**Resistance to dieldrin evolution in African malaria vectors is driven by interspecific and interkaryotypic introgression**

Xavier Grau-Bové¹, Sean Tomlinson¹,², Andrias O. O’Reilly³, Nicholas J. Harding⁴, Alistair Miles⁴,⁵, Dominic Kwiatkowski¹⁴,⁵, Martin J. Donnelly¹,⁵, David Weetman¹, The Anopheles gambiae 1000 Genomes Consortium⁶

1. Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom
2. Centre for Health Informatics, Computing and Statistics, Lancaster University, Lancaster,
3. School of Biological and Environmental Sciences, Liverpool John Moores University, Liverpool, UK
4. Big Data Institute, University of Oxford, Li Ka Shing Centre for Health Information and Discovery, United Kingdom
5. Wellcome Sanger Institute, Hinxton, United Kingdom
6. [https://www.malariagen.net/projects/ag1000g#people](https://www.malariagen.net/projects/ag1000g#people)

**Abstract**

The evolution of insecticide resistance mechanisms in natural populations of *Anopheles* malaria vectors is a major public health concern across Africa. Using genome sequence data, we study the evolution of resistance mutations in the *resistance to dieldrin* gene (*Rdl*), a GABA receptor targeted by several insecticides, but most notably by the long-discontinued cyclodiene, dieldrin. The two *Rdl* resistance mutations (296G and 296S) spread across West and Central African *Anopheles* via two independent hard selective sweeps that included likely compensatory nearby mutations, and were followed by a rare combination of introgression across species (from *A. gambiae* and *A. arabiensis* to *A. coluzzii*) and across non-concordant karyotypes of the 2La chromosomal inversion. *Rdl* resistance evolved in the 1950s as the first known adaptation to a large-scale insecticide-based intervention, but the evolutionary lessons from this system highlight contemporary and future dangers for management strategies designed to combat development of resistance in malaria vectors
Introduction

The recurrent evolution of insecticide resistance in the highly-variable genomes of Anopheles mosquitoes [1–3] is a major impediment to the ongoing efforts to control malaria vector populations. Resistance to dieldrin was the first iteration of this cyclical challenge: this organochlorine insecticide was employed in a pioneering vector control programme in Nigeria in 1954, but resistant Anopheles had already appeared after just 18 months [4] due to a single dominant mutation [5,6]. Dieldrin use ceased in the 1970s due to its high persistence as an organic pollutant and unexpectedly wide toxicity, culminating in a ban in the 2001 Stockholm Convention on Persistent Organic Pollutants. However, resistance has remained strikingly persistent in natural Anopheles populations for more than 40 years [7]. The study of the genetic architecture of dieldrin resistance can thus provide key insights into the evolutionary ‘afterlife’ of resistance mechanisms to legacy insecticides. We address this issue by studying its emergence and dissemination in contemporary African populations of the A. gambiae species complex.

Dieldrin resistance in Anopheles spp. is caused by mutations in its target site, the γ-aminobutyric (GABA) receptor gene – also known as resistance to dieldrin locus, or Rdl [7–9]. Two resistance mutations have been found in anophelines, both in Rdl codon 296 (equivalent to codon 302 in Drosophila [7]): alanine-to-glycine (A296G) and alanine-to-serine (A296S). Populations of Anopheles gambiae sensu stricto (henceforth, A. gambiae) and its sister species A. coluzzii possess both 296G and 296S alleles [7,10], whereas the 296S allele is the only one reported in A. arabiensis and the more distantly-related malaria vectors A. funestus and A. sinensis [7,11,12]. Normally, dieldrin inhibits the activity of Rdl receptors, causing persistent neuronal excitation and rapid death; but codon 296 mutations confer resistance by reducing its sensitivity to the insecticide [13]. However, in the absence of exposure, Rdl mutations appear to carry fitness costs, such as lower mosquito mating success [14] or impaired response to oviposition and predation-risk signals [15,16] (although see [17]). Consequently, with seemingly limited current benefit via exposure to insecticides targeting Rdl, persistence of the mutations in anophelines is puzzling.

We interrogate the Anopheles gambiae 1000 Genomes cohort [3,18] to ascertain how often dieldrin resistance mutations have evolved in the A. gambiae/A. coluzzii species pair, and the mechanisms by which these alleles spread across Africa and may persist. We identify two distinct Rdl resistance haplotypes in these species, defined by hard selective sweeps and the perfect linkage of the 296G and 296S alleles with putatively compensatory mutations. Furthermore, the resistance haplotypes are across genomes from different species (A. gambiae, A. coluzzii and A. arabiensis), and across chromosomes with differing karyotypes in the 2La inversion (the longest inversion in Anopheles genomes) [19] within which Rdl resides. Inter-species reproductive isolation and inversions such as 2La both result in reduced recombination rates [20–23], which would in principle hinder the spread of these adaptive alleles. Here, we provide evidence that Rdl resistance alleles, which our structural modelling shows have divergent effects on the channel...
pore, underwent a rare combination of interspecific and interkaryotypic introgression.

Overall, we show that two founding resistance mutations spread with remarkable ease across geographical distance, species, and recombination barriers. This evolutionary trajectory has parallels with later-emerging target site resistance mutations, such as $Vgsc$ [24–27]. The persistence of dieldrin resistance also challenges the efficacy of current and newly developed insecticides that also target $Rdl$ [28–30], as well as the efficacy of rotative insecticide management strategies [31]. These results thus emphasise the influence of past interventions on current and future programmes of vector population control.

## Results

### Distribution of $Rdl$ resistance mutations across African populations

First, we investigated the genetic variation in $Rdl$ across populations of the *Anopheles gambiae* species complex, including *A. gambiae* and *A. coluzzii* from the *Anopheles gambiae* 1000 genomes project (Ag1000G Phase 2, $n = 1142$) [3], and outgroups from four other species (*A. arabiensis, A. quadriannulatus, A. melas* and *A. merus; $n = 36$) [32]. All genomes and their populations of origin are listed in Supplementary Material SM1.

**Figure 1. $Rdl$ mutations.** A) Frequency of non-synonymous mutations in $Rdl$ across populations of *A. gambiae, A. coluzzii* (Ag1000G Phase 2) and *A. arabiensis*. Only variants with >5% frequency in at least one population are included. B) Distribution of genotypes for the two mutations in codon 296 (A296S and A296G). Note: *A. gambiae* populations denoted with an asterisk (The Gambia, Guinea-Bissau and Kenya) have high frequency of hybridisation and/or unclear species identification (see Methods).
We identified six non-synonymous mutations that are segregating in at least one population at ≥5% frequency (Figure 1A; complete list of variants in Supplementary Material SM2), including the 296G and 296S resistance alleles. 296G is present in West and Central African populations of both A. gambiae and A. coluzzii, with frequencies ranging from 30% (Cameroon A. gambiae) to 96% (Ghana A. gambiae). 296S is present in A. coluzzii specimens from Burkina Faso (63%), as well as A. arabiensis (Burkina Faso, Cameroon, Tanzania) and A. quadriannulatus (Zambia). Resistance alleles occur as both homozygotes or heterozygotes in all species except A. quadriannulatus, which is always heterozygous (Figure 1B).

We also identified two mutations in codon 345 with very similar frequencies to those of each codon 296 mutation: T345M (C-to-T in the second codon position), co-occurring with A296G; and T345S (A-to-T in the first codon position), co-occurring with A296S. The high degree of linkage disequilibrium between genotypes in codons 296 and 345 confirmed that they were co-occurring in the same specimens (Figure 2; e.g., the 296G/345M allele pair had a Huff and Rogers r and Lewontin’s D’ = 1), and was apparent in all individual populations where the alleles were present (Supplementary Material SM3). Codons 296 and 345 are located in the 7th and 8th exons of Rdl, separated by 3935 bp; and they map to the second and third transmembrane domains of the RDL protein, respectively (Supplementary Material SM4).

Rdl resistance mutations evolved on two unique haplotypes in A. gambiae and A. coluzzii

The high frequency of the 296S and 296G alleles in various populations of A. gambiae and A. coluzzii (Figure 1), together with their co-occurrence with nearby mutations (Figure 2), were suggestive of a selective sweep driven by positive selection on the resistance alleles. To clarify this possibility, we inspected the similarity of haplotypes in A. gambiae, A. coluzzii and the four outgroup species (n = 2356 haplotypes) using a minimum spanning network based on 626 phased variants located...
10,000 bp upstream and downstream of codon 296 (Figure 3).

**Figure 3. Rdl haplotypes.** A) Minimum spanning network of haplotypes around *Rdl* codon 296 (626 phased variants located +/- 10,000 bp from the 2L:25429236 position). Only haplotype clusters with a frequency >1% in the cohort are represented (complete networks available as Supplementary Material SM6). Each node in the network is color-coded according to its species composition. Haplotype clusters carrying the resistance alleles *296G* and *296S* are highlighted in blue. Red arrows indicate the direction of non-synonymous mutations (relative to reference genome). B) Frequency of resistance haplotypes per population. Detailed frequencies with absolute counts in Supplementary Material SM14. Note: *gam* = *A. gambiae*, *col* = *A. coluzzii*; *gam* populations denoted with an asterisk have unclear species identification and/or high rates of hybridisation.

