Molecular Evolution of *Drosophila* Cuticular Protein Genes

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**Abstract**

Several multigene families have been described that together encode scores of structural cuticular proteins in *Drosophila*, although the functional significance of this diversity remains to be explored. Here I investigate the evolutionary histories of several multigene families (CPR, Tweedle, CPLCG, and CPF/CPFL) that vary in age, size, and sequence complexity, using sequenced *Drosophila* genomes and mosquito outgroups. My objective is to describe the rates and mechanisms of ‘cuticle-ome’ divergence, in order to identify conserved and rapidly evolving elements. I also investigate potential examples of interlocus gene conversion and concerted evolution within these families during *Drosophila* evolution. The absolute rate of change in gene number (per million years) is an order of magnitude lower for cuticular protein families within *Drosophila* than it is among *Drosophila* and the two mosquito taxa, implying that major transitions in the cuticle proteome have occurred at higher taxonomic levels. Several hotspots of intergenic conversion and/or gene turnover were identified, e.g., some gene pairs have independently undergone intergenic conversion within different lineages. Some gene conversion hotspots were characterized by conversion tracts initiating near nucleotide repeats within coding regions, and similar repeats were found in concertedly evolving cuticular protein genes in *Anopheles gambiae*. Rates of amino-acid substitution were generally severalfold higher along the branch connecting the Sophophora and Drosophila species groups, and 13 genes have Ka/Ks significantly greater than one along this branch, indicating adaptive divergence. Insect cuticular proteins appear to be a source of adaptive evolution within genera and, at higher taxonomic levels, subject to periods of gene-family expansion and contraction followed by quiescence. However, this relative stasis is belied by hotspots of molecular evolution, particularly concerted evolution, during the diversification of *Drosophila*. The prominent association between interlocus gene conversion and repeats within the coding sequence of interacting genes suggests that the latter promote strand exchange.

**Citation:** Cornman RS (2009) Molecular Evolution of *Drosophila* Cuticular Protein Genes. PLoS ONE 4(12): e8345. doi:10.1371/journal.pone.0008345

**Editor:** Cecile Fairhead, Institut de Genetique et Microbiologie, France

**Received** September 26, 2009; **Accepted** November 18, 2009; **Published** December 17, 2009

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**Funding:** The author was supported during the initiation of this work by National Institutes of Health (NIH) grant A155624 to Judith H. Willis, Department of Cellular Biology, University of Georgia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The remainder of the work was completed with no outside funding.

**Competing Interests:** The author has declared that no competing interests exist.

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**Introduction**

Arthropod cuticle is an important ecological innovation of a highly successful invertebrate phylum. It is a strong, light exoskeleton and environmental interface that is predominantly composed of an ordered matrix of chitin fibers and protein [1]. The biomechanical properties of cuticle and the control of its deposition during development have long been of interest. More recently, evidence has emerged that cuticular proteins are relevant to problems in applied entomology, such as the adaptation of disease vectors to human-associated selective pressures [2,3,4]. Cuticular proteins are further of interest because of the high level of gene conversion occurring among certain genes in some insect species [5,6,7,8].

Recent studies have revealed a remarkable diversity of cuticular proteins. By far the largest, and taxonomically most widespread, cuticular protein family is the CPR family, which is characterized by a conserved domain first identified by Rebers and Riddiford [9]. This domain has been revised and extended in the genomic era but is still commonly referred to as the “R&R Consensus”. Examples of this domain have been shown to bind chitin *in vitro* [10,11]. Additional cuticular protein families have been uncovered by methods such as mutant analysis and shotgun proteomics. Some families appear to be narrowly restricted taxonomically (e.g., the apidermin [12] and CPLCW [7] families) and presumably are of more recent origin. Other families that are widely distributed are nonetheless characterized by radiations within particular taxa [6,7]. Thus, the complement of cuticular proteins is dynamic among insect orders, but the pace of change has not been investigated and the functional significance of these gene expansions is unknown.

While functional studies can shed light on the roles of specific proteins during development, an integrated view of how the molecular complexity of cuticle has evolved remains a daunting task for its sheer scale. For example, are major transitions in the cuticular proteome associated with changes in ecology and/or development, and at what taxonomic scale? As they become established in the molecular repertoire, do duplicated genes adaptively differentiate at the protein sequence level, or are differences primarily regulatory? Are some protein families more conserved than others, either at the level of protein sequence, gene regulation, or copy number? An important foothold in this genomic landscape can be gained by identifying genomic patterns of gene gain and loss, and by measuring substitution rates among...
orthologous sequences. This evolutionary perspective provides a context for planning and interpreting comparative functional studies that have broadest impact.

In this paper, I present an evolutionary analysis of cuticular protein families of Drosophila, using the mosquitoes Anopheles gambiae and Aedes aegypti as sister groups, because of the genomic sequence data and gene-family annotations that are available. I compare evolutionary rates of change in gene number across gene families and across taxa, and examine the organization of these gene families in the genome. The questions I address with these data are: How has the number and organization of genes in each gene family changed in a phylogenetic context? Do some gene families or subfamilies change in size more rapidly than others? Has intergenic gene conversion, which has been reported for some cuticular proteins in some species [5,8], occurred more broadly in the evolutionary history of Drosophila cuticle genes? Are certain genes particularly susceptible to such events? Finally, I compare orthologous sequences to investigate whether there has been any general change in the rate of evolution of cuticular proteins along the Drosophila phylogeny. In particular, is there evidence of positive selection driving divergence of protein sequence in a particular lineage? If so, it would imply that cuticular proteins participate in the adaptive divergence of species.

