Report

Current Biology

# Convergent Balancing Selection on an Antimicrobial Peptide in *Drosophila*

## Highlights

- A convergent polymorphism in an antimicrobial peptide is segregating in *Drosophila*
- The polymorphism is predictive of resistance to bacterial infection in two species
- Convergence is observed at this residue five times across the genus *Drosophila*
- Alleles appear to be maintained by balancing selection

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## In Brief

Unckless et al. identify a convergent amino acid polymorphism in an antimicrobial peptide that is segregating in two species of *Drosophila* and is predictive of resistance to infection. They observe a high frequency of the alleles in natural populations and convergence across the genus, suggesting the alleles are maintained by balancing selection.

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# Convergent Balancing Selection on an Antimicrobial Peptide in *Drosophila*

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## SUMMARY

Genes of the immune system often evolve rapidly and adaptively, presumably driven by antagonistic interactions with pathogens [1–4]. Those genes encoding secreted antimicrobial peptides (AMPs), however, have failed to exhibit conventional signatures of strong adaptive evolution, especially in arthropods (e.g., [5, 6]) and often segregate for null alleles and gene deletions [3, 4, 7, 8]. Furthermore, quantitative genetic studies have failed to associate naturally occurring polymorphism in AMP genes with variation in resistance to infection [9–11]. Both the lack of signatures of positive selection in AMPs and lack of association between genotype and immune phenotypes have yielded an interpretation that AMP genes evolve under relaxed evolutionary constraint, with enough functional redundancy that variation in, or even loss of, any particular peptide would have little effect on overall resistance [12, 13]. In stark contrast to the current paradigm, we identified a naturally occurring amino acid polymorphism in the AMP Diptericin that is highly predictive of resistance to bacterial infection in *Drosophila melanogaster* [13]. The identical amino acid polymorphism arose in parallel in the sister species *D. simulans*, by independent mutation with equivalent phenotypic effect. Convergent substitutions at the same amino acid residue have evolved at least five times across the *Drosophila* genus. We hypothesize that the alternative alleles are maintained by balancing selection through context-dependent or fluctuating selection. This pattern of evolution appears to be common in AMPs but is invisible to conventional screens for adaptive evolution that are predicated on elevated rates of amino acid divergence.

## RESULTS

### An Amino Acid Variant in Diptericin Predicts Immune Defense in *D. melanogaster* and *D. simulans*

Diptericin is an antimicrobial peptide (AMP) produced by dipteran flies. We previously discovered a naturally occurring polymorphism at residue 69 of the Diptericin mature peptide that was strongly predictive of resistance to infection by *Providencia rettgeri*, a Gram-negative natural pathogen of *Drosophila* [14]. The ancestral serine residue is phosphorylated and hence negatively charged (99.5% confidence; http://www.cbs.dtu.dk/services/NetPhos/; [15]). The derived arginine allele, carried by 15% of the lines in the mapping panel, is positively charged and is associated with a strong susceptibility to infection (Figures 1A and S1A; [14]).

We confirmed that lines homozygous for the arginine allele are more susceptible to *P. rettgeri* infection than line homozygous for serine. The average pathogen load for an arginine homozygote was 20 times higher than that of a serine homozygote (Figure 1B; $p < 0.001$), and arginine homozygotes were almost four times more likely to die from infection (Figure 1C; $p < 0.001$). Heterozygous flies had statistically intermediate bacterial loads, indicating incomplete dominance of the serine allele (Figure S1B).

We examined the *Diptericin* locus in *D. simulans*, a sister species 1–5 million years diverged from *D. melanogaster* [16]. We found that an arginine polymorphism has convergently arisen at the same residue through independent mutation of the codon (*D. melanogaster*: AGC → AGA; *D. simulans*: AGC → AGG). In both species, the serine/arginine polymorphism is segregating in populations throughout the world, although arginine is rare in *D. melanogaster* and common in *D. simulans* (Figure S2 and Table S1). We infected *D. simulans* with *P. rettgeri* and found that lines homozygous for the arginine allele carried three times higher bacterial loads than lines homozygous for serine (Figure 1C; $p = 0.008$) and virtually never survived infection (Figure 1D; $p < 0.001$). The derived arginine alleles of *D. melanogaster* and *D. simulans* are convergent in phenotype as well as genotype.

