SYMPOSIUM

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

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From the symposium “Evo-Devo Rides the Genomics Express” presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2012 at Charleston, South Carolina.

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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...
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 \textit{N. vectensis} has PG1 (\textit{Anthox6} and \textit{Anthox6a}) and PG2 genes (\textit{Anthox7}, \textit{Anthox8a}, and \textit{Anthox8b}) (Finnerty and Martindale 1997; Chourrout et al. 2006; Ryan et al. 2007) and orthologs of these genes have been identified in the medusozoan \textit{Clytia hemisphaerica} (Chiori et al. 2009).

The relationship of the other \textit{Hox} genes in cnidarians is more controversial. For example, \textit{N. vectensis} has a set of paralogs (\textit{Anthox1} and \textit{Anthox1a}) that have often been affiliated with posterior \textit{Hox} genes (PG9–14) (Fig. 1) (Finnerty and Martindale 1997; Chourrout et al. 2006; Ryan et al. 2007). Three similar genes are found in \textit{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 \textit{Hox} genes (PG4–8) as they do with posterior \textit{Hox} genes (Fig. 1). Therefore, either cnidarians have lost the ancestral central \textit{Hox} gene, or the cnidian–bilaterian ancestor possessed a single gene that gave rise to the bilaterian central and posterior genes as well as the cnidarian genes related to \textit{Anthox1} and \textit{Anthox1a} (Ryan et al. 2006).

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

The criteria used by Kamm and coauthors to define what they call a “\textit{Hox system}” are: (1) \textit{Hox} genes are closely linked within the genome along the same chromosome; and (2) The \textit{Hox} genes are primarily responsible for patterning structures along the primary body axis of a developing embryo (Kamm et al. 2006). In \textit{N. vectensis}, the expression of \textit{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 \textit{in toto}. However, the number of cnidarians examined to date and the extent of experiments involving cnidarian \textit{Hox} genes are both far too few to rule out the existence of a functional \textit{Hox} cluster in the cnidian–bilaterian ancestor.

Recently, the genome of the staghorn coral \textit{Acropora digitifera} was published (Shinzato et al. 2011). \textit{Acropora digitifera} and \textit{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, \textit{Acropora formosa}, was the first to show linkage between the \textit{Hox}-related gene \textit{Eve} and the PG1-related \textit{Hox} gene \textit{Anthox6} in a cnidarian (Miller and Miles 1993). In this article, we characterize the \textit{Hox} cluster of \textit{A. digitifera} and show that the \textit{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 \textit{Hox} cluster.

**Methods**

Homeodomains from \textit{N. vectensis}, \textit{C. hemisphaerica}, \textit{A. digitifera}, \textit{Branchiostoma floridae}, \textit{Homo sapiens}, \textit{Drosophila melanogaster}, \textit{Capitella telata}, and \textit{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 \textit{A. digitifera} scaffold (DF093930) with the following command line: `augustus --species=human -- AUGUSTUS_CONFIG_PATH=augustus.2.5.5/config DF093930.fa`.

**Results**

The \textit{A. digitifera} genome (Shinzato et al. 2011) has the most extensive \textit{Hox} cluster of all reported cnidarians. Phylogenetic analysis of 12 homeobox genes from the genome of \textit{A. digitifera} revealed a total of six \textit{Hox}, one \textit{ParaHox}, three \textit{Max}, one \textit{Eve}, and one \textit{HlxB9} gene (Fig. 1 and Fig. 2). Two of the identified \textit{Hox} genes (\textit{Anthox6} and \textit{Anthox7/8}) along with the homeobox genes \textit{HlxB9} and \textit{Eve} were found to be in the same 5′–3′ scaffold orientation as in \textit{N. vectensis} (Fig. 3A and B). Contrary to \textit{N. vectensis}, the 3′-end of the \textit{A. digitifera} \textit{Hox} cluster contains the central posterior \textit{Hox} gene \textit{Anthox1a}. Interestingly, \textit{Anthox1a} in \textit{N. vectensis} is flanked by the pseudogene \textit{Anthox9}, suggesting additional genes may have been present in the cnidian–bilaterian cluster (Fig. 3).
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 randomly-linked...
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 Hlx9 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
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.

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 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 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 paralogous 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.
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 Hlb9 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*.

**Acknowledgments**

JFR would like to thank Andreas Hejnol for support and insightful conversations regarding this work. The authors would like to thank two anonymous referees for their insightful review of an earlier version of this manuscript, which led to significant improvements.

**Funding**

JFR was supported by the Sars Centre. This research was funded by the National Science Foundation.

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