

ANCIENT ORIGIN OF THE HOX GENE CLUSTER

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The Hox gene cluster has a crucial function in body patterning during animal development. How and when this gene cluster originated is being clarified by recent data from *Cnidaria*, a basal animal phylum. The characterization of Hox-like genes from *Hydra*, sea anemones and jellyfish has revealed that a Hox gene cluster is extremely ancient, having originated even before the divergence of these basal animals.

BILATERIAN

An animal that shows bilateral symmetry across a body axis. Bilaterians include chordates, arthropods, nematodes, annelids, molluscs and others. Echinoderms are included in Bilateria, even though their adults show pentaradial symmetry, because they evolved from bilateral ancestors and their larvae have bilateral symmetry. All living bilaterians are also 'triploblasts'.

BASAL

An evolutionary lineage, or animal within a lineage, that arises close to the root or base within a phylogeny.

DIPLOBLAST

An animal with only two germ layers (ectoderm and endoderm), including the *Cnidaria*, *Ctenophora* and, according to some authors, *Placozoa* and *Porifera*.

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During animal development, the principal axes of the body must be patterned. Positional information must be imparted to different regions so they can be distinguished and so that they can follow the appropriate developmental programmes, allowing anatomical structures to develop in the correct place. In part, this requires a group of transcription factors that are encoded by genes that contain a homeobox (BOX 1). The best characterized of these are the Hox genes: a clustered family of homeobox genes whose distinctive genomic organization seems to be linked to their function. The pathways of Hox gene evolution are quite clear for BILATERIAN animals^{1,2}, but are far less so for more BASAL lineages, such as *Cnidaria* (jellyfish, *Hydra* and corals). The bilaterian data clearly show that a Hox gene cluster arose before the divergence of these 'higher' taxa; therefore, it is to the more basal taxa that we must turn if we are to understand the origins of Hox genes. Genes with sequence similarities to Hox genes have been cloned from various *Cnidaria*, but their relationships to bilaterian Hox genes are unclear. Furthermore, it has not been clear whether the cnidarian Hox-like genes are arranged in clusters. Recent data have greatly clarified the evolutionary relationships between bilaterian and cnidarian Hox-like genes and, in doing so, have pushed back the date for origin of a Hox gene cluster to before the cnidarian-bilaterian divergence.

Cnidarians are one of the most basal groups of animal phyla. They consist of four classes: the well-known Anthozoa (sea anemones, corals), Hydrozoa (hydroids) and Scyphozoa (jellyfish), plus the highly

dangerous Cubozoa (box jellyfish) (FIG. 1). Together with the *Ctenophora* (comb jellyfish), they comprise the DIPLOBLASTS. In metazoan phylogeny, the diploblast lineages arose before the 'higher' animals. The 'higher' animals (such as flies, worms and ourselves) comprise the Bilateria (or TRIPLOBLASTS) (FIG. 2). *Hydra* is probably the cnidarian best known to many biologists, because of its extensive use in experimental biology, particularly in studies of regeneration. *Hydra* is a DERIVED member of the phylum *Cnidaria*³⁻⁶, however, with several peculiar developmental and genomic characteristics. *Hydra* species have proved to be difficult organisms in which to study gene families such as homeoboxes, primarily because their large genome and unfavourably low G+C-content have made it difficult to synthesize and screen genomic libraries. More recently, molecular biologists have turned to other cnidarians, complementing the extensive *Hydra* data and providing some new insights. Homeobox genes have now been cloned from a phylogenetically wide spectrum of cnidarians, including representatives of the three principal classes: Hydrozoa, Anthozoa and Scyphozoa (FIG. 1). Now is a good time to draw the scattered information together and to compare the diploblast and bilaterian data.

Hox, ParaHox or ProtoHox?

