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Report

Axial Patterning and Diversification in the Cnidaria Predate the Hox System

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Summary

Across the animal kingdom, Hox genes are organized in clusters whose genomic organization reflects their central roles in patterning along the anterior/posterior (A/P) axis [1–7]. While a cluster of Hox genes was present in the bilaterian common ancestor, the origins of this system remain unclear (cf. [8]). With new data for two representatives of the closest extant phylum to the Bilateria, the sea anemone *Nematostella* and the hydromedusa *Eleutheria*, we argue here that the Cnidaria predate the evolution of the Hox system. Although Hox-like genes are present in a range of cnidarians, many of these are paralogs and in neither *Nematostella* nor *Eleutheria* is an equivalent of the Hox cluster present. With the exception of independently duplicated genes, the cnidarian genes are unlinked and in several cases are flanked by non-Hox genes. Furthermore, the cnidarian genes are expressed in patterns that are inconsistent with the Hox paradigm. We conclude that the Cnidaria/Bilateria split occurred before a definitive Hox system developed. The spectacular variety in morphological and developmental characteristics shown by extant cnidarians demonstrates that there is no obligate link between the Hox system and morphological diversity in the animal kingdom and that a canonical Hox system is not mandatory for axial patterning.

Results and Discussion

The Hox cluster has been the Rosetta Stone of comparative developmental biology, but its origins are unclear. Hox genes are characteristically organized in clusters whose genomic organization directly reflects domains of expression along the A/P axis [1–7]; this pattern of organization is functionally important and has been conserved across the Bilateria. The central role of Hox clusters in axial patterning in animals with very different body plans, together with functional data from arthropods and chordates, has led to the assumption that much of the morphological variation seen across the animal kingdom can be directly attributed to different numbers of Hox genes or differential use of the Hox system [9–11]. For present purposes, we define a canonical Hox system as a set of closely linked and interacting homeobox genes that are directly related to the Hox classes of *Drosophila* and mammals and that, through their combined actions, are primarily responsible for patterning most or all tissues along the anterior-posterior body axis (cf. [8, 12, 13]).

Cnidarians represent a key transition in the evolution of animal complexity and are therefore critical to understanding the origins of developmental mechanisms such as the Hox system. Although they are among the simplest of true animals at the morphological level, the Cnidaria is among the most taxon-rich phyla and cnidarians have many of the genes traditionally assumed to have arisen in the context of vertebrate complexity [14–17]. Cnidarians have genes clearly related to a number of the key homeobox gene families of bilateral animals, such as Emx, Evx, Hex, Not, and Dlx [18], and some of these are expressed in patterns strikingly like those of their putative bilaterian orthologs (reviewed in [19]). In addition, Hox-like genes have been identified in a wide variety of cnidarians (e.g., [18]) but, in contrast to a number of other key regulatory gene types, their status is often equivocal.

In an attempt to clarify the evolutionary origins of the Hox system, we characterized the Hox-like genes in two representative cnidarians, *Eleutheria dichotoma* (Hydrozoa) and *Nematostella vectensis* (Anthozoa), in terms of sequence relationships, genomic organization, and expression patterns. *Eleutheria* is a typical cnidarian in having both polyp and medusa lifecycle stages, while *Nematostella* represents the basal cnidarian class (Anthozoa). Genes related to the anterior Hox and posterior Hox/Cdx types of bilaterians are present, but most of the Hox-like genes present in cnidarians postdate the Cnidaria/Bilateria split. The organization of these genes differs between the two cnidarians, and we found no evidence for the clustered organization characteristic of true Hox genes. Patterns of expression of the corresponding genes also differ dramatically between *Nematostella* and *Eleutheria* and across a range of other cnidarians. The cnidarian genes therefore do not conform to the Hox paradigm in terms of structure, organization, or expression, and the simplest interpretation of these observations is that the Cnidaria predate the origins of the Hox system. Thus, contrary to expectations, a definitive Hox system is not essential for axial patterning in lower animals. Moreover, the spectacular range of morphological variation across the Cnidaria demonstrates that the canonical Hox system is not mandatory for the elaboration of a wide range of variations on a basic body plan.

