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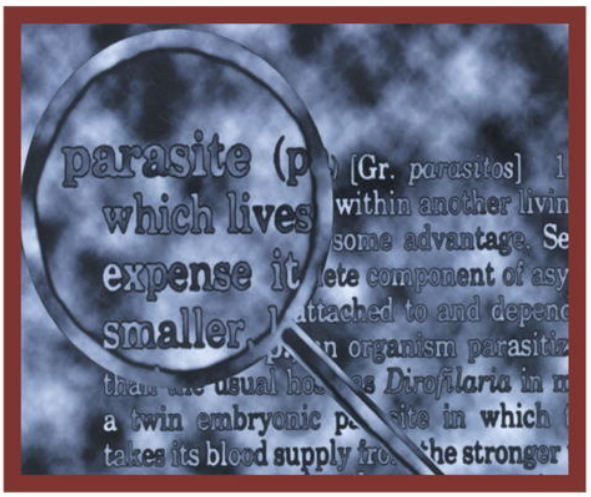

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Review

Hox genes and the parasitic flatworms: New opportunities, challenges and lessons from the free-living

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Abstract

Research into the roles played by Hox and related homeotic gene families in the diverse and complex developmental programmes exhibited by parasitic flatworms (Platyhelminthes) can hardly be said to have begun, and thus presents considerable opportunity for new research. Although featured in some of the earliest screens for homeotic genes outside *Drosophila* and mice, surveys in parasitic flatworms are few in number and almost nothing is yet known of where or when the genes are expressed during ontogeny. This contrasts sharply with a significant body of literature concerning Hox genes in free-living flatworms which have long served as models for the study of regeneration and the maintenance of omnipotent cell lines. Nevertheless, available information suggests that the complement of Hox genes and other classes of homeobox-containing genes in parasitic flatworms is typical of their free-living cousins and of other members of the Lophotrochozoa. Recent work on *Schistosoma* combined with information on Hox gene expression in planarians indicates that at least some disruption of the clustered genomic arrangement of the genes, as well as of the strict spatial and temporal colinear patterns of expression typical in other groups, may be characteristic of flatworms. However, available data on the genomic arrangement and expression of flatworm Hox genes is so limited at present that such generalities are highly tenuous. Moreover, a basic underlying pattern of colinearity is still observed in their spatial expression patterns making them suitable as cell or region-specific markers. I discuss a number of fundamental developmental questions and some of the challenges to addressing them in relation to each of the major parasitic lineages. In addition, I present newly characterized Hox genes from the model tapeworm *Hymenolepis* and analyze these by Bayesian inference together with >100 Hox and ParaHox homeodomains of flatworms and select lophotrochozoan taxa, providing a phylogenetic scaffold for their identification.

Keywords: Platyhelminthes; Homeobox; Hox; ParaHox; *Hymenolepis*

Contents

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1. Introduction

Discovery of the 'homeobox' in the mid-1980s [1] in developmental genes of *Drosophila* first elucidated by Lewis in 1978 [2], and of its unexpected evolutionary conservation [3], led to the re-integration of the fields of evolution and development: two disciplines closely allied at the end of the 19th century that had drifted apart following the advent of population genetics and the subsequent 'new synthesis' in evolutionary biology [4]. Since its discovery, this ~60 amino acid sequence-specific DNA-binding domain has been the cornerstone of the field of evo-devo. Although the homeobox helix-turn-helix sequence motif is found in a great diversity of transcriptions factors, the best known are those of the Hox cluster which are involved in patterning the anteroposterior (AP) axis of developing animals. In *Drosophila*, the homeotic gene cluster (HOM-C) comprises the Antennapedia Complex (ANT-C), with genes expressed in the anterior and central regions of embryos, and the Bithorax Complex (BX-C), with genes expressed in the posterior region. Together, the HOM complex of *Drosophila* is homologous to the duplicated Hox clusters of vertebrates and hence the designation HOM/Hox [5]. They are related to a large number of other classes of homeodomain-containing genes (e.g. ParaHox, NK) that are believed to have diverged from a common ancestor in the distant past. Collectively, the various classes form the Antennapedia superclass (ANTP) and appear to be confined to the animal kingdom [6].

Sequence conservation in the homeodomain region itself has enabled the use of degenerate PCR primers (i.e. oligonucleotides designed to have variable bases in non-conserved positions) to be used to amplify novel homeodomain-containing genes from a large diversity of eukaryotes [7]. Homologies have then been established by identifying gene-specific peptide and intron motifs [8,9], by phylogenetic analysis of the sequences and by comparative analysis of their expression patterns via whole-mount *in situ* hybridization (WMISH). Identification of Hox homologues in diploblastic and early branching metazoans (e.g. acoelomorphs) continues to push the common origin of the Hox genes further back in evolutionary time, requiring hypotheses on the condition of the 'proto-Hox' cluster and its diversification through the duplication and loss of single genes or entire clusters to be continually revised [4,5,10–19]: see Ferrier [20] for the current state of play. Recently, Larroux et al. [21] surveyed the complete genome of a demosponge, finding only a cluster of NK-type genes among the ANTP-like homeoboxes. Their work thus suggests that Hox, ParaHox and other classes of the ANTP superclass originated in the last common ancestor of the diploblastic and bilaterian metazoans from an NK-type gene, citing *Msx* as the sponge orthologue most similar to Hox-like genes of higher metazoans.