The existence of cnidarian homeobox genes has been known for almost a decade⁷⁻⁹. Several of these genes have considerable sequence similarity to the Hox genes of 'higher' animals; indeed, these genes were originally assumed to be cnidarian Hox genes. However, as more

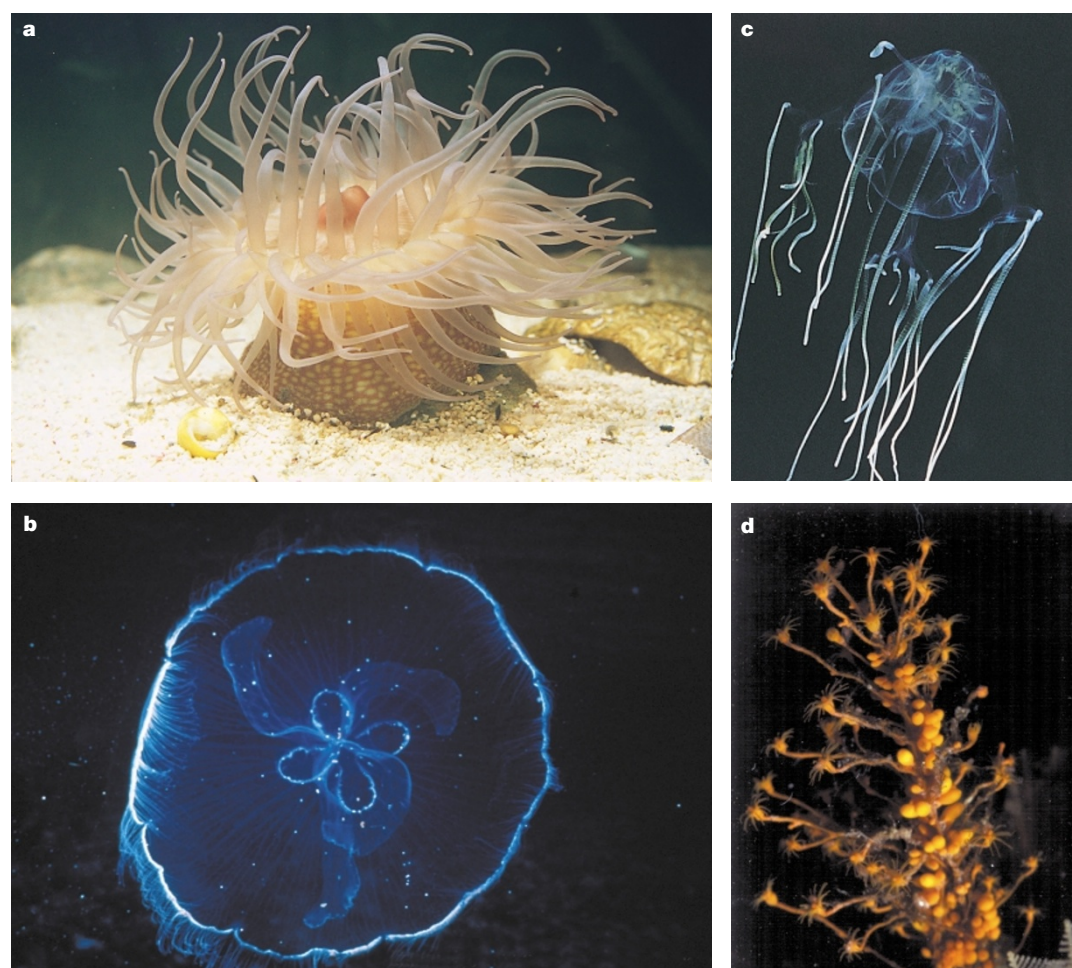


Figure 1 | **Cnidaria**. Representatives of each class of cnidian. **a** | Anthozoa (sea anemone, *Actinia fragacea*). Photo courtesy of Andy Horton at the [British Marine Life Study Society](#). **b** | Scyphozoa (moon jellyfish, *Aurelia aurita*). Photo courtesy of the Florida Keys National Marine Sanctuary. **c** | Cubozoa (box jellyfish) Photo courtesy of the World Life Research Institute, taken by Keith Gillet. **d** | Hydrozoa (hydroid; *Garveia annulata*). Photo courtesy of Ronald L. Shimek.

TRIPLOBLAST

An animal with three germ layers (ectoderm, mesoderm and endoderm).

DERIVED

Evolved to a state that is not like the primitive condition.

CHORDATE

A phylum of animals characterized by possession of a notochord (and post-anal tail, somites/myotomes and gill slits). It includes the urochordates (such as ascidians), cephalochordates (amphioxus) and vertebrates.

AMPHIOXUS

(literally, sharp at both ends.) The common name for the cephalochordate Branchiostoma, which is the closest living invertebrate relative of the vertebrates.

