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SYMPOSIUM

Coral Comparative Genomics Reveal Expanded *Hox* Cluster in the Cnidarian–Bilaterian Ancestor

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Synopsis The key developmental role of the *Hox* cluster of genes was established prior to the last common ancestor of protostomes and deuterostomes and the subsequent evolution of this cluster has played a major role in the morphological diversity exhibited in extant bilaterians. Despite 20 years of research into cnidarian *Hox* genes, the nature of the cnidarian–bilaterian ancestral *Hox* cluster remains unclear. In an attempt to further elucidate this critical phylogenetic node, we have characterized the *Hox* cluster of the recently sequenced *Acropora digitifera* genome. The *A. digitifera* genome contains two anterior *Hox* genes (PG1 and PG2) linked to an *Eve* homeobox gene and an *Anthox1A* gene, which is thought to be either a posterior or posterior/central *Hox* gene. These data show that the *Hox* cluster of the cnidarian–bilaterian ancestor was more extensive than previously thought. The results are congruent with the existence of an ancient set of constraints on the *Hox* cluster and reinforce the importance of incorporating a wide range of animal species to reconstruct critical ancestral nodes.

Introduction

Hox genes are homeobox transcription factors that play a critical role in developmental patterning (McGinnis et al. 1984) and have been identified in every extant phylum outside of Porifera, Ctenophora, and Placozoa (placozoans have *ParaHox* but not *Hox* genes (Jakob et al. 2004; Ryan et al. 2010). The last common ancestor of protostomes and deuterostomes, which gave rise to 99% of all described animal species (Zhang 2011), is thought to have had an extensive cluster of *Hox* genes. Furthermore, since the expression of these genes in the body of many protostomes and deuterostomes is correlated with their position within the cluster, this ancestral condition was likely important for regulation of transcription (reviewed by Akam 1989).

Cnidarians (e.g., corals, sea anemones, hydroids, and medusae) are the only nonbilaterian phyla with *Hox* genes, and therefore critical to our understanding of the early evolution of the *Hox* cluster. The exact relationship of cnidarian *Hox* genes with those of the protostomes and deuterostomes has been difficult to establish and has been debated (Finnerty and Martindale 1997; Gauchat et al. 2000; Yanze et al. 2001; Chourrout et al. 2006; Kamm et al. 2006; Jakob and Schierwater 2007; Ryan et al. 2007; Chiori et al. 2009). In addition, the genomic arrangement of *Hox* genes from sequenced cnidarian genomes have only been analyzed in *Nematostella vectensis* and *Hydra magnipapillata*. The *H. magnapapillata* genome shows no *Hox* cluster (Chapman et al. 2010) and the clustering in *N. vectensis* is limited to anterior *Hox* genes (Chourrout et al. 2006; Ryan et al. 2007; Putnam et al. 2007).

Most studies agree that cnidarians possess representatives of phylogenetically anterior *Hox* genes

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including paralogous group 1 (PG1) and paralogous group 2 (PG2) (Finnerty and Martindale 1997; Chourrout et al. 2006; Ryan et al. 2007; Chiori et al. 2009). For example, the anthozoan *N. vectensis* has PG1 (*Anthox6* and *Anthox6a*) and PG2 genes (*Anthox7*, *Anthox8a*, and *Anthox8b*) (Finnerty and Martindale 1997; Chourrout et al. 2006; Ryan et al. 2007) and orthologs of these genes have been identified in the medusozoan *Clytia hemisphaerica* (Chiori et al. 2009).

The relationship of the other *Hox* genes in cnidarians is more controversial. For example, *N. vectensis* has a set of paralogs (*Anthox1* and *Anthox1a)* that have often been affiliated with posterior *Hox* genes (PG9–14) (Fig. 1) (Finnerty and Martindale 1997; Chourrout et al. 2006; Ryan et al. 2007). Three similar genes are found in *C. hemisphaerica* (Chiori et al. 2009). Our analyses, along with those of others (e.g., Chourrout et al. 2006; Chiori et al. 2009), show that these genes share almost equal phylogenetic affinity with central *Hox* genes (PG4–8) as they do with posterior *Hox* genes (Fig. 1). Therefore, either cnidarians have lost the ancestral central *Hox* gene, or the cnidarian–bilaterian ancestor possessed a single gene that gave rise to the bilaterian central and posterior genes as well as the cnidarian genes related to *Anthox1* and *Anthox1a* (Ryan et al. 2006).

