

Regeneration in spiralian: evolutionary patterns and developmental processes

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ABSTRACT Animals differ markedly in their ability to regenerate, yet still little is known about how regeneration evolves. In recent years, important advances have been made in our understanding of animal phylogeny and these provide new insights into the phylogenetic distribution of regeneration. The developmental basis of regeneration is also being investigated in an increasing number of groups, allowing commonalities and differences across groups to become evident. Here, we focus on regeneration in the Spiralia, a group that includes several champions of animal regeneration, as well as many groups with more limited abilities. We review the phylogenetic distribution and developmental processes of regeneration in four major spiralian groups: annelids, nemerteans, platyhelminths, and molluscs. Although comparative data are still limited, this review highlights phylogenetic and developmental patterns that are emerging regarding regeneration in spiralian and identifies important avenues for future research.

KEY WORDS: *regeneration, Annelida, Nemertea, Platyhelminthes, Mollusca*

Introduction

Many animals are able to regenerate lost body parts, yet little is known about how this capability has evolved (Brockes and Kumar, 2008; Bely and Nyberg, 2010). What is the pattern of regeneration evolution across animals? How common are losses and gains of regeneration ability? Are aspects of the regeneration process homologous across major groups of animals? What evolutionary and developmental mechanisms drive changes in regenerative ability? Despite long-standing interest in the process of regeneration, fundamental questions such as these remain largely unanswered - even largely untackled.

In recent years, important advances have been made in our understanding of animal phylogeny and these have provided long-awaited resolution for the core backbone of the animal tree (Halanych, 2004; Giribet *et al.*, 2007; Edgecombe *et al.*, 2011). This phylogenetic context provides a framework for inferring the broad pattern of regeneration variation across the animals (Bely and Nyberg, 2010). Basally branching lineages such as Porifera, Cnidaria, and Ctenophora generally have high regenerative ability, supporting the idea that the animal ancestor may have had high regenerative ability. Among the Bilateria, though, regenerative ability is much more variable. Some bilaterian groups, and even

entire phyla, appear to have little to no ability to regenerate body structures (e.g., nematodes, rotifers) while other groups have numerous representatives that can regenerate every part of the body (e.g., planarians, annelids). The Ecdysozoa is comprised of groups with no or fairly limited regenerative ability, while the Spiralia (or Lophotrochozoa) and Deuterostomia include both highly regenerative groups as well as ones with more limited regeneration abilities. Is regeneration variation among bilaterians the result of numerous regeneration losses, independent gains of regeneration, or a combination of the two? And what are the biological correlates of changes in regenerative ability? In order to begin to address these questions, a finer-grained picture of the distribution of regeneration must come into focus. Investigating patterns of regeneration variation *within* phyla, in which structural homologies are clear (or at least clearer than among phyla) and divergence times are shallower, is an important next step to better understand how regeneration has evolved.

Interest in regeneration research has also recently surged, fueled largely by its potential biomedical relevance. Through recent efforts, the cellular and molecular underpinnings of regeneration are becoming increasingly well understood in a handful of regeneration model systems, and studies of regeneration in non-model systems are becoming more common as well (Sánchez Alvarado

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and Tsonis, 2006; Brockes and Kumar, 2008; Tanaka and Reddien, 2011). But key questions remain. Which aspects of the regenerative process are shared across major animal lineages? Is there evidence supporting the hypothesis that the process of regeneration is homologous across deeply diverged taxa, such as across phyla? What developmental changes underlie gains or losses of regenerative ability? Beginning to address these types of questions requires thorough characterizations of regenerative processes in a range of animal phyla, and among close relatives that differ in regenerative ability.

The Spiralia are an excellent group in which to investigate the pattern and process of regeneration evolution. Spiralia is a large and morphologically diverse protostome clade composed of about 13 phyla, including annelids, molluscs, nemertean, platyhelminths, bryozoans, and rotifers, among others (Edgecombe *et al.*, 2011). A broad-scale view of this clade suggests there is considerable variation in regenerative ability across phyla, from ones with representatives that can regenerate every part of the body (e.g., annelids, platyhelminths) to those in which no representatives are known to regenerate any major structures (e.g., rotifers) (Bely and Nyberg, 2010). More importantly, the Spiralia include several large phyla in which regeneration varies markedly within the phylum. The apparent evolutionary lability of regeneration within these groups suggests they will be useful for identifying possible correlates of regeneration increases and decreases and to identify the developmental changes responsible for regeneration evolution.

In this review, we provide overviews of the phylogenetic distribution and the developmental basis of regeneration in four major spiralian phyla: the Annelida (segmented worms), Nemertea (ribbon worms), Platyhelminthes (flatworms), and Mollusca. These represent spiralian groups in which the most information on regenerative ability is available, and also groups in which regenerative ability is shown, or suggested, to be variable. Our goal is to assemble data for these groups in order to begin inferring common regeneration patterns and processes, as well as to highlight where important gaps in our knowledge remain. Note that regeneration is a process that can occur at a range of levels of biological organization; here we focus specifically on structure-level regeneration (e.g., regeneration of heads, tails, appendages) and do not cover regeneration at lower levels of organization, such as regeneration of tissues or parts of cells. We also limit our review to regeneration in adults or sub-adults; how regenerative ability varies across developmental stages and how it might relate to regulation in embryos are fascinating questions but ones that are beyond the scope of this review.

Distribution of regeneration across spirilians

In this first section we review information relevant for understanding the distribution and pattern of regeneration evolution in annelids, nemertean, platyhelminths, and molluscs. For the first three phyla, we describe the general body plan; describe general patterns of growth (and degrowth); provide an overview of the current understanding of the phylogenetic relationships within the phylum; review what body structures are known to regenerate (or not regenerate) among members of the phylum; and, where possible, make preliminary interpretations regarding where in the phylum regeneration gains or losses may have occurred. For Mollusca, we include a briefer overview, as regeneration data are much more limited in this group.