PROTOHOX

Hypothetical homeobox gene cluster that duplicated to produce the Hox and ParaHox gene clusters.

homeobox genes were cloned from bilaterian taxa (particularly from CHORDATES and insects), it was gradually realized that Hox genes cannot be defined solely on the basis of DNA sequence. Even in careful molecular phylogenetic analyses using chordate and insect homeoboxes, Hox genes do not form an exclusive set, separate from all other classes of homeobox gene^{10–12}. Instead, there are a few other homeobox genes that are just as closely related to Hox genes as some of the latter are to themselves. Examples include genes of the Gsx, Xlox and Cdx classes. On the basis of DNA sequence alone, these could be mistakenly classified as Hox genes, but they do not reside within the Hox gene clusters of any animal examined. This paradox was clarified by Brooke *et al.*¹¹, who reported that the Gsx, Xlox and Cdx genes form their own gene cluster in the chordate AMPHIOXUS. This gene cluster — termed ParaHox — seems to be an evolutionary sister of the Hox gene cluster. Molecular phylogenetic analyses indicate that the Hox and ParaHox clusters might have arisen by duplication and divergence from a more ancient — and as yet hypothetical — PROTOHOX cluster.

How do these data from **amphioxus** relate to Cnidaria? They imply that although cnidian Hox-like genes could indeed be true Hox genes, or ParaHox genes, they could alternatively be closer to the hypothetical pre-duplication state (ProtoHox). Brooke *et al.*¹¹ did not answer this question definitively, but postulated that cnidarians probably diverged before the duplication event. As more cnidian homeobox sequences have been obtained, and further phylogenetic methodologies brought to bear, this question has been revisited. Recently, two groups have independently published analyses that strongly support the idea that cnidarians diverged after the ProtoHox stage of evolution^{10,13}. In other words, cnidarians have genes that are true Hox and ParaHox genes.

The conclusion that cnidarians possess distinct Hox and ParaHox genes has been controversial, and there are dissenting views¹⁴. In part, this dissent reflects differing hypotheses about the origins of sequence similarity, and the amount of significance to attach to low levels of sequence identity. Our own

Box 1 | What are homeobox, Hox and ParaHox genes?

In his landmark book *Materials for the Study of Variation*, Bateson²⁸ defined the term 'homeosis' to describe the transformation of one body part into the likeness of another. During the emergence of *Drosophila melanogaster* genetics in the twentieth century, several genes were identified that, when mutated, gave homeotic phenotypes. Most of these 'homeotic genes' were found to be arranged in two gene complexes: the Antennapedia and Bithorax complexes^{29–31}. These *Drosophila* genes were all subsequently found to contain a 'homeobox': a 180 bp sequence that encodes the homeodomain protein motif³². The homeodomain is capable of binding DNA, allowing homeobox genes to encode transcription factors. Homologues of homeotic genes, known as 'Hox genes', were quickly found in diverse animals, including vertebrates such as ourselves³³. The Hox genes of vertebrates, similar to those of *Drosophila*, are clustered in the genome. Furthermore, they show co-linearity: the genes at the 3' end function in the anterior of the embryo, the central genes function in the middle of the embryo, and the 5' genes control the development of the posterior of the embryo^{34,35}. Hox gene clusters have now been discovered in other insects^{36–38}, a nematode³⁹, a ribbon worm⁴⁰, amphioxus⁴¹ and sea urchins^{18,42}. Clustering of Hox genes is therefore widespread throughout diverse animal phyla and is likely to be a feature of functional significance in animals.

The homeobox gene family is large and diverse, including many genes in addition to Hox genes. When phylogenetic trees are constructed of the homeobox gene family, based on protein sequence, several genes are found that are particularly closely related to Hox genes, but that do not map within Hox gene clusters (for example, Gsx, Xlox, Cdx and Mox)¹⁰. Of these, Gsx, Xlox and Cdx were found to form a cluster themselves, in amphioxus and probably mammals^{11,43}. Brooke *et al.*¹¹ called this the 'ParaHox' gene cluster, and proposed that an ancestral animal possessed a 'ProtoHox' cluster, which duplicated to produce the separate ParaHox and Hox gene clusters (see lower half of FIG. 3).

unpublished analyses of cnidarian sequences using NEIGHBOUR-JOINING methods reach the same conclusions as Finnerty and Martindale¹³, and Gauchat *et al.*¹⁰: that Cnidaria do have true Hox and ParaHox genes (see TABLE 1 for a summary). A definite Gsx gene sequence is present in a wide range of cnidarians, and both a Cdx gene (also a ParaHox) and Hox genes are probably also present. This implies that the duplication of the hypothetical ProtoHox cluster, which resulted in formation of definitive Hox and ParaHox genes, occurred before the divergence of cnidarians from the bilaterian lineage.

