Chitin Metabolism

Introduction

Chitin is a polysaccharide essential for insect development. It plays a crucial role in the development of the insect cuticle and exoskeleton, the peritrophic membrane of the midgut, and other physiological structures such as the trachea, wing hinges and eggshell [1]. Because chitin is essential for insect development, but is not found in mammals, it could be an attractive target for pest control [1].

The biosynthetic pathway for chitin involves a variety of different enzymes which act on simple sugars such as glucose, trehalose and glycogen to produce intermediates which can then be converted into chitin. In the last step of the biosynthetic pathway, UDP-N-acetylglucosamine is converted to chitin. This reaction is catalyzed by enzymes known as chitin synthases (CHS) [2]. Chitin synthases are the only enzymes in the chitin biosynthetic pathway that act specifically in the synthesis of chitin, and thus they are the best potential targets for an RNA interference (RNAi) based insecticide.

Just upstream of chitin synthase in the chitin metabolic pathway, N-acetylglucosamine-1-phosphate is converted into UDP-N-acetylglucosamine. This reaction is catalyzed by the enzyme UDP-N-acetylglucosamine pyrophosphorylase (UAP) [3]. In addition to its role in chitin synthesis, UAP is also involved in the modification of other carbohydrates, sphingolipids and proteins [4].

While chitin synthesis is essential for the development of insects at all life stages, the degradation of chitin is also an essential process. The insect cuticle, of which chitin is an essential component, protects insects but it also restricts growth. Therefore, for insect growth and development to occur it is essential that the old cuticle be degraded so that new cuticle can form. There are two different pathways through which chitin is degraded. One is through the action of chitinases and the second is through the action of chitin deactylases.

Chitinases are found in species from all kingdoms, including in mammals which aren't known to produce chitin. In organisms that don't produce chitin these enzymes likely act as a method of defense against microorganisms which contain chitin (e.g. fungi). In organisms that do produce chitin as an essential part of their physiology, chitinases are vital for proper development. During insect growth and development, it is imperative that chitin in the insect cuticle and the peritrophic matrix is degraded and replaced. This allows for growth, maturation and tissue repair in the insect. Loss-of-function studies in a variety of insects have shown that inhibiting the degradation of chitin by targeting chitinases is often lethal due to restriction of growth [5–8]. Chitinase has even been successfully targeted by host-induced RNAi in transgenic tobacco and tomato plants as a method of insect control [9].

In a second degradative pathway, chitin is deacetylated to produce chitosan. The metalloenzymes that catalyze this reaction are known as chitin deacetylases (CDAs). The first report indicating CDA activity in insects was in the cabbage looper *Trichoplusia ni* [10]. In *Drosophila melanogaster* the

first three CDAs characterized, *serpentine* (*serp*), *vermiform* (*verm*) and *ChLD3*, were shown to play a role in tracheal development [11,12]. More recently genomic and phylogenetic studies have shown CDAs to be widely present in insects [13,14] and expression and loss of function studies suggest they play an essential role in growth and development throughout insect phyla making them another potential target for pest control [14–19].

When annotating the *D. citri* genome, we found a high level of conservation among genes involved in chitin metabolism particularly when compared to other hemipterans. Overall hemipteran insects appear to have fewer genes involved in chitin metabolism when compared to holometabolous insects, perhaps because hemipterans undergo incomplete metamorphosis and therefore require less chitin synthesis and degradation.

Results and Discussion

Chitin biosynthesis

Chitin synthase (CHS)

Genomic studies of various insects in the dipteran, coleopteran and lepidopteran orders have suggested that there are two CHS genes in holometabolous insects [1,2]. Functional studies suggest that CHS1, also referred to as CHSA, functions to produce the chitin essential for proper cuticle development [1,20,21]. CHS2, also referred to as CHSB, is not required for cuticle development but is instead essential for proper development of the gut peritrophic membrane [1,20,21]. RNAi targeting either CHS gene is lethal in holometabolous insects, causing either molting defects (CHS1) or starvation (CHS2) [1,20,21]. Previous searches of three hemipteran genomes (*Acyrthosiphon pisum, Nilaparvata lugens* and *Rhodnius prolixus)* identified CHS1 but not CHS2, suggesting that CHS2 may have been lost in the hemipteran lineage [22]. This apparent loss of the chitin synthase gene required for peritrophic membrane development is correlated with the reported lack of peritrophic membranes in hemipterans [22,23]. Consistent with this result, we found only a single CHS ortholog in the *D. citri* genome, as well as in the *Bemisia tabaci* genome. We performed phylogenetic analysis with these CHS proteins, as well as the *A. pisum* CHS protein, and found that they cluster in a monophyletic clade with holometabolous CHS1 genes (Figure 1).

Chitin Synthase Orthologs Identified in Insects											
	D. melanogaster	***A. gambiae***	***A. aegypti***	***T. castaneum***	***M. sexta***	***S. exigua***	***A. mellifera***	***N. vitripennis***	***A. pisum***	***B. tabaci***	***D. citri***
CHS1/A	1	1	1	1	1	1	1*	1*	1*	1*	1
CHS2/B	1	1	1	1	1	1	1*	1*	0*	0*	0

*Table 1: Number of Chitin Synthase Orthologs in Insects from Representative Taxa. D. citri numbers were determined based on annotation of D. citri genome v 2.0. * indicates the number was determined from BLAST analysis. All other numbers have been reported in various publications.*

