Proposing a solution to the Articulata–Ecdysozoa controversy

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Recent studies of animal radiation agree on monophyly of the Bilateria, but there is no consensus about the early radiation of the group. Protostomia and Deuterostomia are usually recognized, with two competing theories regarding the division of the Protostomia: one divides them into Spiralia and Cycloneuralia, the other into Lophotrochozoa and Ecdysozoa. The main discrepancy concerns the Arthropoda, which are placed with the Articulata within the Spiralia by the first group, and with the Cycloneuralia within the Ecdysozoa by the second. Here I propose that this discrepancy can be resolved by regarding the Ecdysozoa as the sister group of the Annelida within the Articulata. This implies that segmentation has been lost in phyla such as Nematoda and Priapula, but the Kinorhyncha may show a 'reduced segmentation' with serially arranged muscles associated with a ringed cuticle. Morphological, palaeontological and molecular implications of this theory are discussed. While many morphological and palaeontological data can be interpreted in accordance with the theory, the molecular data remain inconclusive.

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Introduction

Early radiation of the animal kingdom has been the subject of an increasing stream of publications in recent years. Two main approaches can be delineated. The first is rooted in traditional, morphology-based phylogenetics (e.g. Meglitsch & Schram, 1991, Ax 1995–2001, Nielsen 2001) and the second (often termed the 'new' phylogeny) is based on molecular data, especially 18S rRNA and *Hox* genes (e.g. Adoutte *et al*. 2000). Both agree in regarding Metazoa (= Animalia) and Bilateria as monophyletic. However, this has resulted in two somewhat conflicting phylogenies for the Bilateria (Fig. 1). Some of the morphology-based studies recognize the Protostomia and Deuterostomia (with Phoronida and Brachiopoda included in the latter) as sister-groups and divide the former into Spiralia and Cycloneuralia. The molecular-based studies usually recognize the same two main groups, but with the Phoronida and Brachiopoda included in the Protostomia (see below), which is then divided into Lophotrochozoa and Ecdysozoa. The most conspicuous difference between the Spiralia/Cycloneuralia and Lophotrochozoa/Ecdysozoa (henceforth Articulata and Ecdysozoa) hypotheses is the position of the arthropods (Panarthropoda), placed in the Spiralia by the first and in the Ecdysozoa by the second (Fig. 1).

The same approaches can be recognized in most of the recent studies on metazoan radiation (e.g. Zrzavý *et al*. 1998; Cavalier-Smith 1998; de Rosa *et al*. 1999; Giribet & Ribera 2000; Peterson & Eernisse 2001). The morphology-based studies emphasize the strong similarities between annelids and arthropods, especially segmentation, with details such as the addition of new segments from a posterior growth zone, parasegments, coelomic/mesodermal compartments, and metanephridia (Nielsen 2001; Scholtz 2002). Almost all molecular studies based on 18S rRNA sequencing and *Hox* gene structure emphasize that the Ecdysozoa are a monophyletic group (e.g. Giribet & Wheeler 1999; Adoutte *et al*. 2000; Peterson & Eernisse 2001), whereas the Lophotrochozoa (Trochozoa, Lophozoa) are less strongly supported and phyla such as molluscs and annelids often come out as polyphyletic (Winnepenninckx *et al*. 1998; Giribet & Wheeler 1999; Peterson & Eernisse 2001). Studies using immunocytochemical staining of nervous systems support the monophyly of the Ecdysozoa (Haase *et al*. 2001). Further support for the Ecdysozoa comes from studies of the structure of β-thymosin homologues from a sponge, a mollusc, an annelid, an arthropod, a nematode, an echinoderm and three chordates. These showed a highly conserved structure of the actin-binding hexapeptide in all but the arthropod and the nematode, which both showed a triplication with some modifications (Manuel *et al*. 2000).

