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A Brief Review of Metazoan Phylogeny and Future Prospects in Hox-Research[1]

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SYNOPSIS. Underlying any analysis on the evolution of development is a phylogenetic framework, whether explicitly stated or implied. As such, differing views on phylogenetic relationships lead to variable interpretations of how developmental mechanisms have changed through time. Over the past decade, many long-standing hypotheses about animal evolution have been questioned causing substantial changes in the assumed phylogenetic framework underlying comparative developmental studies. Current hypotheses about early metazoan history suggest that three, not two, major lineages of bilateral animals originated in the Precambrian: the Deuterostomes (*e.g.*, seastars, acorn worms, and vertebrates), the Ecdysozoans (*e.g.*, nematodes and arthropods), and the Lophotrochozoans (*e.g.*, annelids, mollusks, and lophophorates). Although information in Hox-genes bears directly on our understanding of early metazoan evolution and the formation of body plans, research effort has been focused primarily on two taxa, insects and vertebrates. By sampling a greater diversity of metazoan taxa and taking advantage of biotechnological advances in genomics, we will not only learn more about metazoan phylogeny, but will also gain valuable insight as to the key evolutionary forces that established and maintained metazoan bauplans.

Approximately 35 fundamentally different body plans (or ''phyla'') are recognized among extant metazoans. Understanding how, when, and why metazoan body plans diversified have been longstanding and challenging questions for biologists. ''Evo-Devo'' research (or research on the evolution of developmental mechanisms) seeks to integrate our understanding of evolutionary history with the observed variation in developmental patterns and mechanisms to help answer some of these questions. Because of their role in regionalization and fate specification along the anteroposterior axis (Akam, 1995) and their ability to cause homeotic mutations (Lawrence, 1992; Gehring, 1994), Hox genes have been a central focus of developmental research examining patterns of body plan formation (*e.g.*, Akam, 1995; Carroll, 1994, 1995; Davidson *et al.*, 1995; Degnan and Morse, 1993; Holland, 1998). These genes are helix-turn-helix transcription factors that act on downstream gene cascades. They are linked in a cluster(s) along chromosomes and are arranged and expressed in a colinear fashion. Generally, genes that are the most similar are next to each other. The Hox gene cluster has been examined (to some extent) in a wide range of metazoans (from sponges to arthropods to vertebrates; *e.g.*, Kaufman *et al.*, 1990; Akam *et al.*, 1994; Degnan *et al.*, 1995; Holland and Garcia-Fernandez, 1996; Popadic *et al.*, 1998). Within non-chordate metazoans, a single Hox cluster is known to range in size from the 3 gene 12 Kb cluster in cnidarians (Finnerty and Martindale, 2001) to the 10 gene >500 Kb cluster in the sea urchin *Strongylocentrotus purpuratus* (Martinez *et al.*, 1999). In comparison, the Hox cluster in *C. elegans* appears highly modified, as it contains only 6 Hox genes with an inversion, and *D. melanogaster*'s cluster contains a large intergenic region (de Rosa *et al.*, 1999).

The purpose of this communication is to provide a phylogenetic context to developmental patterns observed across major metazoan lineages, and to highlight, from

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the evolutionary perspective, future directions of evo-devo study. To this end, we will first review the current understanding of metazoan phylogeny helping to clarify the comparative framework for studies across major metazoan lineages. Then, the sampling of Hox-related genes will be discussed in relation to this framework. In particular, of the three great bilaterian clades, Lophotrochozoans encompass the greatest diversity of metazoan body plans, but have received the least research effort focused on developmental issues. We argue that model systems should be developed in annelids and/or mollusks to develop a more accurate understanding of the evolution of body plans.