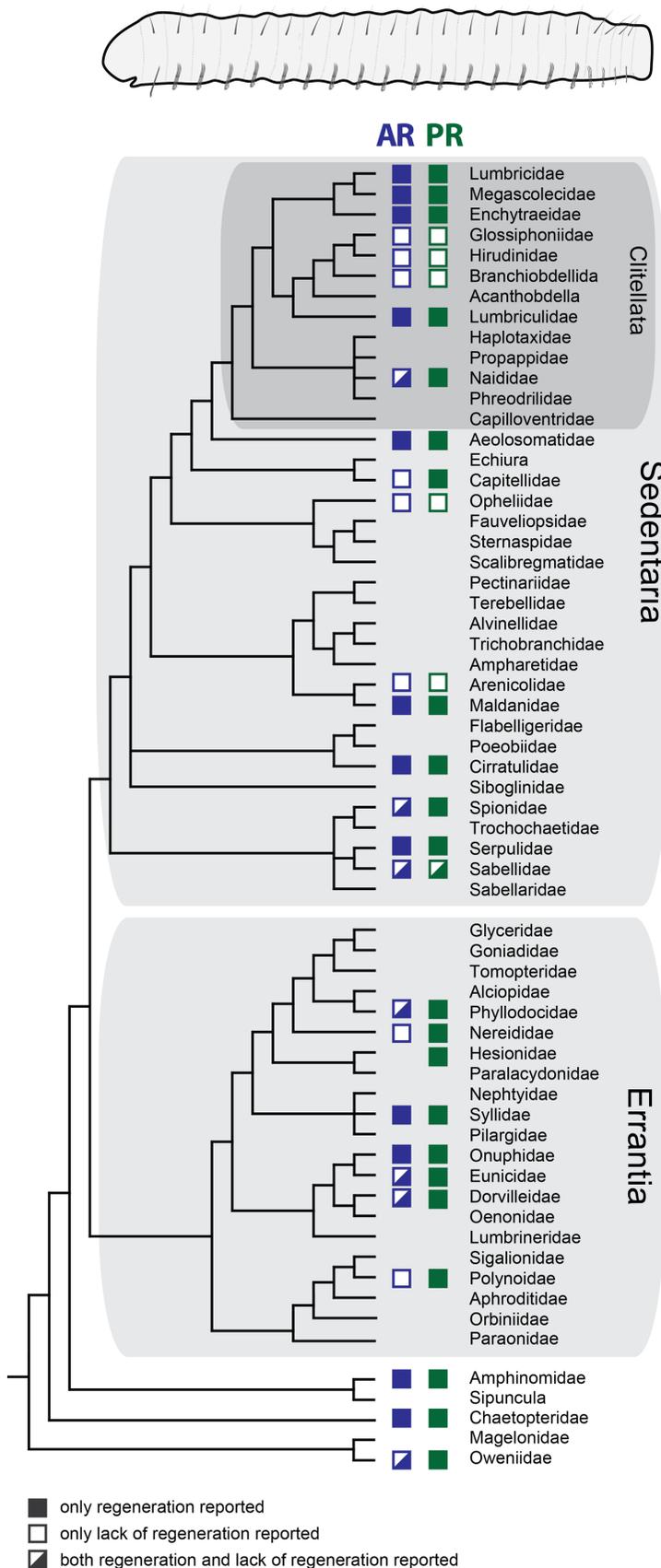
Annelida

Annelids are a large and diverse group of typically segmented worms found in marine, freshwater, and terrestrial environments, with over 17,000 species described (Brusca and Brusca, 2003; Zhang, 2013). The main part of the body is usually composed of a series of repeated segments, ranging from just a few to several hundred (depending on species and age), with an asegmental cap of tissue present at both the anterior and posterior ends. Annelids typically have a large fluid-filled coelom surrounded by a muscular body wall, a complete gut with an anterior mouth (within the anterior asegmental cap) and a posterior anus (within the posterior cap of tissue), and a nervous system composed of paired cerebral ganglia, segmental ventral ganglia, and peripheral segmental nerve rings. Some species possess body wall outgrowths such as lateral, segmentally iterated parapodia (paddle-like outgrowths) used for locomotion; anterior tentacles, palps, or proboscises that aid in sensation, respiration and/or feeding; opercula that seal tube entrances; and lateral or posterior gills that aid in respiration. Based on their body architecture, the most common injuries are expected to involve transverse cuts of the main body (removing head and/or tail) and amputation of the various body wall outgrowths.

Most annelids add segments post-embryonically from a sub-terminal posterior growth zone just anterior to the asegmental posterior cap (pygidium) (Hyman, 1940; Brusca and Brusca, 2003). While a few species reach a fixed number segments, most do not, and some appear to grow continuously throughout their life. Under starvation conditions, degrowth in overall size occurs in at least some representatives (naidids: A. E. Bely, unpublished data; *Lumbriculus*: K. A. Tweeten, pers. comm.) and degrowth resulting in a reduction of segment number may also occur (K. A. Tweeten, pers. comm.).

After many years of uncertainty regarding annelid phylogeny, recent molecular studies have greatly clarified the relationships among annelid groups (Rousset *et al.*, 2007; Struck *et al.*, 2007; Sperling *et al.*, 2009; Struck *et al.*, 2011; Weigert *et al.*, 2014), setting the stage for interpreting regeneration variation in a phylogenetic context. Annelids are classified into ~100 families, and nearly all of the groups included in recent analyses fall into one of two major clades, the Errantia and the Sedentaria, with only a few annelid lineages branching outside of these, as basal lineages (Struck *et al.*, 2011; Weigert *et al.*, 2014). The Errantia include many highly mobile species (though less errant species are also common) and the ancestor of this clade is reconstructed as having pronounced anterior palps and lateral parapodia. Errantia includes groups such as nereids (e.g., *Platynereis*, *Neanthes*, *Nereis*), syllids, eunicids, and glycerids, among many others. The Sedentaria include many infaunal, burrowing species (though highly mobile species also occur), and the ancestor of this clade is reconstructed as having reduced palps and parapodia, and an infaunal, burrowing lifestyle. Sedentaria includes groups such as capitellids (e.g., *Capitella*), spionids (e.g., *Streblospio*), terebellids, serpulids, sabellids, and echiurans, among many others, as well as all of the Clitellata, which include groups such as earthworms, aquatic "oligochaetes", and leeches.

