# Gene duplication in the major insecticide target site, *Rdl*, in *Drosophila melanogaster*

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Edited by Trudy F. C. Mackay, North Carolina State University, Raleigh, NC, and approved July 23, 2013 (received for review June 13, 2013)

**The *Resistance to Dieldrin* gene, *Rdl,* encodes a GABA-gated chloride channel subunit that is targeted by cyclodiene and phenylpyrazole insecticides. The gene was first characterized in *Drosophila melanogaster* by genetic mapping of resistance to the cyclodiene dieldrin. The 4,000-fold resistance observed was due to a single amino acid replacement, Ala$^{301}$ to Ser. The equivalent change was subsequently identified in *Rdl* orthologs of a large range of resistant insect species. Here, we report identification of a duplication at the *Rdl* locus in *D. melanogaster*. The 113-kb duplication contains one WT copy of *Rdl* and a second copy with two point mutations: an Ala$^{301}$ to Ser resistance mutation and Met$^{360}$ to Ile replacement. Individuals with this duplication exhibit intermediate dieldrin resistance compared with single copy Ser$^{301}$ homozygotes, reduced temperature sensitivity, and altered RNA editing associated with the resistant allele. Ectopic recombination between *Roo* transposable elements is involved in generating this genomic rearrangement. The duplication phenotypes were confirmed by construction of a transgenic, artificial duplication integrating the 55.7-kb *Rdl* locus with a Ser$^{301}$ change into an Ala$^{301}$ background. Gene duplications can contribute significantly to the evolution of insecticide resistance, most commonly by increasing the amount of gene product produced. Here however, duplication of the *Rdl* target site creates permanent heterozygosity, providing unique potential for adaptive mutations to accrue in one copy, without abolishing the endogenous role of an essential gene.**

The single point mutation in the *Resistance to dieldrin* (*Rdl*) gene represents one of the most significant cases of target site resistance to an insecticide yet observed. Cyclodiene resistance was reported in 62% of insecticide resistant species in the 1980s, following widespread use of cyclodiene insecticides, including dieldrin, which started in the 1950s (1). The nature of the genetic target, *Rdl,* was discovered after dieldrin was discontinued because of the widespread evolution of resistance in many species. *Rdl* was first discovered in *Drosophila melanogaster* using a positional cloning approach. High homology to human GABA receptors confirmed it was the first insect ligand-gated chloride channel subunit identified (2–4). A point mutation in the chloride channel pore-lining domain, replacing alanine 301 with serine, was present in all resistant *D. melanogaster* strains (5). This mutation provided 4,000-fold resistance when homozygous and lower levels of resistance in heterozygotes (2, 6). The homologous mutation was subsequently found in a large number of cyclodiene-resistant species from many insect orders (7–9), as well as a glycine replacement at the homologous site in some resistant strains of *Drosophila simulans* and other species (5, 10, 11).

Characterization of deficiency lines and inversions in *D. melanogaster* showed that *Rdl* is an essential gene (3). Thus, the Ala$^{301}$ to Ser or Gly mutation in *Rdl* exhibits unique properties, providing high levels of dieldrin resistance without abolishing the role of the RDL receptor (12). Electrophysiological studies showed that the 301 replacement affects cyclodiene sensitivity by two mechanisms: inhibiting direct binding and allosterically modifying the *Rdl* receptor to disrupt the antagonist-favored conformation (13). These differences in channel properties have little effect on overall fitness in *D. melanogaster* other than temperature sensitivity. Resistant adults exposed to temperature stress (38 °C) showed delayed recovery indicated by temporarily impaired flight (14). Laboratory studies of *D. melanogaster* and *D. simulans* showed no decline in Ser or Gly$^{301}$ allele frequencies in population cages after 1 y in the absence of cyclodiene insecticide selection (15). More recently, the Ala$^{301}$ to Ser change was shown to decrease sleep latency (16). Because fitness testing was conducted in the laboratory, the true extent of fitness costs may have been underestimated. Conversely, in the blowfly *Lucilia cuprina*, field studies have shown that in the absence of dieldrin, the *Rdl* resistance allele is at a dramatic selective disadvantage (17). Resistant individuals were more severely selected against during overwintering than other points throughout the year (18).

RDL is highly conserved in insects, and the universal nature of the Ala to Ser/Gly resistance mutation exemplifies this conservation. However, an influx of genomic information from insect species has shown that some lineages have multiple *Rdl* loci, with three copies present in Lepidopteran genomes and two present in the aphid *Acyrthosiphon pisum* (19–21). Before sequencing the *A. pisum* genome, two *Rdl* copies were reported in the peach potato aphids *Myzus persicae* and *M. nicotinae* (10). The presence of two copies in all three species suggests it is an ancient duplication present in the ancestral aphid lineage.

Gene amplification has previously been implicated as a major evolutionary avenue to attaining insecticide resistance, primarily in the form of increased gene expression of detoxification enzymes (22). Organophosphorus chemical resistance in the mosquito, *Culex pipiens,* is mediated by esterase overproduction due to regulatory changes and 250-fold increased copy number of esterase B3 (23). In *D. melanogaster,* an allelic series at the cytochrome P450 *Cyp6g1* locus involves a gene duplication and a variety of transposable element (TE) insertions. The most derived alleles are correlated with increased enzyme production and multiinsecticide resistance, including dichlorodiphenyltrichloroethane (DDT) (24).