METAOZOAN PHYLOGENY

The first formal phylogeny of the Metazoa, and the origin of the term ''phylogeny'' itself, was published by Haeckel in 1866. Subsequent phylogenetic hypotheses were also based on the comparative morphological and developmental work of invertebrate biologists. In particular, Libbie Hyman's (1940–1967) influence on metazoan systematics cannot be understated. Phylogenetic hypotheses in many modern Invertebrate texts (*e.g.,* Brusca and Brusca, 1990; Meglitsch and Schram, 1991) clearly echo ideas from her 1940 diagram (her Fig. 5, Vol. 1, p. 38). Interestingly, on the same pages as her ''hypothetical diagram of the relationships of the phyla,'' Hyman states that she will ''attempt to arrange the phyla in general according to their grade of construction while at the same time avoiding the separation of allied phyla'' (p. 39). It is ironic that this researcher, who laid an important corner stone of invertebrate phylogeny, emphasized ''grade[s] of construction'' (or complexity) over evolutionary history. However, in her defense, Hyman stated her diagram was meant to be a convenient tool and not a rigorous phylogenetic hypothesis.

This emphasis on complexity has lead to delineations within the metazoans based on mesodermal features. The presence/absence of mesoderm is used to distinguish between diploblasts and triploblasts. How the mesoderm is arranged internally to form body cavities or coeloms (*i.e.,* acoel, pseudocoel, schizocoel and enterocoel) was used to divide triploblasts into major lineages (acoels, aschelminths, protostomes and deuterostomes, respectively). Thus, as Figure 1 portrays, metazoan phylogeny has classically been thought to progress from less complex to more complex (body) forms. However, traditional assumptions that complexity has increased over the course of metazoan evolution (*sensu* Hyman, 1940) have recently been called into question (McShea, 1996, 1998). (Willmer [1990] provides a good review of hypotheses based on complexity.)

Following Hyman, the advent of SEM and TEM provided a suite of ultrastructural characters that were utilized in comparative studies. By hypothesizing homology between ultrastructural features from different taxa, workers were able to glean a novel understanding of metazoan relationships (*e.g.,* Barnes, 1985; Nielsen, 1985, 1987). Ultrastructural information also lead to revisions in our understanding about the evolutionary plasticity of morphology. For example, Ruppert (1991) draws on data from microscopy studies and asserts that body cavity types are more evolutionarily labile than previously believed.

The introduction of cladistics methods (Hennig, 1966), nucletoide sequencing, and computers provided powerful new tools, and marked the beginning of a new era of more rigorous phylogenetic investigation. Figure 2 shows a revised view of evolutionary relationships among major groups of metazoans. Sponges and diploblasts (cnidarians and ctenophores) are basal to the triploblastic metazoans (*e.g.,* Eernisse *et al.*, 1992; Eernisse, 1997; Aguinaldo *et al.*, 1997; Aguinaldo and Lake, 1998; Winnipenninckx *et al.*, 1998*b*; Kim *et al.*, 1999). When taken together, the two triploblast ''superclades'' Ecdysozoa (Aguinaldo *et al.,* 1997) and Lophotrochozoa (Halanych *et al.*, 1995) are usually referred to as the Protostomia (*e.g.,* Aguinaldo and Lake, 1998). The Deuterostomia consists of only three recognized ''phyla'' (chordates, hemichordates and echinoderms). Most major rearrangements in our understanding of metazoan phylogeny were initially based on 18S rDNA data. Criticisms of this particular