Annelids vary widely in regenerative ability, from species that can regenerate every part of the body, with some even able to do so from a single isolated segment, to those incapable of regenerating a single lost segment, regenerating the asegmental tip of the body, or even simply wound-healing (Bely, 2006). Regeneration of



anterior (head) and posterior (tail) segments (along with corresponding asegmental extremities) is well documented in a wide range of annelids. Segment regeneration ability across the phylum has been previously reviewed (Bely, 2006, 2010), though recent advances to our understanding of annelid relationships make it worth revisiting the distribution of segment regeneration here (Struck *et al.*, 2011; Weigert *et al.*, 2014).

The ability to regenerate posterior segments is very broadly distributed across the phylum and has been found present in nearly all species in which it has been investigated (Fig. 1) (Bely, 2006), suggesting posterior regeneration ability is likely ancestral for the phylum. Posterior segment regeneration has been documented in at least 23 annelid families, including Errantia, Sedentaria, and at least three lineages reconstructed as basal (Amphinomidae, Chaetopteridae, Oweniidae). Failure to regenerate posteriorly has been documented in only a handful of groups, and interestingly only in the Sedentaria. The non-posteriorly regenerating species include one large clitellate clade (leeches and relatives) as well as members of three polychaete families (Opheliidae, Arenicolidae, Sabellidae). These are likely to represent four independent losses of posterior regeneration in annelids.

The ability to regenerate anterior segments is much more variable across the phylum (Fig. 1) (Bely, 2006). Anterior segment regeneration has been documented in three basal lineages (Amphinomidae, Chaetopteridae, and Oweniidae) and 21 Errantia and Sedentaria families, representing a little more than half of families investigated. However, failure to regenerate anterior segments has been shown in well over a third of the families for which data are available. Although greater sampling is clearly needed, a reasonable interpretation of this distribution is that anterior segment regeneration ability has been lost numerous times within the phylum. If all anterior regeneration failure represents evolutionary loss, then over a dozen losses would be reconstructed. However, alternative scenarios such as numerous independent gains of anterior regeneration ability or a combination of anterior regeneration losses and gains should remain under consideration.

Regarding regeneration of structures other than entire segments, the prostomium, feeding palps, and operculum have also been shown to regenerate among certain annelids, though data on these are much more limited. In species in which complete anterior regeneration has been documented (as described above), regeneration of the anterior asegmental tip (prostomium and peristomium) and any head appendages characteristic of that species is obviously possible. However, only a few studies have carried out amputations removing *only* these structures. Following removal of only the asegmental region, regeneration of the prostomium has been documented in several species of

Fig. 1. Phylogenetic distribution of regeneration across the Annelida. Phylogeny is based on Erséus and Källersjö (2004), Struck *et al.* (2007), Struck *et al.* (2011), and Weigert *et al.* (2014). Regeneration data are from Morgulis (1909), Cresp (1952), Olive and Moore (1975), Nusetti *et al.* (2005), Hentschel and Harper (2006), and Giani *et al.* (2011) or are reviewed in Hyman (1940), Berrill (1952), Herlant-Meewis (1964), Bely (2006, 2010). AR - anterior regeneration; PR - posterior regeneration.

naidid clitellates (including one that cannot regenerate segments), but fails to occur in at least one species of naidid, two species of dorvilleid, and one species of nereid (Pfannenstiel, 1973, 1974; Bely and Sikes, 2010a). Interestingly, in the dorvilleid and nereid studies, regeneration of part of the prostomium was shown to be possible if a part of the prostomium was left behind. The recent finding in a naidid clitellate that the prostomium grows continuously from a basal growth zone anterior to the mouth (Zattara and Bely, 2013) suggests a possible explanation for this requirement for part of the prostomium to be present. If there is a prostomial growth zone in these polychaetes, perhaps this growth zone cannot be regenerated in these particular species but, if still present, can allow for regeneration of the prostomium region that has been removed. Other asegmental structures of the head can also regenerate. For example, the feeding crown of some sabellids, which is derived from the asegmental prostomium, can regenerate if this structure is removed, even in a species incapable of regeneration following removal of even a single anterior segment (Wells, 1952; J. A. C. Nicol, unpublished data in Wells 1952). Regeneration of feeding palps (following removal of only these structures) has been well documented in several genera of spionids (Hentschel and Harper, 2006; Lindsay *et al.*, 2007; Lindsay *et al.*, 2008), as has the regeneration of the operculum in serpulids (Okada, 1933; Szabó and Ferrier, 2014). Amputation and regeneration of parapodia has also been described in one species of nereid (Boilly and Boilly-Marer, 1995). We are aware of no data on regeneration of gills following removal of only these structures.

Nemertea

Nemerteans are elongated, predatory worms with highly flexible bodies found primarily in marine environments, with ~1,300 species described (Brusca and Brusca, 2003; Zhang, 2013). Nemerteans have a thick muscular body with no internal coelom, a complete gut with an anterior or anteroventral mouth and posterior anus, and a nervous system composed of paired cerebral ganglia anteriorly and lateral longitudinal nerve cords connecting a series of nerve plexuses. Nemerteans possess a long, eversible anterior proboscis used for prey capture that is characteristic and unique to this group. The proboscis is usually stored inverted within the body but can be rapidly everted and propelled outward to capture and subdue prey. Based on their morphology, the most likely injuries in nemerteans are expected to be transverse cuts of the elongated body or transverse cuts of the extended proboscis.

Nemerteans can undergo extensive growth and degrowth, indicating dynamic mechanisms of tissue generation and remodeling. Some species hatch as juveniles a few millimeters in length and ultimately grow to remarkable lengths of several tens of meters (McIntosh, 1900). Nemerteans can also degrow, shrinking in size when food is scarce or absent, all the while maintaining proper body proportions (Dawydoff, 1928; Coe, 1929). Some individuals have been maintained without food for over a year, shrinking in size but otherwise appearing healthy over this prolonged period.

Nemerteans have been traditionally classified into three groups, the paleonemerteans, heteronemerteans and hoplonemerteans, and recent molecular phylogenetic studies have largely supported these groupings (Tholleson and Norenburg, 2003; Andrade *et al.*, 2011). Paleonemerteans branch near the base of the nemertean tree and there is some indication that they may represent a paraphyletic assemblage of basal lineages. The heteronemerteans

and the hoplonemerteans are each monophyletic groups and sister to each other. Paleonemerteans include genera such as *Carinoma*, *Tubulanus*, and *Cephalothrix*. Heteronemerteans possess a characteristic planktonic feeding larva, the pilidium, and include groups such as *Lineus*, *Ramphogordius*, *Cerebratulus*, and *Micrura*. Hoplonemerteans have a spiked proboscis and a single anterior opening that serves as both mouth and proboscis pore and include genera such as *Nemertopsis* and *Carcinonemertes*.