In addition to disagreements as to the phylogenetic affinity of cnidarian *Hox* genes, there are differences in opinion as to the extent and nature of the cnidarian–bilaterian *Hox* cluster. Some authors have suggested, based on variations of expression domains in distant cnidarian species, that the cnidarian–bilaterian ancestor lacked *bona fide Hox* genes (Kamm et al. 2006; Schierwater and Kamm 2010). Others have asserted that the *Hox* cluster of the last common ancestor of cnidarians and bilaterians consisted of a maximum of two anterior-like *Hox* genes (Chourrout et al. 2006).

The criteria used by Kamm and coauthors to define what they call a ''*Hox* system'' are: (1) *Hox* genes are closely linked within the genome along the same chromosome; and (2) The *Hox* genes are primarily responsible for patterning structures along the primary body axis of a developing embryo (Kamm et al. 2006). In *N. vectensis*, the expression of *Hox* genes is restricted along the primary body axis (Finnerty et al. 2004; Ryan et al. 2007), but with only a partial cluster, it is perhaps difficult to assess these criteria *in totum*. However, the number of cnidarians examined to date and the extent of experiments involving cnidarian *Hox* genes are both far too few to rule out the existence of a functional *Hox* cluster in the cnidarian–bilaterian ancestor.

Recently, the genome of the staghorn coral *Acropora digitifera* was published (Shinzato et al. 2011). *Acropora digitifera* and *N. vectensis* are both members of the class Anthozoa; however, it is estimated that these two lineages diverged from each other some 500 million years ago (Shinzato et al. 2011). An analysis in another coral, *Acropora formosa*, was the first to show linkage between the *Hox*-related gene *Eve* and the PG1-related *Hox* gene *Anthox6* in a cnidarian (Miller and Miles 1993). In this article, we characterize the *Hox* cluster of *A. digitifera* and show that the *Hox* cluster in the last common ancestor of cnidarians and bilaterians was more elaborate than previously documented. This conserved synteny is consistent with an ancient functional constraint present in this ancestral *Hox* cluster.

Methods

Homeodomains from *N. vectensis*, *C. hemisphaerica*, *A. digitifera*, *Branchiostoma floridae*, *Homo sapiens*, *Drosophila melanogaster*, *Capitella telata*, and *Cupiennius salei* were aligned by eye. See Fig. 2 for accession numbers. We used ProtTest (AIC criteria) to determine that the LG + Gamma model best fit our multiple sequence alignment (Abascal et al. 2005). We ran RAxML version 7.2.8 with the following command line: *raxmlHPC-PTHREADS -T 6 –n hox -s hox.phy -m PROTGAMMALG -k -f a -N 100 -x 43241* (Stamatakis et al. 2008). The resulting tree is shown in Fig. 1. We used version 2.5.5 of Augustus (Stanke et al. 2006) to predict genes on the *A. digitifera* scaffold (DF093930) with the following command line: *augustus –species = human –AUGUSTUS_CONFIG_PATH = augustus.2.5.5/config DF093930.fa*.

Results

The *A. digitifera* genome (Shinzato et al. 2011) has the most extensive *Hox* cluster of all reported cnidarians. Phylogenetic analysis of 12 homeobox genes from the genome of *A. digitifera* revealed a total of six *Hox*, one *ParaHox*, three *Mox*, one *Eve*, and one *HlxB9* gene (Fig. 1 and Fig. 2). Two of the identified *Hox* genes (*Anthox6* and *Anthox7/8*) along with the homeobox genes *HlxB9* and *Eve* were found to be in the same 5′–3′ scaffold orientation as in *N. vectensis* (Fig. 3A and B). Contrary to *N. vectensis*, the 3′-end of the *A. digitifera Hox* cluster contains the central/posterior *Hox* gene *Anthox1a*. Interestingly, *Anthox1a* in *N. vectensis* is flanked by the psuedogene *Anthox9*, suggesting additional genes may have been present in the cnidarian–bilaterian cluster (Fig. 3).