### *Diptericin* Null Alleles in *D. melanogaster* and *D. simulans* Are Associated with Extremely Poor Immune Defense

We previously [14] identified two *D. melanogaster* lines that carry a premature stop codon in the mature peptide and four more that carry a 12-bp deletion removing four residues from the mature peptide (Figure S2A). The two lines bearing the premature stop codon sustained the absolute highest pathogen loads in the initial study, and the three lines carrying the deletion were in the top 7%. In the present study, the two lines homozygous for the premature stop sustained higher pathogen loads than any other lines evaluated (Figure 1B), and no flies from either line survived for more than 48 hr after infection (Figure 1C). Tissue-specific RNAi knockdown of *Dpt* (see Supplemental Experimental

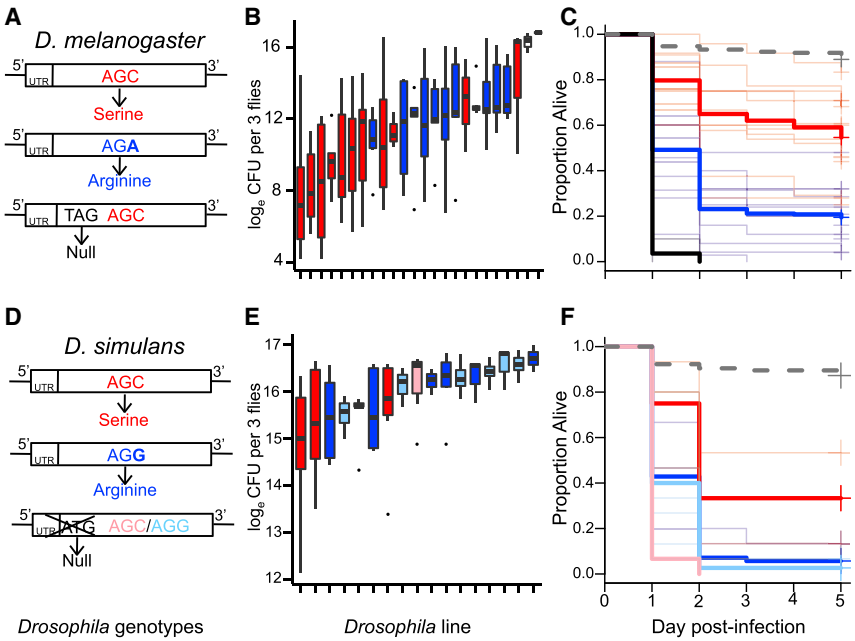


**Figure 1. Convergent Arginine and Null Mutations in *Diptericin* Decrease *D. melanogaster* and *D. simulans* Resistance to *Providencia rettgeri***

(A) Three *Dpt* genotypes of *D. melanogaster*: the ancestral serine residue, the derived arginine residue, and a presumed null genotype. See also Table S1 and Figure S2.

(B) Bacterial load (CFU) is higher in *D. melanogaster* lines carrying null (white) and arginine (blue) alleles than serine alleles (red) at 24 hr post-infection. Error bars represent 25$^{th}$ and 75$^{th}$ percentiles.

(C) *D. melanogaster* lines homozygous for serine (red) survive *P. rettgeri* infection better than lines homozygous for arginine (blue) or null alleles (black). The dashed gray line represents sterile-wound controls.

(D) Four *Dpt* genotypes of *D. simulans*: the ancestral serine residue, the derived arginine residue, and two putative null genotypes. Error bars represent 25$^{th}$ and 75$^{th}$ percentiles.

(E) *D. simulans* lines bearing arginine alleles (blue) and presumed null haplotypes (light blue/red) have higher pathogen loads (CFU) at 24 hr post-infection than lines bearing serine (red).

(F) *D. simulans* lines homozygous for serine (red) survive infection better than lines homozygous for arginine (blue) or presumptive nulls (light blue/red). The dashed gray line indicates sterile-wound controls.