The impetus to characterize homeobox genes in flatworms stemmed initially from two different lines of investigation [22]: still widely considered to represent *the earliest and simplest bilateral animals* [23] in the early 1990s (and, unfortunately, occasionally still today: e.g. [24,25]), the platyhelminths could provide evidence of the antiquity of the various homeobox genes and gene families. In addition, flatworm developmental genes were of interest due to the extraordinary powers of regeneration planarians and many other free-living flatworms exhibit [26,27], making them excellent models for studying the maintenance and fate of totipotent stem cells [26,28], whereas totipotency in parasitic flatworms (e.g. the germinative region of tapeworms) has been comparatively ignored. Thus although a few parasitic flatworms were included in early screens for Hox homologues outside *Drosophila* or mice, the vast majority of our knowledge regarding homeobox-containing genes in flatworms comes from the free-living groups, of which the triclad planarians have been best studied (for reviews see [29–31]).

2. The homeobox gene complement of parasitic flatworms

2.1. Screening for homeobox genes in parasitic flatworms

Homologues of the Hox genes in parasitic flatworms were shown to be present via DNA hybridization studies (at least in cestodes; i.e. *Echinococcus*), as early as 1986 [32]. In 1992, a survey of homeobox-containing genes in *Echinococcus* revealed the presence of the three NK-type homeoboxes as well as a *goosecoid*-type gene, and although PCR survey of the liver fluke *Fasciola* and the planarian *Dugesia* revealed the presence of a number of ANTP-type genes, from these initial results few members of the common ANTP superclass of homeoboxes were predicted to be present in flatworms [33]. In the same year a cDNA screen of the human bloodfluke *Schistosoma mansoni* revealed six homeobox-containing clones [23]: four of the ANTP class and one each of the *engrailed* (*en*) and *paired* (*prd*) classes. The conclusion that these homeobox classes "arose to accommodate features of a simple multicellular body plan rather than the more complex developmental needs of higher animals" [23] was obviously predicated on the assumption that the flatworms represented the most basal branch of the Bilateria, and similar studies on free-living flatworms around this time (e.g. [34,35]) also targeted flatworms as "organisms that may be representative of an ancestor to both the protostomes and the deuterostomes" [35]. This assumption is no longer held (see below). The following year a screen of *Echinostoma trivolvis* recovered four ANTP-like genes provisionally assigned to anterior and central-group genes, and one that showed equal homology to both Hox and ParaHox homeoboxes [35]. Phylogenetic analysis of these

sequences (see Section 2.4) incorporating comparative sequences not available at the time of their publication [35] identifies the genes as orthologues of *labial* (*Lab*), *deformed* (*Dfd*), and *Lox2* (a lophotrochozoan orthologue of *Drosophila abdominal-A*; *Abd-A*), and two unidentified ANTP-type genes, one of which (L19173) shows little affinity to any other sequence. More than a decade later, in the most significant study of Hox genes in a parasitic flatworm published to date, Pierce et al.'s [36] survey of *S. mansoni* also recovered orthologues of *Lab*, *Dfd* and *Lox2* (*Abd-A*) and characterized the full coding sequence of *Smox1* from the previous work of Webster and Mansour [23], enabling it to be identified as an orthologue of *Lox5* (see also Section 2.4 below). Most recently, a similar complement of anterior and central ANTP-class homologues were partially characterized from the recently described [37] human tapeworm *Taenia asciatica*: two paralogues each of *Lab* and *Hox3*, and one orthologue each of *Dfd* and *Lox2*/*Lox4* [24]. These few studies, combined with a number of unpublished homeobox-containing sequences from cestodes on GenBank (i.e. *Echinococcus* and *Mesocestoides* spp.) and of *Hymenolepis* reported herein, demonstrate the paucity of available information.

2.2. Identification of posterior Hox genes and the derived position of the flatworms

Prior to 2001, homologues of the *Drosophila* posterior gene *Abdominal-B* or of the lophotrochozoan posterior genes *Post-1*/*2* (see below) had yet to be found in either parasitic or free-living flatworms. It was thus speculated that their absence in flatworms could indicate a more recent evolution of the BX-C [34]. However, acceptance of the 18S-based 'new animal phylogeny' [38–40] and subsequent studies [29,41] showing that the flatworms were not simple, primitive animals, but rather were members of a much larger clade including the molluscs, annelids and other minor phyla christened the Lophotrochozoa, suggested that the absence of posterior Hox genes represented instead the loss of these genes in the flatworm lineage. At about the same time molecular evidence began to accumulate in the form of ribosomal and protein-coding genes [42–45], Hox gene signatures [46] and mitochondrial codon usage [47], to support the position of the acoelomorph 'flatworms', traditionally considered to be basal members of the Platyhelminthes, as the most basal branch of the triploblastic Bilateria and thus sister to the 'Eubilateria' [48] (i.e. Deuterostomia, Lophotrochozoa and Ecdysozoa). Attention thus turned toward the acoelomorphs (e.g. [19,46]) for studies aimed at understanding the 'proto-bilaterian' Hox complement.

