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Chordate evolution and the three-phylum system

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Traditional metazoan phylogeny classifies the Vertebrata as a subphylum of the phylum Chordata, together with two other subphyla, the Urochordata (Tunicata) and the Cephalochordata. The Chordata, together with the phyla Echinodermata and Hemichordata, comprise a major group, the Deuterostomia. Chordates invariably possess a notochord and a dorsal neural tube. Although the origin and evolution of chordates has been studied for more than a century, few authors have intimately discussed taxonomic ranking of the three chordate groups themselves. Accumulating evidence shows that echinoderms and hemichordates form a clade (the Ambulacraria), and that within the Chordata, cephalochordates diverged first, with tunicates and vertebrates forming a sister group. Chordates share tadpole-type larvae containing a notochord and hollow nerve cord, whereas ambulacrarians have dipleurula-type larvae containing a hydrocoel. We propose that an evolutionary occurrence of tadpole-type larvae is fundamental to understanding mechanisms of chordate origin. Protostomes have now been reclassified into two major taxa, the Ecdysozoa and Lophotrochozoa, whose developmental pathways are characterized by ecdysis and trochophore larvae, respectively. Consistent with this classification, the profound dipleurula versus tadpole larval differences merit a category higher than the phylum. Thus, it is recommended that the Ecdysozoa, Lophotrochozoa, Ambulacraria and Chordata be classified at the superphylum level, with the Chordata further subdivided into three phyla, on the basis of their distinctive characteristics.

1. Introduction

Since Charles Darwin proposed the evolution of animals by means of natural selection [1], the origin and evolution of chordates from common ancestor(s) of deuterostomes have been investigated and discussed for more than 150 years [2–20]. Chordates consist of three distinct animal groups: cephalochordates, urochordates (tunicates) and vertebrates. This review starts with a brief description of how the Phylum Chordata and its three subphyla were originally defined, and then discusses how we should reclassify the major chordate groups.

2. The phylum Chordata and subphylum Vertebrata: their history

Multicellular animals are often divided into vertebrates and invertebrates. Historically, this classification dates back to *ca* 500 BC. During the ancient Hindi era, Charaka distinguished between the Jarayuja (invertebrates) and Anadaja (vertebrates). In the ancient Greek era, Aristotle (*ca* 300 BC) recognized animals with blood (Enaima, or vertebrates) and those without (Anaima, or invertebrates). This recognition persisted even until Linnaeus [21]. It was Lamarck [22] who first explicitly proposed the vertebra-based division of animals, 'Animaux vertèbrès' and 'Animaux invertèbrès', in place of Enaima and Anaima, respectively.

Aristotle had already recognized solitary ascidians as Tethyon around 330 BC. Carolus Linnaeus was a botanist who devised a system for naming plants and animals. In his book *Systema naturae* (12th edn, vol. 1) [21], ascidians were included among the molluscs. Following anatomical investigations of ascidians by Cuvier [23] and others, Lamarck [24] recognized these as Tunicata, namely animals enclosed with a tunic (tunica, in Latin, meaning garment). On the other hand, cephalochordates (lancelets) were first described in mid-to-late eighteenth century as molluscs. Although Yarrell [25] had already noticed that lancelets have an axial rod, calling it 'a lengthened internal vertebral column, although in a soft cartilaginous state', it was Alexander Kowlevsky's discovery that both tunicates and lancelets possess notochords and dorsal neural tubes during embryogenesis, indicating that they are close relatives of vertebrates [26,27].

The term 'Vertebrata' was first coined by Ernst Haeckel in 1866 [28], in which lancelets were of the class Acrania of subphylum Leptocardia and all remaining vertebrates were classified into the subphylum Pachycardia (i.e. Craniota). At that time, the Tunicata was still included, together with bryozoans, in the subphylum Himatega of the phylum Mollusca. Following Kowalevsky's discovery of the notochord in ascidian larvae [26], Haeckel [29] moved the Tunicata from the phylum Mollusca to the phylum Vermes, which also contained enteropneusts (acorn worms), because he thought that tunicates were close relatives to vertebrates. He coined the name Chordonia for a hypothetical common ancestor of the Tunicata and the Vertebrata (including lancelets) by emphasizing the notochord as a significant diagnostic character shared by them. Later, Haeckel [30] redefined Chordonia (i.e. Chordata) to include the Tunicata and the Vertebrata themselves.