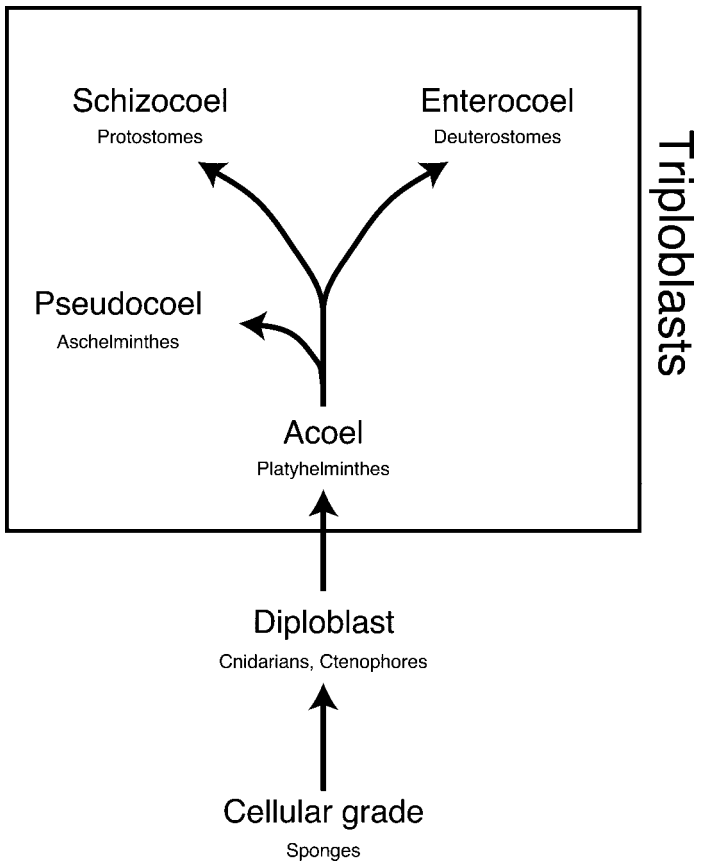

FIG. 1. Traditional concept of the evolution of complexity. Metazoan classification and assumptions about phylogeny have been largely shaped by this hypothesized progression from ''simple'' to ''complex'' which is formulated mainly on mesodermal patterns. Examples of taxa typically associated with each category are shown. Current understanding of metazoan phylogeny suggests the triploblast categories are environmental, not phylogenetic, in nature.

marker (Phillipe *et al.*, 1994; Maley and Marshall, 1997; Abouief *et al.*, 1998) have largely been muted as independent data have confirmed the 18S based findings. In particular, phylogenetic inference based on Hox gene orthologs (de Rosa *et al.*, 1999) and mitochondrial gene rearrangement data (*e.g.*, Boore and Brown, 1998; Boore, 1999; Stechmann and Schlegel, 1999) support the Ecdysozoan and Lophotrochozoan superclades.

Based on 18S rDNA data, Aguinaldo *et al.* (1997) were the first to hypothesize that the pseudocoelomate nematodes are closely related to the arthropods in a monophyletic clade termed the Ecdysozoa. The name Ecdysozoa means ''molting animal,'' in reference to the fact that all the organisms Aquinaldo *et al.* (1997) identified as being within the clade undergo ecdysis. Further support for the ecdysozoan hypothesis has been provided by the identification of clade-specific Hox paralog groups (de Rosa *et al.*, 1999), and recent evidence of a characteristic triplicate repeat in the β-Thymosin homologues of arthropods and nematodes (Manuel *et al.*, 2000).

Other organisms placed in the Ecdysozoa include kinorhynchs, priapulids, nematomorphs, onychophorans and tardigrades (Aguinaldo *et al.*, 1997). Because chaetognaths appear to be allied to nematodes (Halanych, 1996), they are also presumably ecdysozoans. Although ecdysis has not