Among nemerteans regeneration of the proboscis, the tail and the head are well documented in certain groups, and some species can even regenerate an entire individual from a tiny body fragment, but regenerative ability varies considerably across the phylum. Published reports are strongly biased towards heteronemerteans, with less data on hoplonemerteans and virtually no information on paleonemerteans. Regeneration of the proboscis has been reported in a number of species across the phylum (Gontcharoff, 1951; Bierne, 1962; Gibson, 1972) suggesting it could be widespread among nemerteans; we know of no reports documenting failure to regenerate the proboscis. Some ability to regenerate posteriorly also appears to be broadly distributed (Coe, 1934a; Gibson, 1972) (Fig. 2), though posterior regeneration is often poorly characterized, probably due to the lack of easily scorable posterior structures in most species. Among heteronemerteans, posterior regeneration is unambiguously described in species with a posterior cirrus, such as *Zygeupolia rubens* and *Micrura* sp. (Coe, 1934a). In hoplonemerteans, posterior regeneration may be more limited; complete posterior regeneration is only described following amputations in which a very small part of the tail is removed, while regeneration failure or death occurs if a substantial part of the tail is cut off (Coe, 1934a; Gibson, 1972).

As for anterior regeneration ability, scoring for this ability is unambiguous (given the many head structures) but appears to be very limited within the phylum (Fig. 2). Several species can regrow the anterior tip of the body (i.e., structures anterior to the brain) (Coe, 1934a), but complete head regeneration (including of the brain and mouth) has only been reported in species of one heteronemertean family, the Lineidae. These reports clearly document regeneration of the complete head, including brain, mouth, proboscis canal, and sensory organs including ocelli (Nusbaum and Oxner, 1910; Coe, 1929; Gibson, 1972). Interestingly, essentially all such reports are based on species that were recently synonymized to a single species, *Ramphogordius sanguineus* (Riser, 1994). Several other lineids fail to regenerate a head (Gontcharoff, 1951), suggesting head regeneration may actually be unusual in this group. The only report of regeneration of a complete head outside of the Lineidae is that of Kipke (1932) for the freshwater hoplonemertean species *Prostoma graecense*. However, we have been unable to reproduce these results in the very closely related species *Prostoma c.f. eilhardi* (E.E.Z., pers. obs.). Thus, available data suggest full anterior regeneration may be very limited within the nemerteans and that the presence of anterior regeneration in the Lineidae could represent a novel origin. Denser sampling of regenerative abilities within the Lineidae is needed, but if such sampling supports this preliminary conclusion, then the gain of head regeneration within nemerteans would represent an outstanding opportunity to investigate the molecular basis of a novel origin of regeneration.

The high regenerative abilities of *Ramphogordius sanguineus* may be recently evolved, but there is no question that this species is among the champions of animal regeneration. This species is

able to regenerate an entire individual not just from a thin transverse slice, but even just one *quadrant* of a thin slice (Coe, 1930). Furthermore, in a series of early studies, Coe (1929) estimated that a single worm can be repeatedly amputated to obtain over 200,000 worms, each one measuring two hundred-thousandths of the volume of the original individual. Thus, regeneration can be successful even starting with a tiny tissue fragment, a fragment that has a high proportion of wound surface (in quadrant fragments, 4 out of 5 surfaces are wound surfaces), and a fragment in which both the dorsal-ventral and the left-right axes are initially incomplete.

Clearly, much more sampling is needed to determine the broad distribution patterns of proboscis regeneration, tail regeneration, and head regeneration among the Nemertea. The limited data available thus far suggest that proboscis regeneration and tail regeneration could be widespread and possibly ancestral for the phylum, while anterior regeneration may have evolved more recently, notably in the lineid heteronemerteans. Importantly, although nemerteans are frequently reported as being powerful regenerators, we find that a careful review indicates this view has been based almost entirely on reports from what is now recognized as a single species (*Ramphogordius sanguineus*). The nemerteans thus highlight the importance of collecting and drawing conclusions from a broad array of species in order to distinguish between ancestral and

derived regenerative abilities, and not simply generalizing from the species that possess the most extreme regenerative abilities.

Platyhelminthes

Platyhelminthes are a large and diverse group of typically dorso-ventrally flattened worms that include many free-living, primarily aquatic species as well as a large number of parasitic species (Brusca and Brusca, 2003). Nearly 30,000 species have been described (Zhang, 2013). These animals, commonly referred to as flatworms, are bilaterians but lack many traits characteristic of other bilateral animals: they do not have a complete gut (species have a blind gut or no gut at all), have no coelom, do not exhibit mitosis in differentiated somatic cells, and possess no circulatory or excretory systems.

The free-living Platyhelminthes have long been known to possess an unusual system of mesenchymal stem cells known as neoblasts (Baguña *et al.*, 1989; Newmark and Sánchez Alvarado, 2000; Nimeth *et al.*, 2007). As the only mitotically active cells in adult flatworms, neoblasts allow flatworms to persist in a continuous state of cell turnover. Recent studies suggest that a similar stem cell system seems to operate in the parasitic lineages as well, including both tapeworms and blood flukes (Kozioł *et al.*, 2010; Collins *et al.*, 2013). Several free-living platyhelminths (planarians and macrostomids, see below) are known to exhibit continuous growth or degrowth depending on food availability (Baguña *et al.*, 1990; Oviedo *et al.*, 2003; Mouton *et al.*, 2009), suggesting this ability may be prevalent among the platyhelminths. Starved worms undergo a reduction in size yet maintain body proportions and normal organ physiology, with the exception of the reproductive system, which is targeted for apoptosis (Newmark *et al.*, 2008). Upon feeding, flatworms initiate an increase in cell number to reach maximum size along with restoration of reproductive organs and structures.