Here we report the occurrence of a recent duplication spanning the genomic region of *Rdl* in *D. melanogaster*. Because *Rdl* is an essential gene (3) and a major insecticide target site for cyclodiene and phenylpyrazole insecticides, copy number variation allows for evolutionary flexibility, where adaptive mutations may accumulate in one copy, whereas WT function is maintained in the other. The duplication creates a situation of permanent heterozygosity, providing advantages in the presence and absence of insecticide.

## Results

**Identification of the *Rdl* Gene Duplication.** We examined the available genome sequence for the *Rdl* gene in 168 *D. melanogaster* lines [The *Drosophila* Genomic Reference Panel (DGRP) (25)].

Author contributions: E.J.R., P.J.D., and P.B. designed research; E.J.R., R.T.G., C.L., and P.J.D. performed research; E.J.R., R.T.G., J.M.S., and C.L. contributed new reagents/analytic tools; E.J.R., R.T.G., J.M.S., and C.R. analyzed data; and E.J.R., C.R., and P.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1311341110/-/DCSupplemental.

The DGRP was established in 2003 from an outbred population collected at a farmers market in Raleigh, NC. Isofemale lines were inbred for 20 generations (25). Among the inbred lines, there were 10 nonsynonymous polymorphisms in *Rdl* (Table S1; Fig. S1). The *Rdl* resistance mutation, $Ser^{301}$, was present in four lines (Ral-317, Ral-318, Ral-378, and Ral-491); however, in the first three, the mutation was heterozygous with Ala. These three lines also contained a second heterozygous, nonsynonymous mutation, $Met^{360}$ to Ile. $Met^{360}$ is one of four RNA-editing locations in *Rdl.* Editing to Val occurs in 10% of adult head $Rdl^{BD}$ transcripts, where $Rdl^{BD}$ is the most common splice isoform of four alternately spliced transcripts (26).

Genomic heterozygosity within an inbred line may indicate copy number variation (27). We confirmed $Ala^{301}/Ser^{301}$ heterozygosity by HaeII restriction digest of PCR products from Ral-318 and Ral-378 individuals (*Materials and Methods*; Fig. S2). Residual heterozygosity still occurs in these inbred lines (25), so to distinguish this from our prediction that the *Rdl* gene was duplicated, we followed the inheritance of the variants. If they were alleles, alternate segregation would be observed, whereas if it was a duplication, they would cosegregate. Ral-318 and Ral-378 were crossed to an *Rdl* WT line, $w^{1118}$. $F_1$ offspring were tested for genotype at the 301 site using the HaeII restriction digestion assay. One hundred percent were heterozygous for the $Ala^{301}/Ser^{301}$ polymorphism ($n = 32$; Fig. S2), indicating cosegregation of alleles and supporting the duplication hypothesis.

The assembled genome sequence Ral-318 and Ral-378 showed regions of heterozygosity spanning ~110 kb surrounding *Rdl*. Genome coverage showed a greater number of sequence reads across the putative duplication region compared with adjacent regions of the 3L chromosome arm (Fig. 1). Alignment of reads in the region revealed the putative duplication arrangement. The first breakpoint occurred at the site of a *Roo* TE long terminal repeat (LTR) in intron 7 of *nervous wreck* (*nwk*), the gene directly 5′ of *Rdl*. The second breakpoint occurred 113 kb downstream, the site of a second *Roo* TE LTR in intron 1 of the *glutamate receptor IB* (*Glu-RIB*) locus. The rearrangement results in two tandemly arranged copies of *Rdl* and surrounding genes within the 113-kb duplication (Fig. 2). Five genes were fully duplicated across the 1-kb region, as well as partial duplication of the 3′ portion of *nwk* and the 5′ portion of *Glu-RIB* (Fig. 2). Including *Rdl* $Ala^{301}$/Ser and $Met^{360}$/Ile, 15 heterozygous nonsynonymous differences were identified between the duplicated regions (Table S2).

Six primers were designed to regions of *nwk, Glu-RIB,* and the *Roo* LTR (Table S3). Combinations therein and sequencing of positive products confirmed the predicted duplication topology. The presence of an internal *Roo* element within the duplication structure was confirmed with long PCR spanning the *Glu-RIB* intron 1 to *nwk* intron 7 (Fig. S3; Fig. 2). A feature of *Roo* TEs is insertion site duplication, where 5 bp of genomic DNA is duplicated at either side of the point where the TE inserts (28). When ectopic recombination occurs between two different TE insertions, the flanking sequences should differ on each side of the TE. We examined the genomic sequence in Ral-317, -318, and -378 at both *Roo* insertion sites. We found the *nwk-Roo* had a different 5-bp insertion site duplication to the *Glu-RIB-Roo* (AATCT and ACCTG, respectively).