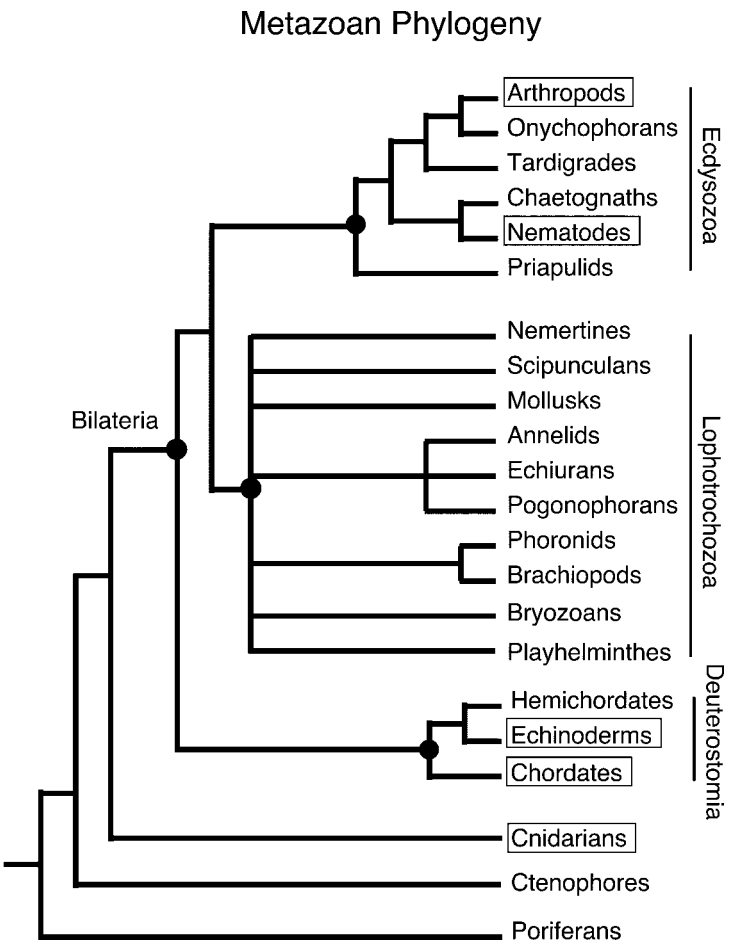

FIG. 2. Current understanding of metazoan phylogeny. Drawing on information from several different sources (*e.g.,* Eernisse *et al.,* 1992; Halanych *et al.,* 1995; Aguinaldo *et al.,* 1997; Eernisse, 1997; de Rosa *et al.,* 1999; see text for additional references), this topology represents a consensus illustrating the relationships between major metazoan taxa. Many lesser-known ''phyla'' (*e.g.,* gastrotrichs, acanthocephalans, placozoans, nematomorphs, etc.) were not included for simplicity or because their phylogenetic affinities are not clear. Taxa in which the Hox cluster has been completely sequenced are boxed. The echinoderm and cnidarian projects are currently underway. A genome project has just been initiated for a flatworm, but since it is not clear when the Hox cluster will be sequenced, it is not boxed here. Also echiurids and pogonophorans are within the annelids (shown separate for simplicity). See text for details.

been reported in chaetognaths, its occurrence in all other members of the Ecdysozoa suggests that this feature was present in the last common ancestor of the clade (Aguinaldo *et al.,* 1997), and predicts that conserved ecdysis mechanisms may be found. Further investigation is necessary to determine whether the cuticle and process of ecdysis are in fact homologous across the Ecdysozoa.

The Ecdysozoa hypothesis has important ramifications, as it means two model organisms (*Drosophila* and *Caenorhabditis*) are more closely related than previously believed. The traditional view of metazoan evolution, which placed the less complex pseudocoelomate nematodes basal to the protostome/deuterostome split, suggested that developmental features common to *Caenorhabditis* and *Drosophila* were likely present in the common coelomate ancestor allowing extrapolation to other coelomates (most notably *Homo sapiens*). However, commonalities between these model organ-

isms must now be interpreted with more caution as they may have arisen following the divergence of the Ecdysozoa.

Analysis of 18S rDNA sequences has also led to the grouping of the lophophorates (brachiopods, bryozoans, and phoronids) with annelids and molluscs in a clade termed the Lophotrochozoa (Halanych *et al.*, 1995). Earlier analyses (Field *et al.*, 1988; Ghiselin, 1988; Lake, 1990) employing only a single partial brachiopod sequence also hinted at this association. The phylogenetic position of lophophorates has been a matter of some debate, with differing interpretations of developmental and morphological traits leading to their assignment as protostomes (Gutmann *et al.*, 1978), deuterostomes (Zimmer, 1973), intermediates between the two groups (Salvini-Plawen, 1982; Siewing, 1976, 1980), or an independent radiation (Willmer, 1990). However, their placement as *derived* protostomes reveals that embryological features (blastopore fate, type of eucoelom formation, cleavage patterns, larval type) are more evolutionarily labile than traditionally believed (Halanych *et al.*, 1995; Valentine, 1997; also see Halanych, 1996).