Although there remains considerable uncertainty regarding the phylogenetic relationships within the phylum, recent molecular analyses have provided important advances in our understanding of relationships within this traditionally problematic group. The organisms traditionally included within the phylum are almost surely polyphyletic; as a result the Acoelomorpha (acoels and nemertodermatids) have been removed and elevated to an independent phylum that falls outside of the Spiralia (Ruiz-Trillo *et al.*, 1999; Philippe *et al.*, 2007; Sempere *et al.*, 2007; Philippe *et al.*, 2011). The phylum Platyhelminthes is now understood to be composed of two monophyletic groups - the Catenulida and the Rhabditophora (Riutort *et al.*, 2012). The Rhabditophora include the bulk of platyhelminth diversity, including groups such as the macrostomids, polyclads, triclads, and the Neodermata, a monophyletic group of obligate parasites that includes flukes and tapeworms. The triclads, commonly referred to as planarians, have received the greatest attention with respect to regeneration. This group includes marine, freshwater, and terrestrial species and three subclades are recognized: the Maricola (marine planarians), the Cavernicola, and the Continenticola (Sluys *et al.*, 2009). The latter group includes the most familiar planarians, namely members of the Planeriidae (e.g. *Planaria*), the Dendrocoelidae (e.g., *Dendrocoelum*, *Procotyla*), and the Dugesidae (e.g., *Dugesia*, *Schmidtea*, *Girardia*), among other groups such as Geoplanidae (land planarians).

Many species of Platyhelminthes demonstrate remarkable regeneration abilities, though a number of groups appear to have much more limited regenerative capacity (Fig. 3). Regeneration

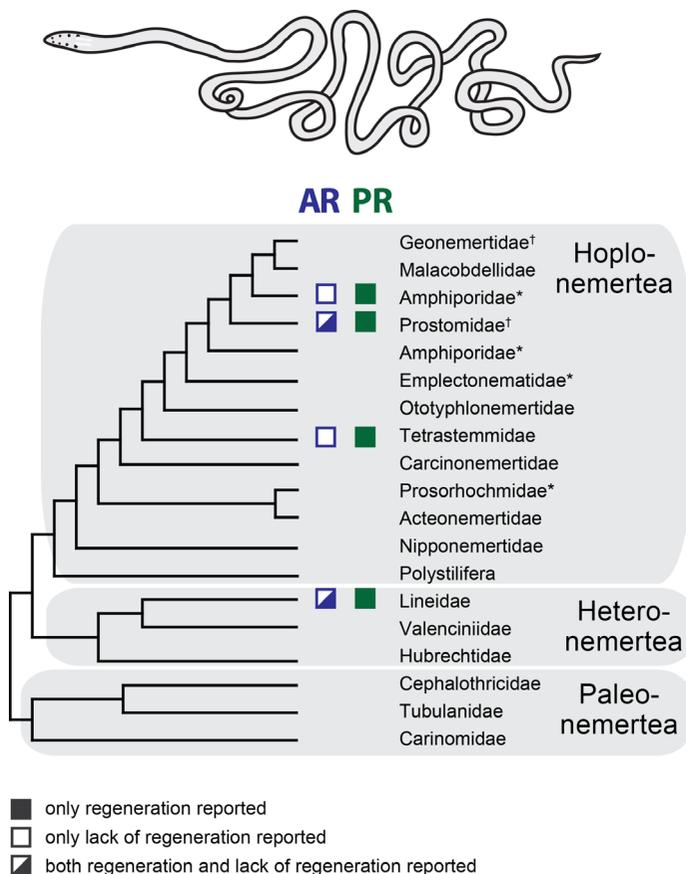


Fig. 2. Phylogenetic distribution of regeneration across the Nemertea. Phylogeny is based on Thollessen and Norenburg (2003) and Andrade *et al.*, (2011). * indicates the family is polyphyletic and † indicates the family name is provisional. Regeneration data are reviewed in the text. AR, anterior regeneration; PR, posterior regeneration.

has been extensively evaluated in the planarians (triclads) where robust structural and whole-body regenerative abilities are distributed among a number of species, including the Planeriidae, Dugesiidae, and Geoplanidae (Brøndsted, 1969; Shirasawa and Makino, 1987, 1991; Newmark and Sánchez Alvarado, 2002). A classic experiment by T. H. Morgan illustrated the remarkable whole-body regeneration capabilities in a planarian species: a tiny fragment just 1/279th of the original worm was able to regenerate an entire individual (Morgan, 1898). However, at least one dugesid (a species of *Phagocata*) is shown to fail to regenerate a new complete head following post-pharyngeal amputation (Umesono *et al.*, 2013), suggesting a possible loss of head regeneration within this group (and possibly even within this genus, since other *Phagocata* species are known to regenerate a head). Among the triclads, several genera within the family Dendrocoelidae have been evaluated for regenerative ability and none are able to regenerate a complete head after amputation posterior to the pharynx (i.e., cutting within the posterior two-thirds of the primary body axis) (Lillie, 1901; Morgan, 1904; Brøndsted, 1969; Sikes and Newmark, 2013). Interestingly, though, in a study that demonstrated intra-population level variation in regeneration rate in a species of Dendrocoelidae, it was found that several post-pharyngeally amputated worms from one population did mount a regenerative response that led to the temporary formation of eyespots, though the small blastemas were then resorbed (Romero *et al.*, 1991). While anterior regeneration is limited among dendrocoelids, members of this group maintain posterior regeneration abilities following cuts along most of the body axis.

More limited records are available for regeneration outside of the planarians (Fig. 3). The macrostomids are variable for the ability to regenerate a head, while posterior regeneration is present in all groups investigated (Egger *et al.*, 2006). The catenulids, the basal platyhelminth lineage, has received little attention. Several species have been shown to have anterior regenerative ability, but, surprisingly, a recent study fails to demonstrate regeneration of posterior body regions (Dirks *et al.*, 2012). Regeneration abilities within the parasitic lineages appear to be limited, though some tapeworms can regenerate posteriorly from the scolex or even just the anterior part of the scolex (Read, 1967; Hart, 1968; Schiller, 1974) and *Schistosoma* trematodes regenerate tegument after sub-lethal doses of the drug praziquantel (Shaw and Erasmus, 1987).

