Hox cluster genes and Hox cofactors in the Asian citrus psyllid, *Diaphorina citri*

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Introduction

The Hox genes are a widely conserved gene family encoding transcription factors best known for their role in specifying regional identity along the anterior-posterior (A-P) axis [1]. One fascinating characteristic of Hox genes is their arrangement in chromosomal clusters in an order that parallels their function along the A-P axis — a phenomenon known as colinearity [2,3]. Ancestrally, Hox genes were located in a single, intact cluster [4,5], but splits have occurred in some lineages, including *Drosophila melanogaster* where Hox genes were first described [6–8].

In insects, Hox genes are generally named after their Drosophila orthologs: *labial* (*lab*), *proboscipedia* (*pb*), *zerknüllt* (*zen*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), *fushi tarazu* (*ftz*), *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*). Non-Hox genes are sometimes found within the clusters, but these are not well-conserved between organisms. There are, however, three known microRNAs (miRNAs) that are conserved in insect Hox clusters. *mir-993* is found between *zen* and *Dfd* [9]. mir-*10* is located between *Dfd* and *Scr* [10], while the bidirectionally transcribed miRNA *iab-4* is located between *abd-A* and *Abd-B* [11–14]. These miRNAs are thought to be involved

in post-transcriptional regulation of Hox genes, although this has not been clearly demonstrated in insects [15].

Hox gene sequences have been identified in several hemipteran insects, including, the milkweed bug *Oncopeltus fasciatus* and the pea aphid *Acyrthosiphon pisum*. These insects seem to have the full complement of Hox genes [16–21]. An additional Hox gene, called *HoxR,* was also found in pea aphid [19], and is thought to have arisen from a duplication of *Abd-B* [22].

Based on what is known in other organisms, hemipteran Hox genes are likely to be clustered, but so far the only published evidence of linkage is that *zen* is located next to *pb* and *ftz* is adjacent to *Scr* in the pea aphid genomic scaffolds [19]. As the quality of genome assemblies improves, the arrangement of Hox genes in hemipteran genomes should become clearer.

Another group of genes important for specification of regional identity are the members of the PBC and MEIS (more recently called MEINOX) family of TALE class homeodomain proteins [23]. These proteins are best known as cofactors to Hox proteins, although they have Hox-independent functions as well. The PBC and MEINOX proteins are very ancient. They are conserved throughout the animal kingdom and a related protein is thought to have been present in the common ancestor of plants and animals [24]. *Drosophila* has one member of the PBC family of TALE proteins called *extradenticle* (*exd*) [25] and one

member of the MEINOX family called *homothorax* (*hth*) [26]. The presence of a single PBC family member seems to be the rule in insects, but most insects have two members of the MEINOX family [19,27]. One of these genes is orthologous to *hth* and the vertebrate Meis genes, while the other is orthologous to the vertebrate Prep/PKNOX genes. These observations suggest that the Prep/PKNOX ortholog was lost at some point in the Drosophila lineage [27].

We have identified and manually annotated the Hox genes, conserved Hox cluster miRNAs, and the MEINOX and PBC family genes in the Asian citrus psyllid, *Diaphorina citri*, the vector of the bacterium that causes citrus greening disease. Most of these genes are expected to be essential for development in D. citri, as they are in other insects, and thus could be good targets for RNAi-based pest control methods.

Materials and Methods

Genes were annotated according to the methods described in [28].

Results and Discussion

Hox genes

We identified single orthologs of each of the Hox genes in the *D. citri* genome. *lab* is not found in the v3.0 genome but was present in earlier genome versions in its expected position adjacent to *pb*, indicating that its absence in v3.0 is due to a local misassembly.

In genome v3.0, the Hox genes (except for *lab*) are located in two clusters on the same chromosome, separated by about 6 Mb. *pb, zen* and *Dfd* are found in one cluster, while *Scr, ftz*, *Antp* and *Ubx*, *abd-A* and *Abd-B* are located in a separate cluster (Fig. 1). Thus, it appears that a split in the Hox cluster has occurred at some point in the lineage leading to D. citri. The ancestral state of the Hox cluster was for all the Hox genes to be transcribed in the same direction. In *D. citri,* we find that *pb* and *Dfd* are oriented in the opposite direction to what is expected. This reversal could be due to genome assembly issues or to actual rearrangements in the Hox cluster.

Among hemipterans, Hox gene function has been best studied in the milkweed bug, *Oncopeltus fasciatus*. RNAi experiments indictate that the function of Oncopeltus Hox genes is broadly similar to their orthologs in holometabolous insects such as Drosophila and Tribolium, although there are many small differences in regulation and function [16,17].The Oncopeltus studies show that RNAi with most Hox genes causes high levels of lethality [16,17]. One exception is RNAi with *pb*, which produces viable nymphs that are unable to feed due to abnormal mouthparts [16] RNAi with *zen* in the brown planthopper, *Nilaparvata lugens*, caused high levels of embryonic death and prevented normal development [29] . These results suggest that Hox genes could be good targets for RNAi-based pest control methods in hemipteran pests such as *D. citri*.

