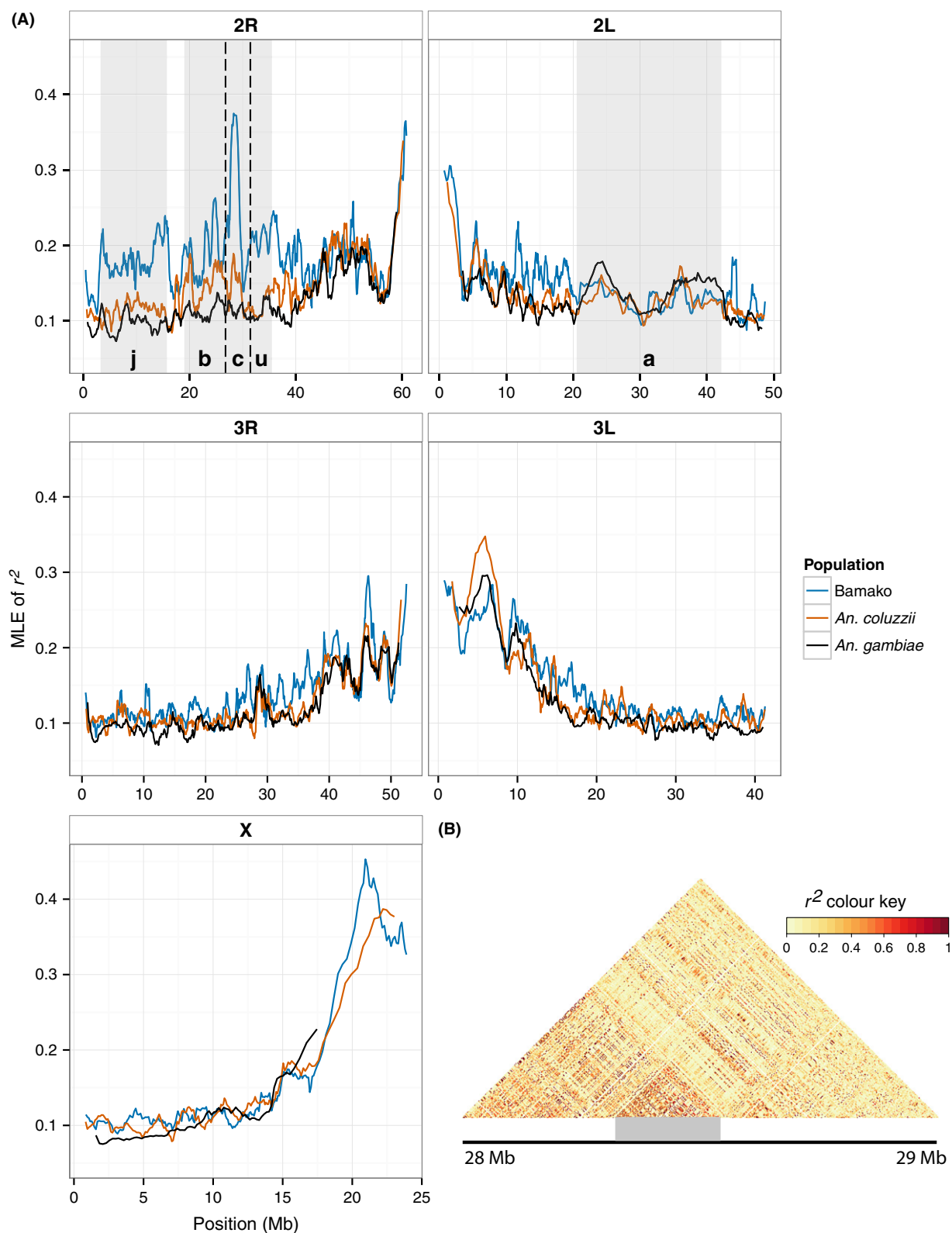
To gain more insight into the distribution and nature of genomic regions of differentiation between Bamako and