The primers designed to detect the *nwk-Roo* TE also amplified a product from the $Rdl^{R}$ *MD-RR* line, first isolated in 1990 (29), suggesting this line carried a *Roo* TE LTR at this site. Sequencing this product showed the same 5-bp target site duplication adjacent to the *Roo* LTR (AATCT) as Ral-317, -318, and -378. Previous characterization of $Rdl^{R}$ *MD-RR* showed that it contained both $Ser^{301}$ and $Ile^{360}$ replacements (6), indicating a common haplotype to the Ral lines. In contrast, the *nwk-Roo* product was not amplified in another single-copy $Ser^{301}$ line from the DGRP, Ral-491, nor did this line contain the $Met^{360}$ to Ile replacement. Long PCR was performed on $Rdl^{R}$-*MDRR* to assess whether a duplication was also present, although we were unable to amplify the *nwk-Roo-GluRIB* product (Fig. S3). Sequencing of introns and cDNA from multiple regions of *Rdl* revealed no heterozygous SNPs, confirming it was homozygous for $Ser^{301}$ and lacked extensive heterozygosity as seen in the duplication lines.

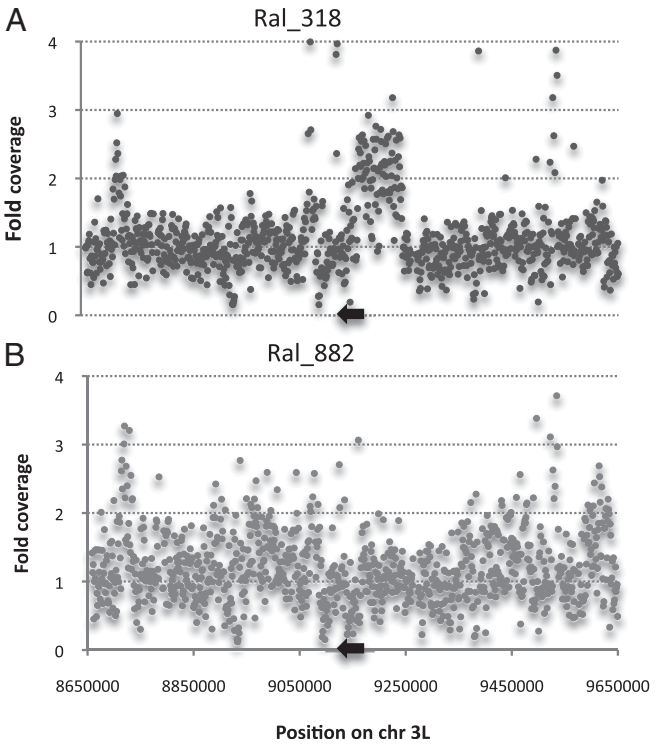


**Fig. 1.** Genome sequence coverage of 1 Mb of Chr3L spanning the *Rdl* locus in DGRP sequenced lines. (*A*) Duplication line Ral-318 and (*B*) single copy line Ral-882. Values are normalized against the average read depth for each line. The black arrow indicates the approximate position of *Rdl*. Fold coverage is greater than 1 across the putative duplication region for Ral-318 compared with the single copy line 882. Similar results were observed for Ral-378.

**Both Copies of *Rdl* Are Expressed.** cDNA was produced from RNA extracted from heads of 1-d-old adults from Ral-318 and -378. The *Rdl* exon 7 and 8 region, spanning the 301 and 360 sites, was amplified and cloned. Forty-five individual clones were sequenced to identify whether both copies of *Rdl* were expressed and if the two derived nonsynonomous variants were found in the same copy. This tissue and life stage were chosen to determine whether $Met^{360}$ to Val RNA editing still occurred correctly in $Ile^{360}$ mutants (26).

The clones fell into two categories: one characterized by the presence of the $Ser^{301}$ and $Ile^{360}$ mutations in the same transcript, and the other category was WT, indicating that both copies of duplicated *Rdl* are expressed. The frequency of sequences containing the $Ser^{301}Ile^{360}$ mutations was 24 of 45 (53%), indicating approximately the same expression level as the WT copy (21 of 45, 47%; $\chi^2 = 0.2$; $P = 0.655$; Table S4). The expressed clones showed variation in RNA editing between the two copies (Fig. 3; Table S5). Editing of codon 360 to Val occurred in $Ser^{301}Ile^{360}$ mutants in 6 of 24 mutant clones as opposed to 1 of 21 WT clones, although this difference was not significant (Fisher exact test, $P = 0.10$). There was a significant loss of $Asn^{294}$ to Asp editing in the $Ser^{301}Ile^{360}$ mutant, with 12 clones edited at the 294 codon in WT, but only 1 edited in mutant clones (Fisher exact test, $P = 0.00013$).

**Resistance to Dieldrin.** Dieldrin toxicity assays were conducted on adults from three naturally derived lines: $Rdl^{R}$ *MD-RR* (single copy; $Ser^{301}$); Ral-378 (duplication; $Ala^{301}Met^{360}/Ser^{301}Ile^{360}$); and Ral-882, (single copy; WT *Rdl*). Dosage–mortality curves were