We identified two distinct groups of haplotypes associated with resistance mutations. First, the *296G* cluster contained haplotypes sharing the *296G*/345M alleles which were widely distributed in Central and West Africa (11 populations of *A. coluzzii* and *A. gambiae*; *n* = 651 haplotypes). The *296G* group showed two sub-clusters associated with the downstream mutations *N530K* and *H539Q* (red arrows in Figure 3A), which were present in a subset of mostly *A. gambiae* populations (Guinea, Ghana, Burkina Faso and Cameroon; Figure 1A); with just a few *A. coluzzii* from Côte d’Ivoire in the *N530K* cluster. Both *N530K* and *H539Q* are in partial linkage disequilibrium with *296G* alleles (Figure 2).

In contrast, the *296S* cluster, defined by ubiquitous co-occurrence of the *296S*/345S allele pair, was restricted to *A. coluzzii* from Burkina Faso (*n* = 94; Figure 3A, B), whereas the *A. arabiensis* and *A. quadriannulatus* *296S* haplotypes appeared as distantly related singletons (not visible on Figure 3, see Supplementary Material SM5 and SM6). We also found four smaller wild-type clusters (*296A*
allele; henceforth wt) that are specific to other geographical locations (Kenya, Mayotte, and The Gambia/Guinea-Bissau). The remaining haplotypes are also wt and group in smaller clusters or singletons with frequencies <1% in the dataset (n = 1476, 62.6% of all examined haplotypes; Supplementary Material SM5 and SM6).

Both the 296G and 296S haplotype clusters are often found in high frequencies within their respective populations. For example, 296S was present in 62.3% of all Burkinabè A. coluzzii, and 296G reached 91.7% in Ghanaian A. gambiae (Figure 3B).

The haplotype clustering analysis shows that all non-synonymous mutations (T345M, T345S, N530K, and H539Q) are associated with either the 296G or the 296S resistance haplotypes. The existence of seven non-synonymous mutations associated in haplotypes that have evolved over the last 70 years is remarkable: mosquito Rdl genes are highly conserved and have accumulated very few amino-acid mutations since anophelines diverged from culicines (for instance, A. gambiae Rdl retains a 97.6% amino-acidic identity with its Aedes aegypti ortholog and dN/dS = 0.052, indicating predominant purifying selection; Supplementary Material SM4). Here, we observe that the resistant haplotypes accumulate an excess of non-synonymous mutations compared to the wt, with non-synonymous to synonymous genetic diversity ratios (πN/πS) being ~18x higher in the 296G cluster (πN/πS = 2.428 +/− 0.009 standard error) than in wt haplotypes (πN/πS = 0.135 +/− 0.001); and ~4x higher in 296S (πN/πS = 0.485 +/− 0.018).

The 296S and 296G alleles are associated with hard selective sweeps

Next, we investigated the signals of positive selection linked to the 296S and 296G resistance haplotypes. First, we found that haplotypes carrying 296G and 296S alleles had longer regions of high extended haplotype homozygosity (EHH) than the wt (Figure 4A), as expected under a scenario of selective sweeps linked to these resistant variants. A closer examination revealed that EHH decays slower at the 3′ region of Rdl (Figure 4A): in both clusters, EHH is above 0.95 (i.e. 95% of identical haplotypes) in the region downstream of codon 296 (exons 7 and 8), but decays more rapidly towards the 5′ of the gene (EHH < 0.20 in exon 6a/6b, EHH < 0.10 in exon 1). The core resistance haplotypes had lengths of 5,344 bp for 296G and 4,161 bp for 296S (defined at EHH > 95%), which were one order of magnitude higher than wt haplotypes (460 bp), and covered all non-synonymous mutations linked to codon 296 alleles (T345M, T345S, N530K, and H539Q).

Next, to estimate the softness/hardness of the sweep, we calculated the profile of Garud’s H statistics [33] and haplotypic diversity along the 2L chromosome arm (Figure 4B-D). Both 296G and 296S haplotype clusters showed signals of a hard selective sweep: (i) they had markedly higher Garud’s H12 (296G: 0.698 +/− 0.001 standard error; 296S: 0.744 +/− 0.006) than wt (0.003 +/− 0.0), which indicates an over-abundance of the most frequent haplotypes in the cohort [33,34]; (ii) lower H2/H1 ratios (296G: 0.052 +/− 0.0; 296S: 0.011 +/− 0.007) than wt (0.756 +/− 0.001), indicative of a hard sweep with decreased background variation [33,34]; and (iii) low haplotypic diversity (296G: 0.501 +/− 0.001; 296S: 0.377 +/− 0.007) compared to the wt (0.998 +/− 0.000).
Unexpectedly, chromosomes containing 296G and 296S alleles also exhibited signals of positive selection at a distant pericentromeric region of 2L (Figure 4B-D), typically associated with strong selective sweeps around two mutations in the Vgsc gene (995F and 995S) [25,27,35], which is the target site of pyrethroids and DDT [24]. Positive selection in Vgsc was particularly strong in chromosomes that also carried 296S alleles ($H_{12} = 0.917 +/− 0.004$ standard error), followed by 296G ($H_{12} = 0.412 +/− 0.001$) and, to a lesser degree, wt ($H_{12} = 0.147 +/− 0.000$). However, neither of the Vgsc resistance alleles (995F and 995S) are in linkage disequilibrium with 296G or 296S (Supplementary Material SM7, SM8). Rather, this apparent association is due to geographical overlap: 296G and 296S are present in West African populations that are near-fixed for Vgsc resistance alleles (>80% 995F in 7 out of 10 populations; Supplementary Material SM8), but are mostly absent elsewhere.

Overall, Rdl resistance alleles are found on two unique sets of highly similar haplotypes (Figure 3),
each of them specific to one allele (296S and 296G), that underwent independent hard selective sweeps (Figure 4).

### Co-segregation of Rdl haplotypes and 2La inversions

*Rdl* lies within the 2La chromosomal inversion, which is the longest in the *A. gambiae* genome (20.5-42.1 Mb) [19]. The 2La inversion emerged in the last common ancestor of the *A. gambiae* species complex [32] and is currently polymorphic in *A. gambiae* and *A. coluzzii* [36], where it is linked to a range of important phenotypes including adaptation to human environments [37], aridity [38], insecticide resistance [39], and susceptibility to *Plasmodium falciparum* [40]. Given that recombination is strongly reduced between chromosomes with discordant inversion karyotypes [21–23], any assessment of the evolution of genes within the 2La inversion, such as *Rdl*, needs to take into consideration whether haplotypes reside in inverted (2La) or non-inverted (2L+a) backgrounds.

#### Figure 5. Genotypes of the 2La inversion

A) Principal component analysis of genotype frequencies of 10,000 random variants located within the 2La inversion (coordinates: 2L:20524058-42165532). Specimens from *Ag1000G* Phase 1 are color-coded by 2La karyotype (homozygotes and heterozygotes), and they are used as a reference to assign 2La genotypes to Phase 2 specimens (grey). Grey dotted lines highlight the separation of three clusters according to 2La karyotype.

B) Frequency of 2La inversion and *Rdl* codon 296 genotypes.

C) Frequency of 2La karyotypes per population and haplotype group.

To address this issue, we estimated the 2La inversion karyotypes for the *Ag1000G* Phase 2 samples using a principal component analysis of allele presence/absence in the inverted region.
(using genomes with known inversion karyotypes as a reference; Figure 5A and Supplementary Material SM1 and SM9). The first principal component clearly discriminated between each of the inversion genotypes (non-inverted 2L+/2L+ homozygotes, inverted 2La/2La homozygotes, and 2La/2L+ heterozygotes). We used this information to compare the frequencies of 2La karyotypes with *Rdl* codon 296 genotypes (Figure 5B), and the karyotype frequencies per population (Figure 5C). The pan-African 296G allele is present in all inversion karyotypes, but is more common in non-inverted backgrounds (73% of 296G/296G homozygotes have 2L+/2L+ karyotypes; Figure 5B), in both *A. gambiae* and *A. coluzzii* populations (Figure 5C). On the other hand, 296S alleles from *A. arabiensis* and Burkinabè *A. coluzzii* occur exclusively within the 2La inversion (100% of 296S/296S homozygotes are in 2La/2La karyotypes; Figure 5B).