Some authors have discussed the morphological consequences of the Articulata–Ecdysozoa discrepancy by comparing similarities/differences of annelids and arthropods vs. arthropods and nematodes, but without reaching any firm conclusion (e.g. Schmidt-Rhaesa *et al*. 1998). The Ecdysozoa
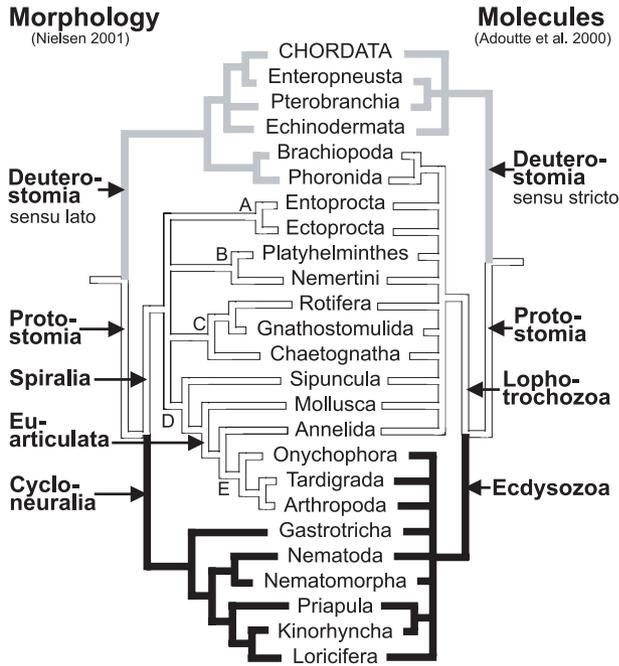
Fig. 1 Bilaterian evolution as deduced from morphological and molecular studies, exemplified by Nielsen (2001) and Adoutte *et al.* (2000), respectively. Note that two studies use the term Deuterostomia in slightly different ways. Clades: A = Bryozoa; B = Parenchymia; C = Gnathifera; D = Schizocoelia; E = Panarthropoda.

are characterized by moulting, which is obviously apomorphic (see below). However, all the lophotrochozoan characters, such as ciliated epithelia, ciliated larvae with apical organ, and lack of moulting, must be plesiomorphic because these characters are found both in cnidarians and deuterostomes. This alone indicates that the Lophotrochozoa may be paraphyletic.

Few papers try to integrate the two datasets; when they do so it is usually in the form of cladistic analyses of combined datasets, without thorough discussions of the morphological implications (e.g. Zrzavý *et al.* 1998; Giribet & Ribera 2000; Giribet *et al.* 2000; Peterson & Eernisse 2001). This is clearly unsatisfactory.

In an attempt at resolving this controversy I here propose a phylogeny which combines the stronger elements from both theories by suggesting that the Ecdysozoa (Panarthropoda + Cycloneuralia) and Annelida are sister-groups, together forming the group Articulata (or Euarticulata) (Figs 2 and 3). The discrepancy about the position of phoronids and brachiopods has been discussed elsewhere (Nielsen 2001) and is summarized in Fig. 4. This resolves the problem of the 'position of the arthropods' by placing annelids, arthropods and nematodes close to each other on the tree. It furthermore makes the 'Lophotrochozoa' paraphyletic, which is already indicated by the difficulties in identifying apomorphies of the group, while maintaining the Ecdysozoa.
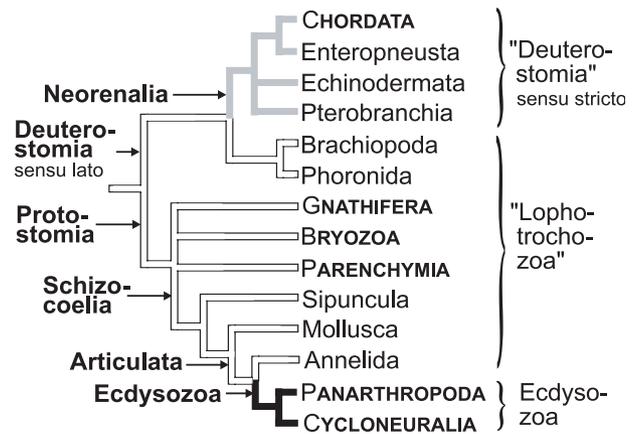

Fig. 2 Bilaterian evolution, as proposed here. The clades Parenchymia, Bryozoa and Gnathifera are explained in Fig. 1.

In the following I discuss the morphological and palaeontological implications of this scheme, with a view to casting some light on the problems and indicating promising areas for future research. The molecular evidence is discussed more briefly, with emphasis on the weakness of most methods in providing consistent and morphologically meaningful pictures of the early bilaterian radiation. I further hope that the main branching pattern proposed here will be tested on the available molecular datasets to assess the likelihood of this hypothesis.