It was eventually found [10,41] that the two great protostome clades, Ecdysozoa and Lophotrochozoa, share unique suites of central and posterior Hox genes with lophotrochozoans characterized by three central genes (*Lox2*, *Lox4* and *Lox5*) and two posterior genes (*Post-1*/*2*), in addition to the five anterior genes also found in ecdysozoans and deuterostomes: *lab*/*Hox1*, *pb*/*Hox2*, *zen*/*Hox3*, *Dfd*/*Hox4* and *Scr*/*Hox5*. Thus unless the flatworms had lost their posterior genes, there was reason to assume that they should possess the same complement as found in other members of the Lophotrochozoa. Indeed, in 2001, posterior Hox homologues were first characterized in planarians: Saló et al. [22] reported unpublished data of an *Abd-B*-related gene (i.e. *GtAbd-Bb*) in *Girardia tigrina* and in the same year Nogi and Watanabe [49] fully characterized two posterior genes from *Dugesia japonica* (*DjAbd-Ba* and *DjAbd-Bb*) that showed clear affinities to *Post-2* (and possibly also *Post-1*; see Fig. 1). Although the ParaHox posterior gene *caudal*/*Cdx* [6] has not yet been found in a parasitic flatworm, it has been characterized in a polyclad flatworm (i.e. *Discocelis tigrina*), and homologues of each of the three ParaHox (*Gsx*, *Xlox* and *Cdx* [11]) genes are found in other lophotrochozoans, in acoelomorphs [19,46] and throughout the Eubilateria, suggesting that they are most likely also present in flatworms.

2.3. Hox genes in the model tapeworm Hymenolepis

In an effort to better understand the molecular basis of development in cestodes, and specifically that relating to the process of segmentation, degenerate primer PCR/RT-PCR-based Hox screens using the model cestode *Hymenolepis microstoma* have been initiated by the author. Using a variety of primers (see e.g. [50] and [30]) directed at the conserved peptides of the homeodomain helices 1 and 3/4, readily identifiable orthologues of the anterior and central genes *Lab/Hox1*, *Dfd/Hox4* and *Lox4/Abd-A* have been characterized, some non-Hox ANTP-type genes (e.g. *NK2*), as well as more highly divergent sequences whose identities have yet to be confirmed. The majority of the ~1000 clones sequenced resulted in homologues of *Lox4/Abd-A* regardless of the primer combination used to produce the cloned products, hindering a more comprehensive survey. It is interesting to note that Pierce et al. [36], also using a degenerate primer approach, recovered the same complement of genes in their survey of Hox orthologues in *S. mansoni*. It may be that the most conserved regions of the homeodomain targeted for priming provides the best match in parasitic flatworms to these particular genes, or that these genes are particularly abundantly expressed making the amplification of other homeoboxes from cDNA difficult. Moreover, as introns are commonly found within the homeodomain region [8], they may be hindering amplification from genomic templates. As an alternative approach, a genomic library for *Hymenolepis* has been constructed and will be used to screen for additional homeoboxes via the hybridization of *Hymenolepis*-specific probes. By this method, the complete set of Hox/ParaHox genes may be recovered with some certainty (i.e. short of characterizing the full genome of *H. microstoma*).

In addition to the general screens described above, particular effort was made to target posterior Hox and ParaHox orthologues in order to yield markers that can be used resolve the polarity of the AP axis in cestodes as discussed below. Attempts to characterize the ParaHox gene *caudal* (*Cdx*) using degenerate primers targeting acoels [46] and protostomes [51] and modifications of such based on comparison of published lophotrochozoan *Cdx* sequences failed thus far to amplify an identifiable *Cdx* or other ParaHox orthologue in *Hymenolepis*, and thus the presence of any ParaHox gene in a parasitic flatworm remains to be demonstrated. However, thanks to the work of Nogi and Watanabe [49], use of their *Abd-B*-directed

primers and modifications thereof produced a clear orthologue of lophotrochozoan *Post-2* in a parasitic flatworm for the first time. In addition, two forms (presumably paralogues) of a posterior-like gene were recovered that may represent orthologues of *Post-1*, albeit their identities appear ambiguous in phylogenetic analysis (see Section 2.4 below).

Full transcripts of the Hox genes thus far identified in *Hymenolepis* have been characterized by the rapid amplification of cDNA ends in preparation for the construction of riboprobes. Messages range in size from ~1250 bps (*Lox2*) to ~2750 (*Post-2*) and the position of the homeodomain within the transcripts varies considerably from being quite near either the 5′ or 3′ end or is located more centrally. Little amino acid identity among the genes is found outside the homeodomain. Efforts to examine their expression patterns via WMISH are currently underway.