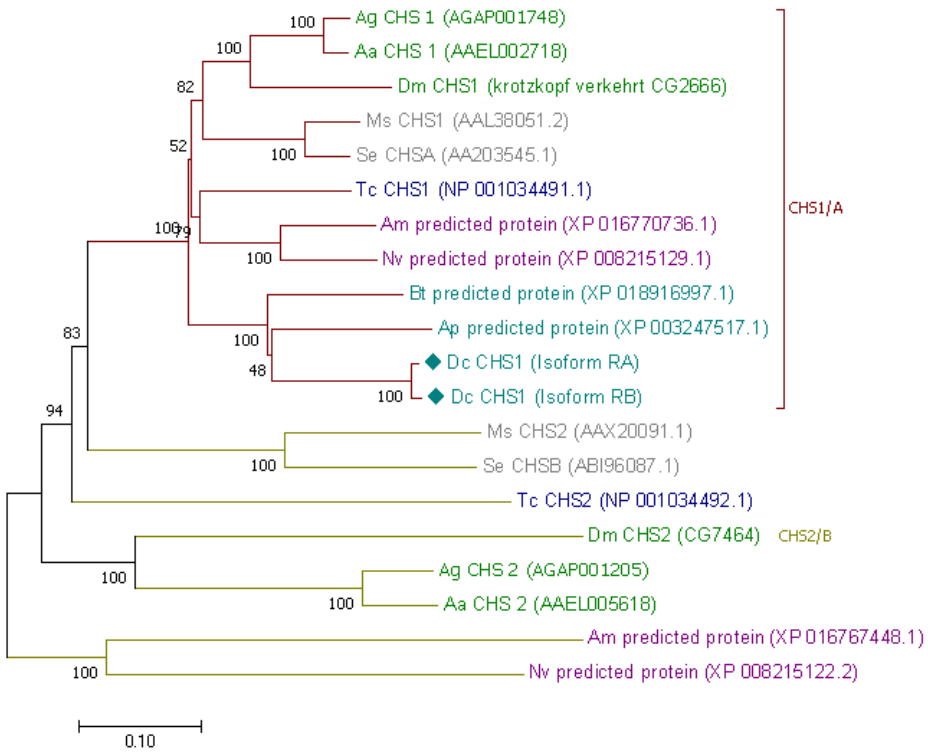

Figure 1: Phylogenetic analysis of CHS proteins from Drosophila melanogaster (Dm), Anopheles gambiae (Ag), Aedes aegypti (Aa), Tribolium castnaeum (Tc), Manduca sexta (Ms), Spodoptera exigua (Se), Apis mellifera (Am), Nasonia vitripennis (Nv), Acyrthosiphon pisum (Ap), Bemisia tabaci (Bt) and Diaphorina citri (Dc). MUSCLE software was used to perform multiple sequence alignments. Tree was constructed with MEGA 7.0 software using the neighbor-joining with bootstrap consensus method. Full length protein sequences were used for phylogenetic analysis. The maroon clade shows monophyletic clustering of CHS1/A genes. Olive branches represent CHS2/B genes. With the exception of D. citri only one isoform for CHS1 is depicted in this tree. Taxon name color represents insect order. Order Diptera: Green. Order Coleoptera: Navy. Order Hymenoptera: Purple. Order Lepidoptera: Gray. Order Hemiptera: Teal. Note: The two Anopheles gambiae CHS genes were misnamed in early insect chitin synthase publications with Ag CHS1 having the CHS2 designation and CHS2 having the CHS1 designation. This was corrected in the Zhang, X. et al 2012 publication.

BLASTp searches of the MCOT transcriptome identified two transcripts that mapped to the CHS genomic locus. MUSCLE alignments of these two translated transcripts indicated they contained identical amino acid sequence with the exception of a small region near the C-terminal end of the protein. These two models were manually annotated as CHS1 isoforms. These manually annotated models were further validated by RNASeq, StringTie and PacBio IsoSeq evidence (Table 2) which confirmed identical exon structure between the two isoforms with the exception of exon 20 where an alternative exon was used. Two isoforms of CHS1 differing only at the C-terminus have also been described in many other insects [2,22,24–29]. Interestingly, while the use of an alternative exon near the 3' end of *CHS1* genes appears conserved across insect taxa, the number of exons is vastly different. In *Drosophila* the alternative exons are exon 7a and 7b [2,30], in *Anopheles* they are in exons 6a and 6b [31], and in *Tribolium* they are exons 8a and 8b [2]. In *D. citri*, however, the alternative exons are exons 20a and 20b, this is due to the fact that the exons in *D. citri* are more

fragmented resulting in a higher number of shorter exons. This is very similar to the exon structure seen in *Manduca sexta* [25].

In recent years there has been an increase of reports illustrating that RNAi of CHS genes have a lethal effect in agricultural insect pests including (but not limited to), *Mythimna separate* [32] *Phthorimaea operculella* [33], *Leptinotarsa decemlineata* [34], *Toxoptera citricida* [35] and our pest of interest *D. citri* [36]. These results further support the hypothesis that CHS genes could be an attractive target for pest control.

D. citri Genes Identified in Chitin Biosynthesis Pathway

Gene/Isoform	**D. citri identifier**	**Gene model**		**Evidence supporting annotation**			
		complete	partial	MCOT	IsoSeq	RNASeq	Ortholog
CHS RA	Dcitr04g09970.1.1	x		x	x	x	x
CHS RB	Dcitr04g09970.1.2	x		x		x	x
UAP 1	Dcitr08g04630.1.1	x			x		x
UAP 2	Dcitr05g05060.1.1	x			x	x	x

Table 2: : Annotated D. citri Genes Involved in Chitin Biosynthesis. Each manually annotated gene has been assigned a gene identifier. For each manually annotated gene it has been denoted as a partial or complete model based on available evidence. Evidence for manual annotation was also recorded. MCOT evidence means a de novo Oases or Trinity model from an independent transcriptome was identified and the sequence from that transcript was used to validate or modify our model. IsoSeq means single reads generated with Pacific Biosciences technology were available and were used to help validate the exon structure of the model. RNASeq means that individually mapped Illumina RNASeq reads were used to help validate or modify our model. Ortholog means ortholog sequences from other insects and information about conserved motifs and domains had to be used to help determine the final annotation.

UDP-N-acetylglucosamine pyrophosphorylase (UAP)

In *Drosophila* there is a single gene encoding UAP. Mutants of *UAP* (also called *mummy*, *cabrio* and *cystic*) have defects in tracheal development, dorsal closure, eye development and nervous system function. Some of these developmental defects are due to UAP's role in chitin synthesis while others are due to the role UAP plays in glycosylation of other proteins [37]. Bioinformatic analysis of representative dipteran, lepidopteran, coleopteran, hymenopteran, hemipteran, phthirapteran, tick and crustacean arthropods suggested that most arthropods have a single *UAP* gene (Table 3). The one exception found in this analysis was *Tribolium* which has two *UAP* genes (*UAP1* & *UAP2*) [37]. RNAi experiments showed that, in *Tribolium,* UAP1 is involved in the biosynthesis of chitin both in the cuticle and the peritrophic membrane, while UAP2 has roles in the modification of other macromolecules [37].

Interestingly BLAST analysis of the *D. citri* MCOT transcriptome and further analysis of the *D. citri* genome v 2.0 identified two potential *UAP* genes.