Origin and evolution of morphological characters of the Articulata (Annelida + Panarthropoda)

Both of the morphologically defined groups Protostomia and Deuterostomia *s.s.* (Fig. 1) show a whole series of synapomorphies which have not been encountered in the other, so separate origins from a common bilaterian ancestor are strongly supported by morphology (Nielsen 2001). One of the most conspicuous protostomian synapomorphies is that the larval apical organ (or areas on its sides) becomes incorporated in the adult brain, which is connected to a ventral nerve cord (paired or fused). This is clearly seen in annelids, whereas arthropods and cycloneuralians lack ciliated larvae and an apical organ. However, the two groups have central nervous systems of the same general architecture with a dorsal or circumpharyngeal brain and ventral nerve cord(s) developing from longitudinal areas along the fusing lateral blastopore lips (or equivalent longitudinal bands). Cleavage patterns show considerable variation in the Protostomia, but it should be emphasized that spiral cleavage is definitely absent in the phyla classified as deuterostomes here (the report of spiral traits in the cleavage of a phoronid by Rattenbury (1954) is now considered as a misunderstanding by all students of phoronid development; Zimmer 1991). Spiral cleavage is clearly seen in several of the protostomian phyla,
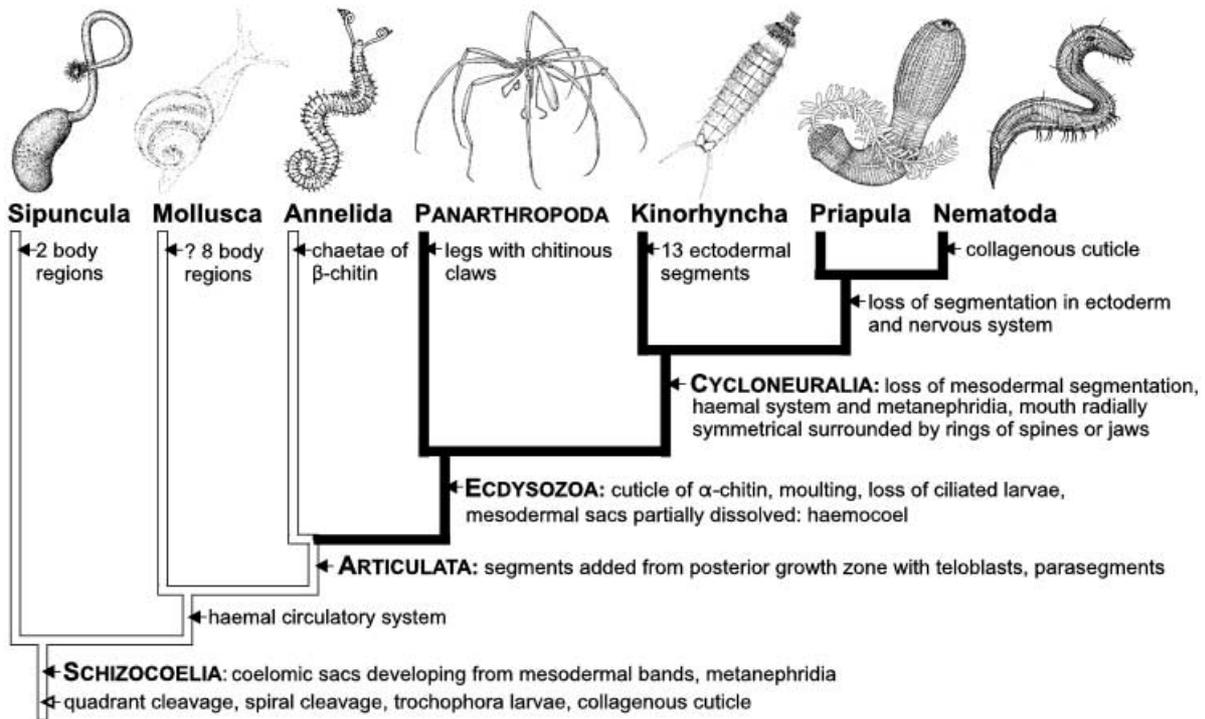
Fig. 3 Evolution of the Schizocoelia; the basal radiation of the Protostomia is seen in Figs 1, 2. Filled arrows, apomorphies.

