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Evolutionary crossroads in developmental biology: annelids

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Summary

Annelids (the segmented worms) have a long history in studies of animal developmental biology, particularly with regards to their cleavage patterns during early development and their neurobiology. With the relatively recent reorganisation of the phylogeny of the animal kingdom, and the distinction of the super-phyla Ecdysozoa and Lophotrochozoa, an extra stimulus for studying this phylum has arisen. As one of the major phyla within Lophotrochozoa, Annelida are playing an important role in deducing the developmental biology of the last common ancestor of the protostomes and deuterostomes, an animal from which >98% of all described animal species evolved.

Key words: Annelida, Polychaetes, Segmentation, Regeneration, Central nervous system

Introduction

"*You have made your way from worm to man,*
but much of you is still worm."
Friedrich Nietzsche, *Thus Spoke Zarathustra*
(trans. R. J. Hollingdale, 1961)

The phylum Annelida gets its name from the latin 'Anellus' (meaning little ring) owing to the overt segmentation exhibited by most members of the group, a feature that annelids share with only two other phyla, the arthropods (see Glossary, Box 1) and the chordates (see Glossary, Box 1) [also see Minelli (Minelli, 2004) for a discussion of what is meant by 'segmentation']. The annelids are one of the few phyla that contains species occupying a range of habitats from marine to freshwater to terrestrial, although the terrestrial species do only occupy rather damp terrestrial niches. This phylum includes the terrestrial earthworm, which was championed by Charles Darwin to the extent that a cartoon published in Punch's Almanack (1882) declared "*Man is but a worm*" (Box 2), as well as the blood-sucking leeches, the predatory ragworm often used as bait by fishermen, and the beautiful, majestic tube-building fanworms. Such a range of habitats along with a wide range of feeding strategies, including predation, deposit feeding, filter feeding, parasitism and symbiosis, as well as the unusual strategy adopted by the bone-eating snot-flower worm, *Osedax mucofloris* (Glover et al., 2005), coincides with an impressive diversity of body forms and development. It is this diversity that makes the annelids a rich source of 'material' for investigating developmental mechanisms as well as the evolution of development.

A variety of species are used in annelid evolutionary developmental biology studies (some of which are shown in Fig. 1), which is one of the strengths of this area of research and is essential for deducing accurate ancestral states for annelids as a whole to allow more robust comparisons with other phyla, as well as for understanding the evolution of diversity. Much of annelid evolutionary developmental biology research, although by no means all of it, has tended to concentrate on three particular taxa: the polychaete (see Glossary, Box 1) *Platynereis dumerilii*; the polychaete *Capitella teleta* (previously known as *Capitella sp.*); and the oligochaete (see Glossary, Box 1) leeches, such as *Helobdella*. Even within this small selection of annelids, a good range of the diversity in annelid biology is evident. Both polychaetes are marine, whereas *Helobdella* is a freshwater inhabitant. The polychaetes *P. dumerilii* and *C. teleta* are indirect developers (see Glossary, Box 1), with a larval stage followed by metamorphosis into the adult form, whereas *Helobdella* is a direct developer (see Glossary, Box 1), with the embryo developing into the worm form without passing through a swimming larval stage. In addition, *P. dumerilii* is a broadcast spawner whereas *C. teleta* retains the embryos in a brood tube, and leeches produce a cocoon. Notably, *P. dumerilii* is considered to be an errant annelid (see below), actively exploring the sea bed to scavenge and predate, and its morphology reflects this, with prominent jaws and an abundance of sensory appendages and structures on its head and prominent trunk appendages for gaining traction. By contrast, *C. teleta* burrows around in the sediment much more and so has a more 'earthworm-like' appearance, without prominent head or trunk appendages that would be a hindrance to this lifestyle. Leeches provide a further contrast with the innovation of their anterior and posterior suckers, used for both locomotion and feeding.

This by no means covers all of the diversity within the annelids, and some of the other species used in evolutionary developmental biology studies, and which are mentioned elsewhere in this review, are listed in Fig. 2 along with the phylogenetic relationships among these taxa. Clearly there is a need for work in yet more species in order to represent properly the evolutionary diversity in the annelids as well as to determine the ancestral states for various developmental processes and features more reliably.

Recent developments in annelid phylogeny

Understanding the relationships among a group of species within a phylum, as well as the phylogenetic relationships relative to other phyla, is an essential prerequisite for any form of comparative, evolutionary research, including evolutionary developmental biology. As segmentation is externally visible in both adult annelids and arthropods, traditional evolutionary scenarios and phylogenies of the animal kingdom proposed a sister group (see Glossary, Box 1) relationship between these two phyla. They were known collectively as the Articulata, with the arthropods evolving from a more annelid-like ancestor. With the advent of molecular phylogenetics, however, this sister group relationship was quickly abandoned (Field et al., 1988) and, whereas the Arthropoda have taken residence within the super-phylum Ecdysozoa (see Glossary, Box 1), alongside nematodes, onychophorans, priapulids and other phyla, the Annelida have come to occupy a position within the Lophotrochozoa (see Glossary, Box 1), alongside molluscs,

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Box 1. Glossary

Arthropods. Members of the phylum Arthropoda, having a hard exoskeleton and jointed appendages. Includes insects such as *Drosophila melanogaster*, along with crustaceans, myriapods and chelicerates.

Chordates. Members of the phylum Chordata, principally distinguished by the possession of a notochord at least at some point in their life cycle. Includes vertebrates, urochordates (e.g. *Ciona intestinalis*) and Cephalochordates (e.g. amphioxus).

Clitellata. Annelids that possess a cocoon-forming clitellum, at least during the reproductive stages of their lives.

Direct developers. Animals that do not possess a free larval stage that is distinct from the adult stage.

Ecdysozoa. A super-phyletic clade of animals that periodically shed their cuticle (moult) via a process called ecdysis.

Echinoderms. Members of the phylum Echinodermata, possessing a calcareous endoskeleton, water vascular system and pentaradial symmetry. Includes sea urchins, starfish, crinoids and sea cucumbers.

Errantia. The clade of annelid families that tends to have an errant lifestyle, actively foraging over the sea-bed.

