Horizontal Transfer of Bacterial Cytolethal Distending Toxin B Genes to Insects

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Associate editor: Harmit Malik

CdtB sequences from *Scaptomyza* species and *D. primaeva* were deposited to NCBI GenBank under accession numbers MH884655–MH884659. *CdtB* codon-optimized oligos used for nuclease assays were deposited under GenBank accessions MH891796–MH891799.

Abstract

Horizontal gene transfer events have played a major role in the evolution of microbial species, but their importance in animals is less clear. Here, we report horizontal gene transfer of *cytolethal distending toxin B* (*cdtB*), prokaryotic genes encoding eukaryote-targeting DNase I toxins, into the genomes of vinegar flies (Diptera: Drosophilidae) and aphids (Hemiptera: Aphididae). We found insect-encoded *cdtB* genes are most closely related to orthologs from bacteriophage that infect *Candidatus* Hamiltonella defensa, a bacterial mutualistic symbiont of aphids that confers resistance to parasitoid wasps. In drosophilids, *cdtB* orthologs are highly expressed during the parasitoid-prone larval stage and encode a protein with ancestral DNase activity. We show that *cdtB* has been domesticated by diverse insects and hypothesize that it functions in defense against their natural enemies.

Key words: **horizontal gene transfer, cytolethal distending toxin, aphids, *Drosophila*, DNase.**

Horizontal gene transfer (HGT) plays an important role in the acquisition of novel traits in microbes, and recognition for its role in the evolution of animals is increasing (Boto 2014; Husnik and McCutcheon 2018). HGT may drive evolutionary innovation because it facilitates immediate acquisition of genes with novel functions, which are then tailored by natural selection in the recipient's genome (Schonknecht et al. 2014).

The evolution of herbivory in insects is occasionally associated with HGT events involving the acquisition of microbial genes for digesting food and overcoming plant defenses (Wybouw et al. 2016). Accordingly, we aimed to identify such HGT events in the drosophilid fly *Scaptomyza flava*, a member of a lineage that recently transitioned from detritivory to herbivory (Whiteman et al. 2011). Using a sequence similarity-based screen, we identified a *cytolethal distending toxin B* (*cdtB*) homolog as the only HGT candidate in the de novo genome assembly of *S. flava* (for these and all methods, see supplementary material, Supplementary Material online).

Cytolethal distending toxins (CDTs) are widespread eukaryotic genotoxins encoded by a gene family restricted to Actinobacteria, Proteobacteria, and bacteriophage genomes (Jinadasa et al. 2011). CDTs are found in diverse pathogens, including *Campylobacter jejuni*, *Salmonella enterica*, and *Escherichia coli*, and may be a cause of irritable bowel syndrome (Pokkunuri et al. 2012). In prokaryotes, *cdtB* encodes the catalytic subunit CdtB of the tripartite CDT holotoxin. CDTs target eukaryotic cells, leading to cell cycle arrest, cellular distention, and death (Elwell and Dreyfus 2000; Lara-Tejero and Galan 2000). The CdtB subunit alone is sufficient for these phenotypes if delivered directly to cells, whereas the CdtA and CdtC subunits are required for binding the toxin to the target cell membrane (Elwell et al. 2001; Jinadasa et al. 2011). The bacterium *Candidatus* Hamiltonella defensa, a bacterial symbiont of aphids, confers resistance to parasitoid wasps if the bacterium is infected with APSE-2 bacteriophage that encode toxin genes, including *cdtB* (Oliver et al. 2010).

To determine if *cdtB* is present in the genomes of other eukaryotes besides *S. flava*, we performed BlastX (Altschul et al. 1997) searches of the NCBI refseq database, genomes of 11 unpublished Hawaiian *Drosophila* species and all available aphid genomes in AphidBase. We found high-confidence hits to *cdtB* homologs in the Hawaiian *D. primaeva* (subgenus *Drosophila*), and in two lineages within the subgenus *Sophophora: D. biarmipes* and ananassae subgroup species *D. ananassae* + *D. bipectinata* (supplementary table S1*a*, Supplementary Material online). We also discovered *cdtB* orthologs in the transcriptomes of two other ananassae subgroup species, *D. pseudoananassae* and *D. ercepeae* (Signor et al. 2013). We found single high-confidence hits to *cdtB* homologs in the genomes of three aphid species, including in the aphid species *Myzus persicae*, *M. cerasi*, and *Diuraphis noxia* (all Macrosiphini) (supplementary table S1*a* and *b*,

Supplementary Material online). As in *C.* Hamiltonella defensa, we did not find evidence of *cdtA* or *cdtC* in insect genomes (Moran et al. 2005).