typical *An. gambiae*, we focused on  $F_{ST}$  outliers based on nonoverlapping 1-kb windows across the genome that passed all filters ( $N = 182\,174$ ). Outliers were empirically defined as  $F_{ST}$  values in the top 1% of the genomewide distribution ( $F_{ST} \geq 0.233$ ). Of the 1822 outlier  $F_{ST}$  windows, the vast majority – 92.5% (1686) – are on chromosome 2R (Fig. 2, bottom panel), despite that 2R represents only 22.5% of the assembled genome (and only 27% of 1-kb windows in the analysis). Moreover, 87% of the outlier  $F_{ST}$  windows on 2R (1474) are located inside of 2R chromosomal rearrangements ( $j$ ,  $b$ ,  $c$ ,  $u$ ), and of those, 81% (1199) fell within the two smallest rearrangements, 2Rc (~4.67 Mb, 815 outlier windows) and 2Ru (~4.02 Mb, 384 outlier windows). The region corresponding to the 2Rb rearrangement (~7.73 Mb) – the only one in which both inverted and standard orientations are segregating in Bamako (the other three 2R rearrangements,  $j$ ,  $c$  and  $u$ , are fixed for the inverted orientation in this taxon) – contained only 20 outlier  $F_{ST}$  windows.

The remaining 7.5% (136) of outlier  $F_{ST}$  windows not located on 2R are distributed across the other chromosome arms: 65 on 2L, 37 on 3L, 28 on 3R and 6 on the X chromosome. Even on these arms, the outlier windows are not dispersed uniformly. On 2L, 61.5% of the windows (40 of 65) are located in two small regions: a ~200-kb region inside of the ~22-Mb 2La inversion spanning chromosomal coordinates ~25.4–25.6 Mb that contains 30 windows, and a ~45-kb collinear region at position ~14.5 Mb that contains 10. Similarly, 60% of the windows on 3R are confined to three small regions totalling ~250 kb, and all but one of the six outlier windows on the X chromosome also are within a single ~250-kb region.

#### Genic differentiation between Bamako and typical *Anopheles gambiae*

Candidate genes overlapping outlier  $F_{ST}$  windows were identified in two steps. An initial list, based on the gene models available in VectorBase (gene set AgamP4.3), was based on the 310 predicted protein-coding genes (and eight noncoding RNAs) that overlap the 1822 outlier  $F_{ST}$  windows identified in the genomewide analysis reported above. This list was refined using a second conservative filtering step imposed in rearranged genomic regions, with the goal of reducing false positives in these regions that may be present owing to reduced recombination in inversion heterozygotes. Towards this end, we identified  $F_{ST}$  outlier windows separately inside each inversion, basing the empirical cut-off on the top percentile of values for windows spanning the focal inversion. The final list of 150 candidate genes (Table S7, Supporting information) is composed of the



**Fig. 3** Analysis of linkage disequilibrium ( $r^2$ ) in Bamako, typical *Anopheles gambiae* and *Anopheles coluzzii*. (A) Sliding window analysis of LD between SNPs separated by 200–300 bp on the same set of reads in population pools (bin, 1000 SNPs; step 100 SNPs). Shaded boxes denote chromosomal inversions. (B) LD within 10 individual Bamako sequences, shown for the portion of chromosome 2R spanning 28 to 29 Mb.

**Table 2** Mean (median) [95 percentile] pairwise  $F_{ST}$  and  $D_{xy}$  values from pools of *Anopheles gambiae* s.l. from southern Mali

	Bamako– <i>gambiae</i>	Bamako– <i>coluzzii</i>	<i>gambiae</i> – <i>coluzzii</i>
$F_{ST}$			
Genomewide	0.084 (0.075) [0.146]	0.108 (0.071) [0.298]	0.120 (0.089) [0.287]
Autosomes, collinear	0.077 (0.072) [0.121]	0.095 (0.067) [0.244]	0.115 (0.087) [0.264]
Autosomes, rearranged	0.112 (0.098) [0.215]	0.125 (0.099) [0.300]	0.111 (0.098) [0.203]
X chromosome	0.068 (0.066) [0.101]	0.156 (0.055) [0.724]	0.178 (0.080) [0.733]
$D_{xy}$			
Genomewide	0.015 (0.013) [0.031]	0.016 (0.014) [0.033]	0.015 (0.013) [0.031]
Autosomes, collinear	0.014 (0.013) [0.030]	0.016 (0.014) [0.033]	0.015 (0.013) [0.031]
Autosomes, rearranged	0.018 (0.016) [0.033]	0.018 (0.016) [0.034]	0.017 (0.016) [0.032]
X chromosome	0.008 (0.006) [0.020]	0.010 (0.008) [0.022]	0.009 (0.007) [0.021]

59 genes overlapping outlier windows in collinear regions from the first list and the 91 genes overlapping windows in inverted regions that were identified as outliers in both the first and the second steps.