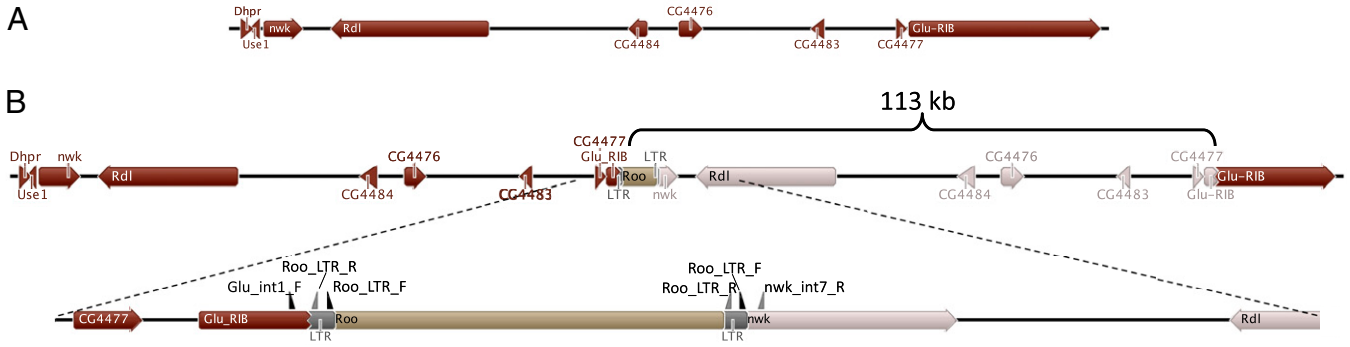


**Fig. 2.** Topology of the duplication. (*A*) Genomic structure surrounding the *Rdl* locus in reference single-copy strains. (*B*) Duplication structure in Ral-317, -318, and -378. The 5′ duplication breakpoint occurs within intron 7 of *nwk*, the gene directly upstream of *Rdl*; 113 kb of sequence is duplicated, ending in intron 1 of *Glu-RIB*. Five genes are completely duplicated (Table S2), and the 3′ portion of *nwk* and 5′ portion of *Glu-RIB* are partially duplicated. Between the partial *Glu-RIB* and *nwk* segments remains a *Roo* TE, as indicated by LTRs and long PCR products (Fig. S3). The region surrounding the *Roo* TE is shown in greater detail, indicating position of primer binding sites (Table S3).

generated (Fig. 4*A*). Compared with WT, $Rdl^R$ showed >45,000-fold resistance to dieldrin. Ral-378, containing both *Rdl* genotypes, showed significant resistance, with 27-fold survival over WT (Table S6).

To isolate the contribution of the *Rdl* $Ser^{301}$ mutation to resistance from within the 113-kb genomic duplication, a transgenic model was generated using the *P*[acman]-*attB* vector. The transgenic construct incorporated 55.7 kb of *Rdl* genomic DNA, but excluded all other genes present in the duplication. Both WT $Ala^{301}$ and mutant $Ser^{301}$ lines were generated in a controlled genetic background, differing by only a single base pair mutation. Three lines were tested for dieldrin resistance: Φ-86Fb-*empty* [*attP* 86Fb line (30)]; Φ-86Fb-P[ac]-$Rdl^{WT}$ (*Rdl* $Ala^{301}$ insertion); and Φ-86Fb-P[ac]-$Rdl^{A301S}$ (*Rdl* $Ser^{301}$ insertion). Thus, the insert lines replicated the *Rdl* duplication, containing two copies of *Rdl*: one transgenic and one endogenous copy.

Φ-86Fb-*empty* and Φ-86Fb-P[ac]-$Rdl^{WT}$ lines showed no difference in survival (Fig. 4*B*; Table S6), indicating an extra copy of WT *Rdl* did not modify resistance in the transgenic Φ-86Fb-P[ac]-$Rdl^{WT}$ line. However, Φ-86Fb-P[ac]-$Rdl^{A301S}$ had increased survival, with sixfold resistance to dieldrin (Fig. 4*B*; Table S6). The moderate resistance observed highlighted the semidominant nature of *Rdl* resistance (2), with combined effects of transgenic $Ser^{301}$ and endogenous WT $Ala^{301}$.

**Recovery from Heat Shock.** To examine temperature sensitivity associated with the *Rdl* $Ser^{301}$ mutation (14), heat shock recovery tests were conducted in lines derived from natural populations and transgenic lines examined previously for dieldrin resistance. Adult females were exposed to 38 °C for 10 min, and recovery time was observed. Among the naturally derived lines, genotype had a significant affect on recovery time [$F(2,6) = 41.861$, $P < 0.0001$]. Pairwise comparison showed a significant difference between WT Ral-882 and Ral-378 ($Ala^{301}Met^{360}$/$Ser^{301}Ile^{360}$) ($P = 0.004$) and Ral-882 and $Rdl^R$ *MD-RR* ($Ser^{301}$) ($P < 0.0001$), with the Ral-378 and $Rdl^R$ comparison yielding $P = 0.051$ (Bonferroni correction). $Rdl^R$ was the slowest to recover from heat shock, with a median recovery time of 10 min. Ral-378 showed an intermediate recovery of 7 min, and WT Ral-882 showed the fastest recovery time (4 min; Fig. 5*A*). Genotype also significantly affected recovery time in the transgenic lines [$F(2,6) = 19.767$, $P = 0.002$]. Φ-86Fb-*empty* and Φ-86Fb-P[ac]-$Rdl^{WT}$ were not significantly different from each other ($P = 0.585$); however, Φ-86Fb-P[ac]-$Rdl^{A301S}$ had a significantly increased temperature recovery time compared with both controls ($P = 0.011$ and $P = 0.003$, respectively; Fig. 5*B*), with a median recovery time of 5.5 min compared with 4.5 (Φ-86Fb-*empty*) and 3.5 min (Φ-86Fb-P[ac]-$Rdl^{WT}$).