Hox cluster miRNAs

There are several conserved microRNAs (miRNAs) located within insect Hox clusters that are believed to have the ability to regulate Hox genes. *miR-10* is conserved in most animals and is located between the *Dfd* and *Scr* orthologs [30]. Three other miRNAs are widely conserved in arthropods [31]. *miR-993* is found between *Dfd* and *zen*, and the bidirectionally transcribed locus producing the *iab-4* and *iab-8* miRNAs is located between *abd-A* and *Abd-B* [32,33]. All of these miRNAs are found in the *D. citri* genome in their expected positions (Fig. 1), with the caveat that *Dfd* and *Scr* are on different scaffolds meaning that the position of *miR-10* between the two genes cannot be established. However, *miR-10* is located within 40 kb of *Dfd* on the expected side.

MEINOX

Like most insects, *D. citri* has two MEINOX genes. One is orthologous to *hth* and the vertebrate Meis genes, while the other is orthologous to the vertebrate Prep/PKNOX2 proteins. In keeping with precedent, we have named the D. citri genes *hth* and *PKNOX2.*

For both the *hth* and *PKNOX2* orthologs, there is *de novo* transcript evidence for two isoforms with different 3' ends. In both cases, one isoform produces a protein with the MEIS domain and a homeodomain, while the other isoform encodes a protein with a MEIS domain but no homeodomain. Drosophila and vertebrates also produce homeodomain-less isoforms of Hth [34] and PKNOX2 [35]. In Drosophila, the HD-less forms of Hth can perform most of the protein's usual functions, but cannot specify antennal development.

extradenticle

The *D. citri* genome contains two apparent *exd* orthologs rather than one as found in most other insects. While one of the genes has a typical structure, the other gene is unusual in that it contains no introns (Fig. 2A). This gene structure suggests that the second ortholog is a retrogene, which forms by retrotransposition of a spliced mRNA. The intron-less locus is found in two independent *D. citri* genome assemblies ([36]and this work), so it is unlikely to be an assembly artifact. The two *exd* genes encode proteins with 84 percent identity to one other. The scattered differences seen between the two proteins are consistent with gene duplication and divergence (Fig. 2B). Moreover, the two genes map to different chromosomes and there are different genes flanking the two loci, strengthening the case for gene duplication. We have therefore named the intronless gene *extradenticle-retrogene* (*exd-r*) and the more typical gene *exd*.

We found no evidence of an *exd* retrogene in other sequenced hemipteran genomes, suggesting that the retrogene insertion occurred fairly recently in the *D. citri* lineage. However, *exd-r* does not seem to have retained the 3' poly(A) region (the vestige of a poly(A) tail) that is still present in some young retrogenes [37]. This is not extremely surprising, as [38] characterized more than 20 young retrogenes in the Drosophila genome and found that almost all of them had lost the poly(A)tail. At the 5' end of *exd-*

r, identity to *exd* at the nucleotide level ends at the translation start site. Such truncation is also common in retrogenes, possibly because of incomplete reverse transcription during the retrotransposition process [39].

Because retrogenes do not usually include the original regulatory elements associated with a gene, they are not likely to retain their original expression pattern. The retrogene will only be expressed if it is activated by regulatory elements close to its new position. RNAseq data shows a moderate number of reads mapping to the *exd-r* locus and to the region just downstream.

If a retrogene has no function because of lack of expression, it will often accumulate mutations to the point that it can no longer produce a functional protein. Such retrogenes are called processed pseudogenes. We examined *exd-r* to determine whether or not it appears to be a pseudogene. The *exd-r* ORF seems to be complete, with no premature stop codons. Surprisingly, when the two *D. citri* Exd proteins are compared to other hemipteran Exd proteins, Exd-r actually has a higher percent identity to the orthologs (>90%) than does the "typical" *D. citri* Exd protein. In fact, Exd-r has higher overall identity to its hemipteran orthologs than it does to the other Exd protein from *D. citri*. Only at their extreme N-termini do the *D. citri* proteins appear more similar to one another (Fig. 2B). Exd-r also lacks most the extended C-terminal region found in other insect Exd proteins (Fig. 2B).