Hemichordates. Members of the phylum Hemichordata. Marine benthic worms with pharyngeal gill slits and deuterostome development, but lacking a notochord. Includes the acorn worms (enteropneusts) and pterobranchs.

Indirect developers. Animals that have a free larva that is distinct from the adult form and which usually undergoes some degree of metamorphosis.

Lophotrochozoa. A super-phyletic clade of animals that contains phyla with either a lophophore (a ciliated, tentacular feeding structure that also has a coelomic cavity derived from the middle coelomic compartment, the mesocoel) and/or larvae based on the trochophore form (see main text).

Oligochaeta. Annelids with fewer, less pronounced chaetae than polychaetes (see below) and no parapodia.

Parapodia. The locomotory body appendages of polychaete annelids.

Phylogenomics. The approach to building phylogenetic trees that uses genome-scale data sets, either from transcriptome or whole-genome data.

Polychaeta. Annelids with many bristles, or chaetae, that are usually used for gaining traction during locomotion and often project from the ends of the parapodia. With the nesting of the clitellates within the polychaetes, the Polychaeta are now considered to be paraphyletic, and the less formal term 'polychaete' might now be more appropriate.

Sedentaria. The clade of annelid families that tends to have a sedentary lifestyle, either being sessile filter feeders or burrowing deposit feeders (although the predatory and parasitic leeches are also members of this clade).

Sister group. A group of organisms that in a phylogeny is the most closely related to whichever group is under discussion.

Box 2. Man is but a worm

A cartoon from Punch's Almanack for 1882 (published in *Punch Magazine* on 6th December 1881) showing Darwin evolving from chaos via an annelid worm, which in this case is his beloved earthworm. At the time of this cartoon it was perhaps thought to be rather tongue-in-cheek, poking fun at the idea that something as grand as a human could have arisen via evolution from such basic forms as worms. However, with modern discoveries in evolutionary developmental biology and genomics, the degree of molecular similarity between animals such as humans and annelid worms is often surprising and undoubtedly more profound than many from the pre-molecular era would ever have imagined. Given the preponderance of worm-like forms across the animal kingdom, it is clear that the last common ancestor of annelids and humans will have been some sort of worm. Man is indeed but a rather fancy worm.

platyhelminths and a handful of other phyla (Halanych et al., 1995; Aguinaldo et al., 1997) (Fig. 2A). This rearrangement has had important implications for our understanding of the evolution of animal segmentation and for comparing developmental mechanisms between the segmented phyla.

Within Annelida, the relationships between the families have been notoriously difficult to resolve, but have recently been significantly clarified (Fig. 2B) (Struck et al., 2011). The elucidation of these relationships impacts our understanding of the polarities of any evolutionary change and the relationships among the lineages being compared, i.e. how close or distant these lineages are or which is more basal than another. The lifestyles of annelids are often referred to as either errant (the Errantia, see Glossary, Box 1) or sedentary (the Sedentaria, see Glossary, Box 1). This distinction was generally thought to have little phylogenetic relevance, until recently. Struck et al. (Struck et al., 2011) used phylogenomics (see Glossary, Box 1) to address the problem of annelid family relationships, and one of the surprising findings is that the Errantia and Sedentaria groupings might have phylogenetic relevance after all. Together, they have been called the Pleistoannelida, and only a handful of annelid lineages fall outside this Pleistoannelid clade (Struck, 2011) (Fig. 2B). An upshot of this is that the Pleistoannelid ancestor is hypothesised to be a surface-dwelling, benthic, mobile polychaete that possesses parapodia (see Glossary, Box 1), with the ancestor of the annelids as a whole also having possessed these annelid appendages (Struck, 2011; Struck et al., 2011; Eibye-Jacobsen and Vinther, 2012). In turn, oligochaetes, hirudinids and the closely associated polychaetes, such as *Capitella*, have lost parapodia, and thus developmental processes involved in making these structures are presumably modified. A second example of loss of a major morphological character within the annelids has come to light with the progress of molecular phylogenetics: the loss of segmentation in the echiurans and possibly sipunculans. The Echiura were traditionally considered to be a distinct phylum of unsegmented,

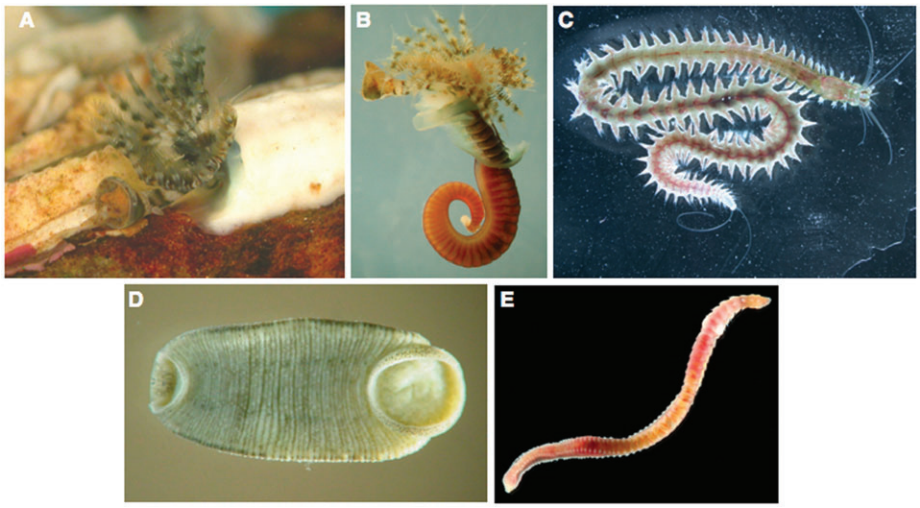

Fig. 1. Representatives of the major groups of Annelida. (**A**) *Pomatoceros lamarckii* (family Serpulidae and a member of the Sedentaria), with its branchial crown (or fan) extending from the calcareous habitation tube. (**B**) An adult *P. lamarckii* extracted from the habitation tube. (**C**) *Platynereis dumerilii* (family Nereididae) is a typical member of the Errantia, with prominent eyes and sensory appendages on the head and well-developed parapodia. Image reproduced with kind permission from Kristin Tessmar-Raible and Detlev Arendt. (**D**) The leech *Theromyzon tessulatum* (family Glossiphoniidae) is a Hirudinid member of the Clitellata group (see Glossary, Box 1; Fig. 2B) within the Sedentaria, and has distinctive suckers at the anterior and posterior. Image reproduced with kind permission from Carmel McDougall. (**E**) *Capitella teleta* (family Capitellidae) is a polychaete within the Sedentaria, with a body form adapted to burrowing, lacking head appendages and prominent parapodia. Image reproduced with kind permission from Aldine Amiel and Elaine Seaver. Images in A and B taken by the author.