UAP Orthologs Identified in Invertebrates													
	D. melanogaster	***A. gambiae***	***A. aegypti***	***A. mellifera***	***N. vitripennis***	***B. mori***	***T. castaneum***	***P. humanus***	***A. pisum***	***B. tabaci***	***D. citri***	***D. magna***	***C. elegans***
UAP	1	1	1	1	1	1	2	1	1	1	2	1	2

Table 3: Number of UAP Orthologs in Invertebrates from Representative Taxa. D. citri numbers were determined based on annotation of D. citri genome v 2.0. All other numbers were obtained from Arakane, Y. et al 2011.

Because lineage specific duplication of *UAP* in arthropods is rare it is possible that the two loci identified in the *D. citri* genome actually represents one gene which exists at two different loci because of an incomplete or inaccurate genome assembly. While this possibility exists, multiple sequence alignments, analysis of conserved residues, and BLAST analysis of neighboring genetic sequences all suggest that misassembly is not likely. Additionally, the MCOT sequences corresponding to UAP1 and UAP 2 both came from Cufflinks gene models inferred from the version 1.1 genome and these sequences are still present in the version 2.0 genome, which was an independent genome assembly, again supporting the accuracy of the current assembly. Thus, we conclude *D. citri* contains two *UAP* genes named *UAP1* and *UAP2* (Table 2). All available evidence suggests the annotation of *D. citri* UAP 1 and UAP 2 represents the full length protein, as amino acid residues known to be important for substrate binding in other insects have been identified in both annotated sequences.

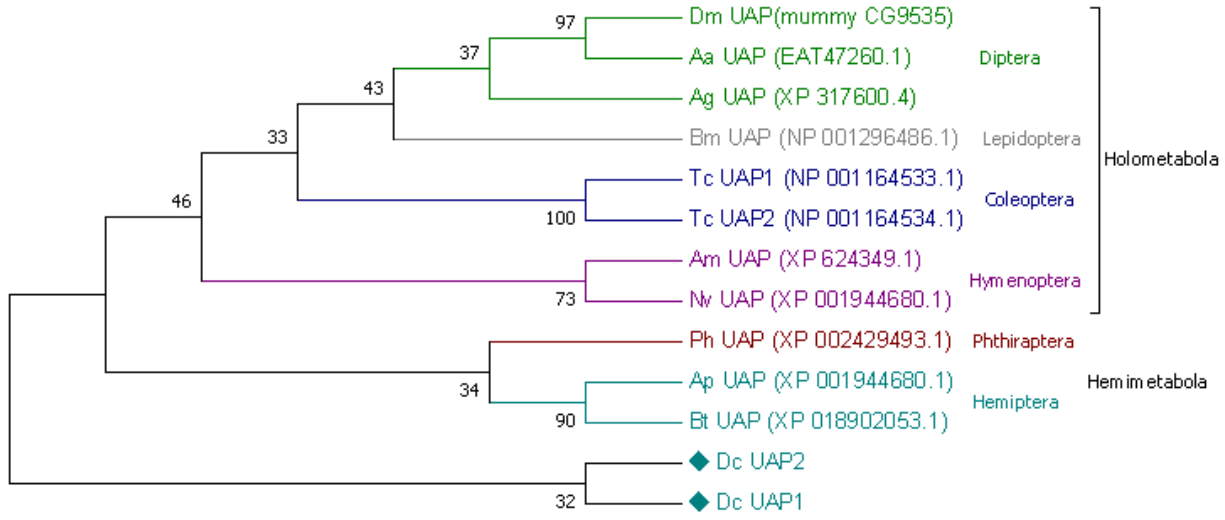

Figure 2: Phylogenetic analysis of representative insect UAP orthologs in Drosophila melanogaster (Dm), Anopheles gambiae (Ag), Aedes aegypti (Aa), Bombxy mori (Bm), Tribolium castaneum (Tc), Apis mellifera (Am), Nasonia vitripennis (Nv), Pediculus humanus (Ph), Acyrthosiphon pisum (Ap), Bemisia tabaci (Bt) and Diaphorina citri (Dc). ClustalW software was used to perform multiple sequence alignments. Tree was constructed with MEGA 7.0 software using the neighbor-joining with bootstrap consensus method. Full length protein sequences were used for phylogenetic analysis. Colors represent insect orders.

Phylogenetic analysis (Figure 2) suggests these two genes are not orthologous to *Tribolium UAP1* and *UAP2* and instead represent a lineage specific duplication. It is important to note that while most insects only have a single *UAP* gene, there is evidence in insects for multiple isoforms of this gene. In *Drosophila* two isoforms for *UAP* have been identified. One isoform contains three coding exons, while the second only contains two coding exons [30]. In *D. citri* both *UAP* genes are single exon genes and we found no evidence to support an annotation of more than one isoform for each gene.

Chitin Degradation

Chitinases

Whole genome searches have shown that in insects there are multiple genes coding for chitinases, typically between 10 and 30 per species. The chitinase proteins produced from these different genes differ in size, domain organization, and in physical, chemical and enzymatic properties. These differences have allowed for the organization of chitinases into several different groups. Initially work in *Drosophila* suggested that chitinases should be divided into five groups [38,39], further work in *Tribolium* [5] and *Anopheles* [40] suggested chitinases should be further divided into eight groups, and work in the pea aphid [41] has suggested an alternative grouping method for chitinases. For the purposes of this discussion we will use the typical eight group methodology and naming convention described in *Tribolium* and *Anopheles*.

Nine chitinase and chitinase-like genes have been identified in *A. pisum* [41] and 12 have been identified in the brown planthopper *Nilaparvata lugens* [14]. This is far fewer than the number of chitinases seen in higher insects (*Drosophila*, 16; *Anopheles*, 20; *Tribolium*, 22), suggesting that there may be fewer chitinase genes in hemipterans (Table 4). BLASTs to the *D. citri* v 2.0 genome and the MCOT transcriptome suggest there are 11 chitinase/chitinase-like genes in *D. citri* (Table 5). The annotation of each of these genes is described below.