Procedures) also resulted in 0% survival (0 out of 40 flies alive), compared to 70% survival (28 out of 40 flies alive after 48 hr) in control flies (Fisher's exact test $p < 0.001$). These data demonstrate that Diptericin plays a vital role in *D. melanogaster* defense against *P. rettgeri* and suggest that the premature stop codon renders the gene nonfunctional.

In an additional convergence, we found a polymorphic loss-of-function allele in *D. simulans*. This mutation is a 6-bp deletion that begins in the 5′ UTR and removes the start codon (Figure 1D). *D. simulans* lines carrying this deletion sustained significantly higher *P. rettgeri* loads ($p = 0.028$; Figure 1E) and mortality after infection ($p = 0.011$; Figure 1F). Thus, both *D. melanogaster* and *D. simulans* are additionally polymorphic for parallel mutations that eliminate Diptericin function and reduce resistance to infection.

## Allele-Specific Protection by Diptericin Is Pathogen Dependent

Previous studies using other bacterial pathogens failed to find an association between alleles of Diptericin and resistance to infection in *D. melanogaster* [9–11]. To test the specificity of the serine/arginine polymorphism, we measured bacterial load after infection with four other pathogens: *Providencia alcalifaciens*, *Providencia sneebia*, *Serratia marcescens*, and *Enterococcus faecalis* (all Gram-negative except the Gram-positive *E. faecalis*). The serine allele provided greater protection against *P. alcalifaciens* ($p < 0.001$; Figure 2) but had no effect on resistance to any of the other bacteria, even though expression of the *Dpt* gene is strongly induced by all four Gram-negative bacteria (Figures S1C and S1D). The two lines carrying the premature stop codon had some of the highest pathogen burdens after infection with *P. alcalifaciens* and *P. rettgeri* but were not exceptional after infection with the other three pathogens (Figures 2 and S1D), indicating that the phenotypic effects of *Diptericin* alleles are pathogen specific.

## There Are at Least Five Independent Mutations to the Arginine Residue across the *Drosophila* Phylogeny

Having observed a convergent serine/arginine polymorphism in *D. melanogaster* and *D. simulans*, we asked how many additional times such variants may have arisen in the subgenus *Sophophora* (genus *Drosophila*). *D. mauritiana* and *D. sechellia*, which are close sister species to *D. simulans*, are both fixed for serine (*D. mauritiana*: n = 107, [17]; *D. sechellia*: n = 18, D. Matute, personal communication). In comparing the reference genome sequences of *Drosophila* species, we found five independent substitutions to arginine at this codon (Figure 3) as well as two independent mutations to glutamine and one substitution to asparagine. This substitution rate is highly elevated relative to expectations under a model of strictly diversifying selection (ratio of posterior odds to prior odds or maximum Bayes factor = 680.94; a value greater than 20 is considered significant).

## Recombination at the Diptericin Locus Obscures the Signatures of Balancing Selection in *D. melanogaster*

Given that arginine is the derived state but provides weaker immune defense, we hypothesized that the polymorphism is segregating in *D. melanogaster* and *D. simulans* due to condition-dependent balancing selection with the arginine allele presumed to be beneficial under some conditions. For example, arginine might provide protection against an unknown pathogen, alter gut microbial composition, or reduce autoimmune damage.

Balancing selection can be classically inferred from sequence data, where deep divergence times between balanced alleles

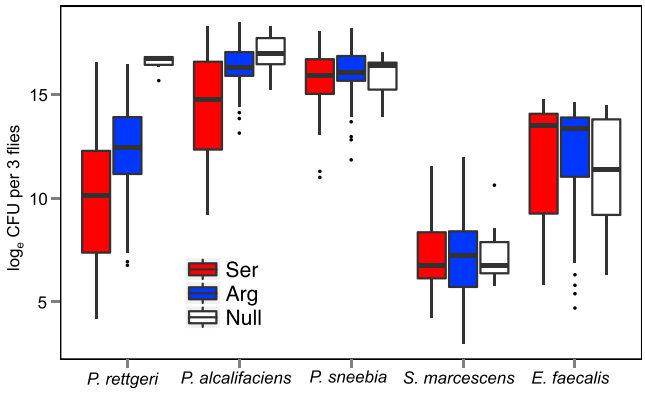


**Figure 2. Effects of *Diptericin* Alleles Are Pathogen Specific**

*D. melanogaster* lines were infected with five different bacteria, and bacterial load (CFU) was measured 24 hr after infection in genotypes homozygous for arginine (blue), serine (red), null (white). There was no effect of the *Dpt* allele on resistance to *P. sneebia*, *S. marcescens*, or *E. faecalis* infections. Error bars represent 25$^{th}$ and 75$^{th}$ percentiles. See also Figure S1.