The lophotrochozoan clade (defined as all the descendents of the last common ancestor of lophophorates, mollusks, and annelids) is more inclusive than originally suspected. (It should be noted that the terms Eutrochozoa [*sensu* Gheslin, 1988] and Spiralia, *sensu stricto,* are less inclusive than Lophotrochozoa, and the terms should not be confused.) Sipunculids have been associated with both mollusks (Scheltema, 1993) and annelids (Boore and Staton, 2001), and echiurids and pogonophorans appear to be annelids (McHugh, 1997; Halanych *et al.*, 1998). Mackey *et al.*'s (1996) report suggests that the pseudocoelomate entoprocts are lophotrochozoans. The nemerteans are also members of the clade, given associations in 18S rDNA topologies (Turbeville *et al.*, 1992). Hox evidence has also placed dicyemid mesozoans (Kobayashi *et al.*, 1999) in the clade. Molecular studies have also provided evidence for the inclusion of platyhelminthes within the Lophotrochozoa. The platyhelminth flatworms were traditionally considered to be basal triploblasts because they had no coelom (Hyman, 1951; reviewed in Willmer, 1990). Analysis of both 18 rDNA and Hox genes (Balavoine and Telford, 1995; Balavoine, 1997) suggest that some platyhelminthes are members of the Lophotrochozoan clade which have undergone secondary simplification (Balavoine, 1998). Recent analysis has also suggested that platyhelminthes may be polyphyletic and that the acoels may be basal bilaterians (Carranza *et al.*, 1997; Ruiz-Trillo *et al.*, 1999; see also Eernisse, 1997), but it is likely that the acoel finding is an artifact of long-branch attraction (hinted at in Campos *et al.*, 1998, Berney *et al.*, 2000). Lastly, Garey and Schmidt-Rhaesa (1998) have proposed that a clade consisting of platyhelminthes, gnathostomulids, rotifers, and acanthocephalans (and probably cycliophorans—Winnepenninckx *et al.*, 1998*a*) is sister to the Lophotrochozoa. Although based on their relative position to bryozoans (which has yet to be determined), these taxa might be within the Lophotrochozoa. In comparison, Eernisse (1997) finds many of these groups, as well as gastrotrichs, are placed as basal bilaterians. Clearly, the status of several traditional ''aschelminthes'' groups awaits further confirmation.

de Rosa et al. (1999) have found that all presumptive Lophotrochozoans surveyed (annelids, molluscs, brachiopods, platyhelminthes, and nemerteans) possess a set of medial and posterior Hox genes not present in either Ecdysozoans or Deuterostomes. The homeodomains of these Hox genes (Lox5, Lox2, Lox4, Post1, and Post2) possess diagnostic peptide motifs which have been conserved throughout the members of the clade. Comparatively ecdysozoans contain 2 diagnostic Hox genes (*Ubx* and *Abd-B*). However, Telford (2000) argues that others have over interpreted the diagnostic ''signatures'' of some Hox genes, and that unique amino acid motifs should be treated as unpolarized characters, rather than synapomorphies, when an outgroup is lacking. Such diagnostic features provide a powerful tool for examination of taxonomic inclusion of these major clades.