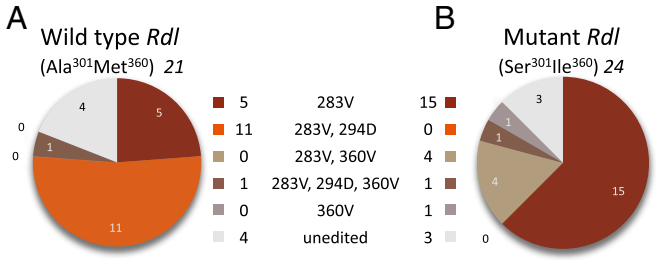


**Fig. 3.** RNA editing frequency. Frequency of RNA editing at three of the four known editing sites ($Ile^{283}$ to Val, $Asn^{294}$ to Asp, $Met^{360}$ to Val) in *Rdl* transcripts within the amplified exon 7–8 region, spanning codons 301 and 360. (*A*) Editing in WT $Ala^{301}Met^{360}$ sequenced clones compared with (*B*) mutant $Ser^{301}Ile^{360}$ clones. Frequencies are shown in Tables S4 and S5.

## Discussion

Investigating the genome sequence of 168 naturally derived inbred lines of *D. melanogaster* revealed a 113-kb tandem duplication encompassing the major insecticide target site, *Rdl.* Two nonsynonymous polymorphisms were present between the duplicated copies: at the insecticide resistance site, $Ala^{301}$ to Ser, and an RNA-edited site, $Met^{360}$ to Ile. Both mutations were found in the same copy, expressed at equivalent levels to the WT copy. The duplication and associated polymorphisms have implications in posttranscriptional RNA editing, dieldrin resistance, and heat shock recovery.

When resistance is dominant, heterozygotes often present intermediate phenotypes in both fitness and resistance to insecticide. This offset may allow resistant alleles to persist in populations in the absence of insecticide (31). Duplications enable the maintenance of permanent heterozygosity and have previously been shown to modify fitness in insecticide resistance context. In *Culex pipiens*, a point mutation in the *acetylcholine esterase-1* (*Ace-1*) locus is associated with high levels of resistance to organophosphates; however, this mutation reduces *Ace-1* activity by 60%, incurring a significant fitness cost (22). Combining a resistant and susceptible allele by gene duplication offsets part of the fitness cost (32). A fitness offset may also be the case for *Rdl* in the duplicated lines, which show expression of both the resistant $Ser^{301}Ile^{360}$ copy and the $Ala^{301}Met^{360}$ WT copy at equal levels and subsequently display intermediate levels of resistance (Fig. 4) and heat shock recovery (Fig. 5). Given that *D. melanogaster* is not generally considered to be a pest, it is likely to be incidentally exposed to insecticides at a lower frequency and concentrations that would be the case for pest species. The intermediate resistance associated with the duplication may be protective against such exposure.

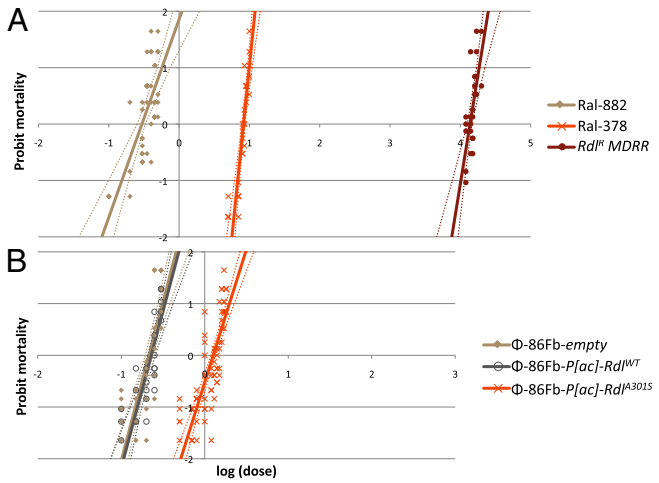


**Fig. 4.** Dosage mortality response curves for dieldrin. (*A*) Natural populations: Ral- 882 (WT, Ala$^{301}$Met$^{360}$); Ral-378 (duplication, Ala$^{301}$Met$^{360}$/Ser$^{301}$Ile$^{360}$); and *Rdl$^R$ MD-RR* (single copy, Ser$^{301}$Ile$^{360}$). (*B*) Transgenic lines: Φ-86Fb-*empty* control, Φ-86Fb-P[ac]-*Rdl$^{WT}$* (WT *Rdl* Ala$^{301}$ insertion) and Φ-86Fb-P[ac]-*Rdl$^{A301S}$* (mutant *Rdl* Ser$^{301}$ insertion). Fold changes are shown in Table S5.

Gene duplications associated with insecticide resistance have been predominantly involved in increased gene expression of metabolic enzymes resulting in enhanced insecticide detoxification (24, 33, 34). However, beyond enhanced expression, gene duplication in a target site provides a unique opportunity for the redundant copy of the duplicate pair to accumulate mutations. Some mutations may be adaptive in certain environmental scenarios that are otherwise detrimental to the original function of the gene (33). Gene duplications are one of the major adaptive forces in eukaryote evolution, but the persistence of duplicate copies is not favored unless each copy acquires a specific role. After duplication occurs, the majority of duplicate pairs result in one gene undergoing rapid deleterious mutations leading to pseudogenization (35). However, a duplicate pair is preserved if beneficial mutations accrue. Either one member acquires mutations that provide a new function (neofunctionalization) or the two copies share the original function of the progenitor gene by subfunctionalizing (35, 36).

