Current Biology 22, 1951–1956, October 23, 2012 ©2012 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2012.08.023

Report

Ghost Loci Imply Hox and ParaHox Existence in the Last Common Ancestor of Animals

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Summary

Hox genes are renowned for patterning animal development, with widespread roles in developmental gene regulation. Despite this importance, their evolutionary origin remains obscure, due to absence of Hox genes (and their evolutionary sisters, the ParaHox genes) from basal lineages and because the phylogenies of these genes are poorly resolved [1–7]. This has led to debate about whether Hox and ParaHox genes originated coincidently with the origin of animals or instead evolved after the divergence of the earliest animal lineages [7, 8]. Here we use genomic synteny and Monte Carlo-based simulations to resolve Hox/ParaHox origins, our approach being independent of poorly resolved homeodomain phylogenies and better able to accommodate gene loss. We show *Trox-2* of placozoans occupies a ParaHox locus. In addition, a separate locus sharing synteny and hence homology with human Hox loci exists in the placozoan genome, but without a Hox-like gene in it. We call this second locus a "ghost" Hox locus, because it is homologous to the human Hox loci, but does not itself contain a Hox gene. Extending our approach to sponges, we discover distinct ghost Hox and ParaHox loci. Thus, distinct Hox and ParaHox loci were present in the last common ancestor of all living animal lineages.

Results and Discussion

Ever since the discovery of the homeobox in the 1980s facilitated rapid comparison of development and its evolution across animal phyla, and then the Zootype hypothesis proposed that axial expression of homeobox genes is a defining character of animals [9], understanding the origin of the Hox gene cluster has been a major goal in deducing the earliest stages of animal evolution. This relates to hypotheses concerning the last common ancestor and whether such an organism was genetically complex (in terms of gene content), with some extant lineages being secondarily simplified. Alternatively, the complexity of higher animals has, broadly speaking, evolved progressively, such that basal lineages can act as good proxies for stages along this evolutionary progression. The Hox genes are a subset of the homeobox gene family involved in patterning animal embryogenesis [10]. The ParaHox genes are the evolutionary sisters of the Hox genes, having evolved via the duplication of a ProtoHox condition that resulted in the paralogous Hox and ParaHox genes at some point deep in animal ancestry [11] (Figure 1; see also Supplemental Information available online). It is widely accepted that Hox genes are involved in anterior-posterior patterning across the major portion of the animal kingdom represented by the bilaterians, but the role of the genes in non-bilaterian animals (the Porifera, Placozoa, Ctenophora, and Cnidaria) is more ambiguous and controversial [1–4]. Despite this controversy about the gene functions, it is clear that in a cnidarian (the sea anemone *Nematostella vectensis*) genomic loci exist with clear synteny, and hence homology, to bilaterian Hox and ParaHox loci [1, 12, 13]. Analyses of gene neighborhoods and assessments of the extent of synteny conservation between taxa can thus provide an important source of information about the evolution of Hox and ParaHox loci that is independent of the molecular phylogenies of the homeobox genes themselves.

The Mode of Hox/ParaHox Origin Permits Resolution of Time of Origin

Given the ProtoHox model (Figure 1), one can potentially assess whether an organism is representative of (or descended from) the ProtoHox condition or the Hox/ParaHox condition, by analysis of the ProtoHox/Hox/ParaHox neighboring genes. We do this here by examining orthologs of neighbor genes that are currently linked to Hox in bilaterians and test whether they are clustered and linked separately from orthologs of genes that are currently linked to ParaHox in bilaterians. If this is the case then one can assume that the animal being examined also possesses distinct Hox and ParaHox loci. On the other hand, if the animal being examined possesses orthologs of Hox and ParaHox neighbors in an intermingled and clustered fashion (rather than intermingled, but randomly dispersed), then this is likely to be indicative of descent from the ProtoHox state.