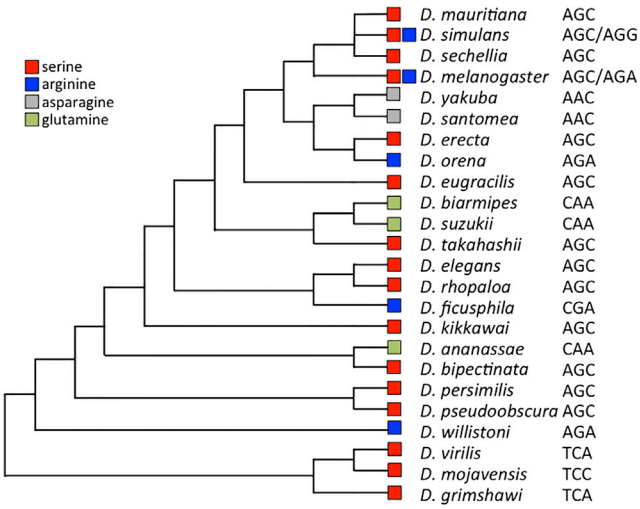


**Figure 3. Convergence across the *Drosophila* Phylogeny**

The amino acid residues at the codon homologous to codon 69 in *D. melanogaster* show the derived arginine state has arisen at least five times independently, glutamine twice, and asparagine once in the *Sophophora* subgenus of *Drosophila* [18]. Codon sequence is given to the right of each species name. See also Figure S3.

may result in elevated nucleotide diversity and an excess of polymorphisms at intermediate frequency [19–21]. However, despite the strong phenotypic evidence that the serine/arginine polymorphism is balanced in *Drosophila*, we do not observe the classically predicted molecular evolutionary signatures. This may be because the convergent alleles are recently derived and thus have short coalescent histories. Alternatively, the large effective population sizes and high recombination rates in *Drosophila* [22–24] may obscure the signature of balancing selection. There is virtually no linkage disequilibrium in the *Diptericin* gene. In an independent set of almost 200 *D. melanogaster* alleles directly sampled from a natural African population [25], we observe unambiguous recombination within 33 bp upstream and 97 bp downstream of the focal polymorphism (Figure S3A; other populations not shown exhibit similar patterns). The meiotic recombination rate in the *Dpt* chromosomal region is in the top 20% genome-wide [26]. Thus, selection may be able to act on the serine/arginine site without leaving a measurable population genetic footprint at flanking positions (Figures S3A and S3B) and eliminating the prospect of testing for balancing selection [21] with population genetic data.

### A Tandem Duplication of *Diptericin* Segregates in *D. simulans*

In *D. simulans*, we found a tandem duplication of *Diptericin* segregating in 23 out of 37 African inbred lines (Figure 4A). These duplicates are annotated in the *D. simulans* genome release 1.4 as *GD11417* (the derived duplicate, hereafter *Dpt A2*) and *GD11418* (the ancestral paralog, hereafter *Dpt A1*; Figure 4A). The phenotypic results described above were obtained from *D. simulans* lines that carry a single copy of *Diptericin*, but we were intrigued by the possibility that duplication may have additional phenotypic consequence. We infected a group of *D. simulans* lines that carried the duplication and compared them to a group that only contained a single copy. Genotype at the *Dpt A1* allele is the strongest predictor of resistance to *P. rettgeri* (Figures 4B and 4C), although lines that carry the duplication have higher survival ($p < 0.001$) and lower pathogen burden ($p = 0.039$) after *P. rettgeri* infection than those that do not. Recent duplicates often experience considerable rates of gene conversion between paralogs. We see conversion tracts in the primary sequence data where both paralogs share the same nucleotide polymorphisms, including the serine/arginine polymorphism (Figure 4D). Figure S4 shows the phylogenetic relationship of all paralogs from a set of inbred African *D. simulans* lines, and the two paralogs are not reciprocally monophyletic even at well-resolved nodes (Figure S4). The non-independence among substitutions arising from paralogous gene conversion violates the assumptions of population genetic tests for balancing selection and other forms of adaptive evolution [27], so these cannot be applied to the *D. simulans Diptericin* gene.