benthic, filter-feeding 'spoon worms'. Molecular studies, however, clearly showed them to be within the Annelida and hence descended from a segmented ancestor (McHugh, 1997; Struck et al., 2007). The same is also likely to be true for the unsegmented sipunculans, but their position within the Annelida is less clearly resolved than that of the echiurans (Struck et al., 2011). These examples of parapodia and segmentation exemplify the need to strive for an accurate understanding of phylogeny as well as the ancestral states of any particular group of organisms. This is needed so that conserved and derived features within certain lineages can be ascertained and the appropriate taxon chosen for making the desired comparisons, whether this is to understand the evolution of novelties or diversity within a group, or to distinguish the extent of conservation with other groups.

Early development and life cycle

Annelida, along with many other lophotrochozoans, is one of the phyla that undergoes spiral cleavage. Spiral cleavage first becomes most obvious at the third cleavage, which generates eight cells (blastomeres), occurring at an oblique angle to the animal-vegetal axis. This cleavage leads to an upper (animal) cell tier that lies over the cell boundaries of the lower (vegetal) tier of blastomeres. Subsequent cleavages continue to produce cell layers that are offset from each other (Fig. 3), as described for *Hydroides elegans* (Arenas-Mena, 2007) and *Capitella teleta* (Meyer et al., 2010). This contrasts with radial cleavage, in which the animal tier of blastomeres is located directly over the cells of the vegetal cell tier, a situation found in deuterostome lineages, such as the chordates and echinoderms (see Glossary, Box 1). Spiral cleavage may well have been the situation in the embryogenesis of the lophotrochozoan ancestor, and the term Spiralia is sometimes used interchangeably with Lophotrochozoa (Giribet, 2008). This, however, would require the secondary loss of spiral cleavage, or rather a switch to radial cleavage, in a small number of lineages, such as brachiopods [discussed in Dunn et al. (Dunn et al., 2008)]. Either way, this form of cleavage and early embryogenesis is representative for a significant portion of the animal kingdom. These early blastomere cleavages, in addition to adopting the spiral formation in Annelida, can be either equal or unequal. Unequal cleavage, such as that found in the leeches and nereids, facilitated the tracking of cells by early embryologists, allowing them to establish some of the earliest lineage maps in developmental biology (Whitman, 1878; Wilson, 1892). This has been greatly extended more recently by the further development of cell labelling techniques (see below).

As mentioned above, Annelida encompasses both direct developers and indirect developers, with the indirect developers tending to have some variation on a trochophore larva, which is the larval form typical for several phyla that constitute the Lophotrochozoa. In general, a trochophore larva has bands of cilia with which it swims and feeds, and the mouth is downstream of the ciliary beating (Fig. 4). There are many variations on this theme, however, both within Annelida and among other lophotrochozoan phyla. Comparisons between these larval forms and with the larval types of other non-lophotrochozoan phyla, particularly deuterostomes such as echinoderms and hemichordates (see Glossary, Box 1), provide a rich source of material for evolutionary comparisons, as well as a source of heated debate about the nature of the bilaterian ancestor and whether it was a direct or indirect developer (Nielsen, 2001; Raff, 2008).

The annelid features described above, including larval form, spiral cleavage and cell lineages, as well as overt segmentation, have stimulated a long history of research on annelid development and its evolution. With the progress of molecular biology, which has impacted both our understanding of phylogeny as well as the range of techniques available for investigating particular questions (see below), various annelid systems are proving to be key components that aid our understanding of a range of developmental