but it is less well documented for example in gnathostomulids, and has been lost completely for example in cephalopods. In general, the two first cleavages in protostomes divide the embryo into four quadrants, viz. left, anteroventral, right and posterodorsal, although the cleavage patterns in several cycloneuralians are unknown or poorly studied. In deuterostomes, the two first cleavages divide the embryo medially

and transversally (Nielsen 2001). The structure of the apical brain of protostome larvae with only 2–3 serotonergic cells, as opposed to many cells in the apical organ in deuterostome larvae, seems to be a new and important character (Hay-Schmidt 2000). The blastopore is unfortunately a highly variable character even within phyla, and cannot therefore be used in assessment of phylogenetic affinities.
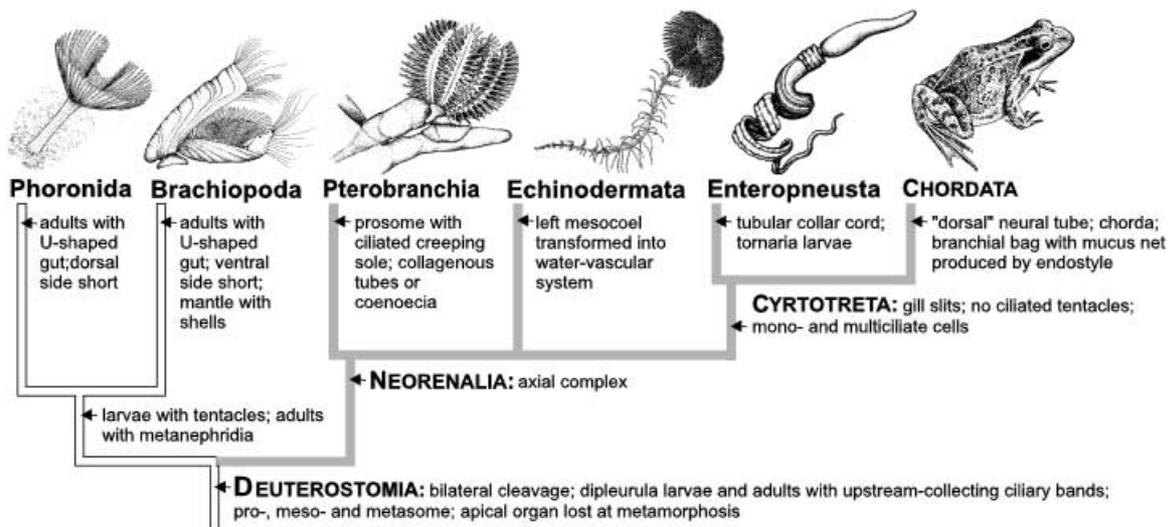

Fig. 4 Evolution of the Deuterostomia *s.l.* (based on Nielsen 2001). Filled arrows, apomorphies.

Within the Protostomia, Bryozoa (Entoprocta + Ectoprocta), Parenchymia (Platyhelminthes + Nemertini) and Gnathifera (Rotifera + Gnathostomulida + Chaetognatha) apparently form monophyletic groups, separate from Schizocoelia (Fig. 1); they are not discussed here.

The 'basal' Schizocoelia (Sipuncula, Mollusca and Annelida) exhibit many plesiomorphic characters, such as ciliated epithelia and planktonic (in several species planktotrophic) larvae with an apical organ, and are characterized by the presence of coelomic sacs, which develop through schizocoely from mesodermal bands and are associated with metanephridia, which usually function as gonoducts too (Nielsen 1997). The close phylogenetic relationships of these three phyla is usually not questioned. The diagram in Fig. 3 shows the steps I envisage in the evolution of the Schizocoelia, demonstrating the proposed connection between the traditional Articulata (Annelida + Panarthropoda) and the Cycloneuralia (less the Gastrotricha, for which I do not propose a phylogenetic position here).

The sipunculans show no sign of segmentation or of a haemal system, i.e. a circulatory system with a haemal fluid moving between basal membranes, usually with a heart formed by areas of coelomic epithelium. Molluscs and annelids have a haemal system, and panarthropods (onychophorans and arthropods) have a special type of body cavity, a haemocoel or mixocoel, which is formed through confluence of haemal and coelomic cavities. The segmented nature of mollusc organization is questionable (Friedrich *et al.* 2002), but this seems of no importance for the present arguments.