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Figure 1. Phylogenetic Analyses Identify Some Cnidarian Hox-like Genes as Relatives of Anterior Hox or Posterior Hox/Cdx Types of Bilateria, but "Intermediate" Genes Are Missing

Numbers on branches reflect the percentages of 1000 ML bootstrap replicates supporting the topology shown. Bayesian posterior probability values are shown below some of the critical nodes. *Nematostella* sequences are coded in red, those from *Eleutheria* in blue. As it is not clear what might be the most appropriate outgroup, and the nature of the outgroup to some extent determines internal topology (since it affects the position of the root), analyses were unrooted. However, for clarity, the tree is shown as if rooted via the *Podocoryne carnea* Gsx sequence (Gsx_Pc; encoded by GenBank #AAG09805), which is the ortholog to *Nematostella* anthox2. Note that although some cnidarian sequences are related to the "anterior" (group 1) Hox or posterior Hox/Cdx classes of bilateral animals, true intermediate (groups 2–8) Hox genes are clearly resolved from the cnidarian sequences. Conversely, independent duplications have increased Hox-like gene complexity within the Cnidaria.

Phylogenetic Analyses Reveal No Clear Orthologies to True Hox Classes

When the Gsx-type genes (which are clearly distinct) are excluded, a total of four Hox-like genes are present in *Eleutheria* [20, 21], and five genes of this type have previously been identified in *Nematostella* [22]. In the present study, we identified three novel Hox-like genes by analysis of the unassembled genomic sequence data now available for *Nematostella*. One of these, designated as anthox8A, encodes an identical homeodomain to anthox8 but differs significantly outside this. In addition, a gene related to anthox6 (anthox6A) and a highly derived gene (anthox9) were identified. The derived position of anthox9 is reflected in its position in the phylogenetic trees; note that although its predicted sequence has the Hox-like characteristic Glu residue at homeodomain position 15, the Ile residue at position 16 appears to be without precedent in the Antp superclass (only LIM and atypical homeodomains have anything other than a Leu residue at position 16 [23]).

To investigate relationships between the cnidarian Hox-like genes and the true Hox classes of higher animals, phylogenetic analyses were conducted with all of available sequences from *Nematostella* and *Eleutheria* (Figure 1 and Supplemental Data available with this article online). The results confirm several key aspects of previously published studies [7, 13, 18, 24] but do not support others. *Nematostella* anthox1/1A and anthox7/8/8A have been duplicated independently, as have several other homeobox gene loci in *Acropora* [25]. Clear support was obtained for common origin for anterior Hox genes and a class of cnidarian genes related to *Nematostella* anthox6 that also includes anthox6A and *Eleutheria* Cnox-5ed. Our analyses also confirm the high similarity between a group of cnidarian genes that includes *Eleutheria* Cnox-4ed and posterior Hox genes, although interestingly *Nematostella* appears not to have a gene of this type. Here we refer to the classes of cnidarian genes that are related to the anterior and posterior Hox/Cdx groups as the "anterior Hox-like" and "posterior Hox/Cdx-like" types, respectively. However, whereas the affinities of these gene types are well supported, other cnidarian Hox-like genes have no clear relationship to true Hox classes. Whereas orthology relationships have sometimes been suggested, a simpler interpretation is that the common ancestor had genes that later gave rise to the anterior and posterior Hox genes, but that intermediate Hox genes