In contrast to the Lophotrochozoans, the deuterostomes have been shrinking. Into the

early 1990s most researchers and evidence suggested that the deuterostomes were composed of chordates, hemichordates, echinoderms, chaetognaths, and lophophorates (although most placed the lophophorates as basal to the true deuterostomes; Willmer, 1990). The placement of the lophophorates has already been discussed above. Chaetognaths, commonly called arrow worms, were considered deuterostomes based on their tripartite coelom and the retention of the blastopore to form the anus. However, two independent 18S rDNA studies (Telford and Holland, 1993; Wada and Satoh, 1994) showed that chaetognaths were not closely related to other deuterostome taxa suggesting that coelomic patterns and blastopore fate are not representative of the relationships of major metazoan lineages (Halanych, 1996). Nielsen's (1995) hypothesis of deuterostome affinities for the ctenophores is inconsistent with available data (Eernisse *et al.,* 1992; Schram, 1991; Eernisse, 1997; Kim *et al.,* 1997; Winnepenninckx *et al.,* 1998*b*).

Of the three recognized deuterstome phyla, echinoderms and hemichordates appear to be the most closely related (Turbeville *et al.,* 1994; Cameron *et al.,* 2000). Metschnikoff (1881) termed an echinoderm-hemichordate group the Ambulacraria drawing attention to similar features in the larvae (Halanych, 1995). Swalla and her collegues (2000) have recently examined chordate origins. Their report that urochordates are comprised of 4 discrete lineages holds interesting implications for understanding the evolution of tadpole morphology and chordate life history.

SURVEYING THE HOX CLUSTER

Two aspect of Hox genes have peaked the interest of phylogeneticists. First, their conservative nature holds information on phylogenetic relationships among major metazoan groups. Although earlier workers alluded to this potential (Ruddle *et al.,* 1994; Dick, 1997), it was not until more recently that researchers began to exploit this information (*e.g.,* Balavoine and Telford, 1995; Balavoine, 1997; Grenier *et al.,* 1997; de Rosa *et al.,* 1999; Anderson *et al.,* 1999; Kobyashi *et al.,* 1999). Secondly, since the discovery that Hox genes cause homeotic mutations, there has been a hope that Hox genes may provide information on how and why metazoan body plans diversify. Earlier work (*e.g.,* Lawrence, 1992; Gehring, 1994) focused on homeotic mutations and mainly compared wildtype to mutated individuals. With the development of molecular and phylogenetic methods, comparative studies were undertaken comparing Hox expression across lineages in a phylogenetic framework. Unfortunately, most of this comparative work has focused on a selective group of taxa (*e.g.,* vertebrates—Holland and Garcia-Fernandez, 1996, and arthropods, esp. insects—Carroll, 1994, 1995; Akam *et al.,* 1994; Akam, 1995, 1998).

In Figure 2, the taxa for which the Hox cluster has been sequenced are boxed. Because of genome projects, the cluster information will be available for *Drosophila, Caenorhabditis* and several chordates. Current work on the Hox clusters of the cnidarian *Nematostella vectensis* (Finnetry and Martindale, 1997, 2001) and the sea urchin *Strongylocentrotus purpuratus* (Martinez *et al.,* 1999) should also soon be available. Therefore, physical maps and *cis*-acting regulatory elements that are in close proximity to the cluster will be known for representatives of the Ecdysozoa, Deuterostomes, and Diploblasts.

With little doubt the study of developmental mechanisms has received far less attention in Lophotrochozoans than in Ecdysozoans and Deuterostomes. Most Lophotrochozoan Hox studies have been limited to PCR surveys for genes (*e.g.,* Webster and Mansour, 1992; Dick and Buss, 1994; Balavoine and Telford, 1995; Irvine *et al.,* 1997; Kmita-Cunisse *et al.,* 1998; de Rosa *et al.,* 1999) and, to the best of the our knowledge and with the exception of leeches, few studies have actually examined Hox gene expression patterns in lophotrochozoans (*e.g.,* flatworms—Bayascas *et al.,* 1997; polychaete—Irvine and Martindale, 2000). Note studies by Bayascas *et al.* (1998) and Degnan and Morse (1993) of RNA transcription levels in flatworms and gastropods, respectively, did not examine the patterns of expression in the organism.