The functional annotation clustering tool of DAVID was able to cluster all but 19 of the 150 candidate genes, revealing two enriched pathways and four enriched annotation clusters with overlapping genic content (Table 3). The KEGG pathways ‘lysosome’ (ko04142) and ‘neuroactive ligand–receptor interaction’ (ko04080) were related to the annotation clusters, which were enriched with terms such as transmembrane helix (cluster 1), G-protein-coupled receptor (GPCR; cluster 2), cytochrome P450 (CYP450; cluster 3), and immunoglobulin domain (cluster 4), a domain potentially involved in protein–protein and protein–ligand interactions. Many of the genes in these clusters are inferred to encode chemosensory receptors (including 12 GPCRs and three ionotropic receptors), ion channels and transporters putatively involved in neuroactive signalling, an activity that may be supported by additional candidate genes predicted to encode proton pumps, ion exchangers and components of the endosomal pathway. It is noteworthy that among the ion channel genes are the GABA-gated chloride channel known as *resistant to dieldrin* (*rdl*), implicated in resistance to that insecticide,

**Table 3** Enrichment analysis of genes in regions of exceptional Bamako–typical *Anopheles gambiae* differentiation

Pathway or annotation cluster	Score	Count
KEGG lysosome	$P = 1.7E-3$	5
KEGG neuroactive ligand–receptor interaction	$P = 3.8E-2$	3
Transmembrane helix	$4.2 (P = 6.3E-5)$	53
G-protein-coupled receptor	$4.1 (P = 7.9E-5)$	12
Cytochrome P450	$2.8 (P = 1.6E-3)$	17
Immunoglobulin	$1.9 (P = 1.3E-2)$	12

and the *para* sodium channel gene, implicated in resistance to DDT and permethrin. Oxidoreductase activity is represented by several desaturases as well as eight CYP450 genes, of which at least four have been previously implicated in insecticide resistance in *An. gambiae* and *An. coluzzii*.

We used OrthoDB (Kriventseva *et al.* 2015) to identify the orthologous group – at the level of Diptera – to which each candidate *An. gambiae* gene belongs, and to retrieve the corresponding functional annotations (indicated in Table S7, Supporting information). The power of this approach is that OrthoDB provides not only integrated functional annotations from UniProt, InterPro and GO, but also (where available from FlyBase) the associated mutant phenotype(s) based on experimentation with the model organism *Drosophila melanogaster*. We found that the *D. melanogaster* orthologs of many of our candidate genes have been implicated experimentally in neurogenesis, neuromuscular synapse formation, neuronal signalling and a variety of behaviours controlled by the nervous system (e.g. locomotion, sleep, learning/memory, courtship). The concentration of Bamako–*An. gambiae* differentiation in 2R inversions and the enrichment of genes implicated in nervous system development and signalling in these regions invite the hypothesis that changes in Bamako’s perception and response to external cues may underlie its adaptation to a marginal rock pool larval habitat.

#### Localized footprints of positive selection

The subset of 1-kb outlier windows of  $F_{ST}$  that also are depauperate of nucleotide diversity within Bamako (i.e. they fall in the top and bottom 1% of genomic distributions of  $F_{ST}$  and  $\pi$ , respectively) may be associated with targets of positive selection in Bamako. Of 103 such windows, all except 5 are on chromosome 2R. The 98 windows on 2R are mainly (86%) distributed in two small clusters: a ~28-kb region near the distal break

point of the 2Rj inversion ( $N = 20$ ), and a ~1.9-Mb region in the distal half of 2Rc ( $N = 64$ ). As the 2Rj and 2Rc inversions are homokaryotypic in Bamako, the reduction in nucleotide diversity in these regions is unlikely to be due to suppressed recombination in the 2Rj and 2Rc regions of the Bamako genome. Nevertheless, LD within euchromatic regions of Bamako reaches its highest value in a 1000-SNP (~1.6 Mb) window centred at ~28.31 Mb inside 2Rc, where mean  $r^2$  is 0.375 in the Bamako pool (Fig. 3A) and individual Bamako mosquitoes show a noticeable haplotype block (Fig. 3B).