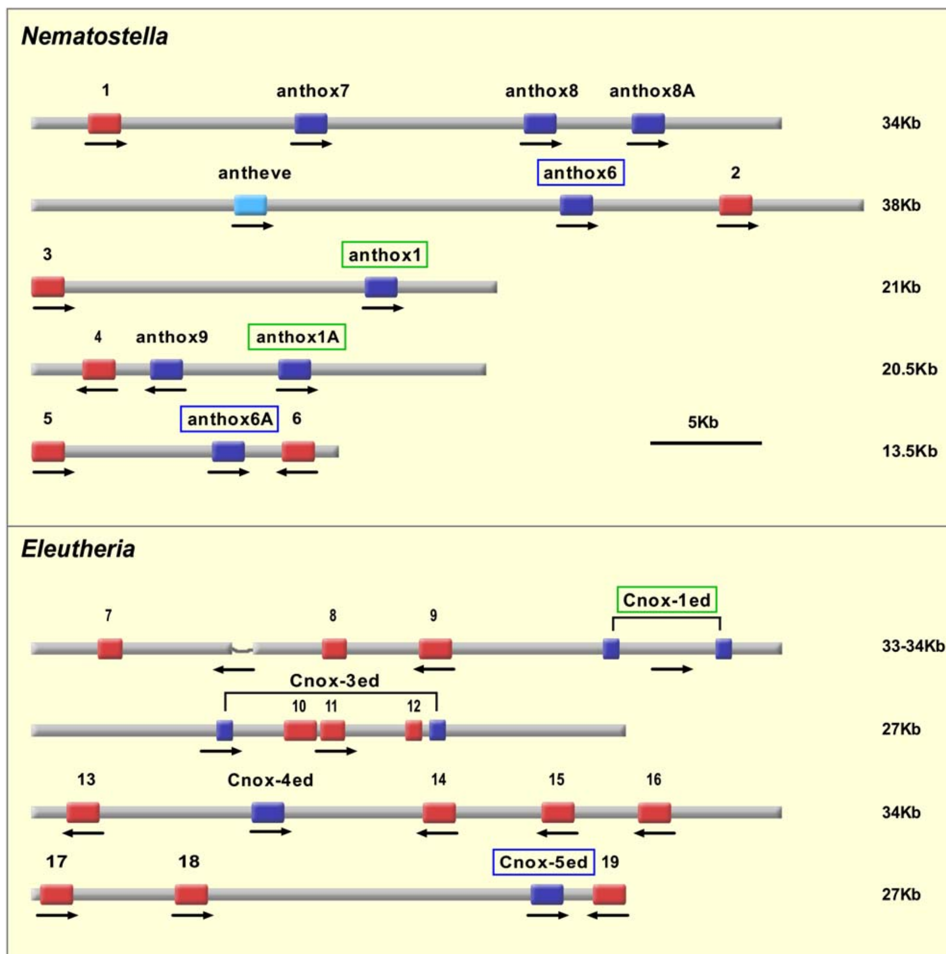

Figure 2. The Hox-like Genes of *Nematostella* and *Eleutheria* Are Organized Differently and Do Not Reflect the Clustered Patterns Characteristic of True Hox Genes

The figure summarizes schematically the genomic organization of Hox-like genes in *Nematostella vectensis* and *Eleutheria dichotoma*. In the case of *Nematostella,* contigs were assembled from GenBank whole-genome shotgun trace files, whereas *Eleutheria* contigs represent genomic fosmid clones. Rectangles show the position of the genes in the genomic context but only approximately represent the sizes of the genes. Details of intron-exon structure have been omitted except in the cases of Cnox-1ed and Cnox-3ed, both of which contain large introns. Arrows show transcriptional orientation of the genes. Hox-like genes are in dark blue with probable orthologs between *Nematostella* and *Eleutheria* framed with the same color. Hox-related genes are in light blue and non-hox genes are in red and numbered: 1, retrotransposon; 2, Rho/Rsa-related gene; 3, putative metalloproteinase inhibitor; 4, putative ANF-receptor; 5, Phosphatidylinositol phosphate kinase; 6, putative lam_G domain; 7, 8, 10–12, fragmented reverse transcriptases; 9, homolog to *Danio rerio* putative protein; 13, reverse transcriptase; 14, resembles metabotropic glutamate receptor; 15, two or more ORFs resembling *Homo sapiens* put. protein; 16, last exon of RRN3; 17, Dfp domain gene; 18, POU gene; 19, 1.2 Kb ORF resembling *Plasmodium* MAEBL (interrupted by vector).