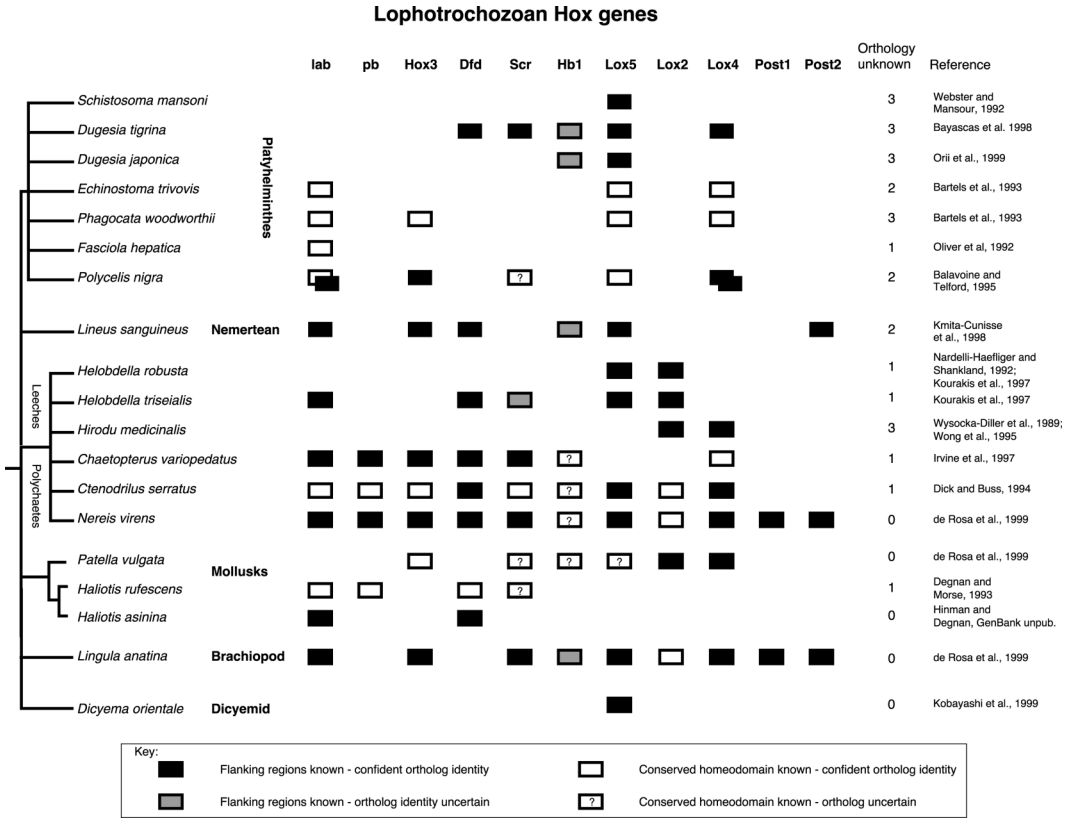

FIG. 3. Compilation of published Lophotrochozoan Hox gene sequences in GenBank as of February 2001. Boxes representing each sequence are aligned under their orthology group. Each sequence is coded according to whether regions flanking the homeodomain are known and whether assignment of orthology is confident. Numbers of homeodomain sequences published, but of unknown orthology, are also listed, though not represented graphically.

Some information has been gathered on the expression of transcription factors associated with segmentation and regeneration in oligochaete annelids (Bely and Wray, 2000). Shankland's group has done excellent work on exploring leech development (*e.g.,* Nardelli-Haeflinger and Shankland, 1992; Nardelli-Haeflinger *et al.*, 1994; Kourakis *et al.*, 1997; Shankland and Bruce, 1998), but Irvine and Martindale (2000) point out some of the shortcomings of leeches as a model for other Lophotrochozoans (including direct development and ''missing'' Hox orthologs). Our knowledge on the mechanics of how Hox genes aid pattern formation of Lophotrochozoan organisms is in its infancy.