Methods

Using published gene family annotations as a seed for BLAST, I searched seven Drosophila taxa and the mosquitoes An. gambiae and A. aegypti for genes of the CPR [13], Tweedle [14], CPF/CPLF [15,16], and CPLCG [7] families. These are the best characterized multigene families that encode structural cuticular proteins. I excluded other known Drosophila cuticular protein ‘families’ that consist of only one or two genes and are not also present in mosquitoes (e.g., Edg91 [17]). I also excluded homologs of putative cuticular proteins identified in An. gambiae (the CPLCA and CPLCP families [7]) that are not yet well characterized in Drosophila.

The annotation of An. gambiae cuticular proteins has been described elsewhere [6,7,16]. Ae. aegypti genes were identified by BLAST as well as by searching Ensembl (http://www.ensembl.org) gene annotations. The Drosophila annotations were based primarily on previous studies [13,14,16] but were amended if necessary based on ortholog alignments. Particularly noteworthy is the inclusion of an additional CPR gene, CG13670, upstream of the genes Cpr66Ca and Cpr66Cb that were annotated by [15].

I annotated only six other Drosophila genomes in addition to D. melanogaster (D. anassae, D. pseudobscura, D. willistoni, D. mojavensis, D. virilis, and D. grimshawi) because this number well represents the phylogenetic diversity of sequenced Drosophila [Fig. 1]. Coding sequences of these Drosophila species were predicted from genomic regions containing BLAST matches using the programs Genscan [18] and SNAP [19], and manually adjusted based on ortholog alignments. This approach serves to identify the number of genes and the coding sequence of the mature predicted proteins (that is, after signal peptide cleavage). It is not intended to serve as a complete annotation strategy, which would be redundant to ongoing efforts by other groups (see http://rana.lbl.gov/drosophila/index.html).

I did not exhaustively annotate the other five sequenced Drosophila genomes because they are closely related to taxa already represented and would provide little additional information while substantially increasing the computational requirements for the analyses. However, I did annotate particular genes in these species when necessary to clarify recurring patterns of intergenic conversion (see Results). Annotated pseudogenes in D. melanogaster and the presumed orthologous sequence in other species are noted in some figures but were not included in total gene counts.

All sequences used in this study are given in Text S1 and inferred orthology is given in Text S2. Neighbor-joining trees

![Figure 1. Schematic representing the estimation of ω, the ratio of nonsynonymous to synonymous substitutions, along branches of the Drosophila phylogeny. A. Based on initial pairwise calculations of Ka/Ks, three distinct ω parameters were estimated for each set of orthologous genes. One ω was estimated for the Sophophora species group, one for the Drosophila species group, and a third ω was estimated for the branch connecting the two species groups. The branches of the tree labeled with each ω class are indicated by colored boxes. B. To test whether the estimated ω for the branch between the Sophophora and Drosophila groups is significantly greater than 1 for a particular set of orthologous genes, ω is recalculated for that branch with all other branches assigned to a single background ω class. doi:10.1371/journal.pone.0008345.g001](image-url)
were created with MEGA3 [20] using Clustal-aligned amino acid sequence, the amino-acid exchange matrix of Jones et al. [21], and pairwise deletion of indels. Bootstrap values represent 1,000 resampled data sets.

Several methods have been described that assess the tempo of gene family evolution. Here I use the CAFE program [22], which parameterizes the rate of gene gain or loss along an organismal phylogeny by fitting a model to the observed distribution of genes in each species and inferring the number of genes at ancestral nodes. The rate is expressed in absolute time assuming independent estimates of divergence are available. The Drosophila phylogeny and branch lengths (in absolute time) follow [23]. The divergence time between An. gambiae and A. aegypti is assumed to be 95 million years based on [24], and 260 million years between An. gambiae and D. melanogaster based on [25].

To evaluate patterns of duplication and/or gene conversion, I used the program Dotter [26] to create dot plots of tandem arrays and the program RDP2 [27] to estimate the probability of gene conversion events. The dot plots shown are graphical depictions of genomic sequence aligned with itself, in which the presence of a dot at any X-Y coordinate indicates nucleotide similarity between sliding windows centered on positions X and Y of the sequence [26]. The dot plots shown in the Results depict patterns of similarity within both the input sequence and its reverse complement, and there is always a dot at positions along the diagonal (X = Y) by definition. Substitutions per site for pairwise comparisons were calculated with DnaSP [28]. I used PAML [29] to estimate Ka/Ks along branches of gene trees. PAML uses a likelihood framework to estimate a parameterization of Ka/Ks, termed Ω, to model sequence evolution. This type of branch-specific analysis is appropriate for estimating rates of ortholog diversification provided that synonymous sites are not saturated. For all analyses, the ratio of transitions to transversions (K) was estimated in order to assess mutational saturation. All multiple sequence alignments were performed with ClustalW using default protein parameters, but were also manually inspected and trimmed of ambiguously aligned regions or regions likely to be concertedely evolving.