In summary, among platyhelminths regenerative ability is extensive in several groups but is not universally so. Posterior regeneration appears to be more widespread than anterior regeneration, but much more sampling is needed to infer the patterns of evolution of regeneration. The limited anterior regeneration ability of certain groups (e.g., dendrocoelids) has been interpreted as a loss, though sampling is still sparse enough that alternative scenarios, such as one or more gains of high anterior regenerative ability among planarians, should be considered. Future efforts should concentrate on assessing regenerative ability in unsampled or poorly sampled platyhelminth lineages, as well as increasing sampling within the triclads, where considerable variation is already known to occur.

Mollusca

The Mollusca are a large group with over ~85,000 species described, including marine, freshwater, and terrestrial species,

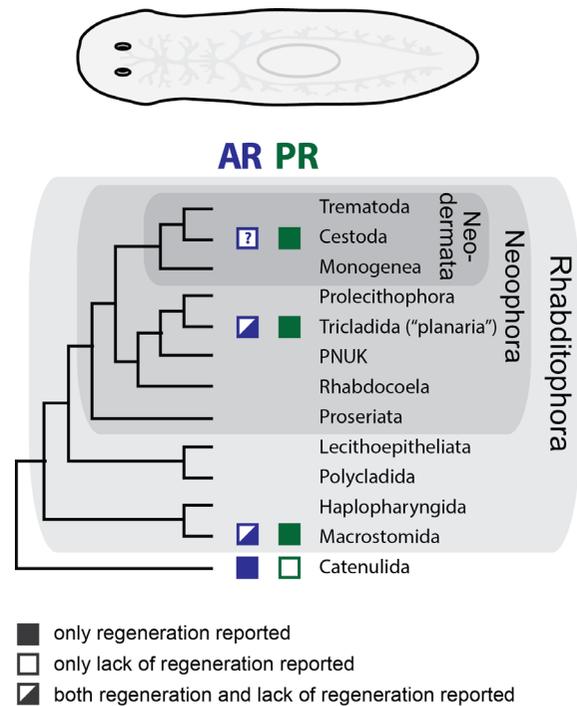


Fig. 3. Phylogenetic distribution of regeneration across the Platyhelminthes. Phylogeny is based on Riutort *et al.*, (2012). PNUK refers to the clade composed of *Piscinquilinus*, *Notentera*, *Urastoma* and *Kronborgia* (*Fecampida* and *Urastomidae*). Regeneration data are reviewed in the text. AR - anterior regeneration; PR - posterior regeneration.

and display extreme morphological diversity (Brusca and Brusca, 2003; Zhang, 2013). While most species have a radula for feeding and a mantle with mantle cavity, and many species also possess a shell, beyond this, few morphological generalizations can be made of the group. The major groups of molluscs are the gastropods, bivalves, cephalopods, scaphopods, monoplacophorans, polyplacophorans, and two groups of aplousobranchs, and important advances in our understanding of their relationships have recently been made (Kocot *et al.*, 2011; Smith *et al.*, 2011). Unlike the annelids, nemerteans, and platyhelminths, molluscs do not have known representatives capable of regenerating every part of the body. However, across the phylum, a number of structures have been shown to regenerate. These include the foot, anterior neural elements, tentacles, and even the entire head of some gastropods (Moffett, 1995, 1996; Gorbushin *et al.*, 2001; Matsuo *et al.*, 2010; Tuchina and Meyer-Rochow, 2010; Hoso, 2012), the siphon and parts of the shell and mantle of some bivalves (Ansell *et al.*, 1999; Tomiyama and Ito, 2006; Liu *et al.*, 2013; Nuñez *et al.*, 2013), and the arms, tentacles, and suckers of many cephalopods (Feral, 1978; Bush, 2012; Fossati *et al.*, 2013) including those of giant squid (Aldrich and Aldrich, 1968). Greater sampling of regenerative ability is needed within each of these groups, as well as in the mollusc lineages in which there are no data on regeneration, in order to obtain a clearer picture of the distribution of regenerative ability across this phylum. Given the morphological disparity of the structures that are known to regenerate, however, it is worth considering that at least some of these abilities represent novel origins of regenerative ability.

Developmental basis of regeneration in spiralian

In this second section we review information pertaining to the developmental processes involved in regeneration in annelids, nemerteans, platyhelminths, and molluscs, focusing on both the cellular basis and the molecular basis of regeneration.

Annelida

Annelids have long been subjects of regeneration research and a number of different annelid groups have been studied (Berrill, 1952; Bely, 2014). Although our understanding of regenerative mechanisms remains limited and fragmentary, important advances have recently been made in clarifying the cellular and molecular mechanisms underlying regeneration in this group.

Following transverse amputation, annelids rapidly seal the wound through contraction of circular muscles and then wound-heal through fusion of the severed body wall epithelium (Hyman, 1940). These processes appear to involve little to no proliferation and, indeed, a recent study shows that cell proliferation is quickly and dramatically shut down throughout most of the body during this early phase after amputation (Zattara and Bely, 2013). During this wound-healing phase and on into slightly later stages of regeneration, there is a large cell migration response to the wound site (Cornec *et al.*, 1987; Bilej, 1994). The functions of different types of migrating cells have variously been inferred to include wound-plug formation, phagocytosis, regeneration initiation, and delivery of a mesodermal or stem cell source, though the definitive roles of migratory cells remain to be clarified. Surgical manipulations have implicated tissue interactions between the severed body wall, nerve cord, and gut in successful regeneration (Fitzharris and Lesh, 1972). After wound-healing, cells near the wound site begin proliferating and, during both anterior and posterior regeneration, form a mass of apparently undifferentiated cells, referred to as a blastema, at the wound site (Hill, 1970; Zattara and Bely, 2011). Regarding the cellular source of the blastema and regenerated structures, several histological studies indicate that all three tissue layers proliferate near the wound site and contribute to the blastema (Clark, 1972; Cornec *et al.*, 1987), and a recent study has shown a definitive contribution of gut endoderm to the regenerated gut (Tweeten and Reiner, 2012). The role in regeneration of a population of large, apparently undifferentiated cells, referred to as annelid neoblasts, that are found among a number of clitellate annelids has long been debated (Stephan-Dubois, 1954; Cornec *et al.*, 1987) and continues to be an area of active research (Tadokoro *et al.*, 2006; Sugio *et al.*, 2008; Myohara, 2012; Sugio *et al.*, 2012). The blastema ultimately differentiates into most of the missing structures, though at least some components of the regenerated nervous system appear to invade the blastema from the stump (Müller *et al.*, 2003; Müller, 2004; Müller and Henning, 2004; Zattara and Bely, 2011).