Estimated Number of Chitinase Homologs											
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	**Group 7**	**Group 8**	**ENGase**	**SI-Clp**	**Total**
D. melanogaster	1	1	1	4	6	1	1	1	*	*	16
A. gambiae	5	1	1	8	2	1	1	1	*	*	20
T. castaneum	1	1	1	14	2	1	1	1	*	*	22
A. pisum	1	1	1	2	1	1	0	1	1	0	9
N. lugens	2	2	1	1	2	1	1	1	1	0	12
D. citri	1	2	1	1	3	1	0	1	1	0	11

*Table 4: Estimated Number of Chitinase Homolgs. D. citri numbers were determined based on annotation of D. citri genome v 2.0. Drosophila, Anopheles and Tribolium numbers were obtained from Zhang J. et al 2011. A. pisum numbers were obtained from Nakabachi, A. et al 2010. N. lugens numbers were obtained from Xi, Y. et al 2014. Orthologs were categorized based on our phylogenetic analysis and literature reports. * indicates that homologs are present in this organism but they have not been reported in publications.*

	D. melanogaster	*A. gambiae*	*T. castaneum*	*N. lugens*	*A. pisum*	*D. citri*
Group 1	Cht5	Cht5-1 Cht5-2 Cht5-3 Cht5-4 Cht5-5	Cht5	Cht5 Cht4	Cht5	Cht5
Group 2	Cht10	Cht10	Cht10	Cht10 Cht1	Cht2	Cht10-1 Cht10-2
Group 3	Cht7	Cht7	Cht7	Cht7	Cht4	Cht7
Group 4	Cht4 Cht8 Cht9 Cht12	Cht4 Cht8 Cht9 Cht12 Cht13 Cht16 Cht23 Cht24	Cht4 Cht8 Cht9 Cht12 Cht13 Cht14 Cht15 Cht16 Cht17 Cht18 Cht19 Cht20 Cht21 Cht22	 Cht3	 Cht7 Cht8	 Cht12
Group 5	IDGF1 IDGF2 IDGF3 IDGF4 IDGF5 IDGF6	 IDGF2 IDGF4	 IDGF2 IDGF4	 IDGF Cht9	 Cht1	 IDGF1 IDGF2 IDGF3
Group 6	Cht6	Cht6	Cht6	Cht6	Cht3	Cht6
Group 7	Cht2	Cht2	Cht2	Cht8		
Group 8	Cht11	Cht11	Cht11	Cht2	Cht6	Cht11
ENGase	CG5613	XP 310876.4	XP 008197368.1	ENGase	ENGase	ENGase
SI-Clp	CG8460	XP 317335.2	XP 971647.1			

Table 5: Phylogenetics Based Comparative Classification of GH18 Superfamily Chitinase and Chitinase-like Family Members. Groupings assigned here are based on publications and our phylogenetic analysis.

Group I Chitinases

In most insects the group I chitinases are named Chitinase 5 (Cht5) and with the exception of mosquitoes, which have seen an expansion in this group, all holometabolous insects examined contain only one Cht5 gene (Table 5). Within hemimetabolous insects *A. pisum* has been found to have one Cht5 ortholog while *N. lugans* [14] and *P. humanus* [41] have two. Cht5 proteins contain one catalytic domain and one C-terminal chitin binding domain (ChBD). In the *D. citri* genome v 2.0 one *Cht5* gene was identified.

Group II Chitinases

Group II Chitinases are typically named Chitinase 10 (Cht10) in insects (Table 5). These chitinases are large molecular weight chitinases which in holometabolous insects have been shown to have four or five catalytic domains and four to seven ChBDs. It has been reported that the number and arrangement of these domains is conserved among the Holometabola [5]. While there is typically only one group II chitinase member, in the *D. citri* genome v 2.0 two genes have been identified that reciprocal blast to insect Cht10 proteins and both cluster with the Cht10 members in our phylogenetic analysis (Figure 3). One of these chitinases, Cht10-1 is a typical Cht10 protein. It is a large gene (21 exons) and the protein contains 5 catalytic domains and 2 ChBDs. The arrangement of these domains does differ from holometabolous insects as there are fewer ChBD domains than expected (Figure 4). The second protein identified as a potential Cht10 in *D. citri*, Cht10-2, is much smaller and only contains one catalytic domain (Figure 4). While it does not show the typical size or domain architecture of Cht10 proteins, phylogenetic and BLAST analysis suggests this is the chitinase for which it shares the most identity (Figure 3). *Cht10-2* has both PacBio IsoSeq and *de novo* transcriptome support (Table 6) indicating that its presence in the genome is not an artifact of genome misassembly.

D. citri Chitinase Genes

Category	**Gene**	**D. citri identifier**	**Gene model**		**Evidence supporting annotation**			
			complete	partial	MCOT	IsoSeq	RNASeq	Ortholog
Group 1	Cht5	Dcitr06g10380.1.1	X		X			X
Group 2	Cht10-1	Dcitr02g11110.1.1	X		X			X
Group 2	Cht10-2	Dcitr12g04430.1.1	X		X	X		X
Group 3	Cht7	Dcitr07g07740.1.1	X		X	X		X
Group 4	Cht12	Dcitr11g03190.1.1	X		X			X
Group 5	Idgf1	Dcitr02g06220.1.1	X		X	X		X
Group 5	Idgf2	Dcitr02g06210.1.1	X		X			X
Group 5	Idgf3	Dcitr02g06590.1.1	X			X	X	X
Group 6	Cht6	Dcitr10g04150.1.1	X		X			X
Group 8	Cht11	Dcitr01g03820.1.1	X			X	X	X
ENGase	ENGase	Dcitr01g14510.1.1	X		X	X	X	

Table 6: Annotated D. citri Genes. Each manually annotated gene has been assigned a gene identifier. For each manually annotated gene it has been denoted as a partial or complete model based on available evidence. Evidence for manual annotation was also recorded. MCOT evidence means a de novo Oases or Trinity model from an independent transcriptome was identified and the sequence from that transcript was used to validate or modify our model. IsoSeq means single reads generated with Pacific

Biosciences technology were available and were used to help validate the exon structure of the model. RNASeq means that individually mapped Illumina RNASeq reads were used to help validate or modify our model. Ortholog means ortholog sequences from other insects and information about conserved motifs and domains had to be used to help determine the final annotation.