2.4. Phylogenetic analysis of flatworm Hox and ParaHox genes

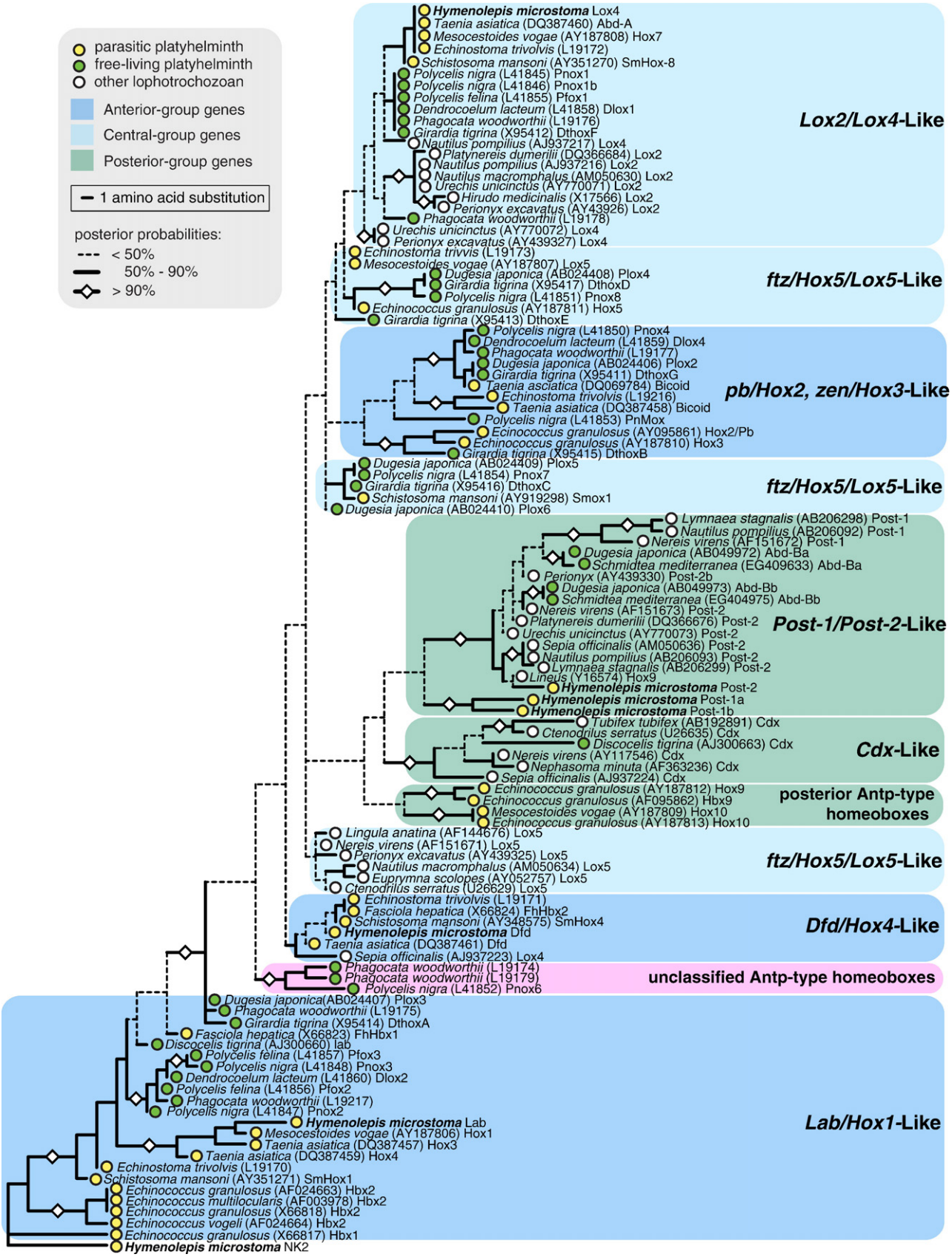
Identifications of the newly characterized Hox genes in *Hymenolepis* are supported by characteristic amino acid residues [10], by the position of introns in the case of *Post-2* (see Fig. 22 in [8]) and by phylogenetic analysis: Fig. 1 shows the results of Bayesian inference analysis of Hox and ParaHox (i.e. *Cdx*) genes from 112 parasitic and free-living platyhelminths and select lophotrochozoans (see figure legend for details of the analysis). Sequences of *Hymenolepis* shown in bold are readily identified within recognizable orthology groupings [10], although the small number of characters and fragmentary nature of the data are insufficient to provide a robust solution (e.g. three times the number of characters to taxa is considered a desired minimum for phylogenetic analysis, whereas the present analysis has over four times the number of taxa to characters). For example, *Lox2*/*Lox4*-like orthologues (lophotrochozoan homologues of *Ubx*/*Abd-A*) of four different genera of parasitic flatworms show 100% identity at the amino acid level. Moreover, they form a sister-group to a digenean (i.e. *Schistosoma*), that combined forms the sister-group to a number of free-living flatworms, that altogether is sister-group to a non-platyhelminth lophotrochozoan. Thus central homeodomain sequences of the *Lox2*/*Lox4*-like genes in flatworms are conserved enough to be both easily identifiable and to recover, to some extent, the phylogenetic history of the group: the fact that the parasitic flatworms share a common ancestor more recent than that shared by both parasitic and free-living flatworms has been strongly supported by molecular data; for a review see [52]. In contrast, most other putative orthologues show high degrees of divergence: *Post-1*-like and *Post-2*-like orthologues of *Hymenolepis*, for example, fail to group with those of other flatworms, albeit the posterior Hox and ParaHox (i.e. *Cdx*) genes each formed well supported clades. The two putative *Hymenolepis* paralogues of *Post-1* failed to group with the lophotrochozoan *Post-1* genes, and instead formed a separate lineage between the *Post-1/2* and *Cdx* clades, making their affinities uncertain. More problematic is a clade of 'unclassified posterior ANTP-type homeoboxes' (Fig. 1) characterized from cestodes that appear most similar (via BLASTx [53]) to the *Hox9* and *Hox10* posterior genes of vertebrates, which in turn are thought to be homologous with the lophotrochozoan *Post-1* gene [41]. Why these sequences fail to group with the *Post-1* genes in the analysis is thus unclear, but raises the possibility that additional posterior paralogues may be present in parasitic flatworms.