(and hence Hox clusters) postdate the split between cnidarians and bilaterians.

Cnidarian Hox-like Genes Are Not Clustered and Their Organization Is Not Conserved within the Phylum

Because the colinear and uninterrupted structure of Hox clusters has been conserved across the Bilateria, we examined the genomic organization of the Hox-like genes in both *Eleutheria* and *Nematostella*—by fosmid cloning in the case of *Eleutheria* and by assembling genomic contigs from GenBank for *Nematostella*. Figure 2 summarizes mapping data for each of these genes.

anthox6 and *Eleutheria* Cnox-5ed are probable orthologs (Figure 1), as are anthox1 and Cnox-1ed, but all of these are flanked by unrelated non-Hox genes in the respective genomes. anthox6A and Cnox-4ed again are flanked by unrelated genes. As in another anthozoan [26], an even-skipped gene is tightly linked to anthox6 in *Nematostella*. This example of tight linkage is in contrast with the mapping data presented for other Hox-like genes and implies that if an equivalent of a Hox cluster were present, we might reasonably expect to have found other linked genes in the range of the assembled contigs. Although we found no evidence for organization characteristic of true Hox genes, the independently duplicated Hox-like genes anthox7, anthox8, and anthox8A (Figure 1) all lie within approximately 20 kb and in the same orientation. Although anthox1A and anthox9 are linked, these may also be paralogs even though the latter sequence is highly derived (the Ile/Leu substitution at position 16 suggests that anthox9 might even