**Introgression of *Rdl*** resistance haplotypes

In order to obtain a more complete picture of possible introgression events, we performed a phylogenetic analysis of haplotype alignments at four loci around *Rdl*: 5' and 3' regions of the gene, and two loci upstream and downstream of the gene body (Figure 6). These phylogenies highlight two events of interspecific introgression (explored below in greater detail): 296G between *A. gambiae* and *A. coluzzii* (as reflected by their identical swept haplotypes; Figure 3), and 296S between *A. coluzzii* and *A. arabiensis*. In addition, they also confirm the spread of 296G haplotypes across different 2La inversion types (interkaryotypic introgression; Figure 5). In the following paragraphs, we characterise these introgressions and attempt to identify the donors and acceptors of each event.

**Interspecific introgression of 296G and 296S haplotypes**

All four phylogenies exhibit two main clades separating *A. gambiae* and *A. coluzzii* haplotypes according to their 2La inversion karyotype, rather than by species (2La in blue, left; 2L+/+ in red, right; ultrafast bootstrap support [UFBS] 91% and 97% respectively; Figure 6A). This clustering is due the fact that the 2La inversion has been segregating in *A. gambiae* and *A. coluzzii* since before the beginning of their speciation [32].

A closer examination shows that *Rdl*-specific phylogenies (Figure 6A, B) have a distinct subclade within the 2La cluster, consisting of *A. coluzzii* 296S haplotypes and *A. arabiensis*, some of which also possess the 296S allele (light blue and green sequences in Figure in Figure 6A; UFBS 97%, 84% for their sister-branch relationship). The deep branching of *A. arabiensis* haplotypes within the *A. gambiae*/*coluzzii* 2La clade is to be expected, as *A. arabiensis* 2La inversions descend from an ancient introgression event from the *A. gambiae*/*coluzzii* ancestor [32]. However, their close phylogenetic relationship with *A. coluzzii* 296S haplotypes is suggestive of interspecific introgression.
Figure 6. Phylogenies of haplotypes around the Rdl locus. A) Maximum-likelihood phylogenetic analysis of variants present at the 3′ region of Rdl (20,000 kbp). Nodes are haplotypes and have been color-coded according to their Rdl genotype (296S, 296G, wt), 2La karyotype (2La, 2L+*) and species. Orange bubbles highlight clades with hypothetical introgression events. Grey bubbles highlight outgroup clades. Statistical supports are shown on selected clades (UF bootstrap). C-E) Analogous phylogenies from the Rdl 5′ region, upstream, and downstream regions within the 2La inversion (+/− 1 Mb of Rdl). Complete alignments and phylogenies in Supplementary Material SM10 and SM11. Species abbreviations: col=coluzzii, gam=gambiae, ara=arabiensis, mer=merus; mel=melas, qua=quadriannulatus. Arrows indicate introgression events.

To confirm this event of introgression and ascertain its direction, we compared the results of two complementary Patterson’s D tests (Figure 7). The D statistic compares allele frequencies between three putatively admixing populations (A, B and C) and one outgroup (O), and can identify introgression between populations A and C (in which case D > 0) or B and C (D < 0; see Methods and [41,42]).
Hypothesis: \( \text{col 296G} \leftrightarrow \text{gam wt} \) (Accepted)

Hypothesis: \( \text{ara 296S} \leftrightarrow \text{col wt} \) ? (Rejected)

B) 296G-2La introgression \( A. gambiae \leftrightarrow A. coluzzii \)

Figure 7. Interspecific introgression. A) Direction of 296S introgression between \( A. arabiensis \) and \( A. coluzzii \) (2La/2L+ background). We test two complementary hypothesis using Patterson’s \( D \) statistics: left, introgression between \( A. coluzzii \) 296S homozygotes (population A), \( A. coluzzii \) wt (B) and \( A. arabiensis \) (296S or wt; C) using \( A. christyi \) as outgroup (O); right, reversing the position of \( A. coluzzii \) and \( A. arabiensis \) as populations A/B and C. The complementary hypotheses can be summarised as follows: if 296S homozygotes from species \( i \) show evidence of introgression with \( wt \) homozygotes from species \( j \) (first test) but not with \( wt \) from species \( i \) (second test), 296S originated in species \( j \). B) Direction of 296G introgression between \( A. gambiae \) and \( A. coluzzii \) (2L+/2L+ background), testing two complementary hypothesis using Patterson’s \( D \) statistics: left, introgression between \( A. coluzzii \) 296G homozygotes (population A), \( A. coluzzii \) wt (B) and \( A. gambiae \) (296G or wt; C) using \( A. quadriannulatus \) as outgroup (O); right, reversing the position of \( A. coluzzii \) and \( A. gambiae \) as populations A/B and C. Color-coded cladograms at the bottom of each plot indicate the groups of specimens used in each test, including the average \( D \) in the \( Rdl \) locus with standard errors and \( p \)-values (estimated from the Z-score of jack-knifed estimates; see Methods). See detailed lists of comparisons and statistical analyses in Supplementary Material SM12 and SM13.

Here, if 296S had emerged in \( A. arabiensis \) and later introgressed into \( A. coluzzii \), we would expect 296S \( A. coluzzii \) specimens to exhibit \( D > 0 \) when compared to 296S \( A. arabiensis \), but also to be more similar to \( wt A. arabiensis \) (from which 296S evolved) than to \( wt A. coluzzii \). As predicted, we identify evidence of introgression between \( A. coluzzii \) 296S homozygotes and both (i) 296S \( A. arabiensis \) (\( D = 0.687 \pm 0.106 \) standard error, \( p = 8.621 \times 10^{-11} \) derived from a Z-score distribution) and (ii) \( wt A. arabiensis \) (\( D = 0.506 \pm 0.123, p = 3.959 \times 10^{-5} \); left panel in Figure 7A). Conversely, if 296S had introgressed from \( A. coluzzii \) into \( A. arabiensis \), we would see evidence of introgression between 296S \( A. arabiensis \) and \( wt A. coluzzii \), but we do not (right panel in Figure

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\[ \text{D} = 0.506 \pm 0.123, \text{p} = 3.959 \times 10^{-5} \]
7A; \( D = -0.033 \pm 0.224, \ p = 0.884 \). These results are robust to various choices of outgroup species (\textit{A. christyi} and \textit{A. epiroticus}), and tests involving a negative control with fixed 2La inversions (\textit{A. merus}) do not show evidence of introgression with \( 296S \) specimens (Supplementary Material SM12). Thus, we conclude that the \( 296S \) allele originated in \textit{A. arabiensis} and later spread into \textit{A. coluzzii}.

\textit{Rdl} phylogenies (Figure 6A, B) also show a sub-clade of highly similar \textit{A. gambiae} and \textit{A. coluzzii} haplotypes within the 2L+\(^a\) cluster, all of them carrying \textit{296G} alleles. This clade corresponds to the swept haplotypes identified above (Figure 3). We established the polarity of introgression using complementary Patterson’s \( D \) tests. Here, we found that \textit{296G} haplotypes from resistant \textit{A. coluzzii} populations (Côte d’Ivoire, Angola, and Ghana) exhibited signals of introgression with \textit{wt A. gambiae} from Gabon (e.g. \( D = 0.542 \pm 0.107, \ p = 3.839 \times 10^{-7} \) compared to Angolan \textit{A. coluzzii}; Figure 7B); but that this signal of introgression disappeared when comparing \textit{wt A. coluzzii} to \textit{296G A. gambiae} from Gabon (e.g. \( D = 0.103 \pm 0.141, \ p = 0.4632 \) compared to Angolan \textit{A. coluzzii}; Figure 7B) or elsewhere (Supplementary Material SM13). These results support the introgression of \textit{296G} from \textit{A. gambiae} to \textit{A. coluzzii}.

The fact that only Gabonese \textit{A. gambiae} have significant support as the \textit{296G} donor population could indicate that they are closer to the founding \textit{296G} haplotype and/or the original introgression event. However, the negative results in other populations harbouring \textit{296G} alleles (Cameroon, Guinea; Supplementary Material SM13) could also be due to methodological limitations of our analysis – e.g., our conservative approach is restricted to specimens that are homozygous for both the inversion karyotype (2L+\(^a\)/2L+\(^a\)) and codon 296 (296G/296G or \textit{wt/wt}); and the similarity between \textit{wt A. gambiae} and \textit{A. coluzzii} relative to the highly divergent swept haplotype can hinder the identification of the original background.

The \textit{296G} haplotype spread from 2L+\(^a\) to 2La chromosomes

The haplotype phylogeny from the \textit{Rdl} 3’ region, where codon 296 variants reside, also revealed that the 2L+\(^a\) clade (non-inverted, red; Figure 6A) contained a sub-cluster of \textit{296G} haplotypes from both 2L+\(^a\) (orange) and 2La orientations (purple; Figure 6A; UFBS 98%). The deep branching of \textit{296G-2La} haplotypes within the 2L+\(^a\) clade implies that \textit{296G} originated in a non-inverted background and later spread to inverted chromosomes via interkaryotypic introgression. Chromosomal inversions are strong barriers to recombination, but double cross-overs or gene conversion events can result in allelic exchange between non-concordant inversions \cite{21, 22} and thus explain this phylogenetic arrangement.