In London, Lankester [31] gave subphylum status to the Urochordata, the Cephalochordata and the Craniata, altogether comprising the phylum Vertebrata. This constituted the first conception of the modern phylum Chordata. Balfour [32] renamed Lankester's Vertebrata 'Chordata', and called the Craniata 'Vertebrata'. This system has been retained for more than a century due to robustness of the shared character set (notochord, dorsal nerve cord and pharyngeal slits) that Lankester defined. Bateson [3] regarded the stomochord or buccal diverticulum of enteropneusts as a notochord, and classified this animal as a member of the Hemichordata ('half-chord'), the fourth subphylum of Chordata. Today, however, molecular phylogenies have established that the Hemichordata is a sister group to Echinodermata [12,17,18].

3. The phylogeny of chordates: traditional and recent views

(a) Traditional view

The prevailing view holds that the phylum Chordata consists of three subphyla: Urochordata (Tunicata), Cephalochordata and Vertebrata (figure 1a). All three groups are characterized by possession of a notochord, a dorsal, hollow neural tube (nerve cord), branchial slits, an endostyle, myotomes and a postanal tail. These characters will be discussed later in relation to evolutionary scenarios for chordates. Meanwhile, the Chordata belongs to the superphyletic Deuterostomia, together with the phyla Echinodermata and Hemichordata (figure 1a). Chordates are thought to have originated from a common ancestor (or ancestors) of the deuterostomes [7,12,17–20]. Reflecting the historical conceptualization of the phylum Chordata mentioned above, a majority of previous researchers of this field have favoured an evolutionary scenario in which urochordates evolved first, then cephalochordates and vertebrates (§4a). In addition, as the term 'protochordate' has often been used, the relationship between enteropneust hemichordates and basal chordates (urochordates or cephalochordates) has frequently been discussed [12,16–18].

(b) Recent view

Molecular phylogeny is a powerful method to resolve phylogenic questions. Its application to eumetazoan phylogeny has resulted in reclassification of metazoan groups not only at the class or family level but also at the phylum level. Bilaterians or triploblasts (metazoans composed of three germ layers: ectoderm, mesoderm and endoderm) are traditionally categorized into two major groups, protostomes (in which the blastopore gives rise to the mouth) and deuterostomes (in which the blastopore gives rise to the anus, and the mouth arises through secondary invagination of the stomodeum; figure 1a), as first proposed by Grobben [33]. Protostomes were further subdivided, mainly based on the mode of formation of the body cavity (or coelom), into acoelomates (with no distinct body cavity) such as platyhelminthes, pseudocoelomates (with a poorly developed body cavity) such as nematodes, and coelomates (with a distinct body cavity) such as annelids, molluscs and arthropods. Molecular phylogeny, first based on comparison of 18S rDNA sequences [34,35] and later protein-coding gene sequences [36,37], however, did not support this classification of protostomes, but instead suggested their division into two major groups, the Ecdysozoa (those exhibit moulting) and Lophotrochozoa (those having lophophores and trochophore larvae; sometimes called Spiralia; figure 1b). The former includes nematodes and arthropods, the latter annelids, molluscs and platyhelminths. The mode of body cavity formation therefore is not critical to protostome phylogeny, but developmental modes such as moulting and spiral cleavage are fundamental to evolutionary scenarios. This Ecdysozoa–Lophotrochoza classification has been supported by other studies, including Hox gene clustering, although there are several groups of which the phylogenic positions are still enigmatic, such as the mesozoans and chaetognaths [20].

On the other hand, recent studies of deuterostome molecular phylogeny, nuclear and mitochondrial genomics, and evolutionary developmental biology, have unambiguously demonstrated that echinoderms and hemichordates form a clade, and that urochordates, cephalochordates and vertebrates form another distinct clade (figure 1b) [12,38–40]. The former is called the Ambulacraria, with similarities in coelomic systems and larvae [41], and the latter Chordata. In addition, within the chordate clade, cephalochordates diverged first, and urochordates and vertebrates form a sister group (sometimes called Olfactores, with similarities in extensive pharyngeal re-modification leading to the formation of new structures [42], which are not found in cephalochordates) [43–45]. This novel view of deuterostome taxonomy and phylogeny became the consensus view, as a great variety of data from different disciplines support arguments for them [46–49].