There are 24 candidate positively selected genes that overlap this set of 103 outlier windows and also are present in the refined list of genes from exceptionally differentiated genomic regions (Table S7, Supporting information). Several of the candidate CYP450s and *para* have been implicated repeatedly in insecticide resistance not only in *An. gambiae* and *An. coluzzii*, but also in other anopheline and insect species (Ranson *et al.* 2011; Tene Fossog *et al.* 2013; Djegbe *et al.* 2014; Edi *et al.* 2014; Silva *et al.* 2014; Toe *et al.* 2015); thus, it is plausible that they may have been recent targets of positive selection in Bamako. CYP6P3 and CYP64 are two of eight consecutive CYP450 genes in the CYP6 subfamily, located within inversion 2Rc (Fig. 4). Both CYP6P3 and CYP64 genes carry nonsynonymous substitutions (CYP6P3, G151E: 2R 28 492 690; CYP6P4, P289L: 2R 28 497 809) that are apparently fixed in our pooled Bamako sample but are not detected in either of the other pools of typical *An. gambiae* or *An. coluzzii*; nor do these Bamako variants appear in other CYP6P3-4 gene sequences from *An. gambiae s.l.* deposited in VectorBase. Immediately downstream of the eight CYP6 genes inside 2Rc is another gene in this candidate set (AGAP13202), intriguing because it carries three nonsynonymous substitutions that are apparently fixed or at high frequency in the Bamako pooled sample (2R: 28 518 614; 28 518 671; 28 519 004) but are not detected in the other pools. Unfortunately, AGAP13202 is one of several genes on this list devoid of any functional annotation, and this gene lacks known orthologs apart from those in other, similarly unannotated, anophelines.

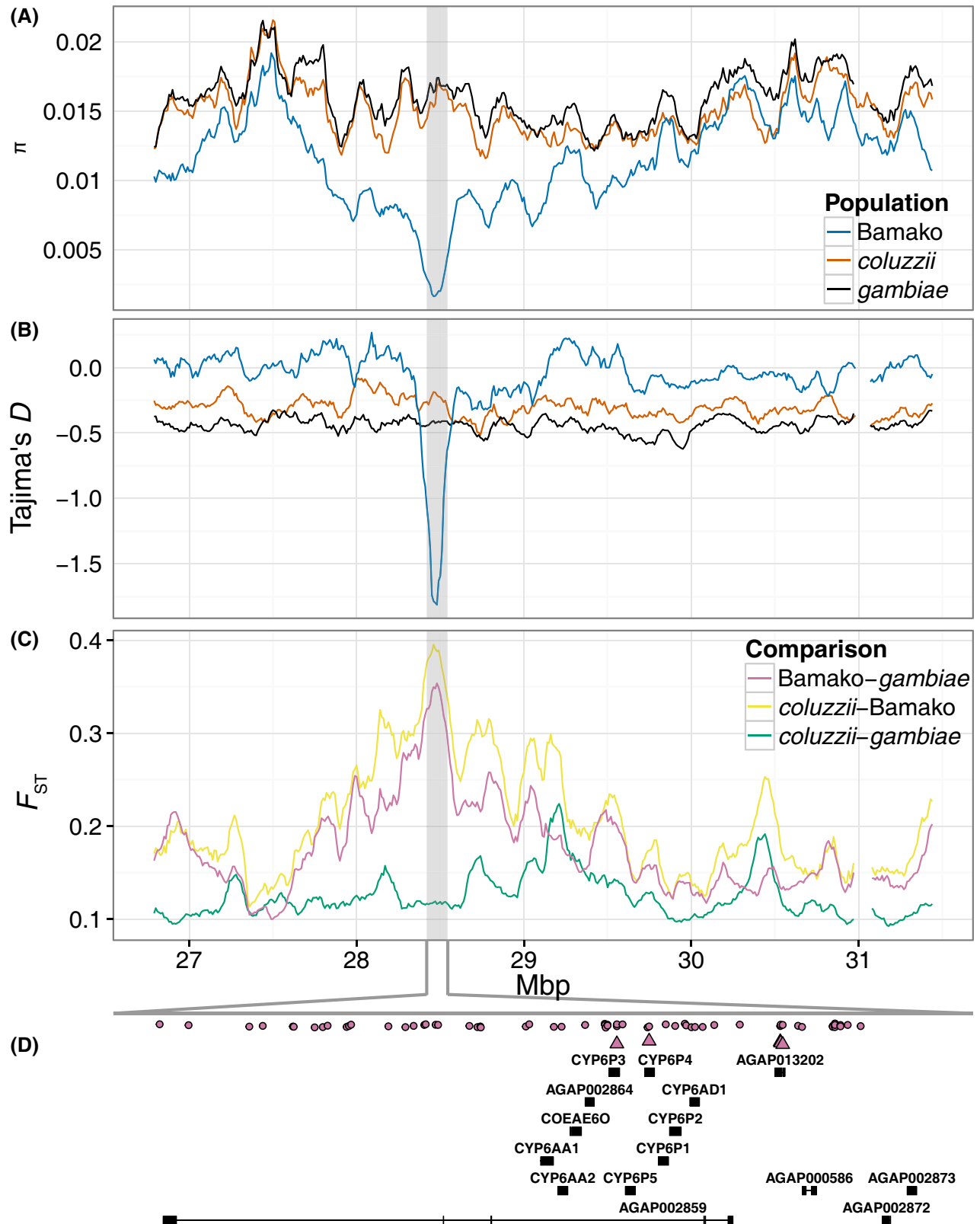
One of only two genes from this list on chromosome 2L – the voltage-gated sodium channel *para* – carries an insecticide resistance mutation well-known in West Africa (L1014F *kdr*). This mutation is at low frequency in the Bamako pool (~3% of Bamako reads) and moderate frequency in typical *An. gambiae* (~35% of reads covering that site), congruent with previous studies from southern Mali (Fanello *et al.* 2003; Tripet *et al.* 2007). We did not detect the L1014F mutation in the *An. coluzzii* pool. An additional seven membrane channel/transporter genes (as well as other candidates on the list)