The reasoning underlying the assumptions above and which underpins the ProtoHox to Hox/ParaHox condition summarized in Figure 1 is as follows. The duplication of the ProtoHox gene or cluster could have occurred via one of several possible routes. First, there could have been a whole chromosome or whole genome duplication, such that at their point of origin the Hox and ParaHox loci were on distinct chromosomes, but surrounded by identical (paralogous) genes. The redundancy between these paralogous neighbors will then tend to be overcome via extensive complementary gene losses from the two daughter chromosomes, such that in time the set of genes neighboring the Hox locus will be distinct from the set of neighbors around the ParaHox locus. A second possible route would be via a smaller scale, subchromosomal duplication. By comparison to data on duplications in modern-day genomes, we can assume that such a small-scale duplication is much more likely to occur within the same chromosome (intrachromosomally) rather than between two different chromosomes (interchromosomally) at the point of origin (see Supplemental Information for further discussion). Thus, following the much more likely intrachromosomal duplication, the newly formed Hox and ParaHox loci will be linked to the same neighbor genes, because everything is still on the same chromosome. The Hox and ParaHox loci are now on distinct chromosomes in modern animals such as humans,

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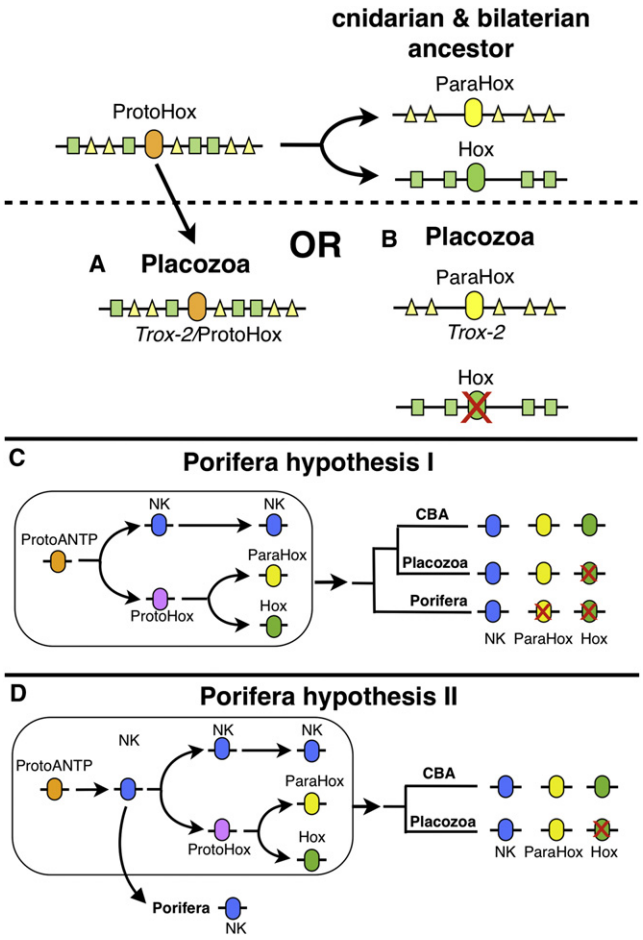

Figure 1. The ProtoHox Hypothesis and Alternative Views of the Placozoan and Poriferan Conditions

The ProtoHox locus duplicated to give rise to Hox and ParaHox loci (top) before the origin of the Cnidaria and Bilateria. Following ProtoHox locus duplication and separation of Hox and ParaHox loci onto distinct chromosomes, the genes that surrounded the ancestral ProtoHox locus became partitioned into two distinct sets of neighbors, one around the Hox locus and one around the ParaHox locus (see Supplemental Information for further details).

(A) *Trichoplax adhaerens Trox-2* gene is a descendent of the ProtoHox condition; or (B) *Trox-2* is a ParaHox gene (*Gsx*) and the Hox gene(s) has/have been lost, leaving a ghost Hox locus. Ovals represent homeobox genes, rectangles represent orthologs of genes neighboring the cnidarian and bilaterian Hox genes, and triangles represent orthologs of genes neighboring the cnidarian and bilaterian ParaHox genes.

(C) Porifera hypothesis I is that the Hox and ParaHox loci evolved before the origin of poriferans, but these homeobox genes were lost on the sponge lineage.

(D) Porifera hypothesis II is that the poriferan lineage arose before the evolution of Hox and ParaHox loci, which evolved by duplication from the NK cluster locus.

amphioxus, and *Platynereis dumerilii* [14]. The most common routes to separating linked genes into two sets of unlinked genes are either via a chromosome fission or via chromosome arm exchanges. These tend to be large-scale multigenic events and so in the Hox/ParaHox context are likely to have separated the Hox and ParaHox loci along with a significant number of neighboring genes. Just as with the first whole chromosome or genome route, this second "intrachromosomal followed by large-scale translocation" route will have resulted in the Hox and ParaHox loci being located on different chromosomes with each surrounded by a distinctive subset of the genes that ancestrally surrounded the ProtoHox locus (Figure 1).