Results of the analysis suggest that many published flatworm homeobox sequences have been misidentified in the literature (e.g. *bicoid* is found only in dipterans). This is not particularly surprising given that the vast majority represent partial homeodomain sequences ~100 bps in length and that relevant comparative data on lophotrochozoan homeoboxes have been published only recently. However, even with additional sequences available, a few of the homeoboxes as well as clades of homeoboxes remain difficult to identify from these limited data. For example, the planarian homeobox *Pnox6* (L41852) could not be readily classified at the time of its publication [56], nor can it be classified herein as its only affinities appear to be two unidentified homeoboxes of another planarian from the earlier work of Bartles et al. [35]. The *Smox1* gene of *S. mansoni* (Genbank: AY919298), among the few available fully characterized transcripts, groups strongly with three other free-living flatworm genes, but shows little orthology to anything else via BLASTx screening (most common returns are vertebrate Hox genes such as *Hox-B7*). Pierce et al. [36] indicated *Smox1* to be a *Lox5* orthologue, supported by phylogenetic analysis and signature residues found outside the homeodomain. In the present analysis, the putative *Lox5* orthologues (including *Smox1*) form a large number of independent clades and individual lineages and thus appear to be either highly divergent or else represent a series of paralogues. The full compliment of flatworm Hox and ParaHox genes, as well as the homologies of many previously characterized flatworm homeoboxes remain uncertain.

2.5. Summary

Despite the small number of studies and published sequences, it is clear that parasitic flatworms have a similarly rich set of Hox genes and of the many related families of homeobox genes, e.g. ParaHox, *Pax*, NK (for an overview see [57]), as other members of the Lophotrochozoa, as well as a number of lineage-specific orthologues and paralogues arising from gene duplication events specific to their clade [24]. Members of the anterior and central-class ANTP-type genes are present, as are both members of the lophotrochozoan posterior genes, now identified in both free-living [49] and parasitic (herein) flatworms. If the Hox genes of flatworms are in fact dispersed rather than clustered within the genome (see below), then the precise complement of genes will be difficult to determine before complete genomes are available and fully annotated. Such efforts are currently underway for both free-living (*Schimdtea mediterranea* [28]) and parasitic (*S. mansoni* [58] and *Taenia solium* [59]) flatworms, and these data will eventually facilitate the characterization of homeoboxes and associated genetic elements from other flatworms. Moreover, once annotated, the genomic arrangement of the genes will be known from representatives of both free-living and parasitic

taxa. It is premature, however, to be overly sanguine about the utility of these data as the construction and annotation of complete genomes is an enormously difficult task relative to the generation of the raw data. The contribution of genome data to evo-devo research in flatworms thus remains a promise for the (perhaps) distant future rather than a tool with imminent utility.



3. Arrangement and expression of Hox genes in parasitic flatworms

3.1. The Hox cluster and colinearity

Hox and other classes of the ANTP superclass are typically arranged in clusters on the chromosome [6], accepted as resulting from tandem gene duplication and subsequent selection pressure for the maintenance of linkage groups (albeit model organisms such *Drosophila* and *Ceanorhabditis* prove atypical in having broken or disrupted clusters). Early, the astonishing discovery was made that their physical arrangement relates directly to their spatial and temporal expression along the AP axis of developing embryos, such that the order in which the genes are expressed, as well as the timing of their expression, relates directly to the order in which the genes are arrayed in the cluster [60]. Encompassing both spatial and temporal aspects of this relationship, the principle of 'colinearity' has long been a paradigm of the field, albeit it is far from universally observed and spatial colinearity can be preserved even when the Hox cluster is known to be disrupted [61]; and conversely, intact, well-ordered clusters may fail to show colinear expression patterns [20,62]. Nevertheless, the mode of evolution, the constraint of temporal colinearity [62] and comparative analysis all suggest that clustering of Hox and other classes of developmental genes represents an ancestral condition that has been highly conserved [6].

Fully characterized Hox clusters in lophotrochozoans have not yet been published (albeit that of a polychaete worm is expected shortly via D Ferrier and AS Monterio, pers. comm.), but the work of Pierce et al. [36] on *S. mansoni* demonstrates that in some platyhelminths the Hox genes may be at least partially dispersed within the genome. Using fluorescence *in situ* hybridization, they localized four Hox genes to chromosomes demonstrating that *SmHox4*/*Dfd* and *SmHox8*/*Abd-A* reside on chromosome 4, whereas *SmHox1*/*Lab* and *Smox1* are located on chromosome 3. Screening of these genes against the *S. mansoni* genome (http://www.sanger.ac.uk/Projects/S_mansoni/) as well as primer walking of BAC libraries showed no evidence of paralogues, suggesting that the 'cluster' has not been duplicated and thus the dispersed Hox genes characterized are the only copies present in the genome. Following from the idea that a clustered arrangement is ancestral, dispersed or disrupted 'clusters' are thought to be typical of highly derived and divergent taxa [20], of which *Schistosoma* is a good example being both a member of the derived Neodermata (a clade encompassing all parasitic flatworms) within the Platyhelminthes, as well as being highly divergent within the Digenea (e.g. uniquely exhibiting dioecy). It remains to be seen if *Schistosoma* is typical of the genomic arrangement of Hox genes in other flatworms or lophotrochozoans.