Microbial contamination of genome assemblies (Koutsovoulos et al. 2016) can be mistaken for HGT events and we used several methods to address this possibility. *CdtB* was identified on scaffolds in species with high-quality genome assemblies (supplementary table S2, Supplementary Material online) proximal to other eukaryotic genes (supplementary table S3, Supplementary Material online), was syntenic across insect species within each lineage (supplementary table S3, Supplementary Material online), and was verified by PCR and Sanger sequencing of both genomic and complementary DNA (supplementary table S4, Supplementary Material online). *CdtB*, when present in an insect genome, was in all transcriptomes except that of *Di. noxia* (supplementary table S1c, Supplementary Material online). These transcriptomes were polyA enriched, which reduces downstream sequencing of transcripts of bacterial provenance since bacteria lack polyA tails (Dreyfus and Régnier 2002). Insect *cdtB* sequences contained motifs unique to eukaryotes (supplementary fig. S1 and supplementary text, Supplementary Material online). Additionally, in species for which we have both transcriptomic and genomic *cdtB* data (except *S. flava*), *cdtB* contains at least two introns, which are rare in bacteria. The absence of *cdtB* transcripts in *Di. noxia*, coupled with a frameshifting deletion and stop codon in the first and only predicted exon suggests that this *cdtB* fragment is a pseudogene.

To identify the lineages involved in HGT of *cdtB* to insects, we assessed phylogenetic conflict between gene and species tree topologies (Gladyshev et al. 2008; Haegeman et al. 2011). We reconstructed a CdtB maximum likelihood (ML) phylogeny using all available CdtB sequences from the NCBI refseq protein database (fig. 1A and supplementary fig. S2, Supplementary Material online). The CdtB phylogeny resolved two insect-encoded subclades: one containing all intron-bearing *cdtB* genes (*Myzus* spp. + all *Sophophora*) and the other containing intron-less *cdtB* genes (*Scaptomyza* spp. + *D. primaeva*) (fig. 1B). All insect CdtB sequences form a clade with CdtB sequences from APSE-2 phage or APSE-2 infected *C.* H. defensa, indicating HGT of *cdtB* from phage or bacteria into insects. In further support of HGT from APSE-2-like ancestors, *D. bipectinata* contains two *cdtB* gene copies in tandem array, one of which is fused with *apoptosis inducing protein 56*, a homolog of an unrelated AB toxin-encoding gene the APSE-2 phage (supplementary table S5, supplementary figs. S3 and S4, and supplementary text, Supplementary Material online). Remarkably, *aip56* is found immediately downstream of *cdtB* in the genome of the APSE-2 phage. The synteny of the two genes in *D. bipectinata* and *C.* H. defensa suggests the two genes were horizontally transferred together from a bacterial or phage ancestor. This chimeric *ctdB+aip56* sequence is expressed as mRNA in *D. bipectinata* as well as two other ananassae subgroup species. We did not find other APSE genes in any of the species investigated. A test forcing monophyly of drosophilid CdtB is slightly worse ($P = 0.059$) than the actual CdtB phylogeny, indicating that intron-less and intron-bearing CdtB were independently transferred into insects.

To better understand the number and timing of horizontal transfer of *cdtB* in insects, we reconstructed drosophilid and aphid species phylogenies and mapped *cdtB* evolution onto these trees using ML ancestral state reconstruction (ASR) (supplementary table S6, Supplementary Material online). In drosophilids, phylogenetic analysis coupled with synteny within clades points to *cdtB* having been acquired three times: 1) within the subgenus *Sophophora* prior to the divergence of the ananassae subgroup (94% posterior clade probability, or PP) about 21 Ma, 2) within the subgenus *Sophophora* following the split between *D. biarmipes* and *D. suzukii* (98% PP) about 7.3 ± 2.5 Ma, and 3) in a subgenus *Drosophila* ancestor common to *S. flava* and *D. primaeva* (13% PP) about 24 ± 7 Ma (fig. 2A). Although the likelihood that *cdtB* was present in a common ancestor of *D. primaeva* and *S. flava* is low based on ASR, synteny indicates a single HGT event in this lineage. None of the genomes (out of ten surveyed) from the more recently derived picture wing Hawaiian *Drosophila* species sister to *D. primaeva* encode a *cdtB* copy, suggesting *cdtB* was lost prior to the picture wing radiation about 7 ± 4 Ma. In aphids, we did not perform ASR due to limited availability of aphid genome assemblies. However, *cdtB* was syntenic in *Di. noxia*, *M. cerasi*, and *M. persicae* (all Macrosiphini), and we infer that *cdtB* was horizontally transferred into their common ancestor about 41 ± 5 Ma. Although a functional copy was retained in *M. persicae* and *M. cerasi*, it was pseudogenized in *Di. noxia* and may have been lost entirely in *Acyrthosiphon pisum* (fig. 2B). Several inferred *cdtB* losses, in both aphid and drosophilid lineages, point to the question of whether the gene may exact fitness costs in insects carrying it.