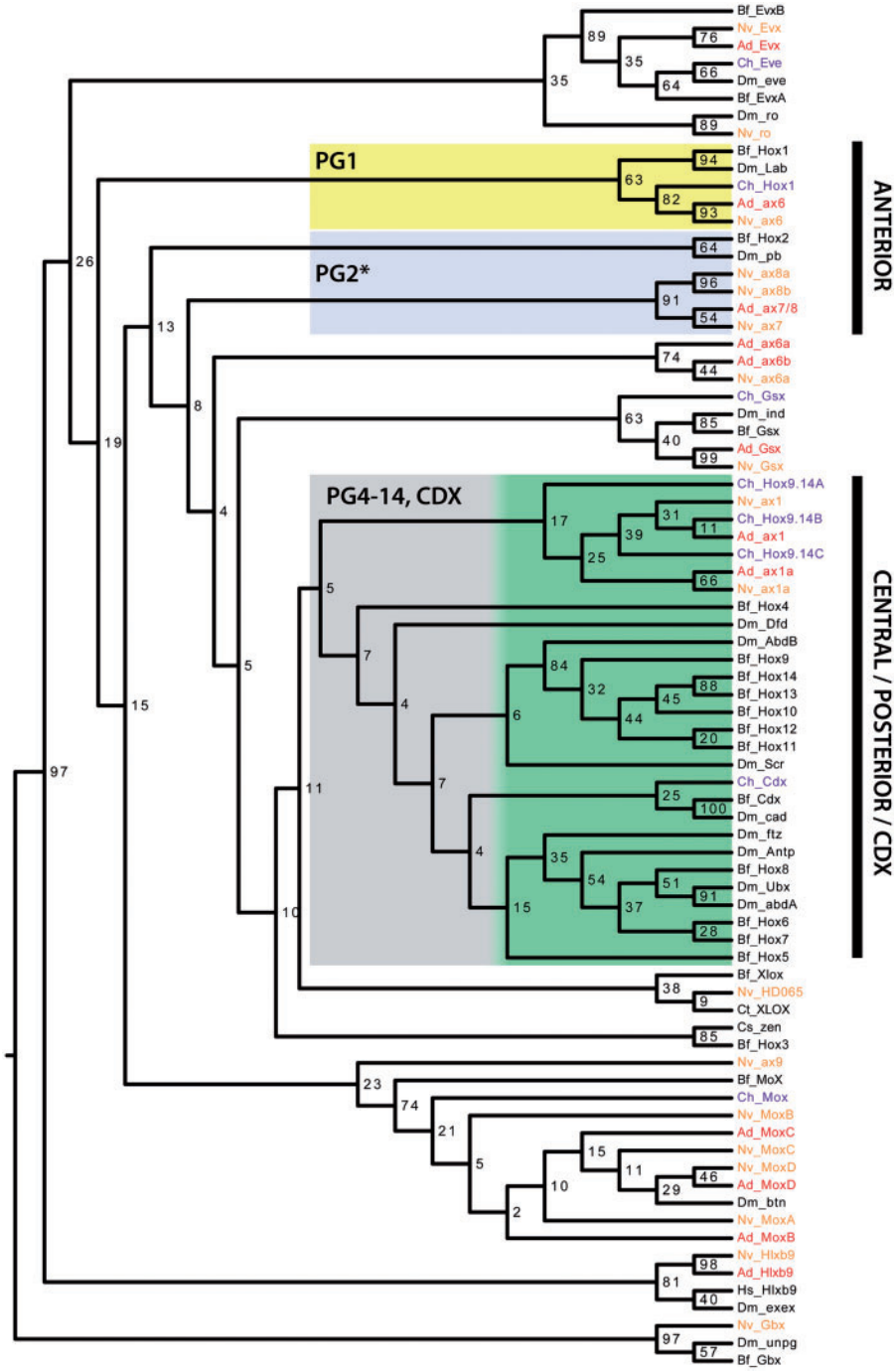


Fig. 1 Phylogenetic analysis of *Hox*, *ParaHox*, *Hox*-related homeodomains using genomic data from *A. digitifera*. Support values at nodes are based on 100 bootstraps. *Nematostella vectensis* taxa are prefixed with Nv and are in orange. *Acropora digitifera* taxa are prefixed with Ad and are in red. *Clytia hemisphaerica* taxa are prefixed with Ch and are in purple. All other taxa represent bilaterian homeodomains (Bf = *Branchiostoma floridae*, Hs = *Homo sapiens*, Dm = *D. melanogaster*, Ct = *C. telata*, Cs = *Cupiennius salei*). **Anthox7*, *8*, and *8a* have been most often associated with bilaterian *Hox2* (e.g., Chourrout et al. 2006; Ryan et al. 2007), but this new analysis suggests the possibility (low bootstrap support notwithstanding) that *Hox2* was lost in the cnidarian lineage and *Anthox7/8* was lost in the Bilateria.