Synteny analyses reveal that Hox and ParaHox loci evolved before the origin of Cnidaria [12, 13]. Homeobox gene content and phylogenies do not conclusively resolve when these loci came into existence relative to the other nonbilaterian lineages (Placozoa, Ctenophora, and Porifera) [1–3, 7, 8]. The single Hox-like gene in the placozoan *Trichoplax adhaerens* has been interpreted in different ways (Figures 1A and 1B), and the absence of Hox/ParaHox genes, but the presence of an NK gene cluster, in the poriferan *Amphimedon queenslandica* has led to alternative hypotheses (Figures 1C and 1D) (see below) [7, 8]. Poor interfamily support values within homeodomain phylogenies make it difficult to resolve between these hypotheses with confidence. Here we use an alternative approach to homeodomain sequence phylogenies to resolve the origin of the Hox and ParaHox loci: genomic synteny.

Trox-2 Is in a Placozoan ParaHox Locus

The genome sequence of the placozoan *Trichoplax adhaerens* contains a single gene with sequence similarity to Hox-like genes [5, 15], *Trox-2*. Opinions have differed as to whether *Trox-2* is orthologous to the ParaHox gene Gsx and hence is an evolutionary sister to Hox genes or instead is the placozoan descendent of the ProtoHox condition that is hypothesized to have been the precursor to the origin of the Hox and ParaHox genes [16](Figures 1A and 1B). To resolve whether the placozoan Hox-like gene, *Trox-2*, is a ParaHox gene or a ProtoHox gene descendent (Figures 1A and 1B), we analyzed the entire genomic scaffold containing *Trox-2* for conserved synteny with the human genome (Figure 2). First, we searched the *Trox-2* scaffold for genes with clear orthology to distinct human genes (see Supplemental Information), to distinguish genes that could be used in our statistical analyses (Figure 2A; Table S1). With this curated list of 27 *T. adhaerens* genes, we tested whether the neighbors of *Trox-2* are significantly similar to the neighbors of human ParaHox loci, or instead are similar to human Hox neighbors, or lack significant synteny to human ParaHox and Hox loci. The *T. adhaerens Trox-2* scaffold shares significant synteny with the ParaHox loci of humans (Binomial and Fisher's exact tests, $p < 0.0005$; Figure 2B, Figures S1A–S1E). This is consistent with two scenarios. Either *Trox-2* is a ParaHox gene, in which case there should be no synteny with human Hox loci (Figure 1B); or *Trox-2* is a ProtoHox descendent, in which case the *Trox-2* scaffold should also have significant synteny with human Hox loci because the ProtoHox neighbors would be expected to have distributed evenly between the descendent Hox and ParaHox loci (Figure 1A). There is a significant lack of synteny with human Hox loci (Binomial and Fisher's exact tests, $p < 0.02$; Figure 2B; Figures S1A–S1E). Synteny of *T. adhaerens Trox-2* neighbors with the human genome strongly supports a ParaHox identity for *Trox-2*. This is consistent with the topology of molecular phylogenetic trees including *Trox-2* [5] and contradicts the hypothesis that *Trox-2* is a direct ProtoHox descendent.

A Ghost Hox Locus Exists in Placozoans

If *Trox-2* is indeed a ParaHox gene and an evolutionary sister (or paralog) to Hox genes, then we would expect there to be a *T. adhaerens* locus with synteny to human Hox loci but which lacks a Hox gene (Figure 1B). To find this "ghost" Hox locus, we used the Putative Ancestral Linkage (PAL) group