However, the phylogeny of \textit{Rdl} 5’ haplotypes (which excludes codon 296 and the adjacent non-synonymous mutations) showed that \textit{296G-2La} sequences (purple) branched within the \textit{wt-2La} clade instead (blue; Figure 6B). Thus, interkaryotypic introgression only affects the swept haplotype at the 3’ end of \textit{Rdl} (Figures 3 and 4), whereas the 5’ region is closer to the \textit{wt}. We can confirm whether the introgression is specific to the 3’ swept haplotype by examining the profile of
sequence divergence along the Rdl gene locus (Dxy; Figure 8). We expect 296G haplotypes to be more similar to wt-2L+a than to wt-2La, given that the 296G allele first evolved in a 2L+a background (blue line, Dxy ratio > 1; Figure 8). In the case of 296G alleles from 2La chromosomes, this expectation holds at the 3′ region of Rdl but not at 5′ nor outside of the gene, where allele frequencies are more similar to the wt-2La (purple line, Dxy ratio < 1; Figure 8).

Figure 8. Interkaryotypic introgression of 296G haplotypes. Ratio of sequence divergence (Dxy) between 296G and wt haplotypes of 2L+a and 2La origin. In this ratio, numerators are divergences between 296G haplotypes (of either 2L+a or 2La origin, in blue and purple respectively) relative to wt-2La haplotypes, and denominators are relative to wt-2L+a. Ratios >1 indicate similarity to wt-2L+a, and values <1 indicate similarity to wt-2La. All values are calculated in windows of 20,000 kbp with 10% overlap.

The presence of alleles from different karyotypic backgrounds in the 296G-2La Rdl sequences is consistent with the sudden decay of haplotype homozygosity immediately upstream to codon 296 (Figure 4A), as the presence of wt alleles of 2La origin at 5′ of the 296G swept haplotypes causes a faster decay in haplotype homozygosity in 2La than in 2L+a haplotypes (Supplementary Material SM14A). Concordantly, haplotype diversity at the 5′ region of Rdl is higher in 296G-2La than in 296G-2L+a haplotypes (Supplementary Material SM14B).

Structural modelling predicts that 296G and 296S disrupt the dieldrin binding site in alternative ways

Finally, we investigated the effects of 296G and 296S resistance alleles on the structure of RDL receptors. The A. gambiae RDL receptor was modelled as a homopentamer based on the human GABA$_\text{A}$ receptor structure [43] (Figure 9). In wt receptors, the 296A residue is located near the cytoplasmic end of the pore-lining second transmembrane helix (M2) and its side chain is orientated into the pore (Figure 9A), whereas codon 345 is located away from the pore, at the cytoplasmic end of the M3 helix with its side chain orientated towards the lipid bilayer. We carried out automated ligand docking for dieldrin in the wt receptor, finding a putative docking site along the receptor pore with estimated free energy of binding ($\Delta$Gb) of −8.7 kcal/mol (Figure 9B). The 296A side chains form a major point of contact with the ligand. A structure of human GABA$_\text{A}$ in
complex with picrotoxin showed that this ligand forms multiple hydrogen bonds with residues lining the pore [43], but dieldrin lacks equivalent hydrogen bond-forming groups. Thus, the close contacts between 296A side chains and dieldrin suggest that van der Waals interactions between these molecules are the predominant binding interaction.

**Figure 9. RDL receptor models with docked dieldrin.** A) Homology model of the *A. gambiae* RDL homopentamer, viewed from the membrane plane (top) and cytoplasm (bottom). The 296A (purple) and 345T (red) positions are shown in space-fill. The dotted outlines depict the receptor regions in panels B-D. B) Docking prediction for dieldrin in the pore of the 296A (wt) receptor. Dieldrin is shown in green, in sticks and transparent surface. Side chains lining the pore are shown as sticks and 296A is coloured purple. C-D) Superimposition of dieldrin docking onto models of the 296G and 296S receptors, respectively. E) Pore radii in 296A, 296G and 296S models.

Next, we superimposed the wt dieldrin docking coordinates onto models of resistant RDL receptors, resulting in disruptions of the predicted form of interaction (Figure 9C, D). The A296G substitution widens the pore at the dieldrin docking site (2.9Å to 3.8Å) and reduces the surface area of contact between the lumen and dieldrin (Figure 9C, E). A296S has the opposite effect: it results in a narrower pore (2Å) and shows an overlap between the serine side-chains and dieldrin, which indicates that steric hindrance could prevent the insecticide from binding at this location (Figure 9D, E).

**Discussion**

**Evolution of Rdl resistance: selective sweeps and multiple introgression events**

Contemporary dieldrin-resistant *A. gambiae* and *A. coluzzii* appear to descend from two unique hard selective sweeps around the A296G and A296S mutations (Figures 3 and 4). Both sweeps occurred independently on different genomic backgrounds (Figure 6), and have undergone at least three introgression events (Figures 6-8): (i) 296G from *A. gambiae* to *A. coluzzii*; (ii) 296G from 2L+ to 2La chromosomes; and (iii) 296S from *A. arabiensis* to *A. coluzzii*.

In the case of 296G, our data supports an origin in *A. gambiae* with 2L+ chromosomes, followed by
interspecific introgression into *A. coluzzii*, and interkaryotypic introgression into 2La chromosomes. The *A. gambiae* origin is inferred from the background similarity between *A. coluzzii* swept haplotypes and *A. gambiae* wt specimens from Gabon (according to Patterson's *D* test; Figure 7B). *A. gambiae* resistance haplotypes have accrued more non-synonymous mutations than *A. coluzzii* (*N530K* and *H539Q*; Figure 1A), which is consistent with a longer evolutionary history in the former. In either case, the swept haplotype currently spans populations of both species across West and Central Africa – mimicking the pan-African selective sweep described for the homologous *Rdl* mutation in *D. melanogaster* [8,9,44]. This result is in line with previous studies that had hypothesized the existence of a pan-African *296G* sweep due to the strong genetic differentiation found in this locus [10].

The interkaryotypic introgression of *296G* haplotypes from non-inverted 2L+*a* into 2La chromosomes (Figures 6 and 7) also facilitated the spread of *296G* resistance alleles, e.g. in *A. gambiae* populations with high frequencies of 2La/2La karyotypes such as Burkina Faso (Figure 5C). While it is generally acknowledged that chromosomal inversions strongly suppress recombination [20], genetic exchange can occur via double cross-over recombination or gene conversion [21,22,45,46]. The reduction in recombination is weaker in regions distant from the inversion breakpoints [21], as it is the case for *Rdl* (located ~4.8 Mb and ~16.7 Mb away from the 2La breakpoints), which results in reduced differentiation at the centre of the inversion [36,38] (Supplementary Material SM15). Few events of adaptive introgression across inversion karyotypes have been described in *Anopheles*. One of such cases are certain loci involved in adaptation to desiccation, which are linked to 2La inversions but are exchanged in 2La/2L+*a* heterozygotes [38,47]. Another example, possibly linked to gene conversion, could be the *APL1* cluster of hyper-variable immune genes: its pattern of sequence variation is more strongly influenced by geography and species (*A. gambiae*/*A. coluzzii*) than by the 2La inversion [48].

On the other hand, the *296S* selective sweep has a more restricted geographical distribution. In the Ag1000G cohort, *296S* is only found in *A. coluzzii* from Burkina Faso (Figure 3). We also identify *296S* alleles in *A. arabiensis* specimens from East (Tanzania), Central (Cameroon) and West Africa (Burkina Faso); as well as two *A. quadriannulatus* specimens from Zambia (which appears to be the first record in this species; Figure 1B).

Interestingly, we find clear evidence of *296S* introgression from *A. arabiensis* into *A. coluzzii* even when comparing to *A. arabiensis* wt specimens (Figure 7A), and despite the fact that none of the *A. arabiensis 296S* share the *A. coluzzii* swept haplotype (Figures 3A, 6A, and Supplementary Material SM6). Thus, lack of genomic evidence from *A. arabiensis* precludes the identification of the actual donor haplotype. A wider sampling of *A. arabiensis* populations will be necessary to complete the picture of *296S* evolution, in order to (i) identify the number of historical *A296S* mutations in this species; (ii) establish whether they were associated with one or more selective sweeps; and (iii) whether any of these hypothetical sweeps introgressed into *A. coluzzii*.
Persistence of *Rdl* mutations after dieldrin withdrawal

*Rdl* is a highly conserved gene, with an extreme paucity of non-synonymous mutations over >100 Mya of evolutionary divergence [1] in culicines and anophelines, and low \( d_{N}/d_{S} \) ratios that indicate a prevalence of purifying selection (Supplementary Material SM4). In this context, the persistence of 296\( G \) and 296\( S \) alleles in natural populations for more than 70 years, in spite of its fitness costs in the absence of insecticide [14–16], has been a long-standing puzzle.