How old are clusters?

Problems of assigning gene homologies on the basis of weak sequence similarity alone can be aided by also considering genomic organization. In particular, physical clustering is a key character of bilaterian Hox genes. It is also a property of ParaHox genes, at least in amphioxus. If cnidarians do indeed have Hox and ParaHox genes, surely we would expect these genes to form physical clusters in the genomes of cnidarians? Although this is a realistic expectation, proof has been extremely elusive. Eight years ago, Miller and Miles¹⁵ published a tantalizing paper showing physical linkage of one Hox-like gene to an Evx class homeobox gene in the coral *Acropora formosa*. This was a highly suggestive finding, because two of the mammalian Hox gene clusters are also linked to Evx class genes. Furthermore, the coral Hox-like gene in question, *antpC*, is most likely to be a true Hox gene (as opposed to a ParaHox gene), as judged by molecular phylogenetics (TABLE 1). Although encouraging, this report did not find linkage between any two Hox-like genes.

Now, from a different species of anthozoan, the elusive linkage data have been obtained. Working with the sea anemone *Nematostella vectensis*, Finnerty and Martindale have detected linkage between two Hox-like genes and an Evx class homeobox gene (personal communication; reported by Pennisi¹⁶). Is this a

cnidarian Hox gene cluster? Two lines of evidence indicate that it might be. First, the linked Hox-like genes of *Nematostella* have both been assigned as Hox genes by phylogenetic analyses (TABLE 1). Second, *Nematostella* also has two genes that are deduced to be

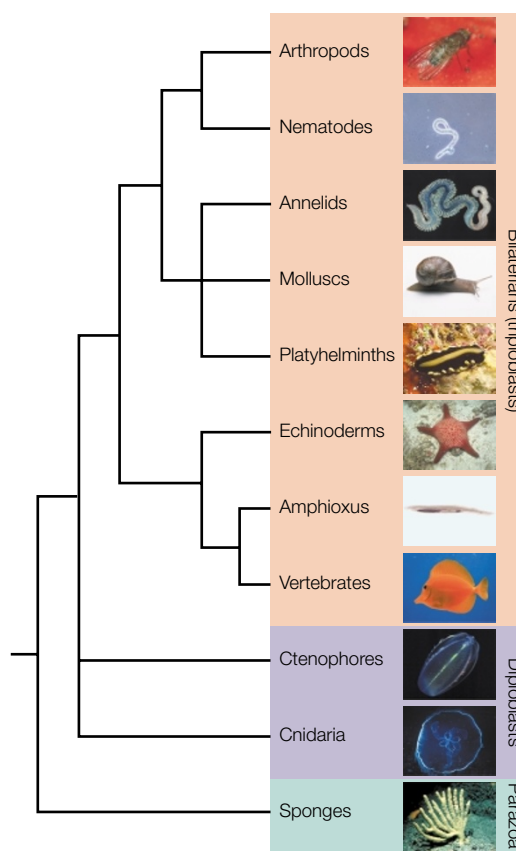


Figure 2 | **Metazoan tree.** A phylogeny of selected metazoans based on ribosomal DNA and Hox gene sequences. Image modified with permission from REF. 44 © (1999) Macmillan Magazines Ltd. Platyhelminth photo courtesy of Allen G. Collins, University of California Museum of Paleontology. Ctenophore photo © Jim Mastro.

NEIGHBOUR-JOINING
A distance-based molecular phylogenetic method involving sequential addition of taxa and minimization of branch lengths that does not assume a molecular clock.