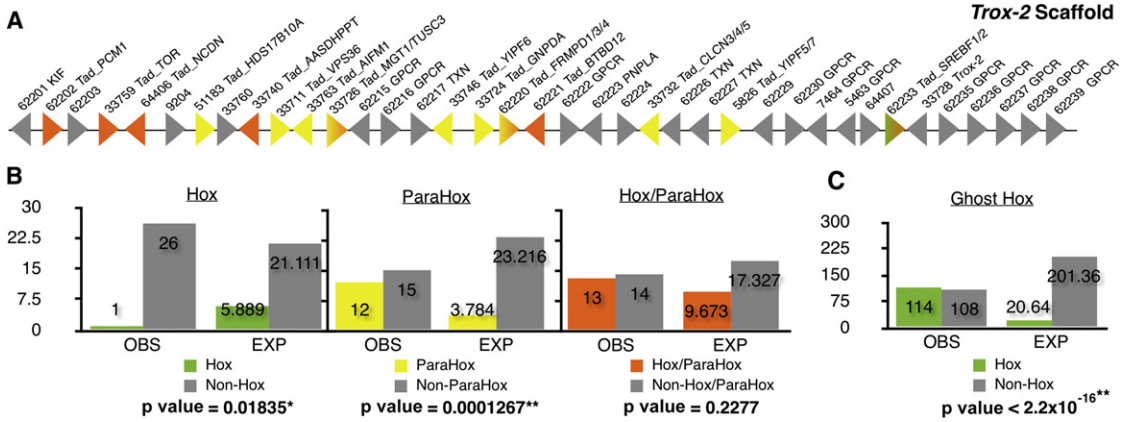

Figure 2. *Trox-2* Is a ParaHox Gene in a ParaHox Locus

(A) Representation of *Trox-2* scaffold. Triangles represent genes, with directionality representing gene orientation. ParaHox neighbor orthologs are defined as *T. adhaerens* genes with human orthologs located on any of the human chromosomes bearing ParaHox loci (Chromosomes 4, 5, 13, and X). Hox neighbor orthologs are defined as *T. adhaerens* genes with human orthologs located on any of the human chromosomes bearing a Hox cluster (Chromosomes 2, 7, 12, and 17). Triangles in gray are genes with no human orthology, triangles in orange are orthologs of human genes not linked to human Hox/ParaHox loci, triangles in yellow are orthologs of human ParaHox neighbors, triangles in yellow-orange are orthologs of human genes that are a mix of ParaHox and non-Hox/ParaHox neighbors, triangle in green-orange is a gene with human orthologs that are a mix of Hox and non-Hox/ParaHox neighbors.

(B) Binomial Exact Tests for synteny of the *Trox-2* scaffold with human Hox, ParaHox, or Hox/ParaHox loci.

(C) Ghost Hox Binomial Exact Test for whether *T. adhaerens* scaffold 3 has significant synteny with human Hox loci. Single asterisks denote statistical significance at 5%, and double asterisks denote statistical significance at 1%. See also Supplemental Information, Figure S1, and Table S1.

information from the cnidarian *N. vectensis* genome [12]. By comparing the *N. vectensis* genome with those of chordates, Putnam et al. [12] deduced a list of 225 genes that neighbored the Hox genes in the cnidarian-bilaterian ancestor. We found 222 *T. adhaerens* orthologs of these cnidarian-bilaterian ancestral Hox neighbors (see Supplemental Information; Table S2). We find a highly significant association of these genes with *T. adhaerens* scaffold 3 (114 genes out of 222) (see Supplemental Information; Figure S1F; Table S2) (Exact Binomial test $p < 2.2 \times 10^{-16}$; Figure 2C). *T. adhaerens* thus has a ParaHox locus in which *Trox-2* resides, and a ghost Hox locus with synteny to cnidarian and bilaterian Hox loci but without a resident Hox gene. This implies that Hox gene(s) have been lost along the placozoan lineage and both the Hox and ParaHox loci evolved before the origin of the Placozoa (Figure 1B).