Mesodermal segmentation with addition of segments from a posterior growth zone is very obvious in annelids and arthropods (Scholtz 2002), although the coelomic sacs have become dissolved into the small nephridial sacs, gonads, various glands, and separate muscles in the arthropods. The embryology of kinorhynchs is unfortunately unknown, but the adults show a serial arrangement of muscles which connect the cuticular segments, and this could be a trace of an ancestral segmentation; the newly hatched juveniles have 11 segments and two additional subterminal segments become added during the five postembryonic moults (Neuhaus 1995). The presence of protonephridia only in the adult could be associated with the loss of coelomic cavities (and consequently of metanephridia). Priapulan embryology is almost unknown; no trace of segmentation has been reported. By contrast, the nematodes have been studied in much detail. The low number of cells (eutely) and direct development without reorganization of the tissues indicate that the latter is highly derived. The cuticle, which is moulted four times during ontogeny, consists of collagen, although chitin has now been detected in structures in the pharynx (Neuhaus *et al.* 1997). The loss of a conspicuous character such as segmentation is one of the stumbling blocks of the present

theory. The reiterated groups of nerves at regular positions along the trunk of *Caenorhabditis elegans* are not an indication of segments added from the posterior end of the body, as shown by their cell lineage (Walthall 1995; Sulston *et al.* 1983).

Moulting of a chitinous or collagenous cuticle, governed by changes in levels of ecdysones (especially 20-hydroxyecdysone), is often mentioned as the conspicuous synapomorphy of the Ecdysozoa, but it also occurs in doliolids and appendicularians. Moulting correlated with changes in the level of 20-hydroxyecdysone has been documented in a leech (Sauber *et al.* 1983). Ecdysones have been found in most animal phyla and also in some plants. Nevertheless, I regard moulting as an important character in the definition of the Ecdysozoa. Some molecular studies (e.g. Littlewood *et al.* 1998) place gnathostomulids and chaetognaths within the Ecdysozoa, but none of these groups moult; also, gnathostomulids have a ciliated ectoderm, so morphologically they do not fit well within this clade (and have been placed in the Gnathifera in Fig. 2). The 'change back' to a collagenous cuticle in nematodes (and nematomorphs) is unexplained. A newly discovered synapomorphy of ecdysozoans is immunoreactivity to antihorseradish peroxidase, which has been found in the nervous system of representatives of Arthropoda, Onychophora, Priapula, Nematoda and Nematomorpha. It is apparently absent in cnidarians, ctenophores, annelids, molluscs, rotifers, chaetognaths, bryozoans, as well as in several deuterostomes (Haase *et al.* 2001).

If a loss of segmentation can be accepted, it thus appears that a coherent scenario for the evolution of Ecdysozoa as the sister group of Annelida can be constructed.

Fossil evidence

The fossil record comprises a wide variety of molluscs, annelids, arthropods and cycloneuralians; there is no reliable record of sipunculans. Early Cambrian faunas of small, calcareous shells include representatives of several living and extinct classes of molluscs (Runnegar & Pojeta 1985). The Cambrian halkieriids (Conway Morris & Peel 1995) have an elongate body with the dorsal side covered by a mail coat resembling the polyplacophoran perinotum and an anterior and a posterior dorsal shell; they probably represent an extinct molluscan clade and bear no resemblance to annelids (Lindberg & Ponder 1996; Nielsen 1997). Annelids are reported from the Lower Cambrian (Chen & Zhou 1997), and a number of well preserved, centimetre-long species are known from the Middle Cambrian Burgess Shale (Briggs *et al.* 1994). There is a wide variety of ecdysozoan fossils of Lower Cambrian age, some of which clearly represent living groups, or at least their stem lineages (Chen & Zhou 1997; Budd & Jensen 2000). The Arthropoda are represented by many trilobites and forms such as *Naraoia*, *Waptia* and