Because of the large number of parameter-rich tests of sequence evolution that can conceivably be performed on a given alignment, it is important to clearly articulate the tested hypotheses and use a hierarchical approach to data analysis that allows suitable likelihood tests. The evolutionary models used in PAML are stated for each analysis in the Results.

Results

Gene-Family Size Evolution

The number of annotated genes of each cuticular protein family varies substantially among Anopheles, Aedes, and Drosophila (Table 1). In contrast, the number of genes in the seven Drosophila genomes I investigated is very similar (Table 2), implying a low rate of gene turnover within the genus. I quantified rates of change in the total number of cuticular proteins using the CAFE program [22]. This method estimates a birth-and-death parameter, λ, defined as the change in gene number per million years. By this measure, the rate of change in the number of cuticular proteins is roughly an order of magnitude lower within Drosophila (3E-04) than it is among the three Dipteran genera (3.5E-03). Although λ = 3.8E-03/10^6 years was the most likely rate for the latter group, even higher parameter values were possible but could not be evaluated because of a methodological constraint [22] requiring that the product of λ and the longest branch length not exceed one.

| Species          | CPR | CPLCG | CPF/CPF | Tweedle |
|------------------|-----|-------|---------|---------|
| Drosophila melanoaster | 102 | 3     | 3       | 27      |
| Anopheles gambiae | 156 | 27    | 11      | 12      |
| Aedes aegypti    | 240 | 16    | 12      | 9       |

1Number of Ensembl-annotated proteins (v. 40) with the Rebers and Riddiford Consensus (CPR family consensus domain, Pfam00379), which should closely approximate the total number of CPR genes.

doi:10.1371/journal.pone.0008345.0001

Drosophila CPR genes often occur on chromosomes as ‘singleton’ genes, linearly distant from other homologs. However, most (>75%) are organized into tandem arrays of genes, with most or all of the genes in the array clustering together phylogenetically (see [13] and Text S3). These arrays are likely to include monophyletic groups that have arisen through tandem duplication from an ancestral gene. Because of the short lengths of paralogous alignments and overall conservation of the defining domain of CPR proteins, bootstrap support for the monophyly of these groups is usually low. Nonetheless, given their physical arrangement and phylogenetic clustering, it is reasonable to treat these arrays as subgroups (Table 3) for further analysis (all CPR subgroups discussed here are referenced by D. melanogaster chromosome band, see [13]).

I first used CAFE to test whether, collectively, tandemly arrayed or singleton CPR genes evolved differently from the overall rate of change in gene number. Neither category deviated statistically from the rate of λ = 3.0E-04 calculated from the data for all gene families in Table 2. This result is somewhat surprising given that tandemly arrayed genes might be expected to have a higher rate of gene duplication than singleton genes, due to unequal crossing over among homologous sequences. However, two individual arrays, the 44C and 65A arrays, did differ significantly (P<0.05) from the overall λ when considered separately. Note that the small array consisting of Cpr50Ca and Cpr50Cb was excluded from this analysis because the former contains a known non-cuticular protein gene (CG13340) within an intron.

The organization of the 44C array in the seven Drosophila species is shown in Figure 2A and a phylogeny of these genes is shown in Figure 2B. The organization and phylogenetic relationships of the 65A array are shown in Figure 3A and Figure 3B, respectively. These figures show that gene gain and loss within the 44C and 65A
arrays is likely greater than suggested by total gene number alone. While the gene trees of other CPR subfamilies show a clear pattern of conserved one-to-one orthology with infrequent gene gain or loss (Text S3), the pattern is strikingly different for these two arrays. In

| Lineage            | 30F | 44C* | 47E | 49A | 62B | 64A | 65A* | 65E | 66C | 67F | 72E | 76B | 78C | 84A | 97E |
|--------------------|-----|------|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|
| D. melanogaster    | 2   | 4    | 7   | 8   | 3   | 4   | 18   | 3   | 3   | 3   | 3   | 3   | 3   | 4    | 2   | 8² |
| D. ananassae       | 2   | 4    | 7   | 8   | 3   | 4   | 19   | 3   | 3   | 3   | 3   | 3   | 3   | 4    | 2   | 8² |
| D. pseudoobscura   | 2   | 4    | 7   | 8   | 3   | 4   | 16   | 4   | 3   | 2   | 3   | 4   | 2   | 7    | 2   |     |
| D. willistoni      | 2   | 5    | 7   | 7   | 3   | 4   | 18   | 3   | 3   | 2   | 3   | 3   | 2   | 8    | 2   |     |
| D. virilis         | 2   | 9    | 7   | 8   | 2   | 4   | 15   | 3   | 3   | 3   | 3   | 3   | 4   | 2    | 8   | 2   |
| D. mojavensis      | 2   | 2    | 8   | 8   | 3   | 4   | 16   | 3   | 3   | 2   | 3   | 5   | 2   | 8    | 3   |     |
| D. grimshawi       | 2   | 4    | 7   | 8   | 3   | 4   | 18   | 3   | 3   | 2   | 3   | 4   | 2   | 8    | 2   |     |

Reconstructed ancestral state: 2 4 7 8 3 3 4 1 8 3 3 4 2 8 2

1 Rate of change in gene number significantly different from assigned \( \lambda = 3.8E-4 \) at \( P < 0.05 \).
2 Found significant at \( P < 0.01 \); however the state reconstructed by CAFE for the D. melanogaster–D. ananassae node was 2 rather than 3, thereby doubling the number of inferred changes. BLAST searches of the melanogaster-group species D. erecta, D. yakuba, D. simulans, and D. sechellia reveal three genes in each species. Running CAFE with these additional data for this subfamily resulted in a reconstructed ancestral state of three genes and no significant deviation from the overall \( \lambda \).