Hox and ParaHox Neighbor Orthologs Are Not Randomly Distributed in a Sponge

We next tested whether Hox and ParaHox loci can be detected even earlier in animal evolution. Porifera constitute the lineage most commonly considered to be more basal than Placozoa and Cnidaria [17, 18] (although see below), and a whole genome sequence from a sponge is available, from *A. queenslandica*. Hox genes are absent from the genome sequence of *A. queenslandica*, as well as from a genome from another non-bilaterian phylum the ctenophore *Mnemiopsis leidyi*, and the genes have not been found in any other members of these phyla [4, 6, 19]. Absence of Hox and ParaHox genes from all sponges that have been examined, including the whole genome sequence of *A. queenslandica* [6, 19], has led to conflicting hypotheses about whether Hox and ParaHox genes evolved before or after the origin of the poriferan lineage (Figures 1C and 1D). Larroux et al. [8] found a cluster of NK homeobox genes in the genome of *A. queenslandica*, which, like Hox and ParaHox genes, are members of the Antennapedia (ANTP)-class of genes [20]. This combination of a cluster of genes with sequence affinity to Hox and ParaHox genes, with the lack of bona fide Hox and ParaHox genes, led Larroux et al. [8] to propose that Hox/ParaHox genes arose from an NK gene cluster after divergence of the poriferan lineage (Figure 1D). Peterson and Sperling [7] used phylogenetic trees to propose an alternative hypothesis, that several homeobox gene families, including the Hox and ParaHox families, were lost during poriferan evolution (Figure 1C).

Using the Hox PAL gene list derived from *N. vectensis*-bilaterian comparisons [12], we searched for orthologs in the *A. queenslandica* genome and found 187 genes. We tested whether these 187 sponge genes are clustered in the *A. queenslandica* genome, as would be expected if this poriferan has a ghost Hox (or ProtoHox) locus, or instead are randomly scattered throughout the genome as might be expected if the Hox locus did not evolve before the origin of poriferans (or the *A. queenslandica* genome has rearranged to the extent that synteny with other phyla has been largely lost). According to simulations (see Supplemental Information; Table S2), the 187 *A. queenslandica* genes show significant evidence of clustering onto a small number of scaffolds (one-tailed test of clustering, $p < 0.001$; Figure 3A).

This clustering of cnidarian-bilaterian Hox neighbor orthologs in this sponge can reflect one of two possibilities; either *A. queenslandica* has a ghost Hox locus or this animal has a ghost ProtoHox locus. To distinguish between these two possibilities, we determined whether *A. queenslandica* has a ghost ParaHox locus that is distinct from the ghost Hox locus, as would be expected if the origin of the Hox and ParaHox loci occurred before the origin of the Porifera. If instead sponge orthologs of ParaHox gene neighbors cluster in a fashion colocalized with the above Hox neighbor clustering, then this would imply the existence of a ghost ProtoHox locus, with the duplication into Hox and ParaHox loci occurring after the divergence of poriferans. To determine whether orthologs of ParaHox neighbors are clustered in *A. queenslandica*, we first constructed a list of human ParaHox neighboring genes