Additionally, a single ParaHox gene (*Gsx*) was identified in *A. digitifera*, where *N. vectensis* has two clustered *ParaHox* genes (*Gsx* and a gene related to *Cdx* and/or *Xlox*) (Ryan et al. 2007). A *Mox* cluster was also identified and contains three genes, where *N. vectensis* has four genes. *Acropora digitifera* and *N. vectensis* have similar *Hox* and *Hox*-related gene complements, but the three tandomly-linked

A

Name	Scaffold GI	5' Scaff Pos.	Strand	Homeodomain Sequence
Ad_Ax1	344215709	303835	plus	GKRKRTAYTRKQLLELEKEFHFNHFLTKERRAEMASQLNLTERQVKIWFQNRRMKWKKTN
Ad_MoxB	344215814	246320	plus	KRKDRTAFTKHQIQELENEFERNNYLTRLRRYEIAVSLDLTERQVKVWFQNRRMKWKRVK
Ad_MoxD	344215814	253095	plus	LRKERTVFTKSQLHQLEKEFGRNNYLTRLRRYEVAVSLDLSERQVKVWFQNRRMKWKRIR
Ad_MoxC	344215814	264146	plus	RRKERTAFTKHQLQELENEFLRNNYLTRLRRYEIAVSLDLTERQIKVWFQNRRMKWKRIK
Ad_Hlxb9	344215894	27918	plus	QRRPRTAFSSQQLLTLERQFQAQKYLTRPQRYELATSLMLTETQVKIWFQNRRMKWKRCG
Ad_Ax6	344215894	214976	minus	SNKKRFTFTQRQLVELEKEFHFSKYLTRTRRIEIASNLDLTETQIKIWFQNRRMKWKREL
Ad_Evx	344215894	226289	minus	TRRYRTAFTREQLSRLEKEFLRENYVSRTRRSELASMLNLSETTIKIWFQNRRMKAKRRR
Ad_Ax7/8	344215894	252838	minus	DKRHRTSYTNKQLLELEKEFHFNKYLCSSRRSEIAKTLSLSERQVKIWFQNRRMKMKKDE
Ad_Ax1a	344215894	264704	minus	KHRRRVAYTRNQILELEKEFHFTRYLTKERRSEMSTMLKLSERQIKIWFQNRRMKWKKDN
AdGsx	344216069	40720	minus	SKRIRTAYTSMQLLELEKEFSQNRYLSRLRRIQIAALLDLSEKQVKIWFQNRRVKWKKDK
Ad_Ax6a	344217215	33972	plus	ADKNRTTYSTRQLVELEKEFHYNRYLCRPRRIEIAQTLGLTEKQVKIWFQNRRMKWKKEN
Ad_Ax6b	344217215	49094	plus	ADKNRTIYSTRQLVELEKEFLYDKYLCRPRRIEIALSLGLTEKQVKIWFQNRRMKWKKEA