Duplications may arise via a number of mechanisms including unequal crossing over or replication slippage (32). For the *Rdl* duplicated lines, our data suggest ectopic recombination in neighboring *Roo* TEs initiated the genome rearrangement. TEs and genome rearrangements have previously been implicated in adaptation to environmental pressures such as insecticide resistance (24, 37, 38). TEs contribute substantially to adaptive evolution and have the capacity to generate deletions, duplications, and regulatory changes with wide-ranging phenotypic effects that cannot be achieved by point mutations (39). *Roo* elements are the most common TE in the *D. melanogaster* genome (40), and are frequent initiators of chromosomal rearrangements such as duplications and deletions (41). PCR analysis indicated a *Roo* LTR was present in intron 7 of *nwk* in the original *Rdl$^R$ MD-RR* line. The *Rdl$^R$* haplotype may therefore be implicated as a precursor for the duplication. Recombination between the *Rdl$^R$ nwk-Roo* haplotype and a downstream *Glu-RIB-Roo* haplotype would result in the generation of the 113-kb tandem arrangement we observe in Ral-317, -318, and -378 (Fig. 6). Observation of different insert site duplication sequences flanking the *Roo* LTRs supports our theory of ectopic recombination between two different *Roo* TEs in generating this duplication structure. Our diagnostic PCRs detect upstream *nwk-Roo* elements at a low frequency in populations of *D. melanogaster* from Australian populations, although no nonduplicated populations have yet been identified with the corresponding downstream *Glu-RIB-Roo* (*SI Text*).

**Permanent Heterozygosity, Resistance, and Temperature Sensitivity.** A functional GABA receptor consists of five subunits. Pentamers formed in homozygous Ser$^{301}$ mutants contain only resistant subunits; however, in duplication lines, Ala$^{301}$ and Ser$^{301}$ alleles from the two *Rdl* copies would result in heteromeric RDL receptors containing a mixture of resistant and susceptible subunits. The same receptor composition also occurs in Φ-86Fb-P[ac]-*Rdl$^{A301S}$*, containing transgenic Ser$^{301}$ and endogenous Ala$^{301}$ *Rdl*, and is functionally equivalent to single copy Ala$^{301}$/Ser heterozygotes, with heteromeric receptors and semidominant, intermediate resistance levels (2).

The role of Ser$^{301}$ in resistance is well established (5, 6). However, to distinguish it as the causal mutation for the phenotypes assayed, transgenic lines containing a 55.7-kb *Rdl*-only duplication were generated. The transgenic construct eliminated other genes and mutations contained in the 113-kb natural duplication, and isolated the Ser$^{301}$ mutation in the absence of other nonsynonymous replacements including Ile$^{360}$, present in duplication lines and the original *Rdl$^R$ MD-RR* line (6). A distinct resistance and temperature sensitive phenotype emerged, even when eliminating background factors in the natural populations, which may consist of many generations of coadapted modifications. The 14 other nonsynonymous changes in the 113-kb duplication, or indeed other genes elsewhere in the genome, may have an additive effect on the two phenotypes assayed here in Ral-378. Additionally, reduced RNA editing efficiency at codon 294, and increased editing at codon 360 (Fig. 3; Table S5) may affect RDL receptor properties and phenotypes alongside the Ser$^{301}$ and Ile$^{360}$ mutations. However, the contribution of Ser$^{301}$ is verified by the transgenic experiments, where the *Rdl* insertion lines differ by a single base pair and illustrate the significance of this point mutation in genetically identical lines.

***Rdl* Copy Number Variation in Insects.** Previous studies reported two copies of *Rdl* in the peach-potato aphid, *Myzus persicae*: one copy with serine at the equivalent 301 site and the other containing alanine. Cyclodiene resistance was attributed to a glycine change in the alanine copy (10). The recent release of the pea aphid (*A. pisum*) genome also revealed two copies of *Rdl*, with Ala in one copy and Ser in the other (21). In Lepidoptera, three copies of *Rdl* are present, and in *Bombyx mori* these copies share 75–91%