## DISCUSSION

A single, convergently arisen amino acid polymorphism in the Diptericin AMP has a large effect on resistance to bacterial infection in *D. melanogaster* and *D. simulans*. Both species also segregate for null alleles that result in high susceptibility to infection. Arginine alleles have arisen independently at least five times across the Sophophora subgenus of *Drosophila*. The presence of this evolutionarily convergent, large-effect amino acid variant does not support the prevailing hypothesis that insect AMPs are functionally redundant or that variation in individual AMPs has little effect on defense. Instead, it suggests that individual sites within AMPs can be targets of natural selection and may be sites of co-evolution between host and pathogen. These new data are more consistent with observations from human defensins, which have been associated with variation in disease susceptibility [28].

Previous studies have not found evidence of recurrent positive selection or balancing selection at the AMP genes of *Drosophila* and other insects [6], although long-term maintenance of allelic

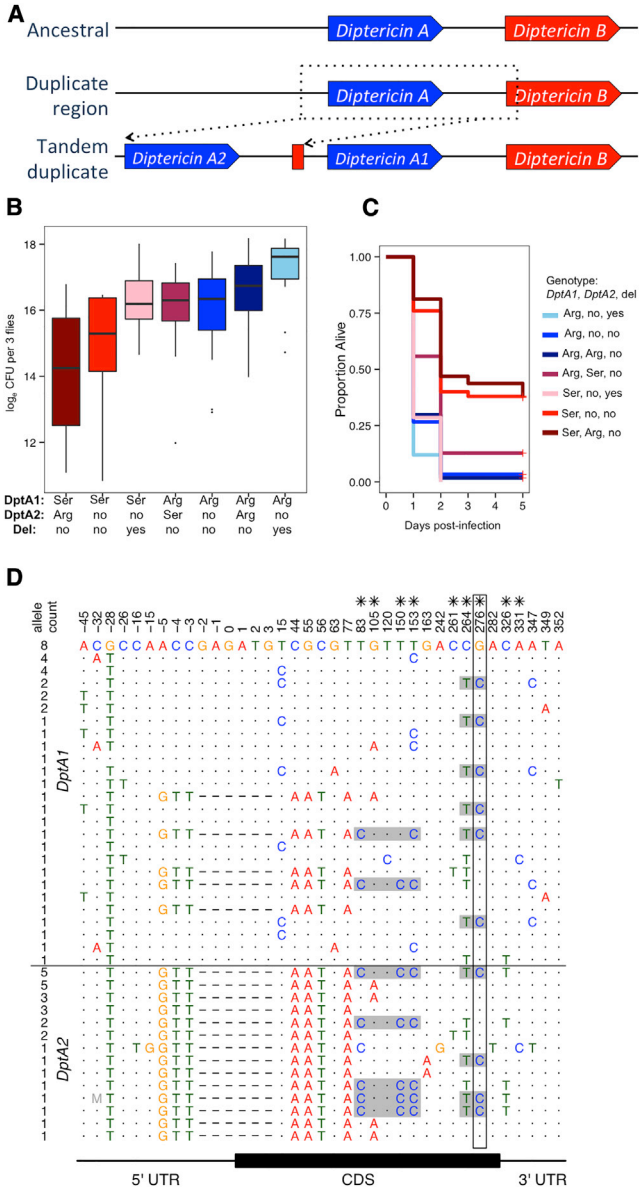


**Figure 4. A Gene Duplication of *Diptericin* in *D. simulans* Influences Resistance to *P. rettgeri* and Has Experienced Recurrent Gene Conversion between Paralogs**

(A) Schematic of the tandem duplication that generated *Dipterican A2*.