Intron-bearing *cdtB* genes, present only in drosophilids and aphids, and not in bacteria or phage genomes surveyed, have three exons that share identical splice junctions (supplementary fig. S4, Supplementary Material online). Because of the vast phylogenetic distance between aphids and drosophilids, it is unlikely that *cdtB* was initially integrated into a common ancestor of these two lineages. Thus, we propose two hypotheses for these shared splice junctions. The first is that this structure is modular and arose through convergent evolution. The second is that the shared splice junctions are a consequence of interinsect HGT, which could be mediated by mites (Houck et al. 1991), bracovirus (by wasp intermediaries), and helitrons (Gasmi et al. 2015). It is also possible that phage directly integrated into insect genomes, since eukaryotic association genes have been discovered in phage that infect *Wolbachia* (Bordenstein and Bordenstein 2016). We provide a hypothetical order and timing of *cdtB* HGT events in figure 2C.

The maintenance of *cdtB* in diverse insect genomes for millions of years suggests that it has an adaptive role. If this is the case, *cdtB* should experience purifying selection and perhaps positive selection in insects. We evaluated this in both *cdtB* lineages in insects (i.e. intron-bearing and intron-less) using divergence-based ML phylogenetic models of codon evolution (Yang 2007). Our results indicate that both insect-associated *cdtB* lineages have largely experienced purifying selection. Additionally, the intron-bearing *cdtB* copies

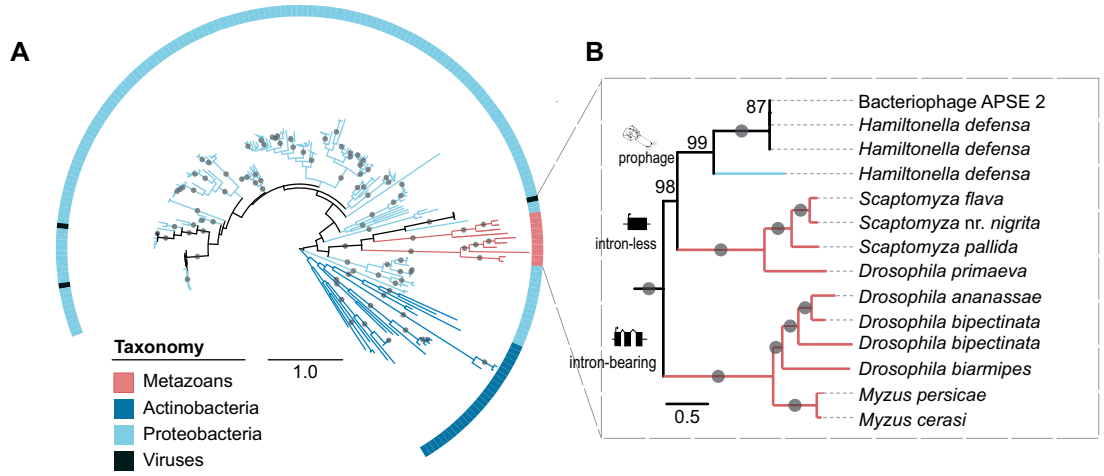

Fig. 1. CdtB protein phylogeny indicates HGT from bacteria or phage to insects. (*A*) ML phylogeny of CdtB from across the tree of life. Tree is midpoint rooted and nodes with 100% bootstrap support are indicated by gray circles. Four clades consisting of highly similar sequences from Proteobacteria were collapsed for clarity. The full phylogeny is available in supplementary figure S2, Supplementary Material online. (*B*) Detailed view of insect CdtB clades. Numbers below branches indicate percent bootstrap support when <100.