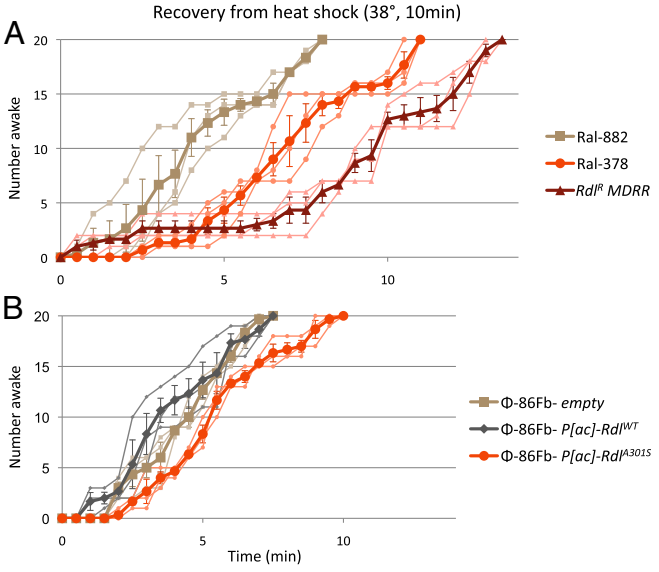


**Fig. 5.** Recovery from heat shock. (*A*) Ral-882 (WT); Ral-378 (duplication, Ala$^{301}$Met$^{360}$/Ser$^{301}$Ile$^{360}$); and *Rdl$^R$ MD-RR* (single copy, Ser$^{301}$). Three individual replicates are graphed with the average (±SEM) superimposed for each line. (*B*) Transgenic lines: Φ-86Fb-*empty*, Φ-86Fb-P[ac]-*Rdl$^{WT}$*, and Φ-86Fb-P[ac]-*Rdl$^{A301S}$*.

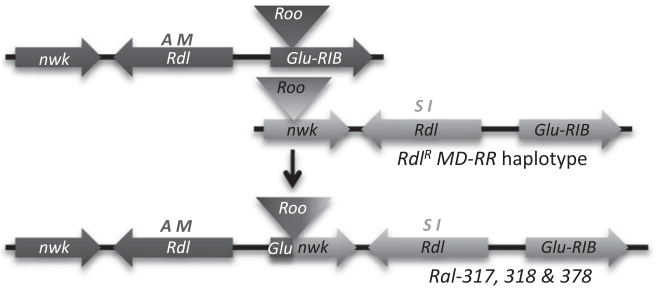


**Fig. 6.** Schematic showing a putative origin of the duplication in the Ral lines, based on ectopic recombination of a heterozygous *Rdl*$^{R}$ *MD-RR*/WT line, containing the *nwk* intron 7 *Roo* TE and the *Glu-RIB* intron 1 *Roo* TE *in trans*.

similarity at the protein level (19). However, one copy has Ala, one has Ser, and the third has a glutamine at the equivalent 301 site (Fig. S4). This site is therefore polymorphic in species with multiple copies of *Rdl*, suggesting a reduction in functional constraint within an otherwise highly conserved domain. It may explain to some extent why the 301 site is amenable to resistance mutations in single copy species.

In *Drosophila*, this is the first example of copy number variation at the *Rdl* locus. Identification of the present *D. melanogaster* duplication was facilitated by the availability of high-depth genome sequences of a large number of inbred lines expected to be homozygous. We did not detect the duplication in a survey of Australian populations (*SI Text*) or in the genome sequences available from 139 African *D. melanogaster* lines (27), suggesting a rare event led to the arrangement discovered in the DGRP. It is possible that other duplications exist at the *Rdl* locus, which were not detectable by our specific diagnostic PCRs.

Since the cessation of cyclodiene insecticide use, the frequency of the *Rdl* Ser$^{301}$ resistance allele varies from undetectable levels in houseflies (42) to relatively high levels in cockroaches and fleas (9, 43). The high stability of these compounds may result in exposure to persistent residues in the environment, maintaining selection for the resistance allele in natural populations. Additionally, increasing household and field use of phenylpyrazole insecticides, which also target the insect RDL GABA receptor, may select for resistance alleles (9, 44). In the early 1990s, the Ser$^{301}$ mutation frequency in populations of *D. melanogaster* in the United States was estimated at 1% (45). This value is similar to the current estimate of 2.4% in the DGRP lines extracted from a population North Carolina and 3% in our survey of Australian populations (25) (*SI Text*).

The duplication identified here creates adaptive potential for accumulation of resistance mutations in one copy that may be detrimental to the endogenous role of *Rdl*. Although the Ala301 to Ser/Gly replacement does not result in lethality and in many species has a negligible fitness effect (15), other replacements may result in enhanced resistance but have a greater impact on fitness. Ser$^{301}$ provides low resistance to the phenylpyrazole fipronil that does not impact the use of this insecticide in the field (9, 43, 46, 47). However, fipronil-resistant strains isolated from two species of planthopper have been shown to contain an Ala to Asn, rather than Ser or Gly, mutation at the equivalent 301 site, found only in the heterozygous state (48, 49). The lack of homozygous Asn individuals was proposed to be a result of lethality, supported by the reduced GABA median effective concentration ($EC_{50}$) observed in electrophysiological data (49). Mutations resulting in lethality can only be viable in a heterozygous state, or more permanently, in a duplication of the type described in this study. Continuing use of phenylpyrazole insecticides may result in increased prevalence of Asn replacements and enhanced resistance in other relevant species. Duplications generating permanent heterozygosity would facilitate the maintenance and spread of such mutations.

## Conclusion

Duplications are a major source of selectable genetic variation and provide evolutionary flexibility for adaptation to new functional niches. Here we characterize a recent duplication in a major insecticide target site, *Rdl,* in *D. melanogaster.* The duplication is associated with insecticide resistance and RNA editing mutations in one copy and one WT copy. The resulting expression of both WT and mutant transcripts results in intermediate resistance to dieldrin while reducing a temperature-sensitive fitness cost, creating a functional, permanent, and heritable heterozygosity. This discovery highlights considerations for continued use of insecticides targeting *Rdl* receptors in insects. A number of pest species exhibit *Rdl* copy number variation and therefore have flexibility to accumulate resistance mutations while retaining sufficient WT function. In species such as *D. melanogaster*, that are ancestrally single copy for *Rdl,* evidence of novel copy number variation at this target site provides a platform for future adaptation to environmental pressures, such as the ongoing use of insecticides.