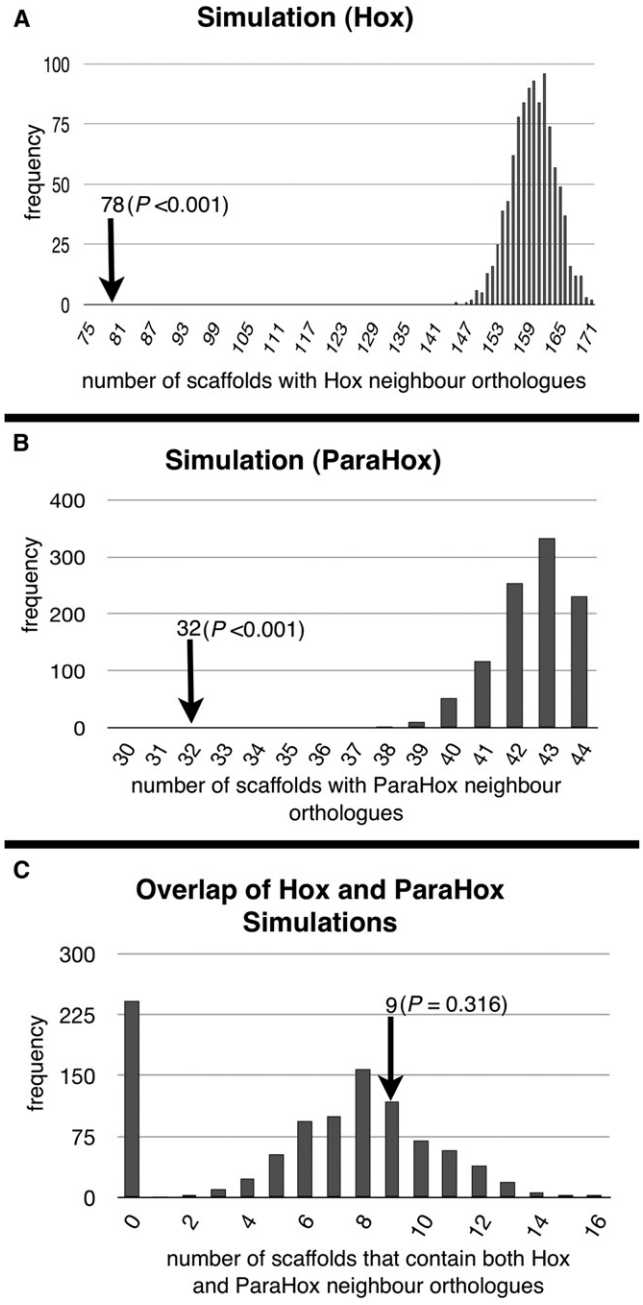

Figure 3. Sponges Have Distinct Ghost Hox and ParaHox Loci

(A) Simulation of randomized location of *Amphimedon queenslandica* orthologs of human Hox neighbors across the sponge scaffolds. The arrow indicates observed number of scaffolds with Hox neighbor orthologs in *A. queenslandica*.

(B) Simulation for ParaHox neighbor orthologs. The arrow indicates observed number of scaffolds with ParaHox neighbor orthologs in *A. queenslandica*.

(C) Overlap plot of both simulations to distinguish whether the Hox and ParaHox neighbor clustering is coincident or distinct. The arrow indicates observed number of scaffolds with colocalization of Hox and ParaHox neighbor orthologs in *A. queenslandica*. See also Supplemental Information, Figure S2, and Table S2.

that are also neighbors in the placozoan *T. adhaerens*, and hence form a ParaHox PAL in the placozoan-cnidarian-bilaterian ancestor. We used the synteny information of Srivastava et al. [15], which matched human genome segments containing the human ParaHox loci with a single scaffold in the *T. adhaerens* genome (scaffold 5). From the 595 genes in these human genomic segments, we found 167 genes on *T. adhaerens* scaffold 5, which when filtered for reciprocal best BLAST hits back to specific human ParaHox segments results in 65 genes in our localized-ParaHox PAL list (l-ParaHox PAL) (see Supplemental Information; Table S2). Using this l-ParaHox PAL list, we detected 44 *A. queenslandica* genes. These 44 sponge genes cluster together on significantly fewer scaffolds than expected for randomly distributed genes (one-tailed test for clustering, $p < 0.001$; Figure 3B).

Sponges Have Distinct Hox and ParaHox Loci

To test whether these clustered orthologs of ParaHox PAL genes colocalize with the clustered orthologs of Hox PAL genes (representing the ProtoHox condition), or they form two distinct loci (representing the Hox and ParaHox condition), we obtained an empirical null distribution of the spread of genes across the *A. queenslandica* genome scaffolds without clustering (see Supplemental Information). The observed number of *A. queenslandica* scaffolds containing both Hox and ParaHox PAL orthologs is nine, which does not differ significantly from the null expectation of random colocalization (one-tailed test, $p = 0.316$; Figure 3C), providing no significant evidence for the ProtoHox hypothesis. We conclude that the clustering of Hox PAL orthologs is distinct from the ParaHox PAL ortholog clustering in *A. queenslandica*, which implies that distinct Hox and ParaHox ghost loci exist in this poriferan. This is consistent with the gene loss hypothesis explaining the absence of Hox and ParaHox genes in sponges (Figure 1C) and is inconsistent with the hypothesis of Hox/ParaHox (or ProtoHox) genes arising from an NK gene cluster (Figure 1D). We find further evidence against the NK-ProtoHox hypothesis (Figure 1D) from an analysis of the genes neighboring the *A. queenslandica* NK cluster, which show no significant linkage with the Hox or ParaHox loci of bilaterians, in contrast to what might have been expected if the Hox/ParaHox/ProtoHox genes had evolved from duplication of the NK locus (see Supplemental Information; Figures S2A and S2B). We also found that the existence of ghost Hox and ParaHox loci is restricted to the animals. Analysis of the genome of a choanoflagellate, *Monosiga brevicollis*, from the sister group to the Metazoa revealed no clustering of the orthologs of the metazoan Hox and ParaHox neighbors (see Supplemental Information; Figure S2C).