carried no nonsynonymous substitutions, but may have been associated with undetected regulatory mutations.

Although the GABA-gated chloride channel gene (*rdl*) was not included among the positive selection candidates, the known association of characteristic mutations in this gene with insecticide resistance in *An. gambiae s.l.* (Du *et al.* 2005) and other insects (Ffrench-Constant *et al.* 2000; Wondji *et al.* 2011; Asih *et al.* 2012), and the previous suggestion of recent independent selective sweeps centred on *rdl* in sympatric populations of *An. gambiae* and *An. coluzzii* from Mali (Lawniczak *et al.* 2010) prompted a closer examination. Two adjacent nonsynonymous substitutions affecting the same amino acid have been reported in *An. gambiae s.l.*: one (Ala296Gly) observed in typical *An. gambiae* and the other (Ala296Ser) in *An. coluzzii* (Du *et al.* 2005; Lawniczak *et al.* 2010). We find that ~25% of reads in the typical *An. gambiae* pool match the Ala296Gly allele, which was not detected in the other pools of *An. coluzzii* and Bamako. The Ala296Ser allele was at high frequency in the *An. coluzzii* pool (~79%), and although it was sampled only once from typical *An. gambiae*, it was detected in ~40% of the pooled Bamako reads covering that site.

## Discussion

Decades of cytogenetic evidence from Mali, based on both larval and adult samples, have supported the existence of an assortatively mating ecotype Bamako, despite the strict sympatry and partial syntopy of two additional reproductive units that are fully interfertile with Bamako: typical *Anopheles gambiae* and *Anopheles coluzzii* (Coluzzi *et al.* 1985; Toure *et al.* 1998). Our analyses based on whole-genome sequencing of these taxa suggest that despite extensive shared variation, Bamako is a distinct evolutionary lineage. In neighbour-joining trees reconstructed from multiple individual genomic sequences of the three taxa, the Bamako individuals all cluster together exclusive of other taxa, even when chromosome 2R rearrangements are omitted from the analysis. Yet, the vast majority of differentiation between Bamako and typical *An. gambiae* occurs in the 2R inversions used to classify Bamako (2Rj, *c* and *u*), especially within the 2Rc region. This finding may have a larger evolutionary significance in the *An. gambiae* complex. A polytene chromosome analysis of this group of species revealed that the central part of chromosome 2R, corresponding to the 2Rc region, has been captured repeatedly by fixed and polymorphic chromosomal inversions in the species complex (Coluzzi *et al.* 2002). The species in this recent radiation are all morphologically indistinguishable at every developmental stage; their most obvious phenotypic differences are associated with preferred larval habitat (*e.g.* ephemeral vs.