The Xenacoelomorpha is a newly recognized phylum some have assigned to the deuterostomes, but this group is not discussed here because its phylogenetic position is still unstable [20,50].
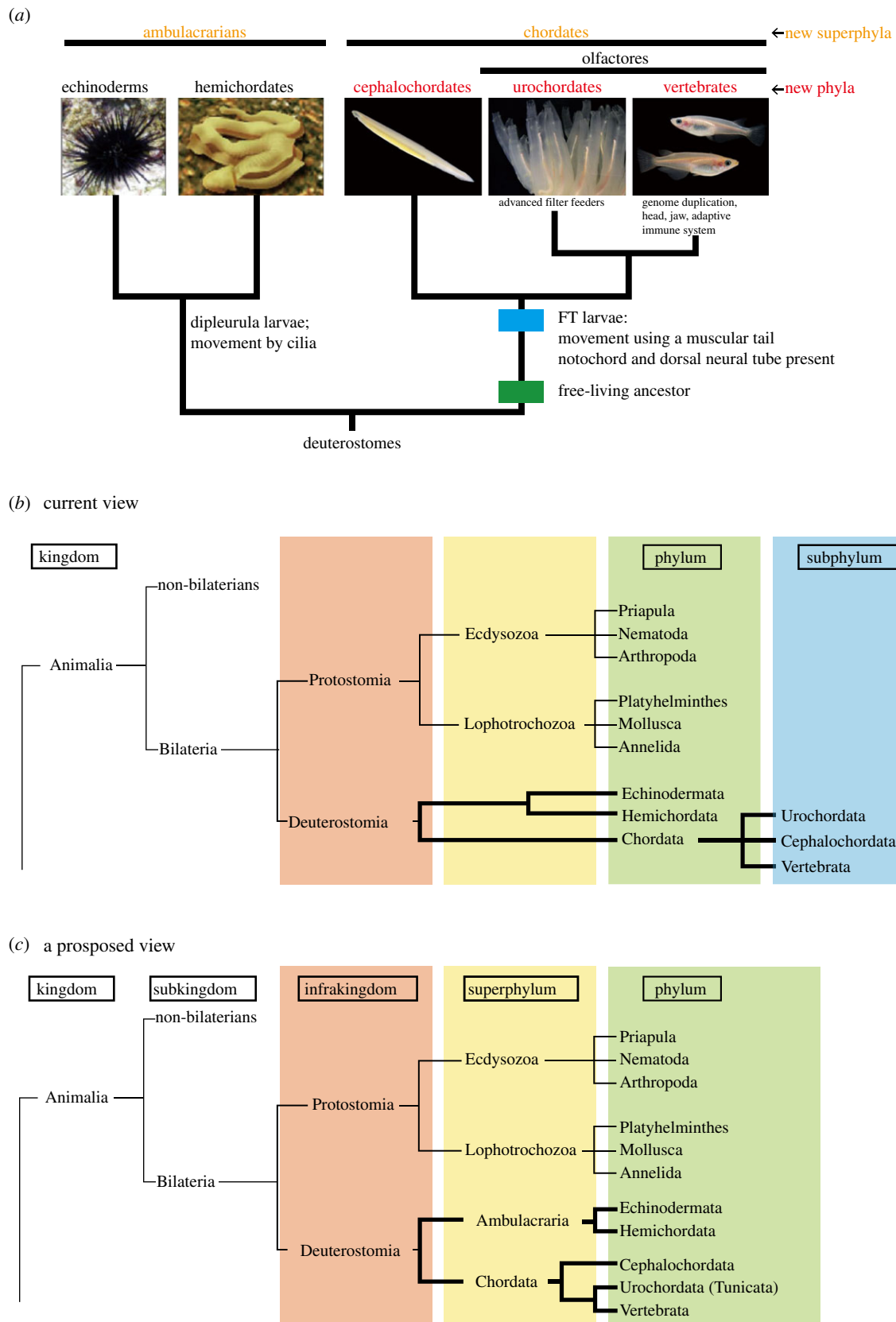
Figure 1. Phylogenic relationships of deuterostomes and evolution of chordates. (a) Schematic representation of deuterostome groups and the evolution of chordates. Representative developmental events associated with the evolution of chordates are included. (b) A traditional and (c) the proposed view of chordate phylogeny with respect to their phylum relationship.