Table 1 | Orthology assignment of cnidarian genes

Class	Species	Gsx/ Hox1–2	Xlox/ Hox3	Hox4–8	Cdx/ Hox9+	References
Anthozoa	<i>Nematostella vectensis</i>	<i>Anthox2</i>				12, 13, 45
		<i>Anthox6</i> <i>Anthox7</i>		?←	<i>Anthox1</i> <i>Anthox1a</i>	
Anthozoa	<i>Metridium senile</i>	<i>Anthox2</i>			<i>Anthox4</i> ?← <i>Anthox1</i>	12, 13, 45
Anthozoa	<i>Acropora formosa</i>					15
		<i>antpC</i>				
Scyphozoa	<i>Cassiopeia xamachana</i>	<i>Scox2</i>				46
				?←	<i>Scox3</i> <i>Scox1</i> <i>Scox4</i> <i>Scox5</i>	
Hydrozoa	<i>Chlorohydra viridissima</i>	<i>Cnox2</i>				9
		<i>Cnox1</i>				
Hydrozoa	<i>Hydra magnipapillata</i>	<i>Cnox2</i>				47
		<i>Cnox4</i>		?←	<i>Cnox1</i>	
Hydrozoa	<i>Eleutheria dichotoma</i>	<i>Cnox2</i>			<i>Cnox4</i>	8, 48
		<i>Cnox5</i>		?←	<i>Cnox1</i> <i>Cnox3</i>	
Hydrozoa	<i>Hydra vulgaris</i>	<i>Cnox2</i>				49
Hydrozoa	<i>Hydractinia symbiolongicarpus</i>	<i>Cnox2</i>				50
Hydrozoa	<i>Podocoryne camea</i>				17, 51	
		<i>Cnox1</i>		?Cnox2?		

Putative homologies between cnidarian Hox and ParaHox genes and their bilaterian counterparts. Pink rows are ParaHox genes (Gsx, Xlox and Cdx); yellow rows are Hox genes (Hox1–2, Hox3, Hox4–8 and Hox9+, adopting the numbering system used for chordates). (?=possible homology with middle Hox genes rather than posterior Hox genes (as judged from neighbour-joining trees) (?Cnox2? in *Podocoryne* denotes its extremely uncertain positioning.)

ParaHox genes. These data therefore support the idea that cnidarians do indeed have Hox genes, and that these genes are arranged in gene clusters, at least in some species. So, clustering of Hox genes is ancient.

Composition of the first Hox gene cluster

The model in FIG. 3 proposes that an ancestral ProtoHox cluster contained four Hox-like genes, plus Evx. This then duplicated to yield the definitive Hox and ParaHox gene clusters¹¹. By incorporating the cnidarian data, this event dates to much earlier in evolution than the original suggestion of Brooke *et al.* and is an important contribution to our understanding of Hox gene cluster origins. The colour coding of FIG. 3 reflects the inferences drawn from molecular phylogenetic analyses. So, Gsx had a common ancestor with Hox1–2, Xlox with Hox3, and Cdx with the posterior Hox groups 9+ (REF. 11). Furthermore, the chromosomal ordering of the genes is maintained in both clusters, with the anterior-expressed genes at one end, followed by the middle-expressed genes, and the posterior-acting genes at the other end (‘spatial co-

linearity’). As shown in FIG. 3, distinct Hox and ParaHox genes existed in the common ancestor of Cnidaria and Bilateria.

One difficulty that is not accommodated by this scheme is the apparent absence of group 3 Hox and group 3 ParaHox (that is, Xlox) genes in cnidarians. Molecular phylogenetics indicates that a group 3 gene may have been present in the ProtoHox gene cluster, pre-dating the origin of the Hox gene cluster. Unless group 3 Hox and ParaHox genes are yet to be discovered in cnidarians, we conclude that these genes were secondarily lost in the cnidarian lineage.

A second issue that remains unresolved at present is the origin of the ‘middle’ Hox genes. No cnidarian gene has been found with unambiguous homology to the middle Hox genes (corresponding to DEUTEROSTOME Hox groups 4–8, or PROTOSTOME Deformed–abdominal A (Dfd–abdA) groups). One intriguing candidate is *Cnox2-Pc* (whose name does not denote homology with the other cnidarian *Cnox2* genes, but rather the chronology of its isolation). This gene may have some affinities with *Hox4/Dfd* genes¹⁷, but this is far from certain (see **supplementary FIG. 1** online and TABLE 1). The lack of unambiguous cnidarian middle Hox genes raises an important question. Does the origin of middle group Hox genes pre- or post-date the divergence of the cnidarian and bilaterian lineages? In FIG. 3, we depict the former possibility, with gene loss occurring in the Cnidaria. A related question is whether the lack of a middle ParaHox gene in any animal also represents the derived condition (FIG. 3). An alternative view that accommodates both observations is that lack of a ‘middle’ gene is the ancestral ProtoHox condition. In this view, the middle genes may have arisen only in the Hox gene cluster, specifically in the bilaterian lineage¹³.