**Fig. 4** Putative selective sweep on chromosome 2R in the Bamako population. Parameters were calculated in 100-kb windows slid 10 kb. (A) Mean nucleotide diversity  $\pi$ ; (B) mean Tajima's  $D$ ; (C) mean pairwise differentiation,  $F_{ST}$ ; (D) genes wholly contained in the pictured region, with exons as black rectangles and introns as lines connecting them. Approximate location of SNPs occurring at high  $F_{ST}$  ( $\geq 0.9$ ) between Bamako and typical *Anopheles gambiae* is indicated by pink dots (synonymous or noncoding) and pink triangles (nonsynonymous). SNPs were identified in the pooled samples at sites that met the coverage minimum and maximums described for the windowed analyses and had a minimum minor allele count of 2.

stable; natural vs. man-made; fresh water vs. brackish). The centrality of the 2Rc region in chromosome changes within and between species prompted Coluzzi *et al.* (2002) to speculate that genic content in this region may play an important role in the choice of oviposition site by gravid females and larval adaptation to breeding sites that differ in abiotic and biotic characteristics such as stability, biotic complexity and salinity. The enrichment in this genomic region of functions related to nervous system development and signalling is broadly consistent with this hypothesis, which now calls for difficult follow-up studies aimed at connecting genotype to specific phenotypes and measuring fitness consequences (Barrett & Hoekstra 2011).

At a variety of loci associated with insecticide resistance (e.g. *Cyp6P3*, *Cyp6P4*, *rdl*, *para*), presumed resistance alleles in Bamako expected to confer a universal benefit upon exposure to particular classes of insecticides are nevertheless segregating at very different frequencies in sympatric samples of typical *An. gambiae* and *An. coluzzii*, underscoring the distinctiveness of the Bamako taxon (see also Main *et al.* 2015). Most striking is the Bamako-specific selective sweep in 2Rc centred on *CYP6P3* and *CYP6P4*, genes fixed for (yet uncharacterized) nonsynonymous substitutions apparently absent from co-occurring population samples of *An. coluzzii* and typical *An. gambiae*. The *rdl* locus also supports the idea of Bamako as a reproductive unit distinct from typical *An. gambiae*. At this locus, a resistance allele common in typical *An. gambiae* is absent from Bamako, which instead carries an alternative resistance allele in common with *An. coluzzii*, albeit at a sharply lower frequency. Conceivably, frequency differences between Bamako and the other taxa with respect to insecticide resistance alleles may be the outcome of different degrees of xenobiotic exposure rather than reproductive isolation. This explanation seems unlikely for several reasons. Insecticides used in public health are applied indoors, on bed nets or walls, where the probability of contact by adults of all three taxa should be equivalent, given their equally high rates of anthropophily (Coluzzi *et al.* 1985). Although insecticides used in agriculture may differentially run off into spatially and ecologically distinct larval breeding sites utilized by these three taxa, habitat partitioning is not absolute (e.g. Manoukis *et al.* 2008). Actual larval habitat use overlaps considerably, implying at least some degree of

common exposure to habitat-associated chemicals and pathogens. An alternative perspective, sometimes forgotten, is that resistance-associated mutations and the genes in which they occur may have other functions important to organismal fitness that are not necessarily related to insecticide resistance (Ffrench-Constant 2013). For example, although *rdl* is associated with resistance to dieldrin and fipronil insecticides, it is also involved in regulation of sleep, aggression and olfactory learning (Liu *et al.* 2009, 2014; Yuan *et al.* 2014). In the same vein, the *TEP1* gene, whose complement C3-like product has been of great interest to vector biologists due to its role in anti-*Plasmodium* immunity, carries alternative alleles that render *An. gambiae* susceptible or refractory to *Plasmodium* infection (Blandin *et al.* 2009). However, it was recently shown that one of the same alleles associated with *Plasmodium* susceptibility (*TEP1*\*S2) also functions during spermatogenesis to confer superior male fertility relative to the refractory (and other) *TEP1* alleles, suggesting possible trade-offs between reproduction and immunity (Pompon & Levashina 2015). Interestingly, in our Pool-seq data the Bamako pool differs from typical *An. gambiae* and *An. coluzzii* at the *TEP1* locus, in that the Bamako sample appears fixed for key nonsynonymous substitutions characteristic of the *TEP1*\*S2 allele, in contrast to the other two taxa whose corresponding sequences are polymorphic but strongly skewed towards refractory alleles (data not shown). Taken together, our data suggest that differences in resistance allele frequencies among the co-occurring taxa reflect an ongoing process of ecological isolation.