Canadaspis (Chen & Zhou 1997) and the Priapula by forms such as *Maotianshania* (Chen & Chou 1997), although the Middle Cambrian *Ottoia* (Briggs *et al.* 1994) looks more convincing. Other ecdysozoan 'lobopod' fossils are of more uncertain affinities. The famous *Hallucigenia* has onychophoran-like limbs and an apparently unarmoured mouth. *Anomalocaris* and its relatives (see Hou, Bergström & Ahlberg 1995) apparently have the anteriormost pair of appendages specialized for manipulation of the food and a radial mouth with an array of spines or scales, and they were actually interpreted as 'aschelminths' by these authors. This could indicate that some of the ancestral cycloneuralian groups were segmented and had limbs, but that these features were lost in most of the surviving lineages. Kinorhynchs are not known as fossils, which may be due to their minute size. Nematodes are not known from Cambrian deposits; they may have originated later, but their absence as fossils may just as well be the result of the collagenous nature of their cuticle, which may not fossilize so easily.

Molecular evidence

The division of Protostomia into Ecdysozoa and Lophotrochozoa is based on molecular evidence, especially on sequences of 18S rRNA (Aguinaldo *et al.* 1997) and *Hox* genes (de Rosa *et al.* 1999; Adoutte *et al.* 2000).

The 18S rRNA sequences have been used extensively to reconstruct phylogenetic relationships among metazoan phyla (e.g. Halanych *et al.* 1995; Garey *et al.* 1996; Aguinaldo *et al.* 1997; Winnepenninckx *et al.* 1998; Giribet & Wheeler 1999; Stechmann & Schlegel 1999; Giribet *et al.* 2000; Peterson & Eernisse 2001). However, comparisons of the various results show considerable discrepancies.

Almost all studies find support for monophyly of the Deuterostomia *s.s.*; most find some support for Ecdysozoa, but the support for Lophotrochozoa is usually weak (Giribet & Wheeler 1999; Giribet *et al.* 2000). Some studies find no support at all for the latter two groups (Abouheif *et al.* 1998) and others find none for any of the groups (Halanych 1998). There appear to be several methodological reasons for these discrepancies, and a number of authors have concluded that 18S sequences for a variety of reasons are poorly suited for phylogenetic reconstruction at deep levels (Huelsenbeck & Bull 1996; Maley & Marshall 1998; Abouheif *et al.* 1998; Halanych 1998; Littlewood *et al.* 1998; McHugh 1998; Blair *et al.* 2002). The analyses of Winnepenninckx *et al.* (1998), Giribet *et al.* (2000) and Peterson & Eernisse (2001) include many sequences of annelids and molluscs, but these morphologically very well established phyla do not show up as monophyletic, and even the class Bivalvia becomes polyphyletic; this indicates severe methodological problems.

Sequences of other molecules have been investigated with varying success. Mitochondrial DNA (mtDNA) has been used in studies of the evolution of phyla and lower systematic categories, but even at the phylum level obviously incorrect trees with high bootstrap support have been obtained (Zardoya & Meyer 2001). Elongation factor-1α has been used in a few studies and found to be 'largely insufficient' for resolving higher animal phylogeny (Berney *et al.* 2000; Littlewood *et al.* 2001). Brown *et al.* (1999) studied sequences of 28S rDNA, histone H3 and U2 small nuclear RNA from a number of annelids, a sipunculan, a turbellarian, and a nematode and obtained three very different trees, both with respect to the position of sipunculans, echiurans and clitellates (as ingroups or outgroups of the polychaetes) and with respect to the interrelationships of the polychaete genera. None of the trees corresponded well with the results of the extensive morphological analysis by Rouse & Fauchald (1997).

It should be remembered that some of the speciation events giving rise to the splits between the living phyla may have been so close in time that few (or even no) substitutions accumulated between the events, for example in the 18S molecule, which will make the resolution of the relationships extremely difficult (or even impossible). It must be concluded that phylogenetic trees showing early animal radiation obtained through sequence studies must be regarded as preliminary, so the phylogeny proposed here cannot be ruled out by the available analyses of 18S or other gene sequences.