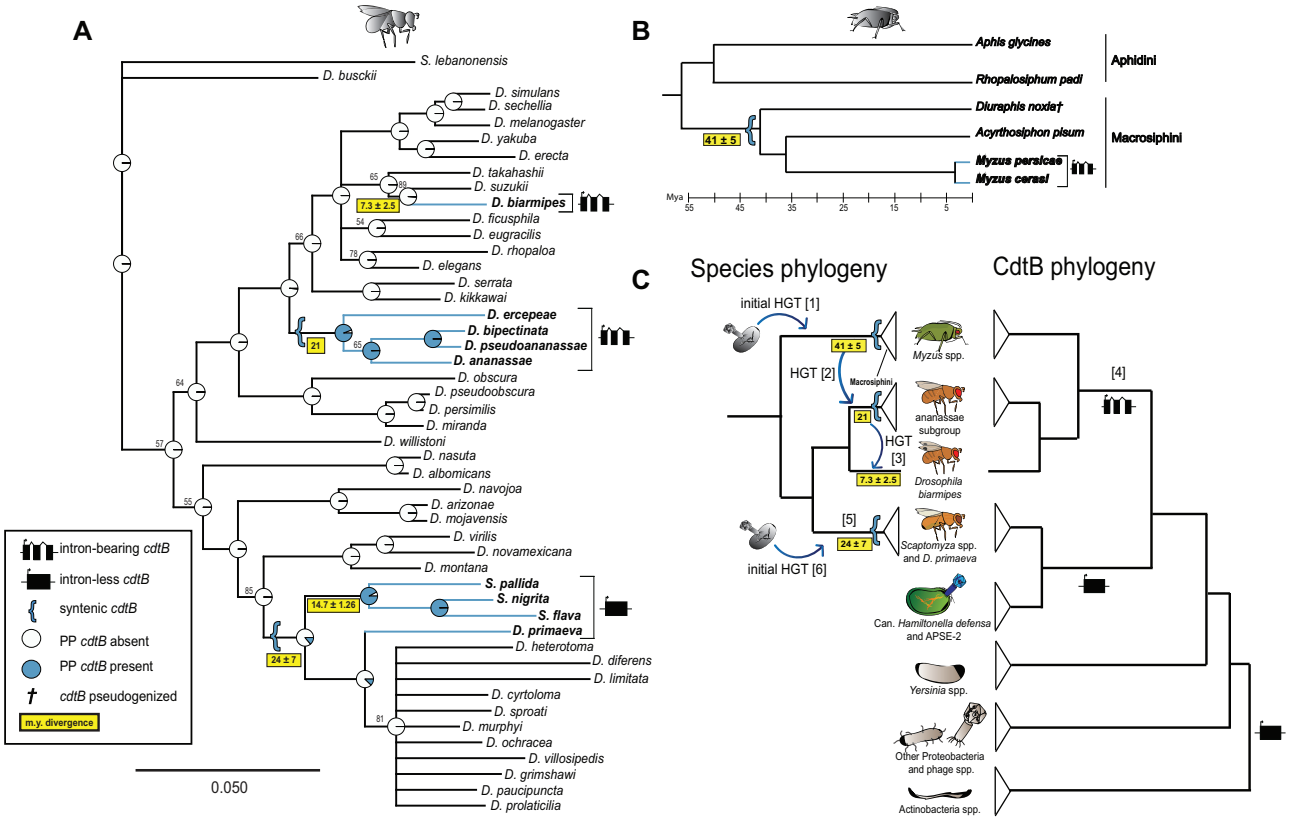

Fig. 2. Species phylogenies show *cdtB* was transferred into, and possibly between, genomes of distant insect lineages. (*A*) ML phylogeny of drosophilid species. Node labels indicate bootstraps if <90% or are collapsed to polytomies if <50%. ASR shows posterior probability (PP) of *cdtB* at nodes. (*B*) Phylogeny of Aphidinae species. Branch lengths drawn approximately to scale using divergence dates from Kim et al. (2011) and Ren et al. (2017). (*C*) Simplified paired CdtB and species phylogenies. Arrows suggest possible HGT directions and bracketed numbers are described here. Possible initial prokaryote or viral to eukaryote HGTs [1, 6]. We hypothesize an initial HGT of *cdtB* from bacteria or bacteriophage integrated into an aphid nuclear genome [1] and was lost or pseudogenized in some aphid lineages (2B). We then posit an interordinal transfer [2] from a *Myzus* spp. ancestor to an ananassae subgroup spp. ancestor, followed by interspecific transfer [3] to a *Drosophila biarmipes* ancestor. This transfer sequence is supported by subclade ages, conserved intron splice sites in [4], and the geographic co-occurrence of these subclades (van Emden et al. 1969; Singh 2015). However, conserved exon structure in [4] could also arise from convergence. Finally, *cdtB* in [5] could have evolved independently or was derived from the same HTG as [4] but failed to acquire introns.