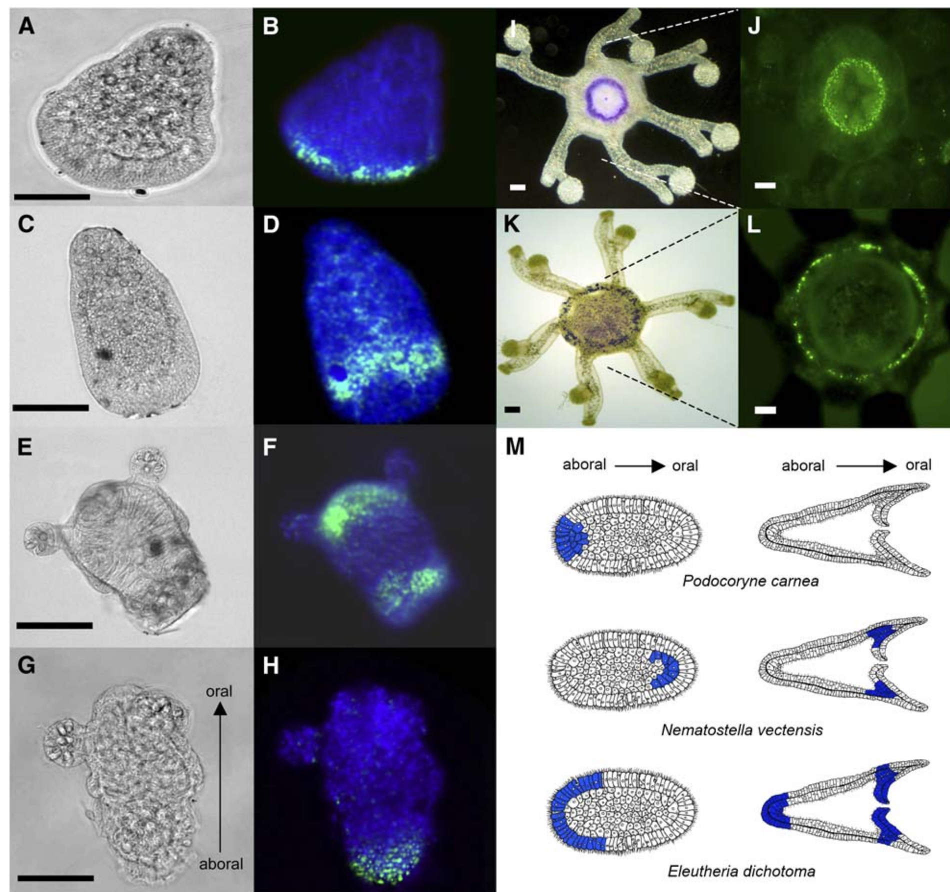

Figure 3. Expression Patterns of Related Hox-like Genes Are Heterogenous among Cnidarians and Do Not Conform to Colinearity Rules

The "anterior Hox-like" genes (= Cnox-5ed in *Eleutheria*, anthox6 in *Nematostella*, and Cnox-1pc in *Podocoryne*) provide a striking example of heterogeneity in expression patterns. In planulae, these orthologs are expressed ectodermally and aborally (*Eleutheria*; [A–D, M]), entodermally and orally [22] (*Nematostella*; [M]), or aborally in both ecto- and endoderm (*Podocoryne* [38]; [M]). In polyps, the corresponding genes are not expressed in *Podocoryne*, but are expressed both orally and aborally in *Eleutheria* (E, F, M) and at the oral end only in *Nematostella* (M).

(A–F) Cnox-5ed in 3-day-old planula larvae (A, B), 4- to 5-day-old planula larvae (signal moves toward the center as an ectodermal ring; [C, D]), and in young primary polyps (oral and aboral; [E, F]).

(G and H) Aboral expression of *Cnox*-4ed in a 10-day-old primary polyp.

(I and J) Ectodermal oral expression of *Cnox*-3ed around the manubrium of a medusa.

(K and L) Ectodermal oral Cnox-1ed expression in the "Cnidoblast channel" of the medusa stage. NBT/X-phosphate (I, K) or fluorescein-labeled probes (B, D, F, H, J, L). Signals in (B), (D), (F) and (H) are overlayed with DAPI staining. Morphologies are shown in light microscopy (A, C, E, G). Scale bar equals 50 μm.

be a pseudogene); there is no support for the alternative hypothesis that these are orthologs of different Hox classes. The linkage of paralogous homeobox genes has clear precedents [25] and should not be confused with the clustering characteristic of true Hox genes. In a true Hox cluster, no non-Hox genes lie within the cluster, so the identification of neighboring non-Hox genes implies that the Hox-like genes are not clustered in *Eleutheria* and *Nematostella*. These observations are consistent with a previous study in which the corresponding *Hydra* genes were shown not to be linked within a range of 150 kb [18]. Hence, although paralogous genes are in some cases linked, three representative cnidarians lack any equivalent of a true Hox cluster.

Noncolinear Expression of Cnidarian Hox-like Genes

Although the cnidarian Hox-like genes are not organized in clusters, preservation of tight linkage and uninterrupted organization appears to be strictly required only for the maintenance of temporal colinearity [7, 27–29]; for example, in the derived tunicate *Oikopleura*, the remaining Hox genes are expressed in a spatially colinear fashion despite the cluster having completely fragmented [30]. If an ancestral Hox cluster had also been fragmented in cnidarians, then conservation of spatial colinearity would be predicted. To test this hypothesis, the spatial expression patterns of *Eleutheria* Hox-like genes were determined and compared with the corresponding *Nematostella* genes [22]. The most informative direct comparisons of expression patterns can be made in the developing planula larvae (which most likely reflects the phylotypic stage). In *Eleutheria*, only Cnox-5ed is expressed in planulae; as the *Podocoryne* ortholog (Cnox-1pc; Figure 3M), this "anterior Hox-like" gene is expressed at the aboral end (i.e., the front end with respect to swimming direction; Figures 3A–3D). In the polyp, Cnox-5ed is expressed at both aboral and oral ends (Figures 3E and 3F), and Cnox-4ed is