The evidence based on *Hox* gene duplications and sequences suffers from a rather different problem. *Hox* 'signatures', in the form of unique gene duplications within the posterior region of the cluster, have been proposed as characterizing Ecdysozoa, Lophotrochozoa, and Deuterostomia (de Rosa *et al.* 1999). However, each of the three groups has a characteristic composition of these genes, so this is an unpolarized character (Telford 2000). The protein sequences of the posterior cluster genes in the available outgroups (Cnidaria and Ctenophora) are simply too few and divergent to resolve the polarity of the gene duplications within the Bilateria (de Rosa *et al.* 1999). Using the alternative approach of paralogue rooting, Telford (2000) showed that many of the amino acids that de Rosa and colleagues used to assign posterior *Hox* genes to different paralogy groups (i.e. to identify *Hox* signatures) characteristic of Lophotrochozoa or Ecdysozoa are probably plesiomorphic and therefore do not provide support for these proposed clades.

In general, expression patterns of developmental regulatory genes provide surprisingly little support for any particular hypothesis of metazoan relationships. These genes, many of which are involved in patterning basic body organization, show two very different phylogenetic patterns: either expression is unique to a phylum (e.g. the role of *brachyury* in notochord development) or it is present throughout the Bilateria (e.g. the role of *Hox* genes in anteroposterior patterning)

(Davidson 2001). Neither of these distributions is helpful in understanding phylogenetic relationships among metazoan phyla.

Mitochondrial gene order is an additional form of molecular evidence, which is of an almost morphological nature. It records supposedly rare genomic rearrangements that are expected to be effectively unique and irreversible events. When such rearrangements can be polarized, they provide powerful evidence of shared ancestry. Unfortunately, so many rearrangements in mitochondrial gene order separate most protostome phyla that it has proven difficult to resolve the succession of rearrangements and therefore shed light on the issue of lophotrochozoan and ecdysozoan monophyly. Stechmann & Schlegel (1999) found almost identical gene orders in the mitochondrial genome of the brachiopod *Terebratulina* and the (partial) genome of the gastropod mollusc *Littorina*, indicating a close relationship. However, other molluscs show more deviating arrangements (Boore & Brown 1994) and a sister-group relationship between gastropods and brachiopods finds no support from other characters. Boore & Brown (2000) argue that mitochondrial gene order argues against a close relationship between arthropods and annelids, but this is again based on characters that are difficult to polarize with confidence. Le *et al.* (2000) and Hickerson & Cunningham (2001) found considerable differences in mitochondrial gene order both between species of the fluke genus *Schistosoma* and between a number of crustaceans, indicating that comparisons of species belonging to different phyla can be dubious if only one or few species have been investigated.

In summary, molecular evidence indicating that most bilaterian phyla belong to three clades is intriguing, but only Ecdysozoa and Deuterostomia *s.s.* are reasonably well supported. Yet there is every reason to believe that the right sort of molecular data and the use of the right types of analytical tools will eventually help resolve the relationships among metazoan phyla (McHugh 1998; McHugh & Halanych 1998; Whelan *et al.* 2001). Not long ago, the early mammalian radiation seemed intractable, yet two recent molecular analyses (Madsen *et al.* 2001; Murphy *et al.* 2001) provide robust support for nearly identical phylogenetic trees. Significantly, both research groups carefully sought out and utilized multiple, easily alignable, protein-coding and flanking noncoding genomic segments that were undergoing appropriate rates of substitution. Although it means starting again from scratch, a similar strategy may be necessary in order to assemble a molecular data set capable of resolving the relationships among the bilaterian phyla.

Conclusions

The so-called 'new' phylogeny of the animal kingdom, which regards the Deuterostomia as the sister group of Lophotrochozoa plus Ecdysozoa, is not globally accepted (see for example discussions in Zrzavý 2001 and Wägele & Misof 2001). Morphological characters do not support monophyly of the Lophotrochozoa, and the molecular evidence is weak. The alternative phylogeny proposed here retains the Ecdysozoa, gives a new conceptual view of the Articulata (i.e. including the Ecdysozoa) and relegates Lophotrochozoa into paraphyly. It is built on morphology, but includes the mainly molecular-based Ecdysozoa. It has not been recovered by the molecular studies but its probability should be tested with the molecular methods. This new alternative raises many questions which can be answered by investigation of readily available animal material. In particular, it emphasizes the importance of new studies of kinorhynchs, particularly their ontogeny, using both traditional and molecular methods to identify genes responsible for 'segmentation'. The discussion would also profit from taking a wider view which includes morphological evidence, instead of framing it simply in terms of 'old' and 'new' phylogeny.

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