Of interest, NIH and the World Health Organization (WHO) have recently begun genome projects on *Schistosoma japonica,* and *S. mansoni.* Although *Schistosoma* Hox genes have been the focus of previous research (Webster and Mansour, 1992), it is not clear if *Schistosoma* will be representative of the Lophotrochozoa. Currently, platyhelminth evolution is in question; the monophyly, origins, and phylogeny of the group are hotly debated (Balavoine and Telford, 1995; Balavoine, 1997; Campos *et al.*, 1998; Carranza *et al.*, 1996, 1997; Ruiz-Trillo *et al.*, 1999; Berney *et al.*, 2000).

Figure 3 summarizes all available information (*i.e.,* sequences in GenBank as of February 2001) for Hox genes in Lophotrochozoans. Although the information is presented in a manner similar to standard Hox cluster illustrations for *Drosophila* or chordates, no gene mapping information exists

for lophotrochozoan Hox clusters. Furthermore, the presence of genes was determined by either PCR screening with degenerate homeobox primers or by screening cDNA libraries. Thus, the spatial arrangement of the genes is merely speculation inferred from other organisms. The information in Figure 3 suggests that the ancestral lophotrochozoan Hox cluster probably consisted of at least 8–10 genes (de Rosa *et al.*, 1999; Irvine and Martindale, 2000). The polychaete *Nereis virens*, perhaps the most thoroughly surveyed Lophotrochozoan, contains at least 11 Hox genes. Although we know some of the genes in the cluster, we do not know their arrangement, *cis*-acting regulatory elements, and if additional genes and/or clusters are present.

Gellon and McGinnis (1998) reviewed Hox transcription mechanisms and concluded that ''evolutionary variation of Hox *cis*-regulatory elements has played a major role in the emergence of novel body plans.'' For example, fly Hox genes share few regulatory regions in comparison to the mouse, where sharing of regulatory elements could help explain conservation of the cluster. Because the unsampled lophotrochozoan taxa have the most diversity in terms of body plans, the group will provide a powerful test of Gellon and McGinnis's hypothesis about the role of regulatory elements in body-plan diversification.

FUTURE RESEARCH

In order to gain a more complete understanding of the evolution of the Hox cluster, future research must begin to employ genomic approaches and must incorporate a greater diversity of organisms. Most Hox genes have been identified using either PCR-based surveys or cDNA library screens coupled with comparisons of sequence similarity. Thus, little positional information or information on *cis*-acting regulatory elements is retrieved. Biotechnological advances have now made sequencing the entire Hox cluster possible even for smaller laboratories (as opposed to major genome centers), and developments in microarray technology will facilitate examination of timing and levels of expression for several genes simultaneously (initially this will only be feasible in model organisms). The combination of sequencing and microarray technology will open up a new realm of experimental studies that not only explore the evolution of the open-reading frame, but the evolution of the entire gene system (ORF, regulatory element, recognition sites, pleiotropic effects, etc.).

Lastly, to understand the evolution of the cluster and how it has shaped body plan evolution, more studies comparing Hox data across taxa must be undertaken. The comparative framework for such studies is phylogeny. However, at present most Hox studies focus on a single species with evolutionary considerations relegated to comparisons to previously published reports. A more desirable and objective approach is to examine multiple species in a single study and then use explicit methods to test alternative hypotheses (*e.g.*, likelihood tests, Huelsenbeck and Rannala, 1997). Such an approach would also provide a context for determining which hypotheses are significantly better than alternatives. As mentioned above, phylogentic representation of Hox genes has been biased with Lophotrochozoans receiving little attention despite having the greatest diversity of recognized body plans. The use of explicit methods for evolutionary comparisons forces us to consider the most appropriate taxa, not just which taxa were most convenient, for the question being addressed.

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