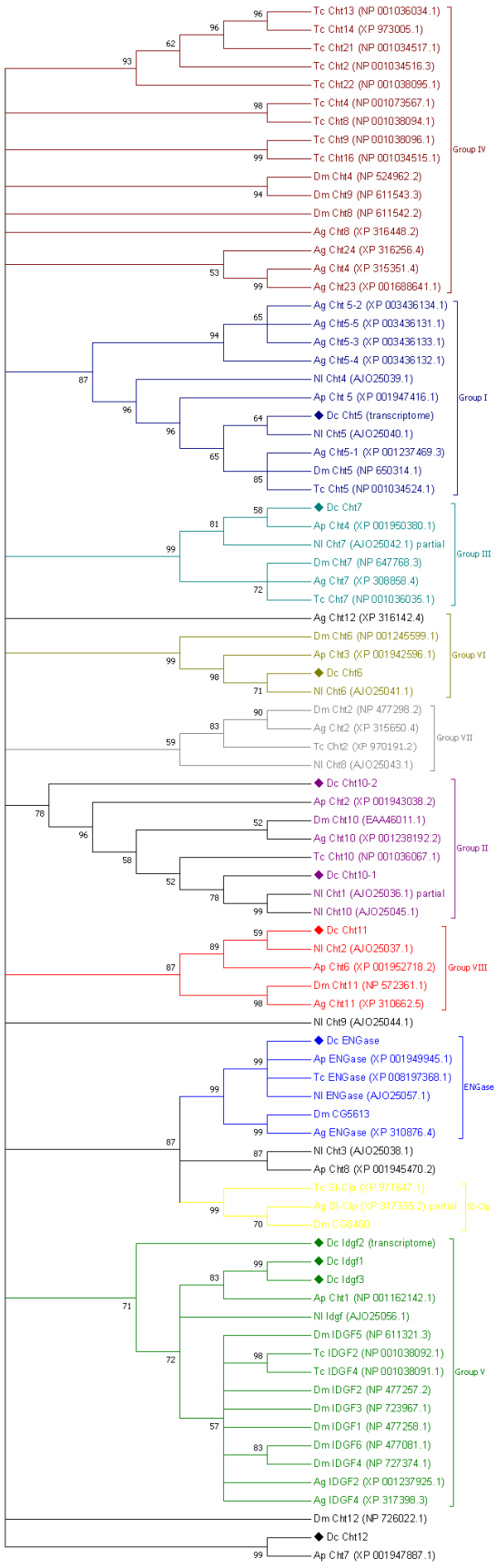

Figure 3: Phylogenetic bootstrap consensus tree of chitinase and chitinase-like family members. ClustalW was used to perform multiple sequence alignments. The tree was constructed with MEGA 7.0 software using neighbor-joining analysis (100 bootstrap). Full lenth protein sequences were used to construct tree. In two cases when full length sequences weren't avaialbe from the genome due to assembly errors, transcriptome sequences were used. Drosophila melanogaster (Dm), Anopheles gambiae (Ag), Tribolium castnaeum (Tc), Nilaparvata lugens (Nl), Acyrthosiphon pisum (Ap) and Diaphorina citri (Dc). Colors delineate established chitinase groups, black genes do not cluster well with any group. In Table 5 and in this description the black genes are listed as Group 4 proteins.

Group III Chitinases

The group III chitinases are typically named Chitinase 7 (Cht7) in insects (Table 5). A group III chitinase has also been identified in the hard tick, *Haemaphysalis longicornis*, indicating an ancient origin of this group of chitinases [42]. This group is typically represented by one member which contains two catalytic domains followed by a ChBD [5]. In *Drosophila*, *Tribolium*, *A. mellifera*, and *Anopheles* Cht7 is reported to also contain at least one N-terminal transmembrane domain [39,40] (Figure 4). In *D. citri* we identified one *Cht7* gene which had strong *de novo* transcriptome and PacBio IsoSeq support (Table 6). As expected this annotated protein contained two catalytic domains followed by one ChBD, however, a transmembrane domain was not identified (Figure 4). Transmembrane domains have not been reported for Cht7 in the hemipterans *A. pisum* and *N. lugens* [14,41] or in the tick *H. lonicornis* [42], suggesting this characteristic may be specific to holometabola (Figure 4).

Group IV Chitinases

In holometabolous insects group IV is the largest and most diverse group of chitinases (Table 6, Figure 3). These chitinases have the greatest variation in domain organization and are sometimes found in clusters in insect genomes suggesting duplication events. As more insect genomes are sequenced and the chitinase gene family is resolved this group will

likely be separated into different groups. In general, most group IV chitinases have a single catalytic domain and lack a ChBD [5] (Figure 4). Within the group IV cluster of our phylogenetic analysis there are no obvious group IV members in *D. citri* (Figure 3). However, *D. citri* does appear to have a *Cht12* ortholog (Table 5). Phylogenetic analysis from different researchers have shown that Cht12 proteins do not consistently cluster within one group although they sometimes cluster with group IV chitinases [40]. Evidence from *B. mori* suggests that the presence of this chitinase in insects may be the result of lateral transfer from a bacterium or baculovirus [43,44]. If such a transfer occurred and this chitinase were free to mutate without evolutionary constraint this could explain why phylogenetic analysis for this particular chitinase is often ambiguous.