B

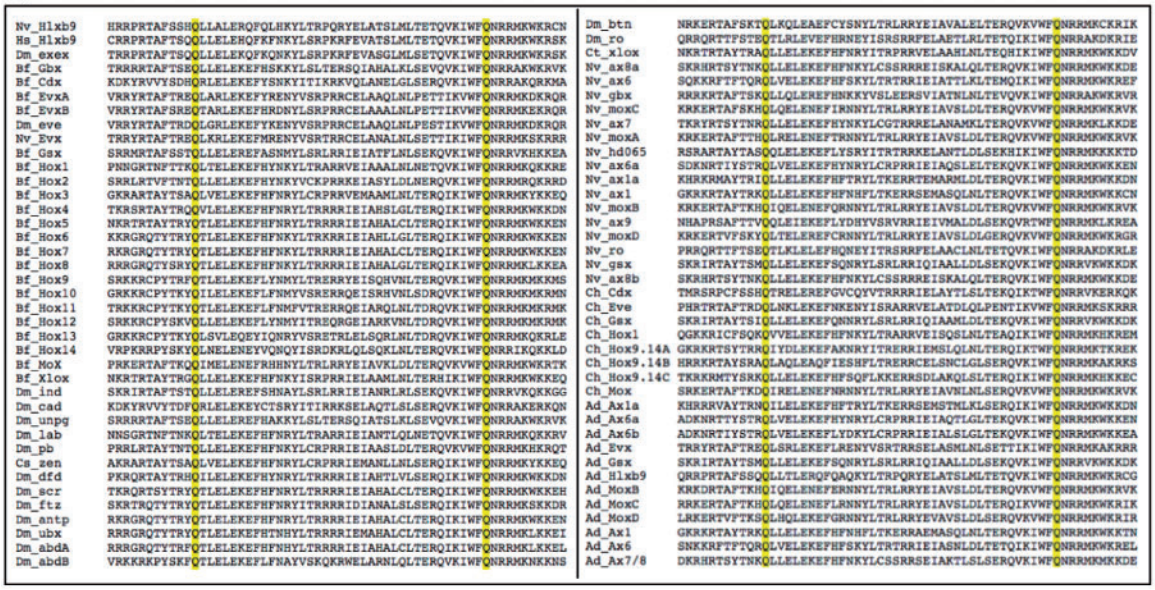


Fig. 2 Information on sequences of the *A. digitifera* homeodomain and the alignment with other taxa. (**A**) Sequences are organized by their linkage in the genome, whereby genes sharing the same color are found on the same scaffold. Genes in white were not linked to other *Hox* or *Hox*-related genes. (**B**) Alignment of homeodomain sequences.

N. vectensis PG2-related genes (*Anthox7*, *8*, and *8a*) are represented by only a single gene (*Anthox7/8*) in *A. digitifera*. This is congruent with the assertion that the *Anthox7*, *8*, and *8a* genes were most likely the result of recent duplications in the *N. vectensis* lineage (Ryan et al. 2007).

The *Hox* cluster of *A. digitifera* is situated on the end of a 270-kb scaffold. We predict 16 genes downstream of the *A. digitifera Hox* cluster (Fig. 4A). Of these, only *Rac3*, *Dars*, *Psma2*, and the *Hox*-related *HlxB9* are linked to the cluster in both anthozoans (Fig. 4). *Dars* and *Psma2* are adjacent to each other in both genomes, but are on opposite sides of the cluster. Seven of the 16 downstream genes appear not to be linked to the *N. vectensis* cluster (Fig. 4C).

Discussion

For the first time, we show that a cnidarian *Hox* gene (i.e., the *A. digitifera Anthox1a* gene) related to the central/posterior *Hox* genes of the Bilateria is linked to a cluster of genes that includes anterior *Hox* genes and an *Eve* gene (Fig. 3B). This new evidence and the genomic linkage between the *Anthox1a* and *Anthox9* genes of *N. vectensis* suggest that a larger cluster almost certainly existed in the cnidarian–bilaterian ancestor (Fig. 3C). The *N. vectensis Anthox9* is thought to be a highly derived pseudogene and is unstable in phylogenies making it difficult to know its true identity (Kamm et al. 2006; Ryan et al. 2007). An ortholog of *Anthox9* was not identified in the *A. digitifera* genome. These data show that the *Hox* cluster of the cnidarian–bilaterian ancestor consisted of at least two anterior-related *Hox* genes, one central/posterior-related *Hox* gene, an *Eve homeobox*, and perhaps another gene related to *Anthox9* (Fig. 3C).

Despite many examples of rearrangements and breakages, the persistence of a *Hox* cluster in disparate bilaterian lineages is attributed to constraints