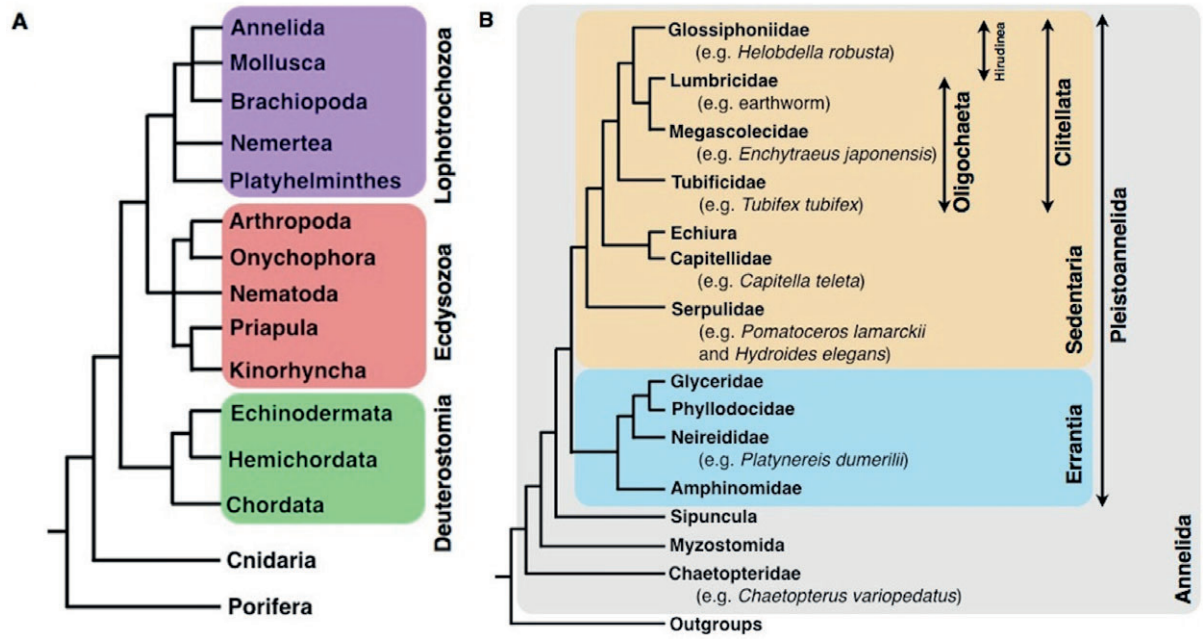

Fig. 2. Simplified phylogenies of Metazoa and Annelida. (**A**) The phylogeny of selected metazoan phyla, with the bilaterally symmetrical (bilaterian) animals divided into the three major clades (super-phyla): Ecdysozoa, Lophotrochozoa and Deuterostomia. (**B**) A phylogeny of Annelida adapted from Struck et al. (Struck et al., 2011) by reducing the number of families shown, in order to highlight the relationships among the annelids mentioned in the text. The bulk of the phylum falls within two major groups: the Sedentaria and Errantia. The leeches (Hirudinea) are grouped with the Oligochaeta, which together constitute the Clitellata, as a subgroup within the Sedentaria. The Sedentaria and Errantia together are the Pleistoannelida (Struck, 2011).

phenomena. What follows is a selective, brief overview of some of these developmental systems, and how annelid research is shaping our understanding of their evolution. This includes evolution of developmental genes and networks and our changing views of their conservation and divergence across the animals; the evolution of segmentation and the central nervous system and the debates about what is conserved and ancestral versus what is derived; and the increasing role for annelids in regeneration and stem cell biology as they provide a range of new, accessible models with a diversity of features for comparison with the more established regeneration and stem cell systems.

Key experimental techniques

Cell labelling and lineage maps

Observations on the early development of annelids began to appear in the late 1800s, and the stereotypical cleavage patterns and unequal blastomere sizes allowed specific cell lineages to be traced by simply observing the early embryo (e.g. Wilson, 1892; Whitman, 1878). Much can still be determined by simply observing early cell divisions, particularly by expanding the range of species being compared (e.g. Arenas-Mena, 2007). However, lineage and fate maps are easier to construct, and can be followed for more extensive periods of time, using techniques to label individual cells and track their progeny. This approach was pioneered for the annelids with the use of horseradish peroxidase injections in the leech (Weisblat et al., 1978). Subsequently, fluorochrome molecules have been used, usually conjugated to dextrans to prevent them from moving into uninjected cells through gap junctions and to allow them to be fixed and visualised (Gimlich and Braun, 1985; Ackermann et al., 2005). Alternatively, the lipophilic dye DiI has been injected (Meyer et al., 2010). Further refinement and improved resolution has been achieved by injecting mRNA coding for nuclear-localised fluorescent proteins (Zhang and Weisblat, 2005), which improves cellular resolution by labelling the nuclei rather than having the signal distributed throughout the cytoplasm. Most recently, the degradation of these injected mRNAs has been circumvented by injecting plasmids, from which nuclear-localised fluorescent protein coding sequences can be transcribed within the injected cells and any descendants that inherit the plasmid (Gline et al., 2009).

Gene knockdown

The injection of nucleic acids, such as mRNAs and plasmids, has also been extended to perform gene knockdown experiments either by injecting morpholinos (MOs) or double-stranded RNAs (dsRNAs). For example, MO injections have been used both in lineage mapping (Zhang and Weisblat, 2005) and in characterising developmental gene functions (e.g. Agee et al., 2006; Song et al., 2002; Kuo and Weisblat, 2011), and dsRNA injection has recently been used to study the novel regeneration gene *grimp* in *Enchytraeus japonensis* (Takeo et al., 2010).

Cell transplantation

The long-suspected importance of the D blastomere and its progeny in organising annelid development, as it does in other spiral cleavers such as molluscs (Lambert and Nagy, 2001), has recently been proven by transplantation studies [the logic for the labelling of individual cells in annelid embryos is summarised in Meyer et al. (Meyer et al., 2010) (Fig. 3)]. In the oligochaete sludge-worm, *Tubifex tubifex*, transplantations of the 2d and 4d micromeres recently proved the axial organising capabilities of these derivatives of the D cell, a property that had been long suspected for this cell in annelids (Nakamoto et al., 2011).

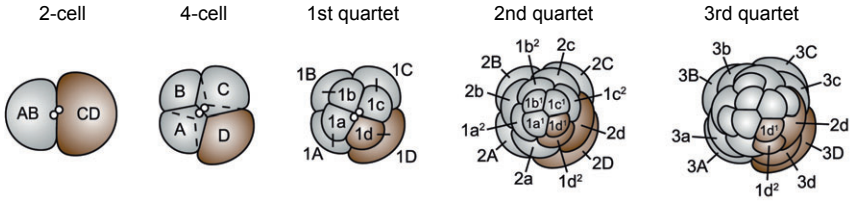

Fig. 3. Spiral cleavage. At the third cell division, the upper (animal) quartet of cells comes to lie over the cell boundaries of the lower (vegetal) quartet of blastomeres. Such spiral cleavages continue through subsequent cell divisions. The logic behind the naming of individual cells, which is facilitated by these stereotypic cleavages, is outlined in Meyer et al. (Meyer et al., 2010). Figure reproduced with permission from Meyer et al. (Meyer et al., 2010). The brown shading distinguishes the D blastomere lineage, including its progeny, to illustrate the 'quadrant' organisation of the early embryo.

Gene expression studies

Many annelids are attractive for gene expression studies because they can produce large numbers of embryos that are often also transparent. This has facilitated the development of techniques such as confocal reflection microscopy and two-colour fluorescence whole-mount in situ hybridisation (WMISH) in the polychaete *Platynereis dumerilii* (Tessmar-Raible et al., 2005; Jekely and Arendt, 2007). These techniques are enabling the detailed comparison of gene expression patterns and the resolution of specific gene combinations or codes ('molecular fingerprints') of individual cells of the embryo. This is made possible by co-labelling the embryo with an anti-tubulin antibody, which labels the nervous system and provides a 3D expression scaffold upon which distinct WMISH experiments can be aligned and directly compared (cellular profiling by image registration, PrimR) (Tomer et al., 2010). Work is well underway to integrate all of this data and make it publicly available via the 4DXpress database (Haudry et al., 2008), and further databases are appearing that will facilitate comparisons of expression data between various animals, including annelids [e.g. the KahiKai database (Ormestad et al., 2011)].