Cnidarians are diverse

Is the morphological diversity of Cnidaria (FIG. 1) reflected in the diversity of the Hox/ParaHox genes? The scheme in FIG. 3 shows some important differences in gene composition among the Cnidaria. These include two anterior Hox genes in *Nematostella* (sea anemone) and possible duplication of posterior Hox genes in *Cassiopeia* (jellyfish). Clearly, there is a propensity for further Hox gene duplications in Cnidaria. This is not unprecedented, as Hox gene gain and loss has been reported in many bilaterian lineages. Examples include loss of a group 4/5 Hox gene in sea urchins, loss of group 7 Hox genes in pufferfish, loss of multiple Hox genes after cluster duplications in vertebrates, expansion of the posterior Hox genes in deuterostomes, and tandem duplication of group 3 Hox genes in insects^{18–22}. So, although Hox gene clusters have been widely conserved, their precise gene composition is variable.

Some remaining questions

Strong statements about homologies between cnidarian and bilaterian homeobox genes are difficult owing to the divergent nature of some of the

DEUTEROSTOME
A bilaterian animal whose mouth forms as a secondary opening, separate from the blastopore. Deuterostomes include chordates, hemichordates and echinoderms.

PROTOSTOME
A bilaterian animal whose mouth and anus develop from the same invagination (the blastopore) during embryogenesis