expressed aborally (Figures 3G and 3H). Cnox-3ed and Cnox-1ed are expressed exclusively in the medusa, in ectodermal regions around the mouth and manubrium (Figures 3I–3L). The embryonic and larval expression patterns of the *Nematostella* Hox-like genes differ markedly to those of their *Eleutheria* counterparts. anthox6 corresponds to *Eleutheria* Cnox-5ed but is expressed at the opposite end of the planula, in the invaginated endoderm at the oral extremity [22]. *Nematostella* lacks a "posterior Hox/Cdx-like" gene; anthox1, which is most similar to Cnox-1ed, is expressed in the ectoderm at the aboral extremity of the *Nematostella* planula. anthox1A, 7, and 8 are expressed in the endoderm along one side of the body column [22], but not in overlapping patterns like those of true Hox genes. Moreover, as is clear not only in our analyses (Figure 1) but also in previous studies [22], those genes with axially restricted expression patterns in *Nematostella* (anthox1/1A and anthox7/8) have been independently duplicated in the Cnidaria, and hence any apparent similarities in expression patterns cannot reflect conservation of function with bilateral animals. In summary, expression patterns of related genes differ dramatically across the Cnidaria, and there is no evidence to support conservation of function with true Hox genes.

Implications for the Origin of Bilaterian Hox Clusters

Whereas the consensus view has been that a Hox cluster was present in the ancestral cnidarian (e.g., [13]), our analyses of sequence relationships, gene organization, and expression data indicate that definitive Hox clusters are not present in cnidarians and are therefore a synapomorphy of the Bilateria. The situation in cnidarians is therefore very different to that even in very derived members of the Bilateria. For example, whereas in urochordates the ancestral Hox cluster has fragmented, the individual genes show high levels of sequence identity and similar (A/P-restricted) patterns of expression to their orthologs in other bilaterians [30]. In cnidarians, not only are the genes dispersed, but also there are no clear relationships in terms of expression patterns or sequence identity. Cnidarians have genes related to anterior and posterior Hox/Cdx genes, but most of the Hox-like genes present are likely to postdate divergence with the bilaterian line, accounting for their unclear relationships to true Hox classes. The Hox cluster presumably arose from the outside in [31]—from a two-gene state via a series of unequal crossing-over events—and the cnidarian "anterior Hox-like" and "posterior Hox/Cdx-like" types may be derived from these ancestral two outer genes. A similar "two-gene" model of Hox cluster origin in Bilateria has been proposed recently to accommodate conflicting views about the nature of cnidarian Hox-like genes [8]. Moreover, whereas the linkage of even-skipped and Hox-like genes in anthozoans [22, 26] (Figure 2) has been interpreted as evidence for a Hox cluster in the common ancestor of Cnidaria and Bilateria [13], our data rather imply that this linkage reflects an even older array of Antp superclass gene precursors [32, 33], predating the definitive Hox system. A survey of the trace archive indicates that most of the non-Hox Antp type genes are present in *Nematostella* (e.g., EHGbox, Evx, Mox, Dlx, Msx, Emx, NK; K.K. and B.S., unpublished data). Thus, the cnidarians must have split off the lineage leading to Bilateria after the ancestors of the Antp subclasses had emerged, but before having a canonical Hox system. The alternative (less parsimonious) scenario is that in those cnidarians examined to date, an ancestral cluster of Hox genes has fragmented, and both individual sequences as well as expression patterns diverged beyond recognition.