Our study provides two key insights to this question. First, we find that, relative to the *wt*, haplotypes with resistance alleles have an excess of non-synonymous genetic diversity (~18x increase in \( \pi_N/\pi_S \) in 296\( G \), ~4x in 296\( S \)). This observation suggests that the emergence of 296\( G \) and, to a lesser degree, 296\( S \), has substantially altered the selective regime of *Rdl* and enabled the accumulation of additional non-synonymous mutations in an otherwise highly constrained protein. A similar change has been recently observed for *kdr* mutations in Vgsc (the target site of pyrethroids), whereby 995\( F \) resistance haplotypes accumulate an excess of amino-acidic substitutions [25].

Second, we identify a high degree of genetic linkage between the 296\( G \)/345\( M \) and 296\( S \)/345\( S \) allele pairs, which is observed in all West African populations where codon 296 mutations are present (Figures 1, 2, and Supplementary Material SM3) due to the fact that virtually all swept haplotypes include both mutations (Figures 3 and 4). This near-universal association is highly relevant because codon 345 mutations are suspected to have compensatory effects that offset the costs of codon 296 variants [49,50]. Studies of fipronil resistance have shown that both the 296\( G \) allele and the combination of 296\( G \) and 345\( M \) alleles resulted in decreased insecticide sensitivity in *A. gambiae* [49], *D. melanogaster* [50], and *D. simulans* [51]. Crucially, Taylor-Wells *et al.* [49] showed that, in addition to fipronil resistance, the *A. gambiae* 296\( G \) allele causes heightened sensitivity to the GABA neurotransmitter (possibly contributing to the observed fitness costs [14–16]); and that the addition of the 345\( M \) mutation reduces these detrimental effects while still conferring resistance.

Interestingly, our structural modelling analyses predict opposite resistance mechanisms for each resistance allele: 296\( G \) results in a wider RDL pore with weaker van der Waals interactions with dieldrin (Figure 9C, E); whereas 296\( S \) narrows the pore and impedes dieldrin docking due to steric hindrance (Figure 9D, E). These two effects suggest the possibility that the mechanisms behind the hypothesised compensatory roles of codon 345 mutations could be different as well, and open a new line of inquiry to investigate the exclusive association of each resistance variant with downstream mutations (296\( G \) with 345\( M \), 296\( S \) with 345\( S \)). Yet, the exact nature of the interaction between these codon 296 and 345 mutations remains unclear. Firstly, residue 345 does not have direct contacts with dieldrin or residue 296 (Figure 9A). Secondly, indirect effects are uncertain too: in human receptors, mutations on the interface between the third and second transmembrane domains (where residues 345 and 296 reside, respectively) affect the transition...
to the desensitized functional state [52]; but residue 345 in *A. gambiae* is not buried in this interface and is instead facing the lipid bilayer (Figure 9A), and the predicted effects of mutations T345M and T345S are not obvious.

Other possible factors behind the persistence of *Rdl* resistance alleles include the long half-life of dieldrin as an environmental organic pollutant; as well as the fact that *Rdl* is the target site of other insecticides such as fipronil, isoxazoline or meta-diamides [28–30]; a secondary target of avermectin [29], and, possibly, of neonicotinoids (imidacloprid), pyrethroids (deltamethrin), [49], and DDT [53].

### Implications for vector control

The apparent ease with which *Rdl* adaptive haplotypes have spread across the barriers to recombination posed by species isolation (*A. gambiae/A. coluzzii* and *A. arabiensis/A. coluzzii*) and non-concordant chromosomal inversions (2L+/2La) mirrors previous findings in *Vgsc* target site mutations [27], and suggests worrying consequences for insecticide deployment programmes. Burkina Faso, where resistance alleles have traversed both barriers to recombination, is a case-in-point example of this risk: the high frequency of 2La inversions (Figure 5C) did not prevent the spread of 296G, and interspecific introgression of 296S from *A. arabiensis* compounded this problem in *A. coluzzii*. In the future, a similar scenario could facilitate the spread of 296S in East African *A. gambiae* and *A. coluzzii*, via adaptive introgression from *A. arabiensis*.

Also noteworthy is the overlap of *Rdl* and *Vgsc* resistance variants in West and Central Africa. The lack of genetic linkage between *Vgsc* and *Rdl* resistance haplotypes suggests that this co-occurrence is purely geographical, and does not fit a hypothetical epistatic relationship (Supplementary Material SM7 and SM8). Yet, this overlap is still relevant for vector control: as pyrethroid resistance increases in *Anopheles* populations [54], the search for substitutes should take into account that some can be rendered ineffective by 296S or 296G (e.g. fipronil [28], avermectin [29], or, possibly, neonicotinoids such as imidacloprid [49]). This risk is currently highest in the West and Central African populations of *A. gambiae* and *A. coluzzii* where both 296G and *Vgsc* 995F [25] are common (Supplementary Material SM8). In the future, the introgression of 296S from East African *A. arabiensis* could further compound current complications caused by the already high frequencies of *Vgsc* 995S in this region [25].

This case study of the mechanisms that underlie persistence of dieldrin resistance is also relevant for integrated resistance management. Strategies such as insecticide rotations or mosaics rely on a gradual decline in resistance over time [31]. Instead, 296G and 296S haplotypes have accumulated additional non-synonymous mutations (Figure 3A), some of which (codon 345) are putatively compensatory. As mentioned above, a similar altered selective regime has also been observed in *Vgsc* haplotypes with *kdr* mutations [25]. Interestingly, a study of Brazilian *Ae. aegypti* found that *Vgsc* *kdr* mutations did not decrease in frequency after a decade without public pyrethroid spraying campaigns [55]. Brazilian *Ae. aegypti* have a longer history of pyrethroid-
based treatments than African Anopheles spp. [55,56]; thus, their resilient kdr mutations could be 
(i) recapitulating our observations with respect to Rdl and dieldrin, and (ii) prefiguring a similar 
persistence of Vgsc kdr in the A. gambiae complex after a future phasing-out of pyrethroids in 
response to their decreasing efficacy [54].

Overall, our results show that the Rdl resistance mutations that appeared after the pioneering 
deployment of dieldrin in the 1950s will still be relevant in the immediate future. Continued 
monitoring is thus necessary to understand the evolving landscape of genomic variation that 
underlines new and old mechanisms of insecticide resistance.

**Methods**

**Data collection**

We used variation data from individual A. coluzzii and A. gambiae mosquitoes from the Anopheles 
gambiae 1000 Genomes online archives, for Phase 2-AR1 [18]. Specifically, we retrieved the 
phased genotype calls, SNP effect predictions, and the array of accessible genomic positions. We 
also obtained the same data for populations of four species in the Anopheles complex (A. arabiensis, 
A. quadriannulatus, A. melas and A. merus) and two outgroups (A. epiroticus and A. christyi), as 
available in the Ag1000G online archive [18]. The complete list of downloaded genomes with 
accession codes is available in Supplementary Material SM1.

The reference gene annotation of A. gambiae was obtained from Vectorbase [57] (GFF format, 
version AgamP4.9). Gene and variant coordinates employed in this study are based on the 
AgamP4 version of the genome assembly.

**Genotype frequencies and linkage disequilibrium**

We retrieved all non-synonymous genomic variants located within the coding region of Rdl 
genomic coordinates: 2L:25363652-25434556) that were biallelic, phased, and segregating at 
>5% frequency in at least one population (henceforth, ‘non-synonymous variants’). Parsing and 
filtering of genotype calls from Ag1000G was done using the scikit-allel 1.2.1 library [58] in Python 
3.7.4.

We calculated the linkage disequilibrium between each pair of non-synonymous variants using (i) 
Rogers’ and Huff r correlation statistic [59], as implemented in scikit-allel (rogers_huff_r); and (ii) 
Lewontin’s D’ statistic [60], as implemented in [25].

**Haplotype networks**

We constructed a network of haplotype similarity using 626 biallelic, phased and non-singleton 
(shared between more than two samples) variants located in a region +/- 10kbp of Rdl codon 296 
(middle nucleotide, coordinate 2L:25429236). We used the presence/absence of each allele within
this genomic region to calculate Hamming distances and build minimum spanning networks [61],
using the *hapclust* function from [25] (with distance breaks >3 variants). Network visualizations
were produced using the *graphviz* 2.38.0 Python library [62], with haplotype clusters being color-
coded according to species, population and presence/absence of the resistance alleles in codon
296 (296S, 2L:25429235; 296G, 2L:25429236) and the 995th codon of *Vgsc* (Figure 3, 
Supplementary Material SM5, and SM6). The network visualization in Figure 3A excludes
singletons and haplotype clusters with a cohort frequency <1%.

We calculated the sequence diversity (\(\pi\)) of each haplotype group in the same region
(*sequence_diversity* function in *scikit-allel*), using a jack-knife procedure (iterative removal of
individual haplotypes without replacement) [63] to estimate the average and standard error. We
also calculated the sequence diversity in non-synonymous coding variants from this region (\(\pi_N\)),
synonymous coding variants (\(\pi_S\)), and their ratio (\(\pi_N/\pi_S\)).