3.2. Hox gene expression in flatworms

Hox gene expression is most commonly studied by WMISH, allowing the visual detection of spatial expression patterns *in situ* by staining with (typically) digoxigenin-(DIG) labelled riboprobes [63]. It is surprising then that WMISH has yet to be employed in studies concerning the Hox genes of parasitic flatworms, and as a result almost nothing is known about their spatial expression patterns. The sole exception is the early study of Martínez et al. [64] who localized *EgHbx3* (an NK-type homeobox) to the stalks of protoscolices of the cestode *Echinococcus granulosus*. Their work was followed up by the characterization of the proximal regulatory domain of this gene, but no further examination of spatial expression patterns of this or other homeobox genes was reported [65]. In planarians, strict spatial colinearity does not appear to be followed, as for example, Nogi and Watanabe [49] found that the anterior boundary of expression of a planarian posterior gene was anterior to those of some central-class genes [30,66]. However, varying degrees of colinearity or the lack of such have been observed using different planarians and in the opinion of Saló and Baguñà [31], more work is needed before sound conclusions regarding colinearity in planarians may be drawn. Moreover, such overlapping boundaries as described by Nogi and Watanabe [49] belie a less strict general pattern of spatial colinearity (i.e. expression of the *AbdB*-like gene extended posterior to that of the central Hox genes) and some disruption of colinearity does not therefore necessarily negate the utility of these genes as cell or region-specific markers. With regard to temporal colinearity, Pierce et al. [36] used semi-quantitative PCR to demonstrate that Hox gene expression occurs throughout the life cycle of *S. mansoni*, and that the genes examined show stage-specific levels of expression. However, it is not known in what way such developmentally-regulated expression relates to their ontogeny. What is needed now are baseline data on the spatial expression patterns of Hox genes throughout the ontogeny of a range of representative species. This will enable information on Hox gene expression to be better generalized across the parasitic flatworms and to be compared with that in free-living flatworms and more distantly related bilaterian taxa.

4. Opportunities and challenges

4.1. Unique developmental sequences, life history strategies and outstanding questions

The four primary lineages of parasitic flatworms: cestodes, trematodes (aspidogastreans and digeneans) and monopisthocotylean and polyopisthocotylean 'monogeneans' (a paraphyletic assemblage; see [52]), exhibit among the most fascinating and

Fig. 1. Phylogenetic analysis of 112 Hox and ParaHox (i.e *Cdx*) genes of parasitic and free-living platyhelminths (and other lophotrochozoan taxa) based on Bayesian inference of an alignment of 25 putative peptides spanning the central region of the homeodomain (from the end of Helix 1 to the beginning of Helices 3/4; i.e. *Drosophila Antennapedia* gene: "HFNRYLTRRRRIEIAHALCLTERQI"). Terminals show species and GenBank sequence accession numbers followed by the original sequence designation where provided in the literature or sequence accession. Unpublished sequences of *Hymenolepis microstoma* (see Section 2.3) shown in bold. Tree rooted with the *NK2* gene of *H. microstoma*. Analysis via MrBayes [54] using the WAG [55] amino acid substitution model with gamma-distributed rate variation. Figure based on 'contype = allcompat' consensus of 500,000 generations (samplefreq = 100; burnin = 300). Note low posterior probabilities supporting most of the backbone of the tree (dotted lines) resulting from the small number of sites in the homeodomain analyzed cf. the number of sequences. Hypothesized orthology groupings shown by coloured boxes (nomenclature follows de Rosa et al. [10]).

complex life cycles in the animal kingdom. Extreme adaptations to parasitism are unique and diverse, involving changes to their morphology, physiology, immunology, development and life history. Completion of the 'typical' digenean life cycle, for example, involves 5–6 discreet ontogenetic stages, 3 host species including both invertebrate and vertebrate phyla, sexual and asexual reproductive modes, passive and active transmission strategies and free-living and parasitic phases. Despite the complexity of these extreme *r*-strategists (i.e. organisms whose reproductive effort is invested in output rather than care of the offspring), the Digenea is by far the most successful group of parasitic flatworms, with over 30,000 described species infecting animals as diverse as chordates, molluscs, arthropods, annelids, echinoderms and diploblasts (cnidarians and ctenophores) [67]. The tapeworms also require multiple hosts for completion of their life cycles (i.e. have complex life cycles) and exhibit a considerable diversity of adaptations, including fully terrestrial as well as aquatic life cycles and both sexual and asexual modes of reproduction in addition to the serial formation of the hermaphroditic reproductive organs (i.e. proglottization) that is the hallmark of the group. In contrast, 'Monogenean' life histories are typically direct and thus comparatively more simple, albeit adaptations that enable increased reproductive output include both viviparity and polyembryony (see below).