(B) Bacterial load (CFU) 24 hr after *P. rettgeri* infection of each *D. simulans* haplotype based on Ser/Arg genotype at *DptA1* and *DptA2* and the deletion in *DptA1*. Error bars represent 25$^{th}$ and 75$^{th}$ percentiles.

(C) Survival of each haplotype defined by Ser/Arg genotype at *DptA1* and *DptA2* and the deletion in *DptA1*.

(D) Shared polymorphism between *DptA1* and *DptA2* reveals recurrent gene conversion (shaded sequence blocks and asterisks). Sites are numbered relative to translational start along the top, with allele counts shown on the left. The serine/arginine polymorphism is boxed. Sites identical to the most common *DptA1* allele are denoted with a period.

See also Figure S4.

variation has been suggested for some vertebrate AMPs [29–33]. Yet selective maintenance of the serine/arginine polymorphism in *Diptericin* is the most plausible explanation for the repeated substitutions to the susceptible arginine across *Drosophila*. The polymorphism might be maintained by either direct fitness tradeoffs or alternating adaptiveness to fluctuating environments, but other potential explanations can be convincingly rejected. The sequence context around the recurrently substituted codon does not appear hypermutable, and distinct nucleotide mutations of the codon repeatedly give rise to parallel amino acid variants. The frequency of the susceptible arginine allele in *D. melanogaster* and *D. simulans* is far too high to be consistent with mutation-selection balance or genetic drift, and arginine can be assumed to be similarly common (if not fixed) in *D. orena*, *D. ficusphila*, and *D. willistoni*.

Our results highlight a general issue in population genetics: although we find phenotypic and molecular evolutionary evidence of a balanced polymorphism, we see no real population genetic signature of balancing selection because of the high rate of recombination at the selected locus. This is likely to be a pervasive problem in *Drosophila* and other organisms that have large effective population sizes. Frustratingly, the small footprint of selection will be winnowed even further for ancient alleles that have had more evolutionary time over which to recombine, and more recently derived alleles may have had insufficient time in which to accumulate the flanking neutral mutations that generate the signature of selection [21]. For these reasons, the general role that balancing selection plays in maintaining genetic variation may be severely underestimated by genome scans and population genetic surveys, especially in organisms with large population sizes.

The frequent incidence of natural loss-of-function alleles of AMPs suggests that AMP function in immune defense is balanced by deleterious effects of AMPs in the absence of infection. Serial pseudogenization and duplication may explain previously observed gene family dynamics. In the *Diptericin* gene family of *D. simulans*, both loss-of-function alleles and tandem duplicates are segregating. One possibility is that during epidemics, null alleles are quickly lost from the population but are regenerated and are possibly even beneficial when pathogen pressure is low. Although the evidence for adaptive divergence after duplication in invertebrates is mixed [5, 34–36], in the case of *Diptericin* in *D. simulans*, it is clearly hindered by gene conversion between the recently duplicated paralogs.

Lazzaro and Clark [7] found evidence of paralogous gene conversion between *Attacin A* and *Attacin B* and a signature of a rapid rise in frequency of the converted region. Like the current observation at *Diptericin* in *D. simulans*, gene conversion and selection reduced sequence divergence between the *Attacin* paralogs. *Attacin A* was also found to be segregating for a loss-of-function allele, as well as a 9-bp insertion/deletion polymorphism, with the insertion present in *D. simulans* and *D. sechellia* but absent from *D. mauritiana* [7]. Using data from Nolte et al. [17], we have found that the 9-bp insertion is polymorphic in *D. mauritiana*, suggesting another incidence either of convergent mutation or long-term maintenance of the polymorphism. The previously unappreciated similarities in the evolutionary patterns of *Diptericin* and *Attacin* genes of *Drosophila*, combined with similar observations in organisms such as mussels and vertebrates [32–36], may reveal general rules of AMP evolution.

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## ACCESSION NUMBERS

D. simulans lines originally collected in Africa were obtained from Charles Aquadro and were sequenced at one or both Diptericin paralogs. The accession numbers for all sequences reported in this paper are GenBank: KU200261–KU200329.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.11.063.

## AUTHOR CONTRIBUTIONS

R.L.U., V.M.H., and B.P.L. conceived, designed, and performed experiments, analyzed data, and wrote the paper.

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