## Materials and Methods

***Drosophila melanogaster* Lines.** *Rdl*$^{R}$ *MD-RR* (Bloomington: 1675) was originally isolated in Maryland in 1990, with 4,000-fold dieldrin resistance, and was used in the initial characterization of the *Rdl* Ser$^{301}$ mutation (2, 6, 29). The sequence of *Rdl* from 168 DGRP genomes (25) was extracted using reference genome coordinates, and polymorphisms were annotated manually. Sequence coverage and read depth of the *Rdl* genomic region was analyzed using MAQ (http://maq.sorceforge.net/maq-man.shtml). Two DGRP lines were used in phenotypic analysis of dieldrin resistance and heat shock recovery: one randomly selected *Rdl* WT line, Ral-882, and one duplication line, Ral-378 (Bloomington: 28255, 28187). An additional DGRP duplication line, Ral-318 (Bloomington: 28168), was used alongside Ral-378 to genetically verify the presence of a duplication by $F_1$ analysis with $w^{1118}$ (Bloomington: 3605), a control line containing WT *Rdl*, used in reciprocal crosses with the putative duplication lines (Fig. S2). Transgenic integration of the *Rdl* locus was performed using the *attB*-P[acman]-Ap$^{R}$ plasmid into chromosome III of the Φ-86Fb-*attP* line (Bloomington: 24749). The Φ-86Fb-*empty* line was used as a control for the two generated insertion lines: Φ-86Fb-P[ac]-*Rdl*$^{WT}$ (WT *Rdl* insertion) and Φ-86Fb-P[ac]-*Rdl*$^{A301S}$ (Ser$^{301}$ mutant *Rdl* insertion).

**Generation of P[acman]-*Rdl* Lines.** Recombineering was carried out according to the methods used in Venken et al. (50). A 55.7-kb fragment surrounding the *Rdl* locus was obtained by the cloning of left and right arms (Table S3) into the attB-P[acman] vector and homologous recombination with BAC-1E12 (RP98 library, *Drosophila* Genomic Resources Centre). The Ala$^{301}$ to Ser replacement was generated using the galK counter selection method (51). Both WT and A301S constructs were integrated into Φ-86Fb-*attP*, and transgenics were identified by eye color and PCR confirmation. Expression of the Ser$^{301}$ transgene was detected by HaeII restriction digest of exon7 from cDNA generated from the Φ-86Fb-P[ac]-*Rdl*$^{A301S}$ line.

**Generation of RNA, cDNA, Cloning, and Sequencing.** RNA was extracted from 1-d-old adult heads from lines Ral-378 and Ral-318 using TRIzol reagent, and cDNA was synthesized with SuperScript III Reverse Transcriptase [oligo(dT)$^{20}$; Invitrogen], following the manufacturer's instructions. PCR primers were designed to amplify exon 7 and 8, spanning the two polymorphisms at codons 301 and 360 (*Rdl_Ex7_F*/*Ex8_R*; Table S3). Products were cloned into pGEM T-easy (Promega) before sequencing (Macrogen).

**Diagnostic HaeII Restriction Digest.** The Ala$^{301}$ to Ser polymorphism occurs in exon 7 of *Rdl* as a result of a T to C base pair substitution, removing a HaeII restriction site (5); 268 bp of exon 7 was amplified (*Rdl_Ex7_F*/*R* (Table S3), and HaeII digest was used to differentiate between Ser$^{301}$ (205 and 63 bp), Ala$^{301}$ (148, 57, and 63 bp), or heterozygous lines (205, 148, 63, and 57 bp; Fig. S2).

**Insecticide Screens.** Dieldrin dosage–mortality analysis was carried out according to the same methods described in a DDT 24-h adult contact assay, where dieldrin powder was dissolved in acetone and coated the inside of scintillation vials (52). Five replicates of at least five doses per strain were tested to generate dose–response curves using PriProbit (ver.1.63; Fig. 4) (53). Resistance ratios and 95% CIs were estimated from dosage–mortality curves as previously described (54) (Table S6).

**Temperature Sensitivity Screens.** Recovery from heat shock was conducted with minor variations to tests performed in ref. 14. Flies were reared at room temperature (22–24 °C). Three replicates of 20 5- to 8-d-old adult females were placed into glass vials equilibrated in a 38 °C water bath and heated for 10 min. Unconscious flies were tipped onto a 2.5-cm-diameter circular arena at room temperature, and flies departing from this area were scored every 30 s for 15 min (Fig. 5). Proportional data were arcsine transformed, and repeated-measures ANOVA was used to assess the significance of the effects of genotype over time. A post hoc test using the Bonferroni correction was used to compare differences between genotypes.

**ACKNOWLEDGMENTS.** We thank Tom Harrop for valuable comments on the manuscript and members of the Social Insects laboratory for advice on statistics. This research was funded by an Australian Research Council grant (to P.B.).

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