As well as visualising the expression of developmental control genes by WMISH and compromising their function by antisense techniques, there is clearly a need to investigate the regulatory elements of these genes in order to establish the links within the gene networks. Traditionally, this has been achieved in other model systems largely via reporter gene transgenics. It is early days for these techniques in annelids, but work in *P. dumerilii* is presently leading the way, with both transient and germ-line transgenics having been obtained (F. Raible, personal communication). The method for introducing these reporter genes, as well as other nucleic acids used in lineage mapping and gene knockdown techniques, is microinjection. This is not easily universally applicable, however, particularly in very small embryos or where internal cells need to be reached without damaging overlying cells. A recent alternative approach to microinjection has made use of lasers to photoporate material into individual cells of the small marine polychaete *P. lamarckii* (Torres-Mapa et al., 2011). This approach has the potential to enable fine-scale manipulations of the early embryogenesis of annelids, as well as other taxa (Kohli et al., 2007; Kohli and Elezzabi, 2008).

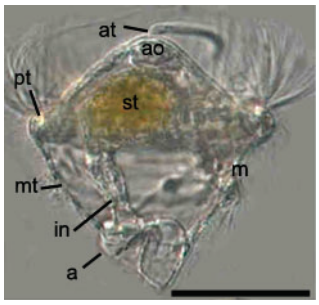

Fig. 4. A trochophore larva of *P. lamarckii*. The trochophore planktonic larval form is typical for many groups within the Lophotrochozoa, although it is often highly modified from the situation found in *P. lamarckii*. The image shown highlights a number of features: a, anus; ao, apical organ (the larval 'brain'); at, apical tuft of cilia; in, intestine; m, mouth; mt, metatroch ciliary band; pt, prototroch ciliary band (the other major ciliary bands, the telotroch and neurotroch, are present but not easily seen on this image); st, stomach, which contains the red algae eaten by this larva and illustrates the transparent nature of these trochophores, which makes them so amenable to investigating embryogenesis and early development. Scale bar: 50 μm. Image reproduced with permission from McDougall et al. (McDougall et al., 2006).

Key recent findings

Insights into evolutionary developmental genomics

Many comparative genomics studies involving annelids have established this group of animals as a relatively conservative lineage that is less derived from the ancestral bilaterian state than many other, more established, model systems. This has important implications for our understanding of both the developmental gene networks that were present in this important ancestor as well as the evolutionary origins of the genes and networks that we study in present-day taxa. These findings also help us understand the degree to which gene networks and developmental gene functions can be compared between model systems, such as fruit flies, nematodes and vertebrates.

Whole genome sequences are already available for two annelids: the polychaete *Capitella teleta* and the leech *Helobdella robusta* (both sequences available at the Joint Genome Institute, http://genome.jgi-psf.org/Capca1/Capca1.home.html and http://genome.jgi.doe.gov/Helro1/Helro1.home.html). In addition, the genome sequencing of *P. dumerilii* is in progress and is close to completion (D. Arendt, personal communication). Furthermore, an ever-increasing range of expressed sequence tag (EST) projects is appearing. All of this enables comparisons of gene content and developmental gene catalogues between annelids and other lineages. The general picture is one of a greater degree of retention of genes in annelids that were present in the ancestral bilaterian than can be found in ecdysozoan model systems, such as fruit flies and nematodes (e.g. Prud'homme et al., 2002; Tessmar-Raible et al., 2007; Simionato et al., 2008; Takahashi et al., 2009; Cho et al., 2010; Shimeld et al., 2010) (Fig. 5A). This means that more extensive comparisons can be made between the gene content and networks of

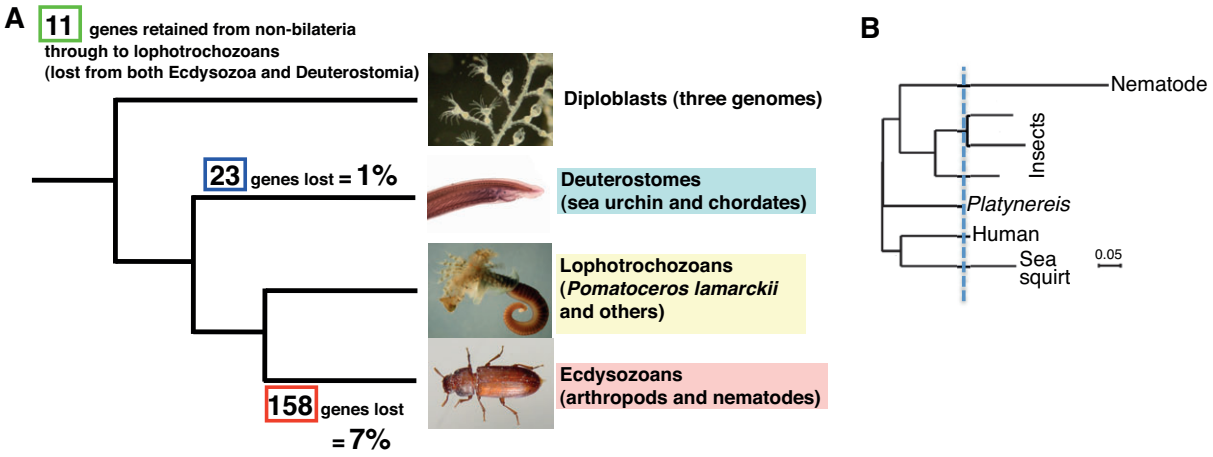

Fig. 5. The relatively conservative nature of annelid gene evolution. (**A**) Loss of ancient genes that were present in the bilaterian ancestor occurs to a greater degree in ecdysozoan genomes, including those of *D. melanogaster* and *C. elegans*, than in deuterostome genomes when assessed from the starting point of a 'random' selection of 2308 genes from an EST screen in the lophotrochozoan annelid *P. lamarckii*. Adapted from Takahashi et al. (Takahashi et al., 2009). (**B**) A global distance tree based on 38,303 noninvariant amino acids from concatenated orthologous protein sequences from a nematode (*Caenorhabditis elegans*), insects (*Drosophila melanogaster*, *Apis mellifera* and *Anopheles* gambiae), the annelid *Platynereis dumerilii*, humans and the sea squirt (*Ciona intestinalis*). Adapted from Raible et al. (Raible et al., 2005). The vertical dashed blue line marks the level of the tip of the *P. dumerilii* branch and illustrates the higher degrees of sequence divergence from the rooted base of the tree found in many other species commonly used in developmental biology.

lophotrochozoan annelids and deuterostome vertebrates than is often possible by comparison with *Drosophila melanogaster* or *Caenorhabditis elegans*, the traditional work-horses of invertebrate developmental genetics (e.g. Denes et al., 2007; Tessmar-Raible et al., 2007).