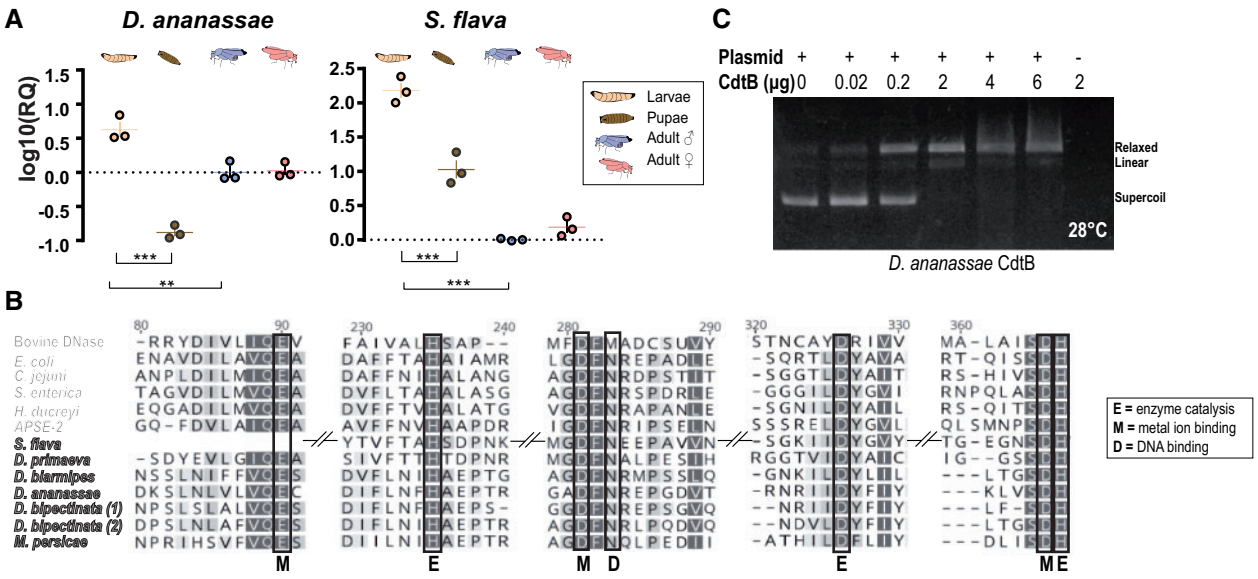

FIG. 3. Insect-encoded *cdtB* is highly expressed during the larval stage in drosophilids and insect CdtB has retained its DNase activity. (*A*) Fold changes in expression of *cdtB* in two representative insect lineages (*Drosophila ananassae* and *Scaptomyza flava*) across development. $P < 0.01$** and $P < 0.0001$***. All pairwise comparisons (except those between males and females) are significantly different, but are not marked for simplicity. (*B*) MUSCLE aligned amino acid sequences of DNase I and CdtB across taxa. Boxed residues are necessary for DNase activity of CdtB. Gray scale corresponds to similarity under the BLOSUM62 scoring matrix. Numbers correspond to alignment residue. Brackets indicate breaks in alignment. Species names in bold are eukaryotic. A complete CdtB alignment can be found in supplementary figure S3, Supplementary Material online. (*C*) Plasmid degradation following exposure to variable quantities of CdtB from *Drosophila ananassae*.

may have experienced positive selection at some codons (supplementary table S7, Supplementary Material online). This further corroborates the functional importance of insect-associated *cdtB*, which is already supported by its retention in so many insect taxa over millions of years.