Regeneration has been studied at the molecular level in several groups of annelids, primarily in nereids (Errantia), enchytraeids (Sedentaria), naids (Sedentaria), and capitellids (Sedentaria). A number of genes have been shown to be expressed broadly within the regeneration blastema, including several stem cell/germ line markers (Giani *et al.*, 2011; Gazave *et al.*, 2013), Hox genes (Pfeifer *et al.*, 2012; Novikova *et al.*, 2013), and a novel gene, *grimp* (Takeo *et al.*, 2010). To date, all of the genes found broadly expressed in the blastema are also expressed during normal growth in the posterior growth zone, suggesting a shared

mechanism between regeneration and growth. In enchytraeids, several genes broadly expressed in the blastema have also been found expressed in isolated cells of the remaining tissue (Takeo *et al.*, 2010; Yoshida-Noro and Tochinal, 2010), and data suggest that some of these cells correspond to germ line cells that apparently migrate to the wound to re-establish the germ line and gonads in regenerated segments (Tadokoro *et al.*, 2006; Gazave *et al.*, 2013). During the segmentation phase of posterior regeneration, Wnt and Hedgehog pathways are implicated in the process (Dray *et al.*, 2010; Niwa *et al.*, 2013) and expression of a *twist* homolog also suggests its involvement in post-amputation segmentation (Pfeifer *et al.*, 2013).

Following amputation, not only are new structures formed at the wound site, but the remaining tissue can become remodeled, a process known as morphallaxis. While morphallaxis has long been recognized as a phenomenon in annelids (Berrill, 1952, 1978), recent studies are now demonstrating some of its molecular underpinnings. Within hours after amputation, expression domains of Hox genes in the nervous system and regional markers in the gut are modified along the anterior-posterior axis to restore the relative pattern in uncut animals (Takeo *et al.*, 2008; Novikova *et al.*, 2013). Rapid morphallaxis is also evident at the level of neural morphology and function (Lybrand and Zoran, 2012).

Nemertea

Despite the formidable regenerative abilities of at least some nemerteans, the process of regeneration in this group has received little attention. The data that are available are based almost exclusively on lineid nemerteans. Following head or tail amputation, the wound is sealed by contraction of the muscular walls and then healed by the formation of a wound epithelium. Studies of histological sections of anteriorly regenerating animals suggest that the wound epithelium has contributions from both ectodermal and mesodermal cells, that wound healing is followed by a phase of cell proliferation, and that a regeneration blastema forms at the wound site eventually differentiating into the missing anterior structures (e.g., brain, mouth, proboscis canal, sensory organs, ocelli) (Nusbaum and Oxner, 1910; Coe, 1929; Gibson, 1972). Beyond this general description, though, very little is known about the cellular underpinnings of regeneration in nemerteans. Based on histological studies, some authors have proposed that cells scattered within the relatively abundant extracellular matrix are pluripotent, contributing to normal growth and migrating to wound sites to give rise to regenerated tissues following amputation (Coe, 1934b). This model would be very similar to the neoblast model in planarians. However, more recent analyses suggest the morphology of the cells within the extracellular matrix does not support the idea that they are undifferentiated stem cells (Turbeville, 1991). What cells contribute to regeneration, as well as to normal growth, in nemerteans remains a fundamental unanswered question. In addition, although a definite blastema forms during anterior regeneration, there is no clear evidence of a blastema forming during posterior regeneration, though the latter process is poorly described. Therefore, the question of how anterior and posterior regeneration differ in nemerteans is another key open question.

Molecular studies of nemertean regeneration are similarly sparse. During anterior regeneration, *pax-6* has been shown to be expressed in the developing central nervous system and ocelli (Loosli *et al.*, 1996), *otx* is expressed in the developing central nervous system

but not ocelli (Charpignon, 2007), and vision-related *six* and *opsin* genes are expressed in the brain and ocelli (Charpignon, 2007). During posterior regeneration, the posterior paraHox gene *cdx* is expressed in the developing gut of posterior regenerates (Charpignon, 2007). No study thus far has addressed what genes could be responsible for initiating and sustaining the regenerative response in nemerteans. Pursuing this question in the highly regenerative *R. sanguineus* would be particularly valuable, especially if coupled with comparative studies of its less regenerative close relatives in order to identify what molecular signatures are found uniquely in the highly regenerative species.

Platyhelminthes

Platyhelminthes are classic invertebrate models for studying regeneration and have received renewed attention in the last few decades. The best-characterized group is the planarians, in which the cellular and molecular bases of regeneration have been intensively studied (Saló *et al.*, 2008; Forsthoefel and Newmark, 2009; Aboobaker, 2011; Tanaka and Reddien, 2011). At the cellular level, planarian regeneration is intricately tied to the group of pluripotent cells referred to as neoblasts. Following amputation, worms form a wound epithelium that signals underlying tissues to initiate a regenerative response (Schürmann and Peter, 1998), though the molecular nature of these signals is still poorly known (Petersen and Reddien, 2009a). Shortly thereafter, neoblasts first proliferate throughout the body and later proliferate specifically at the wound site, producing cells that migrate to form the regeneration blastema (Baguña *et al.*, 1989; Wenemoser and Reddien, 2010; Guedelhofer and Sánchez Alvarado, 2012). The progeny of neoblasts are specified during early phases of regeneration yet still retain some level of developmental plasticity. Ultimately, these neoblasts, which have the potential to give rise to all cell types, replace all body regions (Wagner *et al.*, 2011). While the vast majority of information on regeneration comes from studies in planarians, comparisons of findings in planarians and macrostomids suggest some commonalities in the developmental mechanisms that confer the robust regenerative potential in at least these two groups (Bely and Sikes, 2010b; Simanov *et al.*, 2012).