This conservative nature of annelids, or at least of the polychaete annelids examined, also extends to the less divergent nature of their gene sequences (Fig. 5B) and the retention of more ancestral gene organisations than are found in traditional invertebrate models (Raible et al., 2005; Takahashi et al., 2009). A greater proportion of the introns that were present in the protostome-deuterostome ancestor (PDA; sometimes called the Urbilaterian ancestor) have been retained in *P. dumerilii* than in most other invertebrates examined (Raible et al., 2005). Also, important developmental gene clusters, such as the ParaHox gene cluster [which is the evolutionary sister to the more famous Hox gene cluster (Brooke et al., 1998)], have retained more of the ancestral organisation and gene content than is found in ecdysozoans, such as fruit flies and nematodes (Hui et al., 2009). At the larger scale of genome organisation, at least when comparing synteny of homeobox genes, the polychaete *P. dumerilii* genome appears to be much more comparable to the chordate genome than to the genomes of ecdysozoans (Hui et al., 2012), which in turn seem to have undergone high levels of rearrangement (Zdobnov et al., 2005; Zdobnov and Bork, 2007). This holds out the prospect of discovering higher-order regulatory and organisational properties that have been conserved between annelids and chordates, but lost from the traditional invertebrate genetic models.

The evolution of segmentation

The debate about whether the last common ancestor of bilaterians, or even the last common ancestor of protostomes, was segmented or not is one of the most contentious ongoing discussions within evolutionary developmental biology. This is because the discussions all hinge on a fundamental issue for evolutionary developmental biology, which is being able to distinguish conservation and homology (allied with various levels of secondary divergence and character loss) from convergent evolution of developmental expression and mechanisms, potentially via co-option of homologous genes and networks (Couso, 2009; Chipman, 2010). The pleiotropy of the vast majority of developmental control genes, including those used in segmentation, with their re-use in multiple different (non-homologous) developmental processes, creates further difficulties with regards to deducing homology and the conservation of mechanisms from a common ancestor.

Studies on the developmental genetics of annelid segmentation have thus far been heavily reliant on the candidate gene approach, taking as a starting point the relatively well understood system of segmentation in the fruit fly *Drosophila melanogaster*. Much work has been done to try to deduce which parts of the *Drosophila* segmentation system (including maternal, gap, pair-rule and segment-polarity genes) are used more widely in other arthropods and, hence, are likely to have been used in segmentation in the ancestral arthropod (Peel et al., 2005). This comparative arthropod work has been supplemented by cross-over from the vertebrate segmentation (somitogenesis) field, which has revealed roles for the Notch signalling pathway and its associated gene networks (including genes such as hairy/hes) in the segmentation of vertebrates and at least some arthropods, a notable exception being *D. melanogaster* (Stollewerk et al., 2003; Pueyo et al., 2008), although this conclusion is not without controversy (Kainz et al., 2011). Examination of the expression of these candidate segmentation genes in annelids is still in its relatively early stages and no clear consensus has been reached about the ancestral segmentation mechanism in annelids and how similar (or different) it was to those of arthropods and vertebrates. For example, some authors hypothesise that stripes of engrailed and wingless (in the form of Wnt1) in *P. dumerilii* reveal conservation at least from the last common ancestor of ecdysozoans and lophotrochozoans, with the parasegment boundaries of *Drosophila* being the 'ghosts' of the ancestral segment boundaries (Prud'homme et al., 2003). Others, however, view the diverse expression patterns of the engrailed and

Wnt genes in other annelids as evidence for a lack of homology of segmentation between annelids and arthropods, and any similarity between *Platynereis* and arthropod segment-polarity gene expression as superficial and convergent (Irvine and Seaver, 2006; Seaver and Kaneshige, 2006). There is evidence that the Notch system might be involved in annelid segmentation, at least in a leech (Rivera and Weisblat, 2009), but whether this is representative of the ancestral situation for annelids as a whole remains to be resolved. The Notch-Delta-hes network is also expressed in the posterior growth zone of *C. teleta* (Thamm and Seaver, 2008). Recent evidence for the involvement of the 'segment polarity' genes *ladybird/Lbx* and *hedgehog* in segmentation in *P. dumerilii* is also intriguing (Saudemont et al., 2008; Dray et al., 2010), as is a potential role for the 'Pair-rule' gene *eve* in leech segmentation (albeit in a non-Pair-rule fashion) (Song et al., 2002). However, the troublesome issue of co-option and convergence remains a possibility, and there are clearly differences between the ways in which the genes with roles in fruit fly segmentation are used in annelids (and other arthropods and vertebrates).

There is an obvious need for wider taxonomic sampling of gene expression in annelids and further assays for the functions of these genes, combined with careful analysis of the phenotypes, to precisely define any segmentation role. A challenging alternative would be to take an annelid-focused approach rather than a candidate-gene approach, and perform a segmentation gene screen in annelids. All of this needs to be combined with continued work on the diversity of segmentation (and reconstructions of ancestral states) in both arthropods and vertebrates, as well as deducing the extent of conservation of any segmentation gene networks in unsegmented phyla and the roles that these networks have in these phyla.