Conclusions

The Cnidaria is among the most species-rich and diverse of phyla, indicating that neither true Hox genes nor Hox clusters are strictly required for the elaboration of morphological diversity. Moreover, the Hox system is clearly not mandatory for axial patterning. In the absence of a Hox system, other genes may be able to pattern the primary body axis—for example, *Nematostella* has the full complement of Wnt genes and these may play major roles in patterning the O/A axis [16]. It is also possible that the Hox system represents an intercalation between the "head" and "tail" domains of the common metazoan ancestor of cnidarians and bilaterians [34]. In summary, the evidence implies that true clustered Hox genes evolved in UrBilateria after the Cnidaria diverged. Although it may have facilitated morphological diversification within the Bilateria, the spectacular range of shapes and forms shown by extant cnidarians—from microscopic solitary polyps to colonial siphonophores up to 40 m long—shows the extent of variation possible even in the absence of a Hox cluster.

Experimental Procedures

Library Construction for *Eleutheria dichotoma*

Fosmid libraries were constructed with the CopyControl Fosmid Library Production Kit (Epicentre), with some steps modified to achieve a better efficiency in library construction for this AT-rich species. The detailed protocol can be obtained from the authors upon request. The libraries were screened by PCR for Cnox-1 to -5 fosmid clones and the clones were isolated from the pooled libraries by outdilution. DNA from the clones was isolated and randomly sheared to 1–2 kb fragments. The fragments were end-repaired and ligated into plasmid vectors, and the resulting subclones were sequenced on a MegaBACE1000 system with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham). Sequences were assembled with the SeqMan software (DNAStar, Lasergene). Fosmid sequences were screened for potential genes by blastp and blastx.

Database Search and Contig Construction

To screen the *Nematostella* genome for new Hox-like genes, representative genes from cnidarians and *Amphioxus* were blasted against the *Nematostella* trace archive by means of Discontiguous Megablast. Positive hits were elongated and verified by retrieving and assembling (SeqMan) more sequences with Megablast.

For the construction of large contigs for *Nematostella* Hox-like genes, initial contigs assembled around known sequences were elongated by searching (Megablast) the trace archive by means of the contig ends (100–300 bp segments). The ends used for elongation had to be supported by at least three overlapping sequences. The total coverage of the contigs by sequence length was 10- to 16-fold (the trace files used for the respective contigs can be obtained from the authors upon request).

In Situ Hybridization

In situ hybridization for *Eleutheria dichotoma* was carried out as previously described [35].

Phylogenetic Analyses

The homeodomains encoded by all of the known Hox-like genes from *Nematostella* and *Eleutheria* were aligned with the full suites of *Amphioxus* and *Drosophila* Hox and "ParaHox" sequences (see [36]) and then subjected to Maximum Likelihood analyses with MolPhy version 2.3 as previously described [14] (the Dayhoff matrix was used). 1000 ML bootstrap replicates were used to test tree topology. In addition, Bayesian analyses were conducted to provide further support for aspects of the ML topology. For this purpose, we used the mixed model option in MrBayes version 3.0b4 [37] with the default setting of four Markov chains per run. The analyses were run for a total of 1.5 million generations, sampling every 1000th tree. Log Likelihood values reached a plateau after approximately 20,000 generations. One third of the resulting trees were discarded as the burn-in phase, and the remainder used to estimate posterior probabilities.

Supplemental Data

Supplemental Data include one figure, one table, and nine FASTA files of sequences and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/9/920/DC1/.

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Accession Numbers

Sequences of the *Eleutheria* genomic fosmids have been deposited into GenBank with the following accession numbers: Cnox-1ed DQ451870; Cnox-3ed DQ451871; Cnox-4ed DQ451872; Cnox-5ed DQ451873.

Sequences of the *Nematostella* genomic contigs were assembled from NCBI trace files and are available in Document S2 with the Supplemental Data online.

Note Added in Proof

This paper differs slightly from the version originally published online in that the accession numbers for the sequences of the *Eleutheria* genomic fosmids have been made available and are published here.