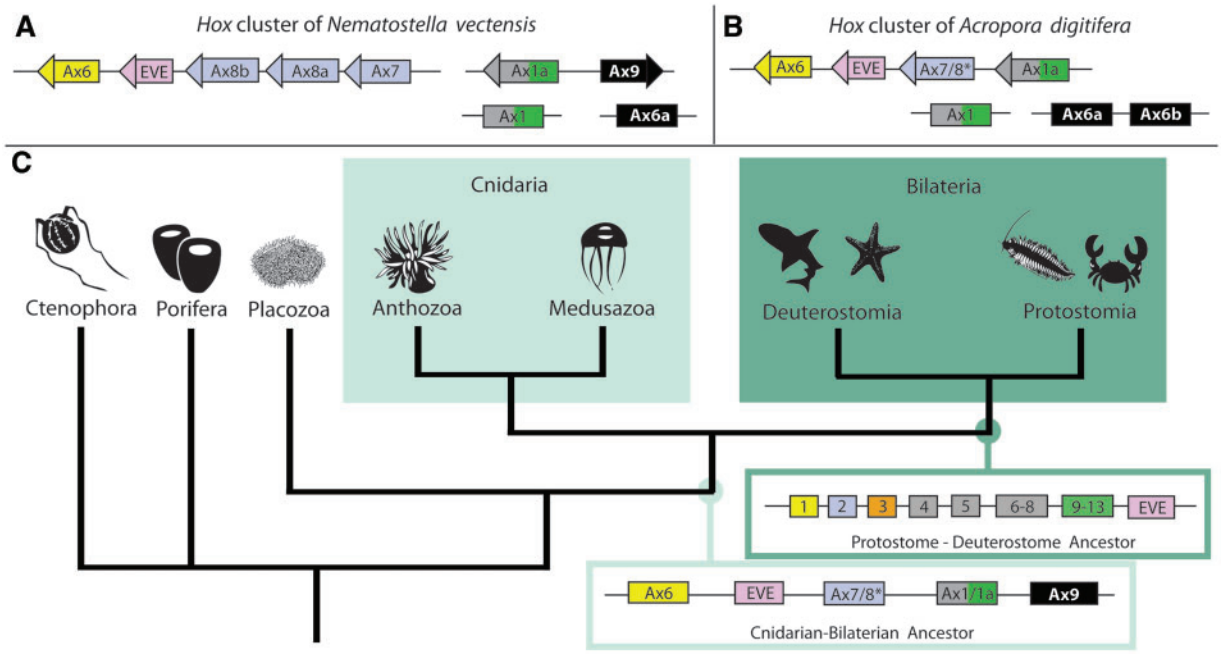
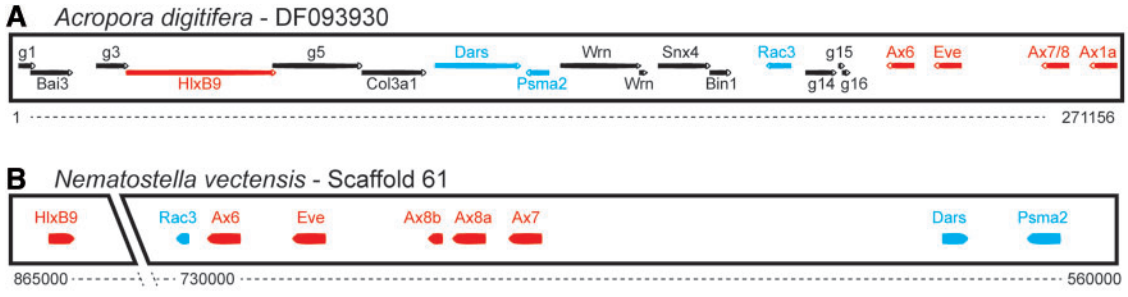