The high level of conservation between Exd-r and its orthologs suggests that Exd-r may still have some function, which is an unusual, but not unheard of, situation for retrogenes. It is more difficult to explain the observation that Exd-r more closely resembles its hemipteran orthologs than its paralog Exd. There are several possible explanations for this situation, although none seem particularly likely: 1) *exd-r* has diverged since the duplication event and accumulated numerous mutations that make it more similar to other orthologs 2) *exd* has diverged more than *exd-r* since the duplication event, resulting in decreased identity to both Exd-r and their orthologs. This would seem to suggest that *exd-r* is still expressed and functional in the original exd domain, freeing *exd* to diverge. 3) the *exd-r* gene might be the result of lateral transfer from another insect, perhaps via a virus or transposable element. The source would likely be another hemipteran based on BLAST searches that show that Exd-r is 91% identical to several different hemipteran Exd orthologs. No one insect stands out as a likely source, but only a few hemipteran genomes have been sequenced so far.

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Figure 1

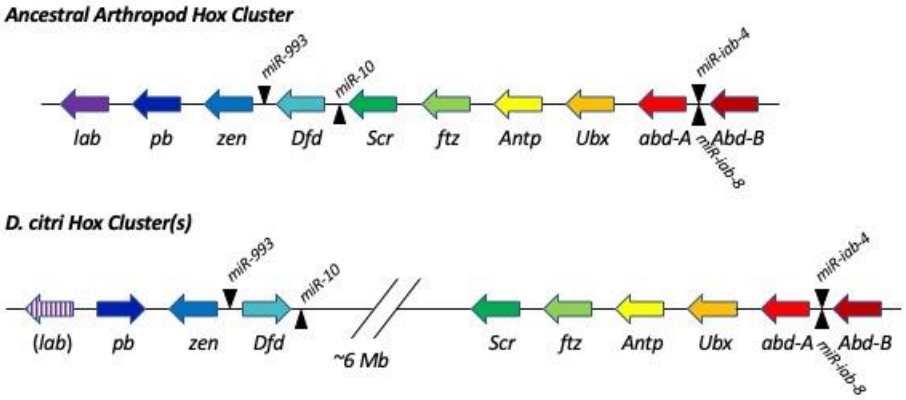
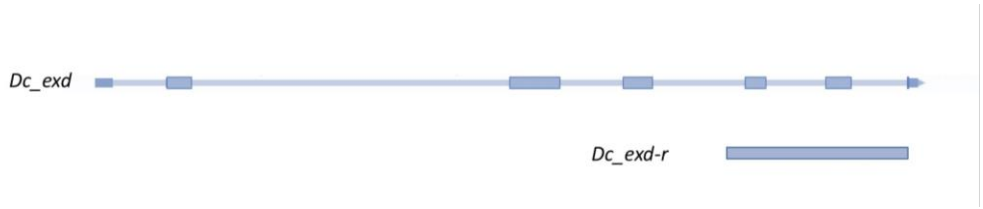


Figure 1. Comparison of Hox gene clusters in *D. citri* with the putative ancestral arthropod Hox cluster. In the ancestral cluster, all ten Hox genes were oriented in the same direction and at least four miRNAs (black triangles) were located within the cluster. In *D. citri*, the Hox genes are split between two clusters on the same chromosome. *lab* is missing from genome v3.0 due to apparent misassembly, but was present in previous genome versions adjacent to *pb*. D*. citri pb* and *Dfd* appear to be oriented in the opposite direction to the ancestral orientation, but this could be the result of local misassembly. *D. citri* has all four of the conserved Hox cluster miRNAs in the expected positions.