A considerable part of parasitological study during the previous century was aimed at simply revealing the enormous diversity of life histories found among parasitic flatworms, and later attempting to synthesize this information within an evolutionary framework. We now possess the tools needed to address the molecular basis for these adaptations and to thus begin to understand the evolution of parasitism in flatworms from the level of the gene. Contributing to this, the study of Hox genes, whilst not a panacea, offers considerable promise in resolving long standing problems in understanding their development and how such morphological diversity has arisen. For example, the seemingly rudimentary question of polarity in the AP axis of tapeworms has been debated since the beginning of the 20th century [68,69] and remains unresolved to this day [70]. Although the conventional interpretation dating back to Leukart [69] posits the scolex (holdfast organ) as the anterior pole (and apparent cephalisation of their nervous system supports this), a number of significant features point to the opposite orientation. The most significant of these are the relative positions of ovaries and testes in cestodes being the reverse to the arrangement typical of trematodes, 'monogeneans' and free-living planarians [70], and the fact that the functional pole of the first stage larvae (oncosphere) bearing hooks and secretory glands is opposite that from which the scolex develops subsequently. A comparative and functional interpretation therefore suggests that the scolex is posterior and the region of growth ('neck') is subterminal, perhaps similar to development in annelids. Questions of this nature can be addressed more directly and definitively by examining developmental genes: expression of posterior genes such as *Post-1/2* and *Cdx*, in combination with anteriorly-expressed 'head' genes (e.g. *Otx* [71,72]), should provide *in situ* markers of the anterior and posterior poles of tapeworms and thus settle a century-old conundrum. Such questions, in part, form the basis of the work initiated using *Hymenolepis* discussed above.

Many other long standing questions may benefit from the study of Hox genes in cestodes. Of particular interest to myself is the evolution of segmentation, the apparent de-coupling of proglottization and somatic compartmentalization, as inferred from examples of secondary loss of the latter process (in e.g. *Anantrum*; see [73]) and whether or not unsegmented tapeworm groups are indeed 'primitive' among cestodes [52,73,74]. A more fundamental question is whether the genetic programme underlying segmentation in cestodes is homologous to other forms of segmentation, such as metamerism in annelids or strobilation in scyphozoan cnidarians (jellyfish), or if it represents a developmental programme uniquely derived. Comparison of Hox gene expression in cestodes with that of the wider Bilateria could provide insight to these questions by providing evidence of broad scale homologies (or lack thereof).

In the Digenea, especially outside the genus *Schistosoma*, almost nothing is known about the molecular basis of development. Examination of their extraordinarily diverse developmental sequences in a phylogenetic context [67,75] suggests that many features, such as the intercalation of a sporocyst stage between the redial and cercarial stages, have been gained and lost numerous times and are thus likely to be under the control of simple genetic switches that initiate or suppress gene expression. In the intramolluscan stages, the relatively superficial difference between rediae (possessing a mouth and simple gut) and sporocysts (lacking such feeding apparatus) may well be controlled in part by the expression of homeotic genes, which could also help explain differences in cercarial morphology and other highly plastic adaptations that have enabled the group to parasitize such a broad spectrum of hosts and ecosystems. Dioecy is another intriguing developmental anomaly in digeneans (exclusive to members of the Schistosomatidae, and to varying degrees in the enigmatic Didymozoidae) that has been addressed from a number of different perspectives in *Schistosoma* [e.g. 76,77], but may also benefit from an understanding of the roles played by homeotic genes.

The monopisthocotylean and polyopisthocotylean 'monogeneans' have been the least studied of the parasitic flatworms with respect to the molecular basis of their development and adaptations to a parasitic lifestyle. One of their most unusual developmental patterns, mentioned above, involves polyembryony in *Gyrodactylus*: a 'Russian doll' scenario in which live young are born replete with immature worms in their uterus that in turn contain young in their uterus, and so on (for a review see [78]). An equally extraordinary and unique form of development involves the pairing and somatic fusing of members of the Diplozoidae which in order to reach sexual maturity must become literally fused in permanent copula, involving considerable somatic developmental reorganization that results in the formation of a single, reproductively mature 'individual' [79].

The parasitic flatworms thus provide a rich set of basic developmental questions awaiting study of their molecular mechanisms. Indeed, with the recent resurgence in stem cell research, the parasitic flatworms, and particularly the cestodes with their 'immortal' germinative region, may prove excellent models alongside their more traditionally studied free-living

cousins. Whilst the Hox family of homeobox genes are specifically involved in the patterning of the AP axis, conservation of the roles and expression patterns of these and the many other classes of homeoboxes have shown fundamental developmental patterns to be conserved throughout the Metazoa and may thus shed light on some of the questions outlined above.

4.2. Lack of comparative models and data

A significant challenge to evo-devo studies on parasitic flatworms is the lack of comparative data. Although 'experimental evo-devo' is beginning to apply functional genomic tools to the study of developmental genes [80], most work to date has been comparative and studies on organisms such as arthropods, for example, benefit from the pioneering work on *Drosophila* which led to the initial discovery of the homeobox [2], and from extensive traditional morphological research on the homologies of arthropod body plans. Thus characterization of homeobox genes in arthropods is comparatively simple thanks to the availability of so many previously characterized sequences and entire clusters of genes and associated genetic elements. Designing primers to target the homeodomain of parasitic flatworms is more difficult as so few genes have been characterized, and of these only a small minority represent complete genomic or mRNA sequences. The situation for the free-living flatworms is certainly better thanks to early and ongoing efforts by workers in the field, but still doesn't benefit from the many practical advantages of working on arthropod and vertebrate taxa. Current initiatives to characterize genomes and transcriptomes in parasitic flatworms will help ameliorate some of these difficulties, but remain a considerable time away from their completion, and longer still from their full assembly and annotation.