One possible function of *cdtB* is that it confers parasitoid wasp resistance to insects, as it does in the bacterial secondary symbionts of pea aphids (Degnan and Moran 2008; Oliver et al. 2009). Given that drosophilid and aphid species are generally at high risk of parasitoid wasp attack (Carton et al. 2008), CdtB may confer protection through DNase activity against wasp eggs or larvae. In a parasitization assay, 100% of *D. ananassae* and *D. biarmipes* survived attack by the generalist wasp species *Leptopilina heterotoma* and specialist *L. boulardi* (Schlenke et al. 2007). It is possible, although speculative, that CdtB facilitates this unusual level of parasitoid resistance. *CdtB* is most highly expressed in larvae of the drosophilid species *S. flava* and *D. ananassae* (fig. 3*A* and supplementary table S8, Supplementary Material online), and an independent transcriptome assembly revealed similar *cdtB* expression patterns in *D. biarmipes* and *D. bipectinata* (Chen et al. 2014), consistent with a protective role.

To determine if insect-encoded CdtB is a functional DNase, we aligned CdtB from insects and bacterial species whose DNase and cytotoxic activity are well characterized and found that residues important for DNase activity are conserved in insect copies (fig. 3*B* and supplementary fig. S5, Supplementary Material online). To examine if this residue conservation corresponded to DNase activity, we heterologously expressed and purified His-tagged CdtB from *D. ananassae* (supplementary fig. S6 and supplementary table S9, Supplementary Material online) in *E. coli*, and separately, the native CdtB copy found in *E. coli* as a positive control, and determined their nuclease activities in vitro. Supercoiled plasmid becomes linearized, relaxed, or degraded entirely when exposed to DNases, which migrate at different rates on agarose gels. We incubated *D. ananassae* CdtB with supercoiled plasmid, which converted to relaxed or linearized plasmid species. *Drosophila ananassae* CdtB had higher DNase activity than *E. coli* CdtB at both 28 and 37 °C (supplementary fig. S7, Supplementary Material online), which may reflect the fact that insects experience a broader temperature range than that typically experienced by *E. coli*, in the homeotherm gut.

In addition to APSE-2 phage in aphid symbionts, there are other, similar examples of protective mutualisms involving endosymbionts that defend insect hosts against enemies. For example, the *Spiroplasma* endosymbiont of *D. neotestacea* encodes ribosome inactivating toxins in defense against parasitoid wasps and nematodes (Ballinger and Perlman 2019). In the case of *cdtB*, HGT could be facilitated by the fact that the protein mediating the mutualism is already somewhat adapted to eukaryotes and can evade the insect immune system (Blow and Douglas 2019). It is possible that HGT of *cdtB* obviates the role of the endosymbiont, and any associated costs of housing a symbiont (Polin et al. 2014).

The domestication of *cdtB* in insects is remarkable given that the toxin originally evolved to destroy, not benefit, eukaryotic cells. Given the wealth of genetic tools and genomic resources available within drosophilids and aphids, horizontally transferred *cdtB* promises to be an exciting, experimentally tractable system for exploring the biology of a novel, eukaryote-adapted toxin.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

We thank Dr Chris Jeans and Brooks Bond-Watts for their preparation of purified CdtB. Coco Verster provided field assistance in acquiring specimens. Raoul O. Martin provided biochemistry advice, Dr Stefan Prost and Timothy O'Connor provided bioinformatics advice, Julianne N. Pelaez assisted in phylogenetic reconstruction, and Dr Anthony T. Iavarone performed mass spectrometry analysis. Dr Artyom Kopp and Dr Doris Bachtrog provided *Drosophila* specimens. The *M. persicae* transcriptome assembly was provided by Dr Alex Wilson and Dr Honglin Feng. Dr Nancy Moran ran BLAST searches of unpublished aphid genomes that corroborated our conclusions from this study. Dr Naomi E. Pierce and Dr Frederick M. Ausubel provided early support for obtaining a genome sequence from *S. flava*. *Funding:* K.I.V. was supported by a National Science Foundation Graduate Research Fellowship and grants from Sigma Xi (University of California – Berkeley chapter) and the Animal Behavior Society. R.P.D. was supported by the Miller Institute for Basic Research in Science at the University of California, Berkeley. Research was supported by the National Institute of General Medical Science of the National Institutes of Health award number R35GM119816 to N.K.W. K.I.V., A.D.G., and N.K.W. are inventors on a preliminary patent application related to this work, entitled "Cytolethal distending toxin B from insects for human cancer treatment."

Author Contributions

K.I.V., A.D.G., M.K., R.P.D., and N.K.W. were involved in conceptualization of the project. K.I.V., J.H.W., R.P.D., M.K., A.D.G., Z.M.A., E.E.A., D.K.P., and N.K.W. conducted the investigations. K.I.V., M.K., R.P.D., and N.K.W. wrote the article. All authors edited and approved the manuscript.

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