3 Includes DmelCpr5C and its ortholog in D. ananassae, which I infer to be an interchromosomal duplication of Ccp84Ac (see Text S2).

Table 3. Number of genes within Drosophila CPR tandem arrays.

doi:10.1371/journal.pone.0008345.t003

Figure 2. The array of CPR cuticular protein genes located approximately at band 44C of D. melanogaster chromosome 2R and the orthologous regions in six other Drosophila species. A. Schematic of the organization of genes in the array, with colored boxes matching colored symbols in the phylogeny according to the legend at left. Names at top are of D. melanogaster genes; plus and minus symbols indicate relative orientation. Genes with dark outlines are predicted to be intronless. B. Neighbor-joining phylogeny (see Methods) of predicted amino acid sequence with bootstrap support indicated.

doi:10.1371/journal.pone.0008345.g002
related paralogs within each lineage could, in principle, be due to high rates of gene duplication and loss within this array. However, a previous study of polymorphism at coding and flanking sites in the *D. melanogaster* 44C array [8] indicates a contribution by interlocus gene conversion as well.

The 65A array includes seven genes that are present in most or all species as one-to-one orthologs, in a conserved order. In the gene tree, these ortholog sets all have bootstrap support greater than 90%. Other sets of genes also occur as one-to-one orthologs but are limited to either the *Drosophila* or *Sophophora* species groups and presumably arose after the divergence of these clades. A third category includes sets of genes that cluster as paralogs within each lineage, similar to that observed in the 44C array. Some of these sets of genes are adjacent whereas others are not, and where they occur in the array is quite variable. Genes in this third category are more closely related to each other across *Drosophila* species than they are to the conserved single-copy genes. This result suggests that a single ancestral gene, or a few closely related genes, gave rise to an eclectic set of descendants within each lineage, due to a higher rate of gene turnover, gene conversion, and/or gene rearrangement than other genes in the same array. Charles et al. [5] previously presented evidence for tandem duplications and intergenic conversion in the evolution of the *D. melanogaster* 65A array, and even detected variation in gene copy number among different strains of that species. The multi-species, whole-genome analysis presented here (Fig. 3B) reveals that these processes are constrained to a particular region of the gene tree, outside of which genes show one-to-one patterns of homology and synteny among species.

Given that the 44C and 65A arrays evolve more rapidly and contain sets of similar paralogs, it is of interest to determine if retrogene formation is an important contributor to their evolutionary history. Retrogens are suggested by the absence of introns present in homologs, divergent or missing promoter sequence, and 3’ thymine runs. Indeed, a modest number of gene predictions within the 44C and 65A arrays lack introns, indicated by thick borders in Figures 2A and 3A, and sites where intronless genes occur in the array are consistent across species. Intronless gene models were manually checked to verify that a valid signal peptide was predicted. In a few cases, candidate TATA boxes were not identified; however, some CPR genes in *An. gambiae* that are detectably expressed also lack introns.

Figure 3. The array of CPR cuticular protein genes located approximately at band 65A of *D. melanogaster* chromosome 3L and the orthologous regions in six other *Drosophila* species. A. Schematic of the organization of genes in the array, with colored boxes matching colored symbols in the phylogeny according to the legend at left. Numbered positions in the array correspond to numbered clades in the phylogeny of part B. Names at top are of *D. melanogaster* genes; plus and minus symbols indicate relative orientation. B. Neighbor-joining phylogeny (see Methods) of predicted amino acid sequence. Arrow indicates the clade within which genes cluster as paralogs rather than as orthologs (see text for details). Bootstrap support is not shown for clarity, but is greater than 90% for all numbered groups and generally low outside these groups.

doi:10.1371/journal.pone.0008345.g003

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TATA boxes [30]. Furthermore, polyT runs were not evident downstream of coding sequences. Thus, the intronless genes in these arrays are more likely to have arisen through ectopic recombination or splice-site mutations. (Note that most CPR genes contain a single intron placed after the first few codons of the signal peptide, and that evidence of intron-regulated gene expression has been found for Csp63A in D. melanogaster [31].) Even if all of the intronless genes in these arrays actually arose through retroposition, the presence of highly similar paralogs is not limited to such genes, nor do all intronless genes cluster within species paralogs. Thus, retrogenes do not appear to contribute substantially to the distinctive pattern of evolution within these arrays.

In summary, comparing the 44C and 65A arrays to the rest of the Drosophila CPR gene family, I conclude 1) that the greater variation in gene number detected by CAFE within these arrays is associated with paralog homogenization; and 2) that there can be pronounced biases as to which genes within arrays are susceptible to sequence homogenization.