4.3. Maintenance and husbandry of parasitic flatworms

Another considerable challenge of working with parasitic flatworms in the laboratory is their husbandry. Maintenance of animals with complex life cycles, particularly where vertebrate hosts are involved, is laborious, costly and subject to strict legal conditions governing animal welfare. Some of the most important species with regard to medical and economic interests are often impractical if not effectively impossible to rear in the laboratory. At the same time, it is difficult to secure the funding and infrastructure to rear non-medically or economically important species (most of which are indeed equally impractical or have yet to have their life cycles fully elucidated). Whereas the characterization of homeotic genes may only require occasional access to specimens, expression studies involving WMISH benefit from a continual source of study material, and the challenges of husbandry may explain in part why almost no such study has been conducted in parasitic flatworms despite the number of Hox genes now characterized. Thus in comparison to models such as *Drosophila*, *Ceanorhabditis* and the exceedingly amiable planarians [26], the parasitic flatworms do not lend themselves readily to evo-devo research.

Among tapeworms, members of the genus *Hymenolepis* have been the models of choice since the early 1950s, and most of our understanding of cestode physiology, biochemistry and host–parasite interactions stems from work on this genus (e.g. [81,82]). Common parasites of rodents utilizing beetles as intermediate hosts, *H. diminuta*, *H. nana* and *H. microstoma* have been passaged in the laboratory for more than 50 years and provide the best practical choices for researchers requiring access to both larval and adult stages. Another tapeworm of mammals, *Mesocestoides* spp., provides an even more attractive model system in some respects due to the biology of their unusual 'tetrathyridia' larvae which multiply asexually in the body cavities of laboratory-maintained rodent hosts [83] and are maintained with relative ease *in vitro*. Moreover, tetrathyridia can be induced to strobilate *in vitro* [84] and this has proven a valuable system for understanding the molecular basis of their development (see e.g. [85]).

Among monopisthocotylean 'monogeneans', *Gyrodactylus* is an excellent model organism requiring nothing more sophisticated than an aquarium of guppies, whereas polyopisthocotyleans are primarily marine and therefore less amenable to the laboratory. The complex life cycle of the digeneans makes their passage in a laboratory setting the most impractical of the parasitic groups. My own institution maintains a WHO centre for schistosome research that utilizes rodents as proxy definitive hosts for human-infecting species together with a wide range of snail hosts (the schistosomes not requiring a second intermediate host). Such a system requires considerable resources, however, and thus certainly does not represent an ideal model system for the individual researcher. However, asexual multiplication in the intra-molluscan stages and enormous egg production are advantages in that they can provide very large numbers of at least these stages with a minimum of effort. Other digeneans, e.g. *Fasciola*, can also be passaged using rodent proxy hosts, albeit inevitably with some of the problems that characterize the use of non-native hosts (e.g. low yields).

5. Summary

Since their discovery in the mid-1980s, studies on Hox and related classes of homeobox genes have had a remarkable impact on our understanding of the molecular basis of development. The parasitic flatworms, however, have remained an almost completely neglected group in the field of evo-devo in contrast to a significant body of literature concerning their free-living ancestors, and indeed the large number of interesting questions that would benefit from understanding the roles of homeobox genes in shaping the complex developmental patterns characteristic of the parasitic groups, including questions pertaining to omnipotent stem cells that thus far have been addressed only in free-living models. A scattering of surveys suggests that the flatworm complement of Hox genes is typically lophotrochozoan, and chromosomal *in situ* hybridization studies in *Schistosoma* [36] suggest the genes are at least partially dispersed within the genome, making the full complement of Hox genes difficult to determine in the absence fully characterized and annotated genomes. Possibly a result of this dispersion, flatworms may not exhibit strict temporal colinearity in the expression of their Hox genes [36], and work on free-living planarians suggests strict spatial colinearity has

been lost in at least some cases [30,49,66]. Thus the clustered genomic arrangement and corresponding colinear expression patterns characteristic of Hox genes of many other animal groups [60] may be at least partially disrupted in flatworms. Moreover, the derived position of the Platyhelminthes as members of the Lophotrochozoa, in contrast to traditional views of their representing the 'protobilaterian' condition, suggests that such features have been lost secondarily. However, as our knowledge of Hox expression in flatworms is rudimentary and lacking entirely among the parasitic groups, such generalizations are highly tenuous and thus likely to be overturned quickly as new results emerge. The enormous diversity in the developmental strategies exhibited by the parasitic groups and the near total lack of understanding for the molecular mechanisms involved represent opportunities ripe for the application of evo-devo methodologies. The lack of available information and the labor, expense and legalities involved in the husbandry of these parasites represent challenges that make the parasitic groups less attractive than many more widely employed model systems. A concerted effort to understand spatial expression patterns through WMISH studies in parasitic taxa is needed to develop at least a baseline understanding of the roles of Hox genes in the developmental patterns of the different groups and to allow for inferences to be made through comparison with the better studied free-living flatworms and other lophotrochozoan taxa. Such studies would benefit from the use of a range of taxa including those targeted specifically for their potential as developmental models.

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