Although the vast majority of genomic divergence between Bamako and typical *An. gambiae* coincides with inversions on 2R, all 2R inversions are shared among taxa. Indeed, with the exception of 2Rj (generally rare in typical *An. gambiae* and *An. coluzzii* populations), 2R inversions characteristic of (and fixed in) Bamako are polymorphic at moderate to high frequencies in the other taxa. For example, in a foundational synthesis of cytogenetic data from Mali (Toure *et al.* 1998), a 1983 collection of ~1400 mosquitoes sampled from the middle of the rainy season in the village of Banambani was karyotyped and analysed in detail. The Bamako subsample carried two chromosome 2R karyotypes (*jcu* and *jbcu*) at frequencies of ~78% and ~22%. However, karyotypes that included the *b*, *c* and *u* inversions also were common in typical *An. gambiae* (e.g. *bcu*, ~17%; *cu*,

~23%) and *An. coluzzii* (e.g. *bc*, ~56%). The most extreme frequency differences were found for the *j* inversion, which was fixed in Bamako while segregating at frequencies of only ~7% and ~3% in typical *An. gambiae* and *An. coluzzii*, respectively. Hence, the role of inversions in helping to prevent suites of locally adapted alleles beneficial to the Bamako ecotype from recombining into other genetic backgrounds is most easily envisioned for 2Rj. In this light, it is striking that most differentiation observed between Bamako and typical *An. gambiae* was observed in 2Rc, but this observation does not negate an instrumental role for 2Rj, even if differentiation is difficult to detect. In addition, because inversion frequencies cycle seasonally (Toure *et al.* 1994, 1998), frequency differences may be more pronounced depending upon the time of year, and this factor may interact with other factors that may contribute to isolation, including spatial heterogeneity and asynchronous variation in relative abundance of the different populations (Toure *et al.* 1998).

The pattern of genomic differentiation that we find between Bamako and typical *An. gambiae* is concordant with previous results based on a 400 000 SNP genotyping array (Neafsey *et al.* 2010). That study, like ours, found that differentiation between this pair of taxa was almost exclusively located in rearranged regions of chromosome 2R (2Rj, ~3.2–3.6 Mb; 2Rb, ~19.6 Mb; 2Rc, ~27–29 Mb; 2Ru, 35.3 Mb) and 2L (2La, ~23.3–25.5 Mb); no differentiation was found on the X chromosome (Table S2, Supporting information of Neafsey *et al.* 2010). On the other hand, a 2013 study employing a tiling microarray to examine the distribution of genomic differentiation between Bamako and typical *An. gambiae* reported strongly contradictory results (Lee *et al.* 2013a), in which the majority of divergence was on the X chromosome, particularly near the centromere – a pattern reminiscent of the X chromosome ‘speciation island’ between *An. gambiae* and *An. coluzzii* (Turner *et al.* 2005). The tiling microarray employed a perfect-match probe design based on the *An. gambiae* (AgamP3) PEST reference genome, a design that could theoretically result in false negatives (if one or both taxa differ with respect to the probe sequence such that hybridization is prevented and true differentiation cannot be detected). However, it is unlikely that such a design could result in false-positive detection of differentiation. To explain the discrepancy between the tiling array and the SNP array studies, Lee *et al.* (2013a) proposed that the SNP array study might have failed to detect true differentiation between Bamako and typical *An. gambiae* in the centromere-proximal X chromosome region due to bias in the array design. Indeed, in the design of the SNP genotyping array, SNPs near the X centromere were

preferentially included that were fixed for different alleles between *An. gambiae* and *An. coluzzii*, instead of polymorphic in both taxa (Neafsey *et al.* 2010). Such SNPs, if they did not happen to be polymorphic between Bamako and typical *An. gambiae*, would have been uninformative. Our whole-genome sequencing data do not suffer from that bias. In centromere-proximal regions, we are able to clearly show the expected centromere-proximal elevation of  $F_{ST}$  between *An. coluzzii* and either Bamako or typical *An. gambiae*. Nevertheless, comparable differentiation between Bamako and typical *An. gambiae* is entirely absent from the corresponding centromere-proximal region. Thus, rather than a technical issue, we favour the hypothesis that the difference in the genomic patterns of divergence between Lee *et al.* (2013a) on the one hand, and both Neafsey *et al.* (2010) and the present study on the other, has to do with differences between the mosquito samples. Although very speculative, we propose that at least a fraction of the ‘typical’ *An. gambiae* samples hybridized to the tiling microarray by Lee *et al.* (2013a) may actually have represented *An. gambiae*–*An. coluzzii* hybrids (possibly advanced generation hybrids). These authors have recently shown that such hybrids occur in nature more frequently than had been appreciated, including in Mali (Lee *et al.* 2013b). Assuming that these hybrids carried some of the X chromosome alleles that are highly diverged between *An. gambiae* and *An. coluzzii*, their inadvertent inclusion in the microarray experiment as ‘pure’ *An. gambiae* samples presumably would have produced the observed elevated divergence on the X chromosome that was interpreted as divergence between Bamako and *An. gambiae*.