**Intergenic Conversion Recurring between Specific Gene Pairs**

To clarify whether the presence of very similar paralogs is distinctive of the 44C and 65A arrays, I systematically examined phylogenetic patterns within all other CPR arrays in Drosophila (Text S3). Two additional arrays at 67F and 84A showed pronounced clustering among the seven genomes initially examined. I then examined these genes in the remaining genomes to determine the consistency of paralog clustering across species. Consistent clustering of paralogs and significant evidence of intergenic conversion was identified within the 67F tandem array for genes Cpr67Fa1 and Cpr67Fa2, but only within the melanogaster species group (Figure 4A) and not between either gene and Cpr67Fb1. Note that in D. simulans, there are three Cpr67Fb-like genes and Cpr67Fb1 is absent, but dot plots reveal the intergenic sequences to be nearly identical as well (not shown). This latter finding suggests that the three gene copies are the result of recent tandem duplication within this lineage rather than intergenic conversion. Only one Cpr67Fb-like gene is present in the other Drosophila species.

Figure 4B illustrates the number of nucleotide differences between paralogous Cpr67Fb-like genes in the melanogaster species group compared with among-orthog group variation. I used a sliding window of 50 bp to measure variation in 25-bp steps along the length of the aligned coding sequence (note that because gaps are excluded, the length of the region compared varies among alignments). There are many fewer nucleotide differences between paralogs than among orthologs, which is unexpected given the implicitly older age of paralogs. These data in conjunction with the phylogenetic pattern strongly imply concerted evolution at these loci.

A formal test of gene conversion between the sequenced alleles of Cpr67Fa1 and Cpr67Fa2 was done with the RDP2 program. For this test, I initially aligned the region extending 500 bp upstream of the start of the second exon to 300 bp downstream of the stop codon. At the 5’ end, this region includes the short intron within the signal peptide, the first exon, and sequence upstream of the predicted promoter. The polyadenylation signal was also included at the 3’ end. I then manually trimmed the alignment edges to be nearly identical as well (not shown). This latter finding suggests that the three gene copies are the result of recent tandem duplication within this lineage rather than intergenic conversion. Only one Cpr67Fb-like gene is present in the other Drosophila species.

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conversion between Cep84Ab and Cep84Ad despite the fact that they are separated by Cep84Ac. In D. persimilis, there has been an inversion of the region containing Cep84Aa and Cep84Ab the breakpoints of which coincide with the repetitive sequence. In contrast, there is no evidence of any non-orthologous interaction involving any of the genes in the 84A array that lack or have minimal repetitive sequence as visualized by dot plot, despite the fact that all genes in this array necessarily share substantial sequence similarity. The implications are that these repetitive sequences promote nonorthologous strand exchange, and that paralogous gene conversion occurs at a high rate in the germ line when nearby genes share this sequence. On the other hand, no nucleotide repeats were found to be associated with gene conversion in the 67F array.

Figure 8 shows an alignment of repetitive sequence from the Cep84Aa and Cep84Ab genes of each Drosophila each species. The
repeats are not simple nucleotide repeats but are instead longer sequences that are inexactly repeated tens of nucleotides apart from each other. The repetitive sequence in this region has diverged among the different species but a core shared sequence can be identified (bracket in Fig. 8). This sequence is GC-rich and bears some similarity to GC-rich motifs associated with guanine ‘quadruplex’ pairing [33] and with recombination hotspots in human [34].

I then investigated whether within-species clustering of paralogs was associated with repetitive coding sequence in the Tweedle gene family. Figure 9A shows a phylogeny of all identified Tweedle genes in the seven Drosophila species. For most genes, putatively orthologous genes cluster together as expected under independent evolution, however, two sets of paralogous genes consistently cluster by species. Both sets of similar paralogs are within a larger array of Tweedle genes at 97C in D. melanogaster. CG5468, CG6447, and CG6448 cluster as paralogs for all seven species (green shading) and CG5471 and CG6460 cluster within the Sophophora species group only (orange shading), as the latter gene is not present in the Drosophila species group. Figure 9B shows a dot plot of a tandem array of Tweedle genes in D. melanogaster that contains both groups. CG5468, CG6447, and CG6448 each contain repetitive sequence near the 5’ end of the coding region whereas the other genes in the array do not. On the other hand, CG5471 and CG6460 have only a minimal amount of repetitive sequence, not visible by dot plot at this scale. These two genes also lack repetitive sequence in D. ananassae (not shown), but do have repetitive sequence in D. pseudoobscura and D. willistoni (Figure 10).

Because An. gambiae cuticular proteins of the CPR and other multigene families show extensive concerted evolution [6,7], I also used dot plots to look for similar patterns of nucleotide repetition associated with gene conversion tracts in this species. Comparable nucleotide repeats within coding regions were found for all sets of highly similar genes in An. gambiae, although the level of repetition
varied substantially (examples are shown in Fig. 11 and Text S4). In contrast, comparable repeats are largely absent for single-copy genes (not shown). Dot plots of some \textit{An. gambiae} gene arrays also reveal extensive tandem duplication and/or inversions involving multiple genes. Thus, while the association between nucleotide repeats and concerted evolution is remarkably similar in both \textit{Drosophila} and \textit{An. gambiae}, specific pairs of interacting genes were not evident in the mosquito and the arrays are structurally more dynamic.