**Positive selection in haplotype clusters**

We analysed the signals of positive selection in three haplotype groups, divided according to
alleles in codon 296: *wt* (\(n = 1476\)), 296S (\(n = 94\)) and 296G (\(n = 651\)) (Supplementary Material
SM5). First, we calculated the extended haplotype homozygosity decay (*EHH*) of each group of
haplotypes, using 22,910 variants (phased and biallelic) located +/- 200 kbp of codon 296
(2L:25429236) (using the *ehh_decay* utility in *scikit-allel*). For each haplotype group, we recorded
the genomic region where *EHH* decay >0.95 and <0.05.

Second, we calculated the profile of Garud’s *H* statistics [33] along the 2L chromosomal arm
(*moving_garud_h* utility in *scikit-allel*; block length = 500 phased variants with 20% step). We
performed the same calculations for the haplotypic diversity (*moving_haplotype_diversity* in *scikit-
allel*). We calculated the Garud *H* and haplotypic diversity estimates in the *Rdl* locus, using a jack-
knife procedure [63] (iterative removal of individual haplotypes without replacement) to calculate
the mean and standard error of each statistic.

**Karyotyping of 2La inversions**

In order to assign karyotypes of the 2La inversion in all specimens from Ag1000G Phase 2, we
used known 2La karyotypes from Phase 1 as a reference [2], and analysed genotype frequencies
within the inversion by principal component analysis (PCA). Specifically, we retrieved the
genotype frequencies of 1142 specimens from Ag1000G Phase 2, 765 of which were also present
in Phase 1 and had been previously karyotyped for this inversion [2]; and selected 10,000 random
SNPs (biallelic, shared between more than two samples, phased, segregating in at least one
population, and located within the 2La inversion 2L:20524058-42165532). SNPs fitting these
criteria were selected using the *scikit-allel* Python library, and the PCA was performed using the
*randomized_pca* utility (with Patterson scaling).
Manual inspection of the principal components (Supplementary Material SM9) showed that PC1 (6.35% of variance explained) was sufficient to discriminate between known karyotypes from Phase 1 using a clear-cut threshold (2La/2La, 2La/2L+α and 2L+α/2L+α). We determined the optimal classification thresholds using the C-Support Vector classification method (SVC, a method for supervised learning) implemented in the scikit-learn 0.21.3 Python library [64]. Specifically, we used the SVC function in scikit-learn (svm submodule) to train a classifier with known karyotypes from Phase 1 (765 observations) and the main principal components of the PCA analysis (10 variables), using a linear kernel and C=1. The selected thresholds were able to classify Phase 1 data into each of the three categories (2La/2La, 2La/2L+α and 2L+α/2L+α) with 100% accuracy (as per the classifier score value), precision and recall (calculated using the classification_report function from the scikit-learn metrics submodule).

Phylogenetic analysis of haplotypes

We obtained genomic alignments of SNPs located from four regions around the Rdl locus, at the following coordinates: (i) 5’ start of the gene (2L:25363652 +/- 10,000 kbp, 696 variants), (ii) 3’ end of the gene (2L:25434556 +/- 10,000 kbp, 428 variants), (iii) unadmixed region 1Mb upstream of Rdl (2L:24363652 + 20,000 kbp; 2903 variants; inside of the 2La inversion), and (iv) unadmixed region 1Mb downstream of Rdl (2L:26434556 + 20,000 kbp, 2594 variants; inside of the 2La inversion). These alignments were built from of phased, biallelic variants within the aforementioned regions, obtained from A. coluzzii and A. gambiae (Ag1000G Phase 2), A. arabiensis, A. quadriannulatus, A. melas and A. merus. We restricted our analysis to haplotypes pertaining to individuals homozygous for the 2La inversion (2La/2La and 2L+α/2L+α), totalling 1684 haplotypes (out of 2356 haplotypes in the original dataset, obtained from 1178 specimens). Invariant sites were removed from the alignments using snp-sites 2.3.3 [65]. All alignments are available in Supplementary Material SM10.

Each genomic alignment was then used to compute Maximum-Likelihood phylogenetic trees using IQ-TREE 1.6.10 [66]. The best-fitting nucleotide substitution model for each alignment was selected using the TEST option of IQ-TREE and according to the Bayesian Information Criterion (BIC), which suggested the GTR substitution matrix with ascertainment bias correction, four gamma (Γ) rate categories, and empirical state frequencies observed from the alignment (F) (i.e. the GTR+Γ+ASC+G4 model in IQ-TREE). We calculated branch statistical supports using the UF bootstrap procedure [67,68] and refined the tree for up to 10,000 iterations, until convergence was achieved (correlation coefficient ≥ 0.99).

Tree visualizations were created in R, using the plot.phylo function from the ape 5.3 library [69] and stringr 1.4.0 [70]. Each phylogeny was midpoint-rooted with phytools 0.6-60 [71] (midpoint.root), and branch lengths in Figure 6 were constrained for display purposes (5 × 10⁻⁵ to 5 × 10⁻³ per-base substitutions range; unmodified trees available in Supplementary Material SM11).
We analysed the signals of introgression along the 2L chromosomal arm using Patterson’s D statistic [41,42]. This statistic requires allele frequencies in four populations (A, B, C and O) following a predefined (((A,B),C),O) phylogeny, where A, B and C are populations with possible introgression events, and O is an unadmixed outgroup. Then, $D > 0$ if there is an excess of allele frequency similarities between A and C (which means either $A \rightarrow C$ or $C \rightarrow A$ introgression) and $D < 0$ for excess of similarity between B and C ($B \rightarrow C$ or $C \rightarrow B$ introgression) [41,42]. We calculated Patterson’s $D$ along blocks of adjacent variants in the 2L chromosomal arm (block length = 10,000 variants, with 20% step length; phased variants only) using the `moving_patterson_d` utility in `scikit-allel`. We also calculated $D$ in the Rdl locus (2L:25363652-25434556), and estimated its deviation from the null expectation (no introgression: $D = 0$) with a block-jackknife procedure (block length = 100 variants; `average_patterson_d` in `scikit-allel`). We then used these jack-knifed estimates to calculate the standard error, Z-score and the corresponding p value from the two-sided Z-score distribution.

Using the procedure described above, we performed multiple analyses of introgression between combinations of populations fitting the (((A,B),C),O) phylogeny. For each analysis, we selected A, B, C and O populations according to two criteria: (i) which interspecific introgression event was under test ($A. gambiae \sim A. coluzzii$ or $A. coluzzii \sim A. arabiensis$); (ii) homozygous karyotypes of the 2La inversion within which Rdl is located (given that it introduces a strong effect on genotype frequencies across the entire A. gambiae species complex [32]) and the resistance haplotype in question; and (iii) exclude populations with high frequencies of hybrids, with controversial species identification, or with extreme demographic histories (Guinea-Bissau, The Gambia, and Kenya) [2,72]. Following these criteria, we then tested the presence and direction introgression between the combinations of populations specified below.

First, we tested the $A. coluzzii \sim A. arabiensis$ introgression of the 296S haplotype in inverted genomes (2La/2La homozygotes; Figure 7A and Supplementary Material SM12). We performed two versions of this test, using either $A. coluzzii$ or $A. arabiensis$ as donors (population C), which can give an indication of the population of origin of the 296S mutation. First, we tested the $A. arabiensis \rightarrow A. coluzzii$ hypothesis using: (i) 296S homozygous $A. coluzzii$ from Burkina Faso as population A; (ii) wt homozygous $A. coluzzii$ from Burkina Faso as population B; (iii) $A. arabiensis$ and $A. merus$ specimens as multiple C populations (donors) C, treating 296S and wt homozygous specimens as different populations; and (iv) $A. epiroticus$ and $A. christyi$ as population O. Second, we tested the $A. coluzzii \rightarrow A. arabiensis$ hypothesis but switching the position of $A. arabiensis$ (now population A and B, for 296S and wt respectively) and $A. coluzzii$ populations (now population C, together with the $A. merus$ negative control). Under this setup, we expect to see evidence of introgression between 296S $A. coluzzii$ and 296S $A. arabiensis$ in both tests (positive controls), but a positive result with any of the wt comparisons can indicate that 296S haplotypes in either species is more similar to wt from the other (and hence, the second species is the species of origin). A detailed account of all
comparisons, populations and complete statistical reports are available in Supplementary Material SM12.

We performed the same series of tests for the \textit{A. gambiae} ~ \textit{A. coluzzii} introgression of the 296G cluster in individuals without the 2La inversion (2L\textsuperscript{+}/2L\textsuperscript{+} homozygotes; Figure 7B and Supplementary Material SM13A, B) and with the 2La inversion (Supplementary Material SM13C, D). In these tests, homozygous individuals from various \textit{A. gambiae} and \textit{A. coluzzii} populations were alternatively used as groups A/B (A if 296G, B if wt) and C (296G and wt, separately); and wt outgroups were selected according to their 2La karyotype (2L\textsuperscript{+}/2L\textsuperscript{+}: \textit{A. quadriannulatus} and \textit{A. melas}; 2La/2La: \textit{A. merus}). A detailed account of all comparisons, populations and complete statistical reports are available in Supplementary Material SM13.