Figure 2

A



B

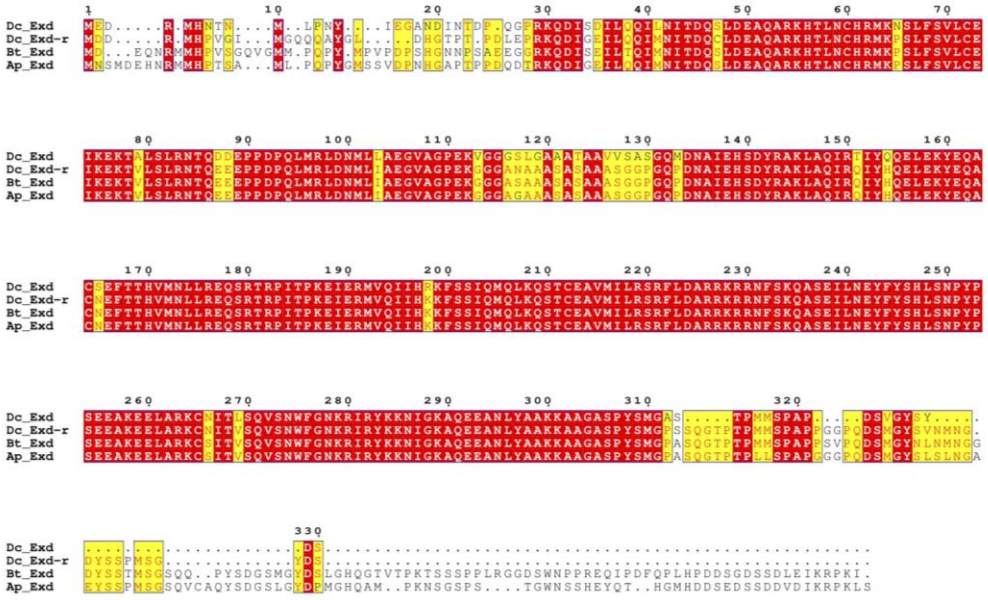


Figure 2. *extradenticle* (*exd*) genes in *D. citri*. A) Structural comparison of the *D. citri exd* and *extradenticle-retro* (*exd-r*) transcripts. *exd* has 7 exons and six introns, while *exd*-r has a single long exon with no introns. B) Multiple Sequence Alignment of *D. citri* Exd (Dc_Exd), Exd-r (Dc_Exd-r), *Bemisia tabaci* Exd (Bt-Exd) and *Acyrthosiphon pisum* Exd

(Ap_Exd). Residues identical in all 4 proteins are shaded red, while those identical in 3 out of 4 are shaded yellow.

Table 1

Hox genes	*Dm*	*Am*	*Tc*	*Ap*	*Dc*
labial	1	1	1	1	1
proboscipedia	1	1	1	1	1
zerknüllt (*Hox 3*)	3	1	2	1	1
Deformed	1	1	1	1	1
Sex combs reduced	1	1	1	1	1
fushi tarazu	1	1	1	1	1
Antennapedia	1	1	1	1	1
Ultrabithorax	1	1	1	1	1
abdominal-A	1	1	1	1	1
Abdominal-B	1	1	1	2	1

Gene counts were obtained from the following sources: *Drosophila melanogaster* (*Dm*) – Flybase; *Apis mellifera* (*Am*) – Dearden et al. 2006; *Tribolium castaneum* (*Tc*) – Shippy et al. 2008; *Acyrthosiphon pisum (Ap*) – Shigenobu et al. 2010, O'Neill, 2012; *Diaphorina citri* (*Dc*) – this work.

Hox cluster miRNAs	*Dm*	*Am*	*Tc*	*Ap*	*Dc*
miR-10	1	1	1	1	1
miR-993	1	1	1	1	1
miR-iab-4	1	1	1	1	1
miR-iab-8	1	1	1	1	1

Gene counts were obtained from the following sources: *Drosophila melanogaster* (*Dm*), *Apis mellifera* (*Am*), and *Tribolium castaneum* (*Tc*) – Pace et al. 2016; *Acyrthosiphon pisum (Ap*) – Legeai et al. 2010, Miura et al. 2011; *Diaphorina citri* (*Dc*) – this work.

Hox cofactors	*Dm*	*Am*	*Tc*	*Ap*	*Dc*
extradenticle	1	1	1	1	2
homothorax	1	1	1	1	1
PKNOX2	0	1	1	1	1

Gene counts were obtained from the following sources: *Drosophila melanogaster* (*Dm*), *Apis mellifera* (*Am*) and *Tribolium castaneum* (*Tc*) – Mukherjee and Bürglin, 2007; *Acyrthosiphon pisum (Ap*) – Shigenobu et al. 2010; *Diaphorina citri* (*Dc*) – this work.

Gene	**D. citri OGS3 identifier**	**Gene model**		**Evidence supporting annotation**			
		complete	partial	MCOT	IsoSeq	RNASeq	Ortholog
labial†		X		X			X
proboscipedia	Dcitr07g11290.1.1 Dcitr07g11290.1.2	X		X	X	X	X
zerknullt	Dcitr07g11300.1.1	X				X	X
Deformed	Dcitr07g11310.1.1	X		X	X	X	
Sex combs reduced	Dcitr07g04890.1.1	X					X
fushi tarzu	Dcitr07g04910.1.1	X		X			
Antennapedia	Dcitr07g04960.1.1	X			X		
Ultrabithorax	Dcitr07g05020.1.1	X		X	X		X
abdominal-A	Dcitr07g05110.1.1	X				X	X
Abdominal-B	Dcitr07g05150.1.1	X		X	X		
miR-10		X*					X
miR-993		X*					X
miR-iab-4		X*					X
miR-iab-8		X*					X
extradenticle	Dcitr08g10400.1.1	X		X		X	
extradenticle-pseudo	Dcitr01g03720.1.1	X					X
homothorax	Dcitr03g04000.1.1	X		X	X	X	X

	Dcitr03g04000.1.2						
PKNOX2†	Dcitr00g14970.1.1		X	X	X	X	X

* For miRNA genes, a complete gene model indicates that the entire hairpin-forming portion of the gene (the pre-miRNA) has been annotated

† These genes were annotated in genome v2.0, but are not properly assembled in genome v3.0.