### Rates of Protein Evolution

The first part of this paper examined patterns of stasis and change in the number of genes within gene families. I then examined patterns of concerted evolution within tandem arrays of CPR genes. I now turn to investigating the evolutionary rate of change in the coding sequences themselves, which is usually quantified as the ratio of nonsynonymous substitutions (\(K_a\)) to synonymous substitutions (\(K_s\)). Estimating the \(K_a/K_s\) of orthologous sequences reveals how the same gene inherited from a common ancestor has diverged among the descendant species. \(K_a/K_s\) can be derived directly from \(K_a\) and \(K_s\) estimates for any pairwise comparison, but for sequences evolving along a phylogeny the ratio is parameterized as \(\omega\) in PAML [29]. For a given phylogeny, the likelihoods of different evolutionary models can be compared using assigned or estimated \(\omega\) parameters. This approach provides a statistical framework for evaluating explicit models of sequence evolution that are of biological interest.

I first estimated \(\omega\) along the seven \textit{Drosophila} species phylogeny for 116 'single-copy' genes (those judged to have one-to-one orthologs in other species by sequence and synteny) from all four gene families. In addition to identifying specific genes likely to be under positive selection during the evolutionary history of \textit{Drosophila}, this analysis can address whether, on average, different cuticular protein families evolve at similar or different rates. This is an interesting topic given that these gene families differ in age and in the complexity of their conserved domains and likely have distinct functions in cuticle. However, initial pairwise comparisons of \(K_a/K_s\) between orthologous sequences revealed a complication, which was that evolutionary rates within the \textit{Drosophila} species group consistently differed from those of the Sophophora species group, regardless of cuticular protein family. I therefore estimated separate \(\omega\)'s for the two species groups. I also estimated a third \(\omega\) for the branch connecting the \textit{Drosophila} and Sophophora groups because pairwise comparisons indicated consistently high rates of evolution along this branch (i.e., three branch labels in PAML, with Model = 2 and Nsites = 0; see Fig. 1). The distribution of \(\omega\) estimates by gene family is shown in Figure 12. The range of evolutionary rates was similar for both singleton and tandemly arrayed CPR genes, and these rates were comparable to the Tweedle and CPF/CPFL families as well. The CPLCG family had the highest mean \(K_a/K_s\), but the sample size is only three genes and the values are well within the range of the larger gene families. Thus, there does not appear to be a qualitative difference among these \textit{Drosophila} cuticular protein families in rates of amino-acid evolution.

The results also confirmed that orthologous genes tend to have lower \(K_a/K_s\) within the Sophophora species group than within the \textit{Drosophila} group (Fig. 12), implying higher rates of cuticular
protein evolution in the latter group. However, this difference between species groups is primarily driven by shorter synonymous-site branch lengths within the Drosophila species group rather than longer nonsynonymous-site branch lengths (Table 4). That is, the total amount of amino-acid change has been similar within each group, but since synonymous substitution rates were lower in the Drosophila species group, the orthologous sequences in those species are therefore less conserved per unit of synonymous change. Codon bias is unlikely to explain this difference in Ks because the two species groups have very similar codon usage both for these genes (not shown) and, with the exception of *D. willistoni*, in general [35]. Furthermore, the proportion of indels in aligned sequences is also higher within the Sophophora group compared with the Drosophila group. Table 5 shows the mean pairwise percentage of alignment sites with a gap, calculated separately for comparisons within Drosophila, within Sophophora, and between the two clades. For both the CPR and Tweedle families, the proportion of gaps was significantly higher in Sophophora by t-test (P<0.01 and P<0.05, respectively). These data suggest that demographic histories or mutation probabilities have differed between species groups.

In contrast, PAML analysis strongly supported the initial observation of high evolutionary rates along the branch connecting the Drosophila and Sophophora species groups. Thirty-six out of the 116 single-copy genes examined had ω estimates along this branch equal to or greater than one, including all three CPF/CPFL genes. For those genes with an estimated ω less than one, the rate was nonetheless 3.3 times higher, on average, than the ω along other branches. Thus, the divergence of these two taxa was associated with a very broad acceleration in amino-acid substitutions within the **Table 4.** Mean evolutionary rates among orthologous single-copy genes in each species group, for all protein families combined.

| Rate | Sophophora | Drosophila |
|------|------------|------------|
| Ka   | 0.035      | 0.041      |
| Ks   | 0.676      | 0.383      |
| ω*   | 0.056      | 0.103      |

*Note that the mean of ω estimates need not equal the ratio of mean Ka to mean Ks.

doi:10.1371/journal.pone.0008345.t004

**Table 5.** Mean proportion of aligned sites containing gaps, within and between Drosophila and Sophophora species groups.

| Gene family | Sophophora | Drosophila | Inter-group comparison |
|-------------|------------|------------|------------------------|
| CPR**       | 0.077      | 0.036      | 0.079                  |
| Tweedle*    | 0.066      | 0.041      | 0.073                  |

*P<0.05 of equal means by t-test.
**P<0.01 of equal means by t-test.

doi:10.1371/journal.pone.0008345.t005
cuticular proteome. This finding is unlikely to be an artifact of applying a complex model to a short and/or mutationally saturated branch of a gene tree, because estimates of \( k \) were generally well below 2 and the synonymous branch lengths were generally moderate and consistent. Genes with unusually long synonymous branch lengths were discounted, as were cases of ambiguous orthology (such as concertedly evolving genes). Moreover, the topology of the species tree is very well supported by independent data [35].