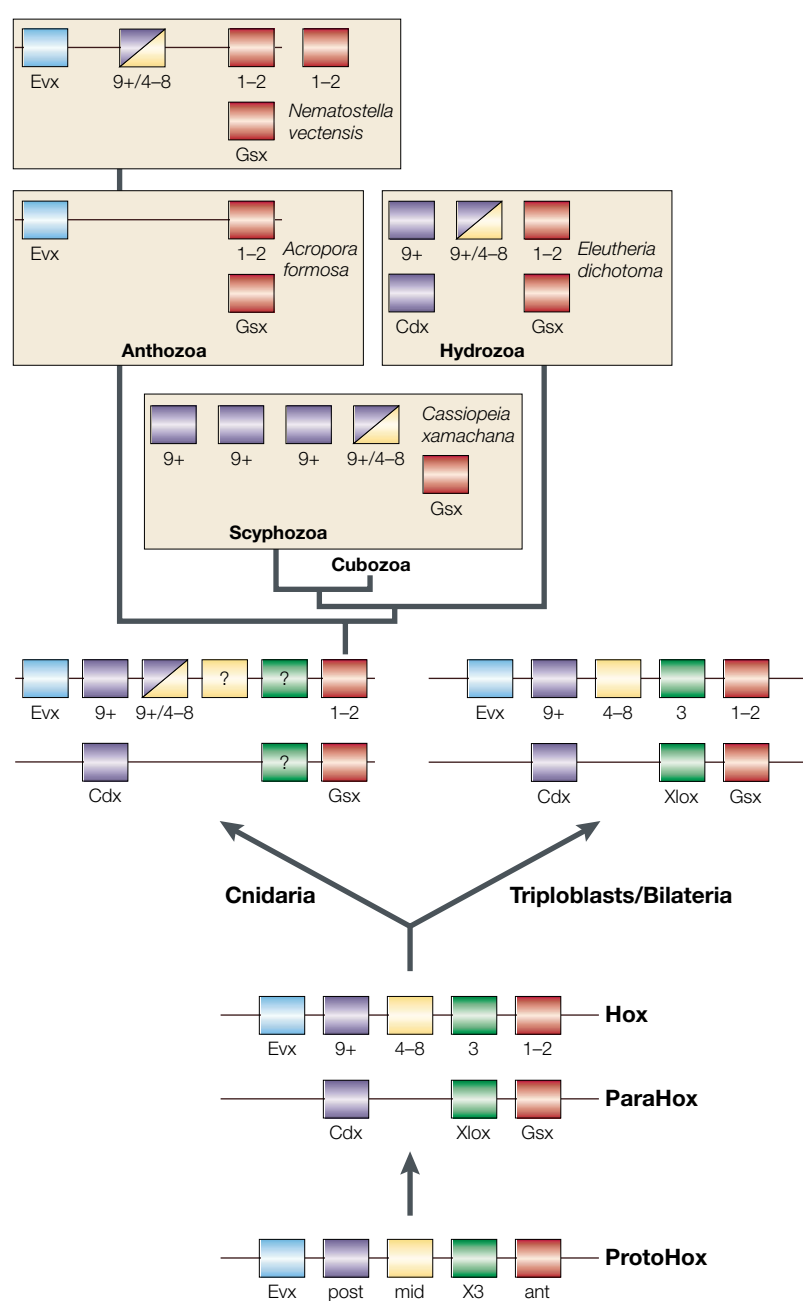


Figure 3 | Hox/ParaHox gene cluster evolution. The lower half of the figure shows hypothetical clusters; the upper half (pink boxes) shows the cnidarian data, superimposed on a phylogeny of the cnidarian classes. Continuous lines denote physical linkage, whereas the absence of lines indicates that linkage has not been shown. The figure proposes that the ancestral ProtoHox cluster had four Hox-like genes (post, posterior; mid, middle; X3, Xlox-Hox3; ant, anterior), plus Evx. The Hox and ParaHox clusters arose by duplication of the ancestral ProtoHox cluster. The colour coding reflects the phylogenetic relationship between sequences (for example, Gsx in the ParaHox cluster and Hox groups 1–2, in red, both derive from ant (anterior) in the ProtoHox cluster). The bilaterian clusters are representative of those of vertebrates (Hox) and cephalochordates (ParaHox). Some cnidarians have several genes in certain classes, such as two group 1–2 genes in *Nematostella vectensis* and at least three group 9+ genes in *Cassiopeia xamachana*. The ‘9+/4–8’ genes represent the ambiguous classification of these genes. No group 3 Hox or ParaHox genes (green) have been found in Cnidaria. There is uncertainty over whether middle Hox genes (yellow) were in the ancestral ProtoHox cluster, and have been lost in Cnidaria.

sequences. Therefore, although it is possible to paint the broad picture, every single detail in the proposed evolutionary scheme may not be correct. Homology assignments can be aided by linkage data, and also by consideration of sequence characters outside the homeodomain. One example is the HEP (H2.0, Engrailed, Paired) motif^{23,24}, a transcriptional repressor domain that is remarkably similar in the sequence of cnidarian Cnox2 and Gsx¹³ (supplementary FIG. 2 online). Therefore, more sequence data from cnidarian homeobox genes and more linkage information are needed to confirm — or reject — the details of the scheme that we present here.

It also remains to be clarified whether the cnidarian ParaHox genes are linked into a cluster distinct from the Hox genes. This question could be readily addressed in either the anthozoan *Metridium senile* or the hydrozoan *Eleutheria dichotoma*, as two ParaHox genes have been cloned for each of these cnidarian species (Gsx and a putative Cdx; TABLE 1). And last, if we accept that Cnidaria do have distinct Hox and ParaHox genes, when did they arise from the ProtoHox cluster? Are there any extant groups of animal that diverged before this event? The sister group to the diploblasts and triploblasts is the Porifera, or sponges⁵ (FIG. 2). Do these animals possess true Hox/ParaHox genes, or do they possess Hox-like genes descended from the ProtoHox stage? Only one Hox-like gene fragment has been reported from a sponge²⁵; therefore, it is not possible to answer this question definitively. Furthermore, this sequence looks remarkably like an ascidian posterior Hox gene, so there is a real possibility that it represents a contamination, perhaps from a colonial ascidian growing in association with the sponge. It may be relevant that other groups have not identified any Hox-like genes in another sponge species, despite considerable effort²⁶. If sponges will not give us insight into the pre-Hox condition, what about the other principal diploblast phylum, Ctenophora? The only published Hox-like gene fragment from a ctenophore has a strong similarity to a middle Hox gene²⁷. This is an interesting finding, especially as definitive middle-class Hox genes have not been found in Cnidaria (TABLE 1). This gene sequence indicates that Ctenophora, like Cnidaria, may have diverged after the origin of true Hox genes.

Whatever the ancestor of jellyfish and ourselves looked like, we can be sure that it had a Hox cluster.

Update — note added in proof

Acropora formosa is synonymous with *Acropora muricata* according to REF. 52.

Links

DATABASE LINKS Amphioxus

FURTHER INFORMATION An introduction to cnidarian phylogeny and classification | A useful list of cnidarian links and resources on Dr R. Steele's homepage

ENCYCLOPEDIA OF LIFE SCIENCES Evolutionary developmental biology: Hox gene evolution

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