A Last Common Ancestor with Hox and ParaHox Was Followed by Gene Loss

The assumption underlying all our analyses is that the Hox and ParaHox loci evolved by duplication of a ProtoHox locus such that neighbors of the ProtoHox cluster distributed relatively equally with the postduplication Hox and ParaHox loci (Figure 1). If instead the Hox/ParaHox genes evolved by some mechanism like a retrotransposition or a small-scale DNA-based transposition, then the daughter gene would have inserted into a distinct genomic location without necessarily taking neighbors from the parent (ProtoHox) locus (Figure S3). We consider this less likely than our ghost loci hypothesis (see Supplemental Information for further discussion), which merely implies duplication and gene loss, a phenomenon that is known to be common [21–25] and which is consistent with gene phylogeny topologies [7].

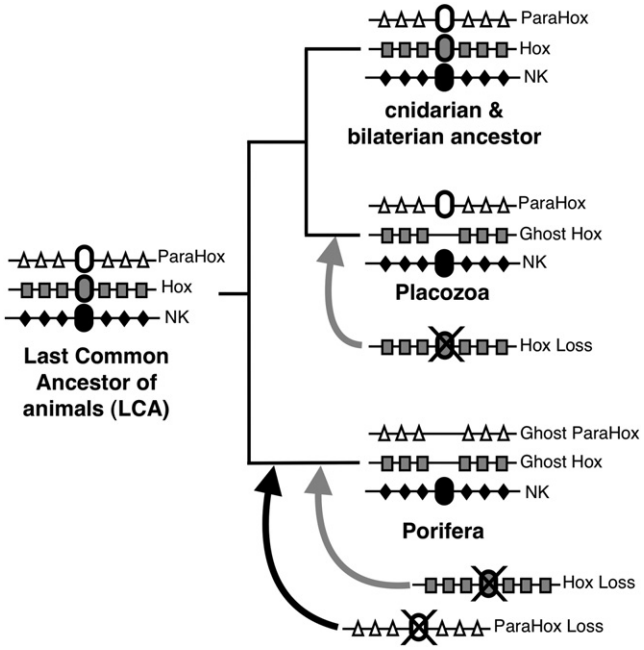

Figure 4. Last Common Ancestor of Animals had Hox, ParaHox, and NK Loci

Placozoans have lost their Hox gene(s) but retained a ghost Hox locus, and *Trox-2* is a ParaHox gene in a ParaHox locus. Poriferans have lost Hox and ParaHox genes but retained distinct ghost Hox and ParaHox loci. Cnidarian and bilaterian ancestors had Hox, ParaHox, and NK loci as did the Last Common Ancestor of animals. Gene symbol shapes as for Figure 1. See also Supplemental Information and Figure S3.

The phylogeny of the basal animal lineages is controversial [18] and is important for understanding events in early animal evolution such as those described here. The Ctenophora are the only nonbilaterian animal phylum for which a whole genome sequence is not yet publicly available, although one has been sequenced [4], and so they could not be included in the present analyses. Although some authors contend that Ctenophora might be the basal-most animal lineage [26], further analyses reject this hypothesis [18], retaining poriferans as the basal animal lineage. This is consistent with traditional morphological and embryological analyses, such as the possession of a nervous system by ctenophores [27] that is absent from both placozoans and sponges. The view that poriferans are the basal-most lineage of living animals is thus the arrangement that we adopt here (Figure 4).

Consequently, our discovery of ghost Hox and ParaHox loci in a sponge, and a ParaHox locus containing *Trox-2* alongside a ghost Hox locus in a placozoan, implies that the last common ancestor of animals possessed distinct Hox and ParaHox loci (Figure 4). This, in turn, implies loss of these homeobox genes during the evolution of some basal animal lineages, which, in terms of these developmental control genes, have been simplified relative to the last common ancestor of animals.

Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.08.023.

Acknowledgments

We thank Nik Putnam for providing the map between *N. vectensis* PAL ID numbers and accession numbers. We acknowledge funding from the BBSRC and University of St Andrews School of Biology. Data deposition - Python code for the simulation analysis as well as all of the alignment, SMART, and phylogenetic tree data for the scaffold 38 analysis are available at http://biology.st-andrews.ac.uk/cegg/downloads.aspx.

Received: January 2, 2012
Revised: August 9, 2012
Accepted: August 13, 2012
Published online: September 27, 2012

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