Fig. 3 The anthozoan complement of *Hox* genes and the implications of the evolution of the *Hox* cluster. Comparing the genomic linkage of *Hox* genes in the sea anemone *N. vectensis* and the staghorn coral *A. digitifera* confirms that cnidarians once had a *Hox* cluster that contained both anterior and posterior/central class *Hox* genes. (**A**) The *Hox* cluster of *N. vectensis* includes the anterior *Hox* genes *Anthox6* (PG1), *Anthox8b* (PG2), *Anthox8a* (PG2), and *Anthox7* (PG2) as well as the *Eve* homeobox gene. (**B**) The *Hox* cluster of *A. digitifera* includes the anterior *Hox* genes *Anthox6* (PG1) and *Anthox7/8* (PG2), and the posterior/central class *Hox* gene *Anthox1a* (PG4–14), as well as the *Eve* homeobox gene. Another gene *HlxB9* (also named *MNX*) is found upstream of *Anthox6* in the *Hox* cluster of both genomes (data not shown). (**C**) The metazoan tree of life with inferred ancestral *Hox* clusters. The ancestor to protostomes and deuterostomes is thought to have had two anterior class *Hox* genes (*Hox1* and *Hox2*), one paralagous group 3 gene (*Hox3*), three central class genes (*Hox4*, *Hox5*, and *Hox6–8*), one posterior class *Hox* gene (*Hox9–14*), and one *Eve* homeobox gene. Because of the extended cluster in *A. digitifera*, we can now say that the cnidarian–bilaterian ancestor had, at least, two anterior class *Hox* genes (*Anthox6* and *Anthox7/8*), a central/posterior class *Hox* gene (*Anthox1/1a*), and the *Eve* homeobox gene. It is unclear at what point the genomic rearrangement involving the *Eve* homeobox gene occurred. The origin of the PG3 *Hox* genes also is not clear. **Anthox7/8* has been categorized as a PG2 *Hox* gene in previous publications, but it is possible, based on our current phylogenetic analysis, that *Anthox7/8* descended from a *Hox* gene that was lost in bilaterians. Based on the genomic orientation of these genes, we also believe the ancestor likely had a fourth *Hox* gene potentially related to *Anthox9*. Abbreviations: PG = paralogous group, Ax = Anthox.

on the developmental regulation of these genes. The absence of *Hox* clustering in the the *H. magnipapillata* genome (Chapman et al. 2010) and the partial clustering in *N. vectensis*, a genome remarkable for its large-scale conserved synteny with vertebrate genomes (Putnam et al. 2007), presented the possibility that these regulatory constraints were established after cnidarians and bilaterians diverged. Nevertheless, the presence of an extensive *Hox* cluster in a third cnidarian lineage suggests that this regulatory constraint dates back prior to the last common cnidarian–bilaterian ancestor. The conservation of the clusters in the two anthozoan lineages despite the many genomic events that appear to have occurred in the region (Fig. 4) reinforces this view.

Our current understanding of the early evolution of the *Hox* cluster is still at an early stage. As more cnidarian genomes are sequenced, and as experimental techniques are established for these new model systems (including *A. digitifera*), the structure and function of the cnidarian–bilaterian ancestor's *Hox* cluster will become even clearer. A better understanding of the similarities and differences between the *Hox* clusters of the cnidarian–bilaterian ancestor and the protostome-deuterostome ancestor will help explain the origin of bilaterian-specific complexities. Furthermore, more comprehensive surveys into the independent variation of *Hox* genes in cnidarian lineages will lead to a better understanding of the role these genes have played in establishing the vast diversity of body plans exhibited in the Cnidaria.



C

A. digitifera	*N. vectensis*
Ax1a	Scaffold 3
g1	Scaffold 413
Bai3	Scaffold 286
Col3a1	Scaffold 36
Wrn, Wrn, Snx4	Scaffold 243
Bin1	Scaffold 229
g5, g14, g15	no match

Fig. 4 Genes associated with the *Hox* cluster of *A. digitifera* and their location in the *N. vectensis* genome. (**A**) The *Hox* cluster of *A. digitifera* occurs on the scaffold with the NCBI accession DF093930 (total size = 271,156 bp). Genes on this scaffold were predicted with the Augustus gene finder (Stanke et al. 2006). If the resulting protein had a reciprocal best BLAST hit with a human RefSeq, the gene name associated with that RefSeq is used in the figure; if not, the genes were numbered from g1 to g16. Predictions and spatial relationships are roughly to scale. (**B**) The *Hox* cluster of *N. vectensis* occurs on the scaffold with the JGI ID# 61 (total size = 1,073,712 bp) in v1.0 of the JGI assembly. This scaffold includes three nonhomeobox genes (*DARS*, *PSMA2*, and *RAC3*) that are also associated with the *A. digitifera Hox* cluster. *Nematostella vectensis* genes not associated with the *A. digitifera Hox* cluster are not displayed. The broken line and broken box indicate that the distance between the HlxB9 gene and the rest of the scaffold is not to scale. (**C**) Those genes in the *A. digitifera* cluster not on scaffold 61 in *N. vectensis* are listed along with the corresponding JGI scaffold number. The coordinates below the A and B panels indicate the scaffold coordinates of the region shown. Genes in red are *Hox* and *Hox*-related genes. Genes in blue are nonhomeobox genes that are associated with the *Hox* cluster in both *A. digitifera* and *N. vectensis*.

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