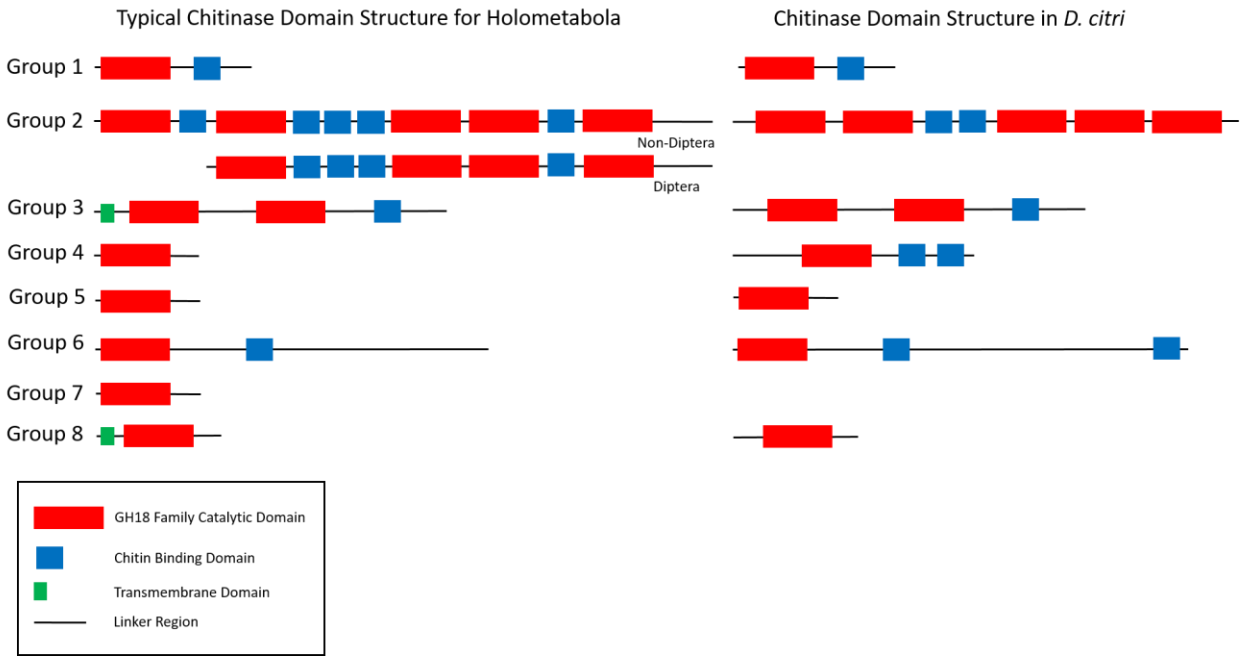

Figure 4: Chitinase Domain Organization in Holometabola and D. citri. Chitinases are categorized by group based on phylogenetic analysis, sequence similarity, and domain organization. The typical domain structure for holometabola was determined from work done in Drosophila (Zhu, Q. et al 2003), Tribolium (Arakane, Y. and Muthukrishnan, S. 2010) and Anopheles (Zhang, J. et al 2011). D. citri domain analysis was performed using InterPro. Group 1 is represented by Cht5.. Group 2 is represented by Cht10-1. Group 3 is represented by Cht7. Group 4 is represented by Cht12. Group 5 represents 3 proteins, Idgf1, Idgf2 and Idgf3. Group 6 is represented by Cht6. Group 8 is represented by Cht11. Note: there is not a Group 7 Chitinase in D. citri. Domain analysis in other hemimetabolous insects can be found in Nakabachi, A. et al 2010 and Xi, Y., et al 2014.

Group V Chitinases

The group V Chitinases were first identified for their role in the growth of imaginal disc tissue in *Drosophila* and hence have been named imaginal disc growth factor (Idgf) 1-6 (Table 5). While 6 Idgf genes have been identified in *Drosophila* a variable number have been identified in other insects and phylogenetic analysis suggests independent gene duplications of group V chitinases has happened several times in insect lineages [41]. In *D. citri* we identified three Idgf genes (Table 4). These genes have been named *Idgf1*, *Idgf2* and *Idgf3* (Table 5). Note that the *D. citri* Idgf genes do not represent one-to-one orthologs with *Drosophila Idgf1*, *Idgf2* and *Idgf3* genes as phylogenetic analysis suggests that Idgf genes have duplicated independently in these two insect

lineages (Figure 3). In fact, in *D. citri* one of these duplications may have occurred relatively recently as *Idgf1* and *Idgf2* are adjacent to one another on a contig. As seen in other insects all three *D. citri* Idgf genes have only one catalytic domain and they do not contain a ChBD (Figure 4).

Insect chitinase proteins contain 4 conserved motifs within the catalytic domain. The second motif (CR_2: FDGxDLDWEYP) is the best studied and site directed mutagenesis has determined the critical residues [45]. In all three *D. citri* Idgf proteins the aspartic acid (D) closest to the tryptophan (W) in the CR_2 motif is replaced by alanine (A). This mutation causes the enzyme to be inactive [45] and is known to exist in all Idgf proteins examined [5]. The identification of this mutation in all three *D. citri* Idgf genes confirms their identification as Idgf genes.

The annotation for *D. citri Idgf1* is supported by both *de novo* transcriptome evidence and PacBio IsoSeq models (Table 6). *Idgf2* is located a short distance downstream of *Idgf1* and is supported by MCOT evidence. The final *D. citri* Idgf gene, *Idgf 3*, is strongly supported by previous versions of the genome, PacBio IsoSeq evidence, individual RNASeq reads and ortholog sequences (Table 6).

Group VI Chitinases

In insects the group VI chitinases are usually named Chitinase 6 (Cht6) (Table 5). It has been reported that these chitinases have a similar domain structure to Cht1 proteins with a N-terminal catalytic domain and one ChBD. However, they differ from Cht1 proteins because they have a long stretch of Ser/Thr rich amino acids at the C-terminus [5]. In the *D. citri* genome v 2.0 one *Cht6* gene was identified. This protein differs from the typical domain architectures described above as it contains one catalytic domain and two ChBD, one of which is at the very C-terminal portion of the protein (Figure 4). This is also what has been reported in the hemipterans *N. lugens* and *A. pisum* [14,41]. The *D. citri* Cht6 protein does contain a long stretch of amino acids after the first ChBD which contains approximately 25% Ser/Thr residues (data not shown). Therefore, it seems likely that holometabolous insects have lost the most C-terminal ChBD that has been reported in hemipterans.

Group VII Chitinases

Group VII chitinases are typically named Chitinase 2 (Cht2) in insects (Table 5). A group VII chitinases was not identified in the *D. citri* genome v 2.0 nor was one identified in *A. pisum* [41] (Table 4). However, a chitinase does cluster with group VII chitinases (named Nl Cht8) in the hemipteran *N. lugens* [14]. The domain structure of Nl Cht8 is consistent with holometabola group VII chitinases with only one catalytic domain and no ChBD [14] (Figure 4). Our results suggest the group VII chitinase may have been lost only in the lineage leading to sap sucking hemipterans.

Group VIII Chitinases

Group VIII chitinases are typically called Chitinase 11 (Cht11) in insects (Table 5). To our knowledge in all insects examined to date there is only one group VIII chitinase member. We too identified only one group VIII chitinase in the *D. citri* genome v 2.0. In *Tribolium* and *A. pisum* [41] this

chitinase has been reported to have a transmembrane domain in the N-terminus of the protein. A transmembrane domain was not identified in the group VIII orthologs in *N. lugens* [14] or *D. citri* (Figure 4).

ENGase and S1-Clp Chitinase-like Proteins

While endo-β-N-acetylglucosaminidase (ENGase) and stabilin-1 interacting chitinase-like proteins (S1-Clp) were not included in initial phylogenetic analyses of holometabolous chitinase proteins [39,40] they are part of the GH18 chitinase-like superfamily [46] and therefore have been included in more recent phylogenetic analysis [14,41]. ENGase orthologs have been found across a wide variety of insects including in hemipteran insects [14,41]. However, orthologs of S1-Clp have not been found in the hemipteran insects *A. pisum*, *N. lugens* or *R. prolixus* [41]. S1-Clp is not absent in all hemimetabolous insects as an ortholog has been identified in *P. humanus* [14,41]. In the *D. citri* v 2.0 genome we identified one ENGase ortholog (Table 4) which had strong PacBio IsoSeq and *de novo* transcriptome evidence (Table 6). As expected we were unable to identify and S1-Clp ortholog in *D. citri* (Table 4).