To test whether any of these 36 genes had \( \omega \) significantly greater than one, implying positive selection, requires a two-\( \omega \) evolutionary model in PAML. In this model, one parameter represents the branch of interest and the other parameter represents the rest of the orthologous gene tree (i.e., Model = 2 and Nsites = 2). The likelihood of the gene trees is then estimated with \( \omega \) fixed at 1 or \( \omega \) free to vary, and the two likelihoods compared by a \( \chi^2 \) test [36]. Using this test, I identified 13 genes with Ka/Ks statistically greater than 1 during the divergence of the two species groups (Table 6).

The CPR genes identified tend to be highly diverged from paralogs as well, i.e. they lie on long branches in the Drosophila CPR phylogeny [6,13], and their products include the eye lens protein drosocystallin [37,38] and the Drosophila resilin protein [39].

### Discussion

There are scores of proteins that contribute to cuticle in Drosophila, about which relatively little is known. The goal of this study was to characterize evolutionary patterns within the Drosophila cuticular proteome, in order to gain insight into how this protein diversity has evolved, both within the genus and compared with other Diptera. The results show that the rate of change in the number of cuticular protein genes is much slower (in absolute time) within Drosophila than along the branches connecting Drosophila with the mosquitoes An. gambiae and A.e. aegypti. Thus, the differences in gene number between genera do

### Table 6. Genes with statistically significant evidence of positive selection along branch connecting the Sophophora and Drosophila species groups.

| Family | D. melanogaster gene name | ln(likelihood), \( \omega \) fixed = 1 | ln(likelihood), estimated \( \omega > 1 \) | 2ln(likelihood)* |
|--------|---------------------------|----------------------------------|----------------------------------|------------------|
| CPR    | resilin                   | −5503.0                          | −5487.0                          | 31.89            |
| Tweedle| TweedleX                  | −3593.5                          | −3581.5                          | 23.88            |
| CPR    | Cpr100A                   | −2010.7                          | −2001.8                          | 17.73            |
| CPR    | Cpr76Bd                   | −15292.6                         | −15284.1                         | 16.99            |
| CPR    | Cpr47Ee                   | −3945.6                          | −3939.3                          | 12.44            |
| CPR    | Cpr65Ee                   | −1336.6                          | −1330.7                          | 11.79            |
| Tweedle| TweedleV                  | −1936.6                          | −1930.8                          | 11.59            |
| CPR    | Cpr72Es                   | −2067.2                          | −2061.7                          | 10.99            |
| CPR    | cry (crystallin)          | −4135.8                          | −4130.6                          | 10.44            |
| CPR    | Cpr30B                    | −1654.3                          | −1649.9                          | 8.83             |
| CPR    | Cpr66Cb                   | −1392.0                          | −1389.3                          | 5.31             |
| CPR    | Cpr72Ec                   | −3220.8                          | −3218.7                          | 4.08             |
| CPR    | Cpr49Af                   | −1697.7                          | −1695.7                          | 4.08             |

*Critical value of \( \chi^2 \) distribution is 3.84 for \( \alpha = 0.05 \) and 1 degree of freedom.

doi:10.1371/journal.pone.0008345.t006
not merely reflect greater divergence times, but instead suggest periods of more rapid evolutionary change and subsequent stasis. For comparison, the rate of change in chemosensory gene number is as high or higher within Drosophila as it is among Drosophila and Anopheles, based on CAFE results for data reported by [40]. In that case, the most likely value of $\lambda$ was 7.0E-3 within Drosophila and greater than or equal to 3.8E-3 among Drosophila and Anopheles (again, higher values for the latter group could not be evaluated as noted previously). Thus, these two categories of gene family appear to have had different rates of evolution during the diversification of Drosophila. It will be of great interest to determine whether differences in cuticular protein complement are associated with the greater morphological complexity of mosquito larvae and pupae relative to Drosophila.

An important feature of gene-family evolution highlighted in this study is that, in Drosophila, gene turnover and/or gene conversion are primarily concentrated in a few tandem arrays of CPR genes. The functions of these gene products during cuticle assembly may dictate their susceptibility to duplication or loss. Future comparative analyses of the regulation and function of cuticular proteins, expanding on work already begun on the 65A array [5,31,41,42,43], might help explain this evolutionary pattern. Alternatively, local sequence and/or chromatin features, such as intergenic repetitive elements, could contribute to variation in rates of structural mutation. Such factors seem unlikely in the case of the 65A array, in which well-conserved genes and highly labile genes are interspersed together, but may play a role in the evolution of the 44C array.

Nucleotide sequence repetition within coding regions was associated with recurring gene conversion between particular genes within the 84A array of CPR genes and within the 97C array of Tweedle genes. These patterns led me to extend this correlation between coding sequence repeats and concerted evolution to sets of highly similar cuticular protein genes in An. gambiae as well. While the exact nature and extent of sequence repetition clearly varies among sets of concertedly evolving genes (Text S4), this strong and consistent association in both Drosophila and Anopheles suggests a common molecular mechanism, one that may be present in other Diptera. Given the importance of this order in agricultural systems and for human health, there will doubtless be additional genomic data in the near future with which to evaluate this hypothesis. In addition to these genomic data, population-level and chromatin-level studies of this phenomenon are also needed to understand the process of gene conversion at these sites. However, the possibility that the mechanism is related to known associations between GC-rich sequence and both quadruplex pairing [33] and recombination [34] seems promising.