**Sequence divergence between 2La karyotypes**

To ascertain whether 296G karyotypes from 2La chromosomes were introgressed from a 2L\textsuperscript{+} background, we calculated the absolute sequence divergence (D\textsubscript{xy} [73]) around the \textit{Rdl} locus between all combinations of the following groups of haplotypes: (i) between 296G-carrying haplotypes from 2L\textsuperscript{+}/2L\textsuperscript{+} homozygotic genomes, (ii) wt haplotypes from 2La/2La; (iii) 296G haplotypes from 2La/2La, (iv) wt haplotypes from 2La/2La (Figure 8). D\textsubscript{xy} estimates were calculated along the 2L arm using the \textit{windowed_divergence} utility in \textit{scikit-allel} (window size=20,000 bp with 10\% overlap). At each window, we also calculated the ratio between the following D\textsubscript{xy} estimates: (i) 296G-2L\textsuperscript{+} ~ wt-2La / 296G-2L\textsuperscript{+} ~ wt-2La\textsuperscript{+}; and (ii) 296G-2La ~ wt-2La / 296G-2La ~ wt-2L\textsuperscript{+}. Thus, windows with ratios >1 are more similar to the wt-2L\textsuperscript{+} background, and windows with ratios <1 are more similar to the wt-2La background.

**Alignment of \textit{Rdl} orthologs**

We retrieved \textit{Rdl} orthologs from the following species of the Culicidae family (available in Vectorbase): \textit{A. gambiae}, \textit{A. arabiensis}, \textit{A. melas}, \textit{A. merus}, \textit{A. christyi}, \textit{A. epiroticus}, \textit{A. minimus}, \textit{A. culicifacies}, \textit{A. funestus}, \textit{A. stephensi}, \textit{A. maculatus}, \textit{A. farauti}, \textit{A. dirus}, \textit{A. atroparvus}, \textit{A. sinensis}, \textit{A. albimanus}, \textit{A. darlingi}, \textit{Ae. aegypti}, \textit{Aedes albopictus}, and \textit{Culex quinquefasciatus}. We retained (i) those orthologs that resulted in complete predicted peptides (defined as having the same start and end codons as the \textit{A. gambiae} \textit{Rdl}), and (ii) the longest isoform per gene (except for \textit{A. gambiae}, where all three isoforms were retained). These sequences were aligned using \textit{MAFFT} 7.310 (1,000 rounds of iterative refinement, G-INS-i algorithm) [74]. Pairwise sequence identity between peptide sequences was calculated using the \textit{dist.alignment} function (with a identity distance matrix, which was then converted to a pairwise identities) from the \textit{seqinr} 3.4-5 library [75], in R 3.6.1 [76]. Pairwise \(d_N/d_S\) ratios were calculated from a codon-aware alignment of CDS sequences, using the \textit{dnds} function from the \textit{ape} 5.3 R library [77]. The codon-aware alignment of full-length CDS was obtained with \textit{PAL2NAL} [78], using the peptide alignment as a reference. Tables of pairwise identity and \(d_N/d_S\) values have been created with \textit{pheatmap} 1.0.12 [79].
Homology modelling and automated ligand docking

The structure of human GABA\textsuperscript{A} receptor bound with picrotoxin (PDB accession: 6HUG) provided the template for generating a homology model of the homopentameric \textit{A. gambiae} RDL receptor (UniProtKB accession: Q7PII2). Sequences were aligned using \textit{Clustal Omega} [80], and 50 homology models were generated using \textit{MODELLER} 9.23 [81]. A single best model was chosen based on the internal scoring values from \textit{MODELLER} and by visually inspecting models in \textit{Swiss-PdbViewer} [82] to eliminate candidates with structural problems. The A296G and A296S mutants were generated using \textit{Swiss-PdbViewer} to introduce the amino acid substitutions and to energy minimise the resulting structures using 50 steps of conjugate gradient energy minimization. The pore radii of the channel models were calculated using \textit{HOLE} 2.0 [83]. The 3-dimensional structure of dieldrin was generated ab initio using \textit{MarvinSketch} 19.22 of the ChemAxon suite [84]. \textit{AutoDockTools} 1.5.6 [85] was used to define rotatable bonds and merge non-polar hydrogens. Automated ligand docking studies with the wild-type GABA receptor model were performed using \textit{AutoDock Vina} 1.1.2 [86] with a grid of 20 × 20 × 20 points (1Å spacing) centred on the channel pore. Figures were produced using \textit{PyMOL} [87].

Availability of code and data

Python (3.7.4) and R scripts (3.6.1) to reproduce all analyses in this manuscript are available on GitHub: https://github.com/xgrau/rdl-Agam-evolution

All genome variation data has been obtained from the publicly available repositories of the \textit{Ag1000G} project Phase 2-AR1 [18]. Accession codes are available in Supplementary Material SM1 and download instructions can be found in the above-mentioned GitHub repository.

Author contributions

XGB, MD and DW designed the study. XGB carried out the analyses of sequence diversity, selection and introgression, with assistance and code contribution from ST, NJH and AM. AOR carried out the structural modelling analyses. The \textit{Ag1000G} Consortium undertook collection, preparation, sequencing, and primary analysis of the samples. All authors read and approved the final manuscript.

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Supplementary legends

Supplementary Material SM1. Data sources. List of genome samples from Ag1000G Phase 2-AR1 (table A), the Phase 1-AR3 subset (table B) (both of which contain A. gambiae and A. coluzzii specimens), and outgroup species (table C; includes A. arabiensis, A. quadriannulatus, A. christyi, A. epiroticus, A. merus and A. melas). For each sample, we include their country and population of origin, accession numbers (based on Ag1000G for Phase 1 and 2, and on NCBI SRA for outgroups), and the estimated 2La karyotypes.

Supplementary Material SM2. List of genetic variants in Rdl. A) List of all variants present in the Rdl gene (AGAP006028), including their genomic coordinates, reference and alternative alleles, coordinates of the mutation along Rdl CDS and peptide sequences, effect on the peptide sequence (aminoacid substitution), and frequencies in each of the populations of the cohort (Phase 2 and outgroups). B) Genotypes of Rdl non-synonymous mutations in each sample (for the six mutations reported in Figure 1), where 0=wt homozygote, 1=heterozygote, 2=alternate allele homozygote.

Supplementary Material SM3. Linkage disequilibrium in Rdl. Linkage disequilibrium between non-synonymous mutations in Rdl, separated by population. Only populations where non-synonymous variants are shown are displayed. For each population, we display Huff and Rogers’ r (left) and Lewontin’s D’ (right).

Supplementary Material SM4. Alignments of Rdl orthologs. A) Alignment of Rdl orthologs from 12 species from the Culicidae family: A. gambiae (Anogam) A. arabiensis (Anoor), A. atroparvus (Anotr), A. darlingi (Anodar), A. dirus (Anodir), A. epiroticus (Anoei), A. farauti (Anofar), A. funestus (Anofun), A. merus (Anomer), A. minimus (Anomin), and Ae. aegypti (Aedaeq). Pfam-predicted protein domains, transmembrane regions and the 296 and 345 codons are shown on top of the alignment (coordinates based on the A. gambiae ortholog). B-C) Pairwise sequence identity and d_s/d_f between Rdl orthologs, including all A. gambiae isoforms (RA, RB, RC).

Supplementary Material SM5. Haplotype classification and population frequency. A) Clustering of haplotypes according to the minimum spanning networks (built from 626 phased variants located around codon 296; Figure 3 and Supplementary Material SM6). For each cluster, we report their population and country of origin, species, and allele present in Rdl codon 296 (296G, 296S, wt) and Vgsc codon 995 (995F, 995S and wt). Cluster “A” includes haplotypes with 296G alleles, cluster “34” includes 296S alleles; all other clusters are wt. B) Absolute frequency of 296G, 296S and wt haplotype clusters per population.

Supplementary Material SM6. Minimum spanning networks of Rdl haplotypes. Minimum spanning networks of haplotypes around Rdl codon 296 (626 phased variants located +/- 10,000 bp from the 2L:25429236 position), including all non-singleton haplotype clusters. Purple arrows indicate the direction of non-synonymous mutations (relative to reference assembly). A) Nodes are color-coded according to genotype in Rdl codon 296. B) Nodes are color-coded according to genotype in Vgsc codon 995. C) Nodes are color-coded according to species.

Supplementary Material SM7. Linkage disequilibrium of Rdl and Vgsc. Linkage disequilibrium between non-synonymous mutations in Rdl and Vgsc, calculated using Huff and Rogers’ r (A) and Lewontin’s D’ (B). Resistance variants in both genes are highlighted in orange (Vgsc) and cerise red (Rdl).