Evolution of the central nervous system

There is general agreement that the gene networks used to pattern the anterior-posterior (AP) regionalisation of the CNS are broadly conserved across bilaterian animals. This is based on the observation that orthologous genes have similar regionalised expression domains and functions in arthropods and vertebrates as well as in annelids (e.g. Hui et al., 2009; Steinmetz et al., 2010; Steinmetz et al., 2011). However, the issue of conservation (versus convergence) of the mechanisms patterning the other main axis of the CNS, the dorsal-ventral (DV) or mediolateral axis (when development is traced from the neural plate stage), has been less clear-cut. Some similarity between CNS mediolateral patterning in flies and vertebrates was evident from comparisons of several homeobox-containing genes, including those encoding Nk2.2, Gsx, Msx and Nk6 (Weiss et al., 1998; Cheesman et al., 2004), with an upstream signalling role for the bone morphogenetic protein (BMP) system (Mizutani et al., 2006), notwithstanding the hypothesised DV inversion between the fly and vertebrate conditions (Arendt and Nubler-Jung, 1994; De Robertis and Sasai, 1996). However, the view that this represented a conserved, but inverted, mechanism from the CNS in the bilaterian ancestor was questioned on the basis of gene expression data in the hemichordate *Saccoglossus kowalewski* (Lowe et al., 2006). In this acorn worm, neural differentiation markers are expressed in regions around the entire body wall of the embryo, where the cell bodies of the more nerve-net-like system of this hemichordate were thought to be located (a so-called 'skin brain') (Holland, 2003). Also, these genes are not repressed by BMP signalling in this hemichordate (Lowe et al., 2006), so that these worms do not have a restricted neural domain and lack an epidermal/neural distinction, which in chordates and flies is determined by BMP signalling. The conclusion from the hemichordate work was that the fly and vertebrate nervous system centralisation arose independently, with convergent evolution of the restriction of neural differentiation genes into a confined neural domain under the influence of BMP signalling. Two subsequent lines of evidence threw doubt on this convergence hypothesis, one of these lines coming from annelid data (Denes et al., 2007) and the other from a further examination of the nervous system of *S. kowalewski*, which revealed a previously unappreciated degree of centralisation that evidently seems to be occurring independently from the 'conventional' battery of CNS mediolateral patterning genes (Nomaksteinsky et al., 2009). The annelid data, from *P. dumerilii*, revealed an extensive similarity, and hence presumed conservation, between the mediolateral patterning genes of vertebrates and their expression in *P. dumerilii*, with both systems also being under the control of BMP signalling. The conserved components of this ancestral medio-lateral patterning network were deduced to be *Nk2.2*, *Nk6*, *Pax6*, *Pax3/7*, *Dlx* and *Msx* with some further similarities between *Gsx*, *Pax2* (*Pax 2/5/8* in *P. dumerilii*), *sim* and *Dbx* (Denes et al., 2007). This represents a much greater degree of similarity between the mediolateral patterning of the CNS of the annelid *Platynereis* and vertebrates than between flies and vertebrates, and the supposition is that the extent of this similarity is too detailed and too large to be easily explained by convergence. The bilaterian ancestor is thus believed to have had a CNS, and this CNS was patterned along its mediolateral axis in the trunk by a network of *sim*, *Nk2.2*, *Pax6*, *Nk6*, *Pax3/7*, *Pax2/5/8*, *Msx*, *Gsx*, *Dlx* and *Dbx*, all under the control of BMP (Denes et al., 2007).

Just as the hemichordate seems to show an evolutionary divergence from this ancestral system, as does *Drosophila* to a lesser degree, a recent annelid example also illustrates the relative ease and high frequency of divergence from these ancient, conserved mechanisms. In the leech *Helobdella*, the BMP signalling network has been rewired. The *BMP5-8* orthologue is now the principal BMP signal in the DV patterning system of the leech, instead of the *BMP2-4* orthologue that is used by most other animals examined to date (Denes et al., 2007; Mizutani and Bier, 2008; Kuo and Weisblat, 2011). Also, the BMP antagonist *gremlin* is used instead of the more typical BMP antagonist *chordin* in primary DV patterning in this annelid (Kuo and Weisblat, 2011). This illustrates the common occurrence of divergence in evolutionary developmental biology, the high degree of lability over these time scales, and the fact that some lineages are more derived from the ancestral states than others in particular aspects of their development.

As well as the ancestral mechanisms for axial development of the CNS being deduced with the aid of annelid data, the differentiation genes that are active in particular cell types are also being revealed. Comparisons between *P. dumerilii* and vertebrates have revealed an extensively similar, and hence presumably conserved, molecular anatomy (or fingerprint) for sensory-neurosecretory centres (Tessmar-Raible et al., 2007) and mushroom bodies/cerebral cortex (Tomer et al., 2010). The neurosecretory centre in *P. dumerilii* now appears to be homologous to an equivalent region in the vertebrate hypothalamus in the forebrain. Overlapping expression domains of *Nk2.1*, *retina homeobox* (*rx*), *orthopedia* (*otp*) and *ventral anterior homeobox* (*vax*) (*nk2.1a*, *rx3*, *otp* and *vax1* for zebrafish) contribute to delimiting this neurosecretory region, which includes cells producing vasotocin-neurophysin adjacent

to extraocular photoreceptor cells, as well as RFamidergic neurons (Tessmar-Raible et al., 2007). Data from *P. dumerilii* have also contributed to the idea that invertebrate mushroom bodies are homologous to the cerebral cortex area of the mammalian forebrain. Tomer et al. (Tomer et al., 2010) analysed the expression of >20 *P. dumerilii* genes, orthologues of which are known to pattern the mammalian cortex. This work was enabled by the use of an image registration system (PrimR, discussed above), which uses the axon scaffold to provide landmarks against which individual images of different gene expression patterns can be compared. These authors also attempted to address directly the thorny issue of distinguishing conservation from convergence of these developmental gene networks by statistical analysis. Although this type of analysis may still be misled by a lack of detailed knowledge of the connectedness of the developmental genes being examined, and hence an inevitable under-appreciation of how independently each gene can be considered, this work nevertheless provides an important framework for future studies and highlights a clear need to establish the nature of the connections between the components of the developmental networks in the multiple developmental systems that are being compared. Thus, despite the clear differences between invertebrate mushroom bodies and the mammalian cerebral cortex, and the variations in morphology, function and gene expression that have accumulated in the distinct lineages over the intervening 550-600 million years of evolution, a homologous relationship can still be detected between these animal forebrain structures (Tomer et al., 2010).