The adaptive significance of concerted evolution of cuticular proteins remains to be clarified. Gene conversion events may be selectively neutral or may be an example of gene amplification in response to some selective pressure, such as the rate of protein production. A number of examples of adaptive gene amplification have been identified, primarily in cases of microbial adaptation to nutrient limitation and in human cancers [44]. A more relevant example is the developmentally programmed amplification of chorion genes within follicle cells during Drosophila oogenesis [45]. The rapid synthesis of specific proteins at discrete points during development characterize both chorion and cuticle synthesis and it is reasonable to hypothesize that concerted evolution with the Drosophila CPR family is adaptive for this purpose. In this regard, it is noteworthy that conversion tracts occasionally extend both upstream and downstream of genes, yet only the portion of the second exon containing the (chitin-binding) R&R Consensus is consistently homogenized within each species (see Fig. 4B.)
Fig. 5B and Fig. 6). This suggests that there is no intrinsic bias in the direction that strand exchange proceeds, but rather that mutations that homogenize this portion of the gene are more likely to be fixed. Whether genes undergoing gene conversion are highly expressed at the per-cell level remains to be clarified, although products of the 44C array are known to be abundant in third instar larvae [46].

Other examples of interlocus gene conversion reported in insects include the homogenization of heat shock protein genes in the mosquito *An. albimanus* [47,48] and in *Drosophila* [49]. These proteins are also likely to be highly expressed at certain times and, given their role in protein folding, might be reasonable candidates for selection for homogeneity. Dot plots of heat shock genes (not shown) did not reveal any repetitive sequence comparable to that observed in this study. These examples, along with the concerted evolution of *Cpr67Fa1* and *Cpr67Fa2* described here, further demonstrate that nucleotide repeats are not required for concerted evolution. Rather, a diversity of factors is likely to influence the underlying rates and fixation probabilities of conversion events among different genes. More extensive population-genetic data from a set of more closely related *Drosophila* species would help confirm these results and potentially identify sites under selection.

This study identified a consistent increase in the rate of cuticular protein sequence evolution along the branch connecting the Sophophora and Drosophila species groups. Indeed, a number of proteins had Ka/Ks significantly greater than one, implying positive diversifying selection, perhaps in response to new developmental or ecological conditions. This finding demonstrates that cuticular protein evolution at the amino-acid level is a potentially important component of ongoing adaptation of insect species. The genes identified here as having evolved under positive selection are favorable candidates for functional/ecological studies of species divergence.

Numerous genome-scale analyses have sought to infer general patterns in the evolution of duplicated genes, such as changes in ploidy or gene number, rates of nonsynonymous substitution, and divergence in gene expression. While these studies have provided extraordinary insights, focusing on the evolution of individual families can more directly connect the molecular histories of duplicated genes to their biological functions and effects on fitness. Insect cuticle promises to be an increasingly powerful model for studying, at hierarchical scales, the origins of biological novelty.
Supporting Information

Text S1 Sequences of genes/proteins used in this study. *Drosophila melanogaster* names are those used by Ensembl, whereas predicted sequences from other *Drosophila* species have names combining a four-letter species code and an arbitrary number. File is a spreadsheet with separate pages for each family of cuticular protein genes examined in the text. Sequences are entered in fasta format and can be copied or saved as text.

Found at: doi:10.1371/journal.pone.0008345.s001 (0.36 MB XLS)

Text S2 Tables showing inferred orthologous genes, or inferred co-orthologous groups of genes when one-to-one matching is not possible.

Found at: doi:10.1371/journal.pone.0008345.s002 (0.04 MB XLS)

Text S3 Phylogenies of CPR genes from seven *Drosophila* species, grouped by tandem array. Each slide shows a phylogenetic tree representing tandemly arrayed CPR genes from seven *Drosophila* species as indicated by the legends. A tree is shown for each tandem array listed in Table 3, except for those at chromosomal band 44C and 65A of *D. melanogaster*, which are shown in Figure 2 and Figure 3, respectively. All trees are neighbor-joining trees constructed from predicted protein sequences using the JTT cost-exchange matrix, as described in the methods. There is no methodological difference between trees presented with circular versus rectangular branches. Rather, the former is used when the number of genes is too large to be easily presented in rectangular format.

Found at: doi:10.1371/journal.pone.0008345.s003 (1.76 MB PPT)

Text S4 Dot plots of tandem arrays of CPR genes in *Drosophila* species and in *Anopheles gambiae*. The first slide shows dot plots of the 84A region in ten *Drosophila* species, to show the generality of
the pattern shown in Figure 3 of the manuscript. The remaining
slides show patterns of repetitive sequence within tandem arrays of
CPR genes in *Anopheles gambiae* (see cited references in text).

**Acknowledgments**

The author thanks Judith H. Willis, Monica Poelchau, and reviewers for
comments on the manuscript.

**Author Contributions**

Conceived and designed the experiments: RSC. Performed the experi-
ments: RSC. Analyzed the data: RSC. Contributed reagents/materials/
analysis tools: RSC. Wrote the paper: RSC.

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**Figure 12.** Graphs showing the range of $\alpha$ estimates for orthologous ‘single-copy’ genes of each cuticular protein gene family, as described in the text. CPR genes occurring in tandem arrays or as isolated genes (‘singletons’) are shown separately. For each gene, the values in the Sophophora (S) and the Drosophila (D) species groups are shown separately, connected by a line to more clearly illustrate the trends among genes. The red lines indicate the mean values.

doi:10.1371/journal.pone.0008345.g012
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