Chitin deacetylases

Initial bioinformatics and phylogenetic studies using holometabolous insect CDA sequences [13,47] and more recent, more comprehensive CDA sequences from holometabolous and hemimetabolous insects has classified CDAs into five different groups [14]. The general findings of these studies suggest that many holometabolous insects have at least one representative of each of the five CDA groups while hemimetabolous insects are lacking CDA members in Group 2 and Group 5. Genomic analysis of CDA genes in the *D. citri* genome v 2.0 support this finding (Table 7).

Group 1 chitin deacetylases

In *Drosophila* there are two group 1 CDA genes, *serp* and *verm*. In most other insects the convention has been to name the group 1 CDA genes *CDA1* and *CDA2*. In *Drosophila serp* and *verm* are directly adjacent to one another on a chromosome. This clustering of *CDA1* and *CDA2* is also seen in *Tribolium* [13]. The *D. citri CDA1* and *CDA2* orthologs are also located within about 50 kb

Estimated Number of Chitin Deacetylase Homologs						
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Total**
D. melanogaster	2*	1	1	1*	1	6
A. gambiae	2*	1	1	1	0	5
T. castaneum	2*	1	1	1*	4	9
B. mori	2	1	1	1	3	8
A. mellifera	2*	1	1	1*	0	5
N. vitripennis	2	1	1	1	0	5
P. humanus	2	0	1	1	0	4
R. prolixus	2	0	1	1	0	4
A. pisum	2	0	1	1	0	4
N. lugens	2	0	1	1	0	4
D. citri	2*	0	1	1*	0	4

*Table 7: Estimated Number of Chitin Deacetylase Homolgs. D. citri numbers were determined based on annotation of D. citri genome v 2.0. All other ortholog numbers were obtained from Dixit, R. et al 2008 and Xi, Y. et al 2014. Orthologs were categorized based on our phylogenetic analysis and literature reports. * indicates that isoforms have been found for at least one member of that group. In group 1 isoforms have been identified for CDA2 and for group 4 isoforms have been identified for CDA5.*

of one another. The conserved clustering of these two genes suggest there may be evolutionary constraint on their physical location. Expression studies in both *Tribolium* [47] and *N. lugens* [14] indicates CDA1 and CDA2 are expressed at the same time and in the same tissue further supporting the idea that these two genes may be co-regulated. *CDA2* has been shown to have multiple isoforms in several holometabolous insect species including *Drosophila* [30], *Tribolium* [13,47], *Anopheles* [13], *A. mellifera* [13], *Christoneura fumiferana* [16], *Leptinotarsa decemlineata* [19] and *Hyphantria cunea* [17]. However, the only comprehensive analysis done for CDAs in a hemipteran insect was unable to confirm or refute the existence of isoforms of *CDA2* in *N. lugens* [14]. Our RNAseq and PacBio IsoSeq data clearly supports the existence of at least 2 isoforms (Table 8). These two isoforms differ from one another due to the use of one alternative exon, exon 3a and 3b. This same isoform exon architecture is seen in *Tribolium* [13], *L. decemlineata* [19], and *H. cunea* [17] CDA2 isoforms. CDA1 and CDA2 both contain the expected ChBD, LDLa domain, and deacetylase catalytic domains (Figure 5).

While loss of function analysis in *D. citri* was beyond the scope of this work, RNAi of Group 1 CDAs in a variety of insects including *Tribolium* [15], *L. decemlineata* [19], *C. fumiferana* [16], *H. cunea* [17], *Stegobium paniceum* [18]and *N. lugens* [14] suggests that loss of function of CDA1 or loss of function of one of the two CDA2 isoforms can result in lethality and therefore could be potential targets for pest control methods.

D. citri Chitinase Genes								
Group	**Gene**	**D. citri identifier**	**Gene model**		**Evidence supporting annotation**			
			complete	partial	MCOT	IsoSeq	RNASeq	Ortholog
Group 1	CDA1	Dcitr04g03590.1.1 Dcitr04g03590.1.2	X		X	X	X	X
Group 1	CDA2	Dcitr04g03540.1.1 Dcitr04g03540.1.2	X			X	X	X
Group 3	CDA4	Dcitr02g03950.1.1	X		X	X		X
Group 4	CDA5	Dcitr01g12310.1.1 Dcitr01g12310.1.2 Dcitr01g12310.1.3 Dcitr01g12310.1.4 Dcitr01g12310.1.5		X	X	X	X	X

Table 8: Annotated D. citri Genes. Each manually annotated gene has been assigned a gene identifier. For each manually annotated gene it has been denoted as a partial or complete model based on available evidence. Evidence for manual annotation was also recorded. MCOT evidence means a de novo Oases or Trinity model from an independent transcriptome was identified and the sequence from that transcript was used to validate or modify our model. IsoSeq means single reads generated with Pacific Biosciences technology were available and were used to help validate the exon structure of the model. RNASeq means that individually mapped Illumina RNASeq reads were used to help validate or modify our model. Ortholog means ortholog sequences from other insects and information about conserved motifs and domains had to be used to help determine the final annotation.

Group 2 chitin deacetylases

One Group 2 CDA member has been identified in most holometabolous insects examined including members of the orders Diptera, Coleoptera, Hymenoptera and Lepidoptera (Table 7). However, Xi *et al* were unable to find Group 2 orthologs in any of the hemimetabolous insect genomes examined (*A. pisum*, *N. lugens*, *R. prolixus* and *P. humanus*) [14]. Our data supports their conclusions that plant-sap/blood sucking hemimetabolous species from the Hemiptera and Phthiraptera orders do not contain group 2 CDAs as we were unable to identify one in the *D. citri* v 2.0 genome (Table 7, Figure 6). The absence of a CDA3 gene in hemimetabolous insects is perhaps unsurprising as CDA3 expression in *Tribolium* peaks during the pupal stage [47]. Since hemimetabolous insects undergo incomplete metamorphosis they do not exhibit a pupal life stage.