Recent years have seen major advances in understanding the molecular basis of planarian regeneration. To initiate regeneration of appropriate structures, flatworms reestablish axial polarity soon after amputation through several conserved signaling pathways. Extracellular signal-related kinase (ERK) and Wnt/ β -catenin signaling pathways have been shown to establish molecular gradients for the establishment of the anterior-posterior axis in anteriorly-regenerating planarians (Gurley *et al.*, 2008; Petersen and Reddien, 2008; Petersen and Reddien, 2009b; Gurley *et al.*, 2010; Tasaki *et al.*, 2011; Umesono *et al.*, 2013). Several recent studies in three different planarian species with limited regeneration ability indicate aberrant Wnt signaling inhibits regeneration in regeneration-deficient tissues (Liu *et al.*, 2013; Sikes and Newmark, 2013; Umesono *et al.*, 2013), suggesting the importance of polarity reestablishment for successful regeneration. While these signals specify the epimorphic regeneration of proper structures at wound sites, blastema formation does not always result in the generation of all missing structures and can be asymmetric. Thus, planarians typically also undergo subsequent morphallaxis to replace body proportion and symmetry, though the molecular mechanisms by which morphallaxis occurs are not well understood (Reddien and

Sánchez Alvarado, 2004).

While considerable strides have been made in understanding the developmental underpinnings of regeneration in planarians and macrostomids, little is still known about regeneration mechanisms in most basal platyhelminth lineages and in the more derived parasitic lineages (Neodermata). While neoblast-like cells have been identified in all major flatworm groups investigated (Newmark and Sánchez Alvarado, 2000; Ladurner *et al.*, 2005; Koziol *et al.*, 2010; Dirks *et al.*, 2012; Collins *et al.*, 2013), there is a particular need for studies of regenerative mechanisms across a broader array of platyhelminths, and in particular in the basal catenulid lineage, as these will be key for inferring regenerative mechanisms that are ancestral for the phylum. Platyhelminths include both highly regenerative groups and others, such as the parasitic flatworms, which are much more limited in their ability to regenerate, presenting excellent opportunities for identifying developmental mechanisms responsible for regeneration limitation and regeneration loss. While studies have begun to identify aberrant signals that limit regeneration in some planarians, the molecular mechanisms that interact to alter regeneration abilities remain unknown. Identifying these mechanisms will be key for elucidating how regeneration has evolved among the Platyhelminthes.

Mollusca

Little is known about the cellular and molecular processes of regeneration in molluscs. Most studies describe only the outward appearance of regeneration. A recent study of octopus arm regeneration (Fossati *et al.*, 2013) demonstrates that following wound healing, a thin layer of undifferentiated cells appears at the wound site. A mass of mesenchymal cells accumulates at the wound site, forming a blastema, and this mass is underlain by highly vascularized tissue. The regenerating tip continues to grow, lengthening the regrowing arm as it forms differentiated structures. Cell proliferation assays on histological sections of the regenerating tip indicate that the blastema is highly proliferative and that high levels of proliferation persist as the regenerating tip continues to grow. There is a clear need for greater study of the developmental processes involved in mollusc regeneration.

Conclusions & future directions

A number of conclusions can be drawn from this comparative and developmental review of regeneration in spiralian. First, there is high value in considering regeneration in a phylogenetic context. Although annelids, nemerteans, and platyhelminths are often hailed as being some of the most highly regenerative animal groups, available data make it clear that high regenerative ability (e.g. the capability to regenerate all parts of the body) is present in only a subset of representatives of each of these phyla. Perhaps the most extreme example of this is the nemerteans, in which the widespread reputation for high regenerative abilities is based almost entirely on the abilities of a single species! Importantly, interpreting regeneration in a phylogenetic context indicates numerous increases and/or decreases in regeneration ability have occurred across these groups. This highlights that when comparisons of regeneration are made across phyla, the possibility that regeneration may not be homologous across them, and thus that similarities may be convergent, needs to be considered. Second, in all three worm-shaped phyla (annelids, nemerteans, platyhelminths), posterior regeneration is

considerably more widespread than anterior regeneration. The cause of this trend should be further investigated, as should the possibility that posterior regeneration could be homologous across spiralian phyla. Third, a blastema stage is described during regeneration in all four of the phyla reviewed here, including during head and tail regeneration in annelids and platyhelminths, head regeneration in nemerteans, and arm regeneration in cephalopod molluscs. Yet if some of these regenerative abilities represent novel origins (as, for example, seems likely for head regeneration in lineid nemerteans), then this raises the interesting possibility that blastema formation itself may have evolved independently several times, perhaps through recruitment of similar underlying mechanisms. Fourth, in all three phyla with extensive regenerative abilities (annelids, nemerteans, and platyhelminths), the capacity for continuous growth as well as degrowth are documented. This raises the question of whether an underlying set of processes, such as those that confer the ability to continuously regulate morphogenetic processes, potentiates both growth/degrowth mechanisms and regeneration.

Major gaps in our knowledge are highlighted as well by this review. With respect to the distribution of regeneration ability, there is clearly a very large amount of missing data. For many subclades within these phyla, regeneration data come from just one or a few species, and for many lineages we could find no evidence of regeneration at all. Furthermore, as has been suggested for annelid regeneration (Bely, 2010), there has likely been a publication bias toward reporting on high regenerative abilities and under-reporting regeneration failure. Thus, the picture we currently have of the distribution of regeneration could well be biased, and a significant proportion of lineages for which data are unavailable may be expected to have limited regeneration abilities. For understanding regeneration evolution, value needs to be placed on reporting regeneration failure, not just regeneration success, and it is important that amputation experiments be performed in such a way that negative data are as strong as possible (e.g., with adequate controls, replicates, testing under different conditions). As phylogenetic sampling for regeneration increases, inferences regarding the pattern of regeneration evolution and the ancestral capabilities for each phylum will become strengthened, and it will become possible to directly test for correlates of increases and decreases in regenerative ability.

Another major gap in our knowledge centers on our understanding of the diversity of developmental mechanisms for regeneration within a phylum. Because the vast majority of data come from just one to a few species per phylum, we still have little understanding about what developmental processes of regeneration are conservative and what features are variable across groups regenerating the same body parts. Similarly, we know little about what changes in developmental mechanisms are responsible for differences in regenerative ability. The few studies that have been performed comparing regeneration among close relatives have demonstrated the power of this approach. As broadly applicable developmental tools and techniques to probe the cellular and molecular basis of regeneration become more available, pursuing such studies will be increasingly feasible and is likely to reward us with a much deeper understanding of the mechanisms underlying regeneration evolution.

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