In the present study, we compared patterns of genomic differentiation among ecotypes sampled at the same time from a common geographic area in Mali, a design that helps to minimize the impact of spatial or temporal variation on patterns of genomic variation. However, our study had a number of limitations that complicate interpretation of the genome scans. Perhaps the most obvious is that by their very nature, polymorphic chromosomal inversions reduce recombination, causing difficulties in distinguishing true targets of positive selection from linked false positives. Assuming that at least some important phenotypic differences among ecotypes are manifest at the level of gene expression (Gilad *et al.* 2006; Fuller *et al.* 2016; Mack *et al.* 2016), transcriptional profiling could constitute a complementary approach to identifying candidate genes that would not be subject to the same limitation. Another important limitation is the near-complete lack of prior phylogeographic or demographic information concerning the Bamako ecotype, and the absence of

other geographic samples available for study. The Bamako ecotype has been understudied, at least in part due to its relative rarity (even in Mali) and its consequent minor contribution to the overall malaria vectorial system. However, in the absence of expanded sampling and attention to population demographic history, robust inferences about the nature and the genetic basis of ecotypic differentiation will not be possible.

Studying the genome architecture and genetic mechanisms that facilitate adaptive radiations is fundamental to understanding the origins of biological diversity. In the case of anopheline mosquito vectors of human malaria, this approach can also provide insights into the evolutionary forces that affect vectorial capacity (Cohuet *et al.* 2010) and malaria epidemiology. Although *An. gambiae* may be the most intensively studied, nearly all of the ~10% of anopheline species that transmit malaria in nature are the products of recent and rapid species radiations. A better understanding of their evolutionary ecology is important both for the development and the success of vector control strategies in the global fight against malaria.

## Acknowledgements

We thank H. Uyhelji and the Notre Dame Genomics Core Facility for assistance with library construction; R. Waterhouse for assistance with OrthoDB; C. Witzig for programmatic advice and scripting assistance; M. Hahn for helpful discussion and feedback; and six anonymous reviewers. This work was supported by NIH grant R01AI076584 (NJB, RRL), the FNIH through a program of the Bill & Melinda Gates Foundation (NJB, MCF), a Richard and Peggy Notebaert Premier Fellowship (RRL) and a Jack Kent Cooke Foundation Graduate Scholarship (RRL).

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## Data accessibility

Pooled sequence data generated for this project have been deposited in the Sequence Read Archive under BioProject PRJNA311062. VCF files generated for this project are available on Dryad at doi:10.5061/dryad.m2821. Individual sequence data generated for this project were previously submitted to NCBI under BioProject ID PRJNA254046.

R.R.L., M.C.F. and N.J.B. designed project; M.B.C. and S.T. contributed samples; A.S., M.C.F. and S.J.E. designed alignment and variant calling pipelines; R.R.L. and M.C.F. performed analysis; R.R.L., M.C.F. and N.J.B., with input from co-authors, wrote manuscript.

## Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1** Provenances and karyotype of *Anopheles gambiae s.l.* mosquitoes from southern Mali used for Pool-seq.

**Table S2** Chromosome 2 inversion frequencies within pools of *Anopheles gambiae s.l.* from Mali.

**Table S3** Provenances of individually sequenced *Anopheles gambiae s.l.* mosquitoes used in this study

**Table S4** ADMIXTURE likelihood and cross-validation errors

**Table S5** Nucleotide diversity ( $\pi$ ) values for different *Anopheles gambiae s.l.* taxa and data partitions based on Pool-seq.

**Table S6**  $F_{ST}$  values for different *Anopheles gambiae s.l.* contrasts and data partitions based on Pool-seq.

**Fig. S1.** Sequencing coverage of pooled samples.

**Fig. S2.** Whole-genome scans of diversity and pairwise divergence based on individual Bamako, typical *Anopheles gambiae* and *Anopheles coluzzii* sequences.

**Fig. S3.** Genome-wide distributions of nucleotide diversity  $\pi$  in pooled samples.

**Fig. S4.** Genome-wide distributions of  $F_{ST}$  in pooled samples.

**Table S7.** Candidate gene annotations from VectorBase and OrthoDB.