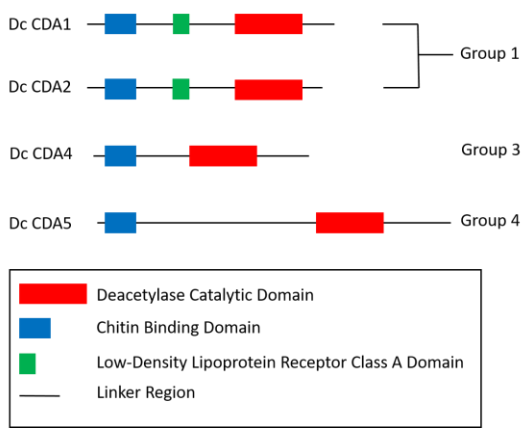

Figure 5: Chitin Deacetylase Domain Organization in D. citri. Chitin deacetylases are categorized by group based on phylogenetic analysis, sequence similarity, and domain organization. D. citri domain analysis was performed using InterPro. CDA1 is represented by D. citri MCOT00151.1.CO sequence (546 amino acids) because the genome annotation for CDA1 is partial. CDA2 is represented by CDA2 isoform RA (532 amino acids). CDA4 is 491 amino acids. CDA5 is represented by MCOT06229.1.CO sequence because the genome annotation for CDA5 is partial (749 amino acids).

Group 3 chitin deacetylases

One group 3 CDA, CDA4, was identified in the *D. citri* genome v 2.0 (Table 7). In *Tribolium* [13] and *N. lugens* [14] it has been reported that Group 3 CDA proteins contain a ChBD and deactylase catalytic domain, but unlike the Group 1 and 2 CDAs lack the LDLa domain. Our analysis indicates that a similar domain architecture is seen in *D. citri* as our annotation contains a ChBD and catalytic domain but lacks the LDLa domain (Figure 5). PacBio IsoSeq and *de novo* transcriptome evidence strongly support this annotation (Table 8).

Group 4 chitin deacetylases

Most insects examined to date have one Group 4 CDA, typically called *CDA5* (*CDA4* in *N. lugens*) (Table 7). While only one gene exists in this group, this gene has been shown to have multiple isoforms [30,47]. Based on results from *D. citri de novo* transcriptomes, individual RNASeq reads and PacBio IsoSeq reads, five different isoforms were identified and annotated at the *CDA5* locus in *D. citri*. Unfortunately, most of these gene models are missing both a portion of the 5' and 3' part of the gene due to genome assembly errors. Despite being annotated as partial transcripts, three of the five transcripts contain both the ChBD and the catalytic domain which are known to exist in CDA5 insect orthologs (two of the transcripts were missing the ChBD domain) (Figure 5).

Group 5 chitin deacetylases

To our knowledge Group 5 CDAs have not been found in any hemipteran insects examined to date. We were also unable to find a Group 5 CDA in the *D. citri* genome v 2.0 (Figure 6). Group 5 CDAs may also be missing from some mosquito and hymenopteran species [14] suggesting gene loss of this group can CDAs occur (Table 7). Group 5 CDAs are gut specific CDAs which are known to be expressed in the gut [47] and associated with the peritrophic membrane [14]. Since hemipterans do not have a peritrophic membrane [23], it is not surprising that these CDAs are absent in hemipteran insect species.

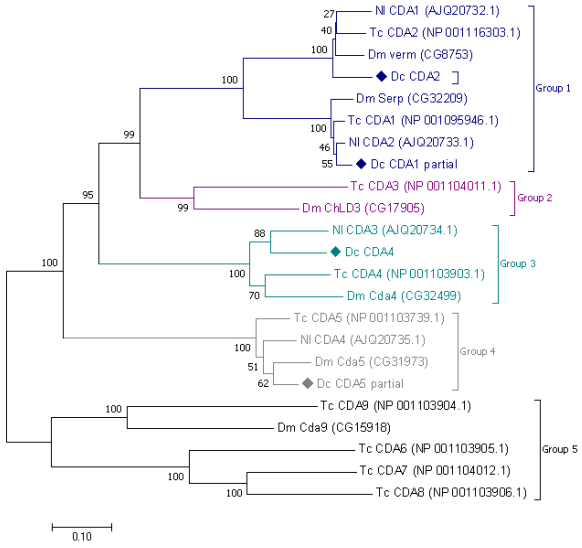

Figure 6: Phylogenetic tree of chitin deacetylase family members. ClustalW was used to perform multiple sequence alignments. The tree was constructed with MEGA 7.0 software using neighbor-joining analysis (100 bootstrap). Full lenth protein sequences were used to construct tree, unless otherwise noted on the tree. Drosophila melanogaster (Dm), Tribolium castnaeum (Tc), Nilaparvata lugens (Nl), and Diaphorina citri (Dc). Colors delineate established chitin deacetylase groups.

Overall the CDA genes appear to be well conserved in *D. citri*, both in number and in gene/protein architecture (Table 7 and Figure 5). As seen in other hemipteran insects *D. citri* only contains Group 1, 3 and 4 CDA genes (Figure 6). Within in these groups one-to-one orthologs were found resulting in the annotation of 4 CDA genes in *D. citri*. This is consistent with the total gene number found in the hemimetabolous insects *A. pisum*, *N. lugens*, *R. prolixus* and *P. humanus* [14]. Additionally, conservation of isoforms for both CDA2 and CDA5 was found. Domains were conserved as expected across the 4 groups and the 5 different small motifs which have been previously identified in insects CDAs [14] were also identified in *D. citri* orthologs (data not shown).

Annotation of the chitin metabolism genes in *D. citri* shows a substantial decrease in gene number when compared to holometabolous insects. In most situations either a one-to-one ortholog was found or a gene absence was noted. For example, *D. citri* does not contain a CHS2 gene and it lacks Group 4 and Group 7 Cht members and Group 2 and Group 5 CDA members. The only exception to this rule was for UAP where a duplication has occurred causing *D. citri* to have more UAP genes than most holometabolous species. While *D. citri* contains substantially fewer chitin metabolism genes when compared to holometabola, when compared to hemimetabolous insects this is not the case. Like *D. citri* most hemimetabolous insects seem to have dramatically fewer chitin metabolism genes which is likely due to their lack of peritrophic membrane and their drastically different morphogenesis. While the conservation of ortholog number is not seen across insect taxa, in general for the orthologs that are conserved this conservation includes conservation of domain architecture and conservation of isoform exon structure.

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