Supplementary Material SM8. Co-segregation of Rdl and Vgsc mutations. A-B) Frequency of alleles in Vgsc codon 995 and Rdl codon 296 per population, calculated per chromosome. Note: A. gambiae populations denoted with an asterisk (The Gambia, Guinea-Bissau and Kenya) are listed separately due to their high frequency of hybridisation and/or unclear species identification (see Methods). C) Geographical co-occurrence of Rdl and Vgsc mutations, at 10% and 30% frequency thresholds (chosen for illustrative purposes). Dots indicate presence. D) Euler diagrams and contingency table depicting the co-occurrence of Vgsc 995F and 995S alleles with Rdl 296G, 296S and wt alleles within chromosomes analysed in this study (n = 2356). For chromosomes carrying each of the Rdl haplotype groups, we include the percentage of associated genotypes at Vgsc codon 995. E) Number of chromosomes carrying 296S or 296G mutations (x axis) against number of 995F mutations (y axis), per population (only values >0 included). F) Contingency tables of Rdl and Vgsc resistance mutations co-occurrence, per
population. Only populations were resistance alleles in are segregating in both genes are included. \( p \) values and odds ratios \( [OR] \) correspond to Fisher’s exact tests (one-sided, testing for a greater co-occurrence of \( Rdl \) codon 296 and \( Vgsc \) 995 resistance alleles).

**Supplementary Material SM9. PCA of 2La karyotypes.** Principal component (PC) analysis of allele presence/absence from 10,000 random variants located within the 2La inversion (coordinates: 2L:20524058-42165532). Specimens from \( Agt100G \) Phase 1 and \( A. arabiensis \) are color-coded by 2La genotype (homozygotes and heterozygotes, blue-purple), and they are used as a reference to assign 2La genotypes to Phase 2 specimens (grey). Panels A and B show PC1, PC2 and PC3; panel C shows the fraction of variance explained by each PC. The 2La karyotypes of all Phase 2 specimens are available in Supplementary Material SM1.

**Supplementary Material SM10. Alignments of \( Rdl \) haplotypes.** A) 5’ start of the gene (2L:25363652, 696 variants). B) 3’ end of the gene (2L:25434556, 428 variants). C) Unadmixed upstream region within the 2La inversion (1 Mb upstream of \( Rdl \); 2903 variants). D) Unadmixed downstream region within the 2La inversion (1 Mb downstream of \( Rdl \), 2594 variants). The name of each sequence name includes the specimen (codes from Supplementary Material SM1; e.g. AA0040-C), haplotype (a or b), population of origin (e.g. GHcol), genotype at codon 296 (gt0=wt, gt1=296G, gt2=296S), and 2La background (kt0=2L+/2L+, kt1=2La/2L+, kt2=2La/2La).

**Supplementary Material SM11. Phylogenies of \( Rdl \) haplotypes.** Phylogenetic trees from alignments around the \( Rdl \) locus (Supplementary Material SM10), in Newick format and including ultrafast bootstrap (UFBS) statistical supports. The name of each sequence (e.g. “AA0040-Ca_GHcol_gto_kt0”) indicates the specimen (codes from Supplementary Material SM1; “AA0040-C”), chromosome (“a” or “b”), population of origin (“GHcol”), allele at codon 296 (gt0=wt, gt1=296G, gt2=296S), and 2La karyotype (kt0=2L+/2L+, kt1=2La/2L+, kt2=2La/2La).

**Supplementary Material SM12. 296S introgression between \( A. coluzzii \) and \( A. arabiensis \).** A) Profile of Patterson’s \( D \) in 2La/2La backgrounds, using \( A. coluzzii \) specimens as populations A and B (296S and \( wt \), respectively); \( A. arabiensis \) as population C (296S as positive controls, \( wt \) as test), \( A. merus \) as a negative control for population C (\( wt \)); and either \( A. christyi \) or \( A. epiroticus \) as outgroups (\( wt \)). B) Profile of Patterson’s \( D \) in 2La/2La backgrounds, using \( A. arabiensis \) specimens as populations A and B (296G and \( wt \), respectively); \( A. coluzzii \) as population C (296S as positive controls, \( wt \) as test), \( A. merus \) as a negative control for population C (\( wt \)); and either \( A. christyi \) or \( A. epiroticus \) as outgroups (\( wt \)).

In all panels, the hypothesis under test can be summarised as follows: if 296S homozygotes from species \( i \) show evidence of introgression with \( wt \) homozygotes from species \( j \) but not with \( wt \) from \( i \), it means that 296S originated in species \( j \). Left plots depict the entire 2L chromosomal arm (orange lines demarcate 2La inversion), and rightmost plots focus on the \( Rdl \) locus (\( Rdl \) gene coordinates highlighted in red). \( D \) was calculated in sliding blocks (standardized \( D \) and \( p \)-value that reflects deviation from the null expectation of \( D = 0 \)).

**Supplementary Material SM13. 296G introgression between \( A. gambiae \) and \( A. coluzzii \).** A) Profile of Patterson’s \( D \) in 2L+/2L+ backgrounds, using \( A. coluzzii \) specimens as populations A and B (296G and \( wt \), respectively); \( A. gambiae \) as population C (296G as positive controls, \( wt \) as test); and either \( A. quadriannulatus \) or \( A. melas \) as outgroups (\( wt \)). B) Profile of Patterson’s \( D \) in 2L+/2L+ backgrounds, using \( A. gambiae \) specimens as populations A and B (296G and \( wt \), respectively); \( A. coluzzii \) as population C (296G as positive control, \( wt \) as test); and either \( A. quadriannulatus \) or \( A. melas \) as outgroups (\( wt \)). C) Profile of Patterson’s \( D \) in 2La/2La backgrounds, using \( A. coluzzii \) specimens as populations A and B (296G and \( wt \), respectively); \( A. gambiae \) as population C (296G as positive controls, \( wt \) as test); and \( A. merus \) as an outgroup (\( wt \)). D) Profile of Patterson’s \( D \) in 2La/2La backgrounds, using \( A. gambiae \) specimens as populations A and B (296G and \( wt \), respectively); \( A. coluzzii \) as population C (296G as positive controls, \( wt \) as test); and \( A. merus \) as an outgroup (\( wt \)).

In all panels, the hypothesis under test can be summarised as follows: if 296G homozygotes from species \( i \) show evidence of introgression with \( wt \) homozygotes from species \( j \) but not with \( wt \) from \( i \), it means that 296G originated in species \( j \). Left plots depict the entire 2L chromosomal arm (orange lines demarcate 2La inversion), and rightmost plots focus on the \( Rdl \) locus (\( Rdl \) gene coordinates highlighted in red). \( D \) was calculated in sliding blocks (standardized \( D \) and \( p \)-value that reflects deviation from the null expectation of \( D = 0 \)).
of 10,000 phased variants (with 20% overlap). For each comparison, we report the mean value of \( D \) in the \( Rdl \) locus and use a block-jackknife procedure (block length = 100 variants) to estimate its standard error, a Z-score (standardized \( D \)) and \( p \)-value (that reflects deviation from the null expectation of \( D = 0 \)).

**Supplementary Material SM14. Diversity of 296G haplotypes in 2L+\(^a\) and 2La backgrounds.** A) Profile of \( EHH \) decay for each group of 296G haplotypes (296G in 2L+\(^a\)/2L+\(^a\), 2La/2L+\(^a\) and 2La/2La backgrounds), built from 16,623 phased variants located +/- 150,000 bp from codon 296 (2L:25429236 position). B) Profile of haplotypic diversity along chromosomal arm 2L (sliding blocks of 500 variants with 20% overlap). C) Absolute sequence divergence (\( D_{xy} \)) between 296G alleles of 2L+\(^a\) background and \( wt \) resistance haplotypes of 2L+\(^a\) and 2La backgrounds. D) Absolute sequence divergence (\( D_{xy} \)) between 296G alleles of 2La background and \( wt \) resistance haplotypes of 2L+\(^a\) and 2La backgrounds. All values are calculated in windows of 20,000 kbp with 10% overlap.

**Supplementary Material SM15. Genetic differentiation in the 2La inversion.** Differentiation (Hudson’s \( F_{ST} \)) along the 2L chromosomal arm between \( A. gambiae \) and \( A. coluzzii \) species, separated by their 2La karyotype (2La/2La or 2L+\(^a\)/2L+\(^a\)). Panel A shows comparisons with \( A. gambiae \) with 2L+\(^a\)/2L+\(^a\) karyotypes, and panel B for \( A. gambiae \) with 2La/2La karyotypes. \( F_{ST} \) estimates have been calculated in adjacent blocks of 5,000 phased variants with 20% overlap. Sub-panels at the right focus on the \( Rdl \) genomic locus. Note that interkaryotype comparisons have higher \( F_{ST} \) in the 2La region than inter-species comparisons.
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