The molecular fingerprint for the ancient neurosecretory region of the forebrain also includes a microRNA (miR-7), as does the mushroom body-cortex fingerprint (miR-9 and miR-9*) (Tessmar-Raible et al., 2007; Tomer et al., 2010). A role for microRNAs in ancient, conserved tissue- and cell-specific molecular fingerprints is not restricted to these two cases. Studies of microRNA expression in the annelids *P. dumerilii* and *C. teleta* has helped to deduce the presence of particular tissue types that were present in the PDA and that were patterned by a selection of widely conserved microRNAs (Christodoulou et al., 2010). The tissue types include foregut, motile cilia, neurosecretory brain tissue, sensory brain tissue and sensory organs, in addition to more general tissue types with microRNA components, such as musculature, general CNS tissues and the gut.

The evolution of regeneration

The typical model systems used in studies of regeneration biology are selected vertebrates, platyhelminth flatworms and cnidarians, such as *Hydra* (Sánchez Alvarado and Tsonis, 2006; Bely and Nyberg, 2010). It has been known for some time, however, that a variety of regenerative capacities are exhibited by annelids, ranging from the regeneration of complete animals from single isolated segments (e.g. *Chaetopterus*) through more restricted capabilities (e.g. *Platynereis* and *Capitella*) to a lack of ability to regenerate at all (as is the case for leeches) (Bely, 2006). Annelids are thus a rich source of material for investigating the evolution of regeneration capabilities, particularly because there are many species known to show differential abilities to regenerate depending on whether anterior or posterior segments are to be replaced. Also, several groups exist in which closely related species show different responses to amputations [summarised in Bely et al. (Bely, 2006)], which would enable comparison of phylogenetically close systems. This was recently exploited by Bely and Sikes (Bely and Sikes, 2010), who demonstrated that in a species of naidine oligochaete that cannot undergo anterior head segment regeneration, but which can reproduce asexually via fission, a latent regenerative ability can be revealed when the amputation is made in the proliferative region that transiently appears during reproductive fission (fission-zone regeneration) (Bely and Sikes, 2010). This regenerative ability in these annelids might be connected with *nanos* activity (Bely and Sikes, 2010). A further link between the molecular pathways involved in reproduction via fission and regeneration was provided by the observation that *engrailed* and *Otx* are involved in both processes in the naidine oligochaete *Pristina leidyi* (Bely and Wray, 2001).

Regeneration in conventional model systems, such as *Hydra* and platyhelminths, has revealed the involvement of stem cells, known as neoblasts in the flatworms, which reside in the adult body and are active in supplying the range of cell types needed to reconstruct lost tissues (Sánchez Alvarado and Tsonis, 2006). Neoblasts are also evident in at least some annelids (Sugio et al., 2008). Furthermore, there are molecular similarities in the development of annelid neoblasts and the adult stem cells of other taxa, which could well be indicative of homology between the neoblasts of some annelids and those of platyhelminths. Vasa genes (*Ej-vlg1* and *Ej-vgl2*) have been shown to distinguish the neoblasts of the fragmenting potworm *Enchytraeus japonensis*, as they do in *Hydra* stem cells (Sugio et al., 2008). These somatic stem cells are, however, not responsible for the regeneration of every component of the worm; germ cells are produced from a distinct germ-line stem cell, which, in addition to expressing the *vasa* genes, also expresses *piwi* (*Ej-piwi*), thus allowing the distinction between somatic and germ-line stem cells to be made in a regenerating species (Tadokoro et al., 2006). This molecular profile of *vasa* and *piwi* genes in primary germ cells, and a putative link between germ cells and somatic stem cells, is also evident in *P. dumerilii* (Rebscher et al., 2007), and *piwi* expression in primordial germ cells and regeneration has also been confirmed in *C. teleta* (Giani et al., 2011). *E. japonensis* is also amenable to regeneration gene discovery, via cDNA subtraction, to complement the candidate gene approach that stimulated the *vasa* and *piwi* research. A novel gene (*grimp*) was shown to be required for neoblast proliferation via knockdown by RNAi (Takeo et al., 2010).

Regeneration in annelids can also be used to investigate other developmental processes besides regeneration itself, such as segmentation, nervous system development and mesoderm formation. This avenue has been pursued in *P. dumerilii* and has revealed the role of 'segment polarity' genes in regenerating segment formation (see above) (Saudemont et al., 2008; Dray et al., 2010) and possibly other genes orthologous to those involved earlier in arthropod segmentation, such as *caudal* (*cad/Cdx*) and *eve* (de Rosa et al., 2005). Although the expected role for the NK homeobox genes in mesoderm formation was confirmed by examining the expression of these genes in tail regeneration as well as during embryogenesis in *P. dumerilii*, a surprising widespread involvement in segmentation was also discovered (Saudemont et al., 2008), which should stimulate a wider examination of these genes in further taxa.

Conclusions and future directions

In addition to the specific developmental systems briefly reviewed above, data from annelids are also playing a significant role in understanding the evolution and development of many other organs and tissues in animals, including the eyes (Arendt

et al., 2004), gut (Arendt et al., 2001; Boyle and Seaver, 2008; Saudemont et al., 2008; Hui et al., 2009; Boyle and Seaver, 2010), appendages (Panganiban et al., 1997; Winchell et al., 2010; McDougall et al., 2011), mesoderm (Kerner et al., 2006; Dill et al., 2007; Saudemont et al., 2008; Kerner et al., 2009) and more. The prominent role of annelids in evolutionary developmental biology is now clear, and this is likely to be extended in the future. This is particularly desirable because annelids are proving to have retained more of the ancestral complement of genes and mechanisms that are also conserved in vertebrates, but which have been lost from the traditional protostome model systems, such as *D. melanogaster* and *C. elegans*. Further annelid genome sequences and transcriptomes will continue to appear, which will help to distinguish how widespread this conservative nature is within annelids relative to other taxa. This genome-scale data will also underpin the work on the evolution of the extensive biodiversity within this phylum itself. The development and use of gene knockdown (and overexpression) techniques is clearly needed across a wide range of annelid species. This will enable more confident deductions about the ancestral developmental mechanisms of annelids and will facilitate more robust comparisons with the developmental mechanisms operating in other phyla.

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