4. Evolutionary scenarios of chordates

We discuss here four major scenarios proposed to explain chordate origin and evolution: the paedomorphosis hypothesis, the auricularia hypothesis, the inversion hypothesis and the aboral-dorsalization hypothesis. The first of these debated whether adults of ancestral chordates were sessile or free-living. The next three discussed, in terms of embryology or evolutionary developmental biology, how the chordate body plan, especially its adult form, originated from the common ancestor(s) of deuterostomes. Therefore, the four are not always independent, and supporting arguments for them frequently overlap.

4

(a) The paedomorphosis scenario: was the ancestor sessile or free-living?

Various authors have addressed the question of whether the chordate ancestor(s) were sessile or free-living [8,12,14–20]. Extant hemichordates consist of two groups with different lifestyles: the sessile, colonial pterobranchs and free-living enteropneusts (acorn worms). According to one scenario, ancestral deuterostomes were sedentary, tentaculate animals with pelagic larvae, like modern pterobranchs, which evolved into sedentary ascidians (urochordates) [4,5,51] (see [15] for further details). The motile, free-living lifestyle of cephalochordates and vertebrates was believed to have evolved from a motile larval stage of the sedentary, tentaculate ancestor (like tadpole-type larvae of ascidians) by paedomorphosis, a form of heterochrony roughly equivalent to neoteny, in which the larval stage became sexually mature and replaced the adult [15]. Tunicate larvaceans, in which adult organs develop in the trunk region of tadpole-like juveniles, may be a good example of a paedomorphogenetic transition.

According to the alternative scenario or progressive evolution of motile adults, the chordate ancestor was free-living and vermiform, and the sequence of ancestral forms is thought to have consisted of motile, bilaterally symmetric organisms, as opposed to larvae [12,17,18,52–54]. Motile forms such as enteropneust hemichordates and cephalochordates are typically considered close to the main lineage, whereas urochordates are viewed as more distant.

Historically, the first scenario of sessile ancestry received much support, as ascidians had long been believed to be the most basal chordates. It was just 10 years ago that molecular phylogeny first gave support for the second, free-living ancestor scenario, by positioning cephalochordates as basal among chordates [43,44]. Since then, accumulating evidence supports a free-living ancestor of chordates (figure 1a).

(b) The auricularia hypothesis

The auricularia hypothesis, originally proposed by Garstang [4], attempted to explain how the chordate body plan originated from a deuterostome common ancestor, by emphasizing the significance of changes in larval forms [10,15]. According to this view, the pterobranch-like, sessile animals with dipleurula (auricularia-like) larvae led to the primitive ascidians (as the latest common ancestor of chordates) through morphological changes both in larvae and adults. (This hypothesis therefore falls under the sessile ancestor scenario mentioned above.) Adults changed their feeding apparatus from external tentacles to internal branchial sacs. In larvae, the ancestor's circumoral, ciliated bands and their associated underlying nerve tracts moved dorsally to meet and fuse at the dorsal midline, forming a dorsal nerve cord in the chordate body. At the same time, the aboral ciliated band gave rise to the endostyle and ciliated tracts within the pharynx of the chordate. Nielsen [10] proposed a revised version of this hypothesis in which the chordate central nervous system evolved from the postoral loop of the ciliary band in a dipleurula larva.

In view of the mode of dorsal neural tube formation in lancelet embryos, it becomes evident that neural tube formation occurs by rolling up of presumptive neurectoderm soon after gastrulation or simultaneously with the later phase of gastrulation. This stage of amphioxus embryos has no structure related to the circumoral ciliary bands. Indeed, it is more plausible to consider the dorsal, hollow, neural tube as evolutionarily independent of the ciliary band of dipleurula larvae (see also discussion in §4d). In this sense, the auricularia hypothesis appears to have faded in light of recent evo–devo studies of deuterostomes [18,55].

(c) The inversion hypothesis

Recent debates on the origin of chordate body plans have focused most attention on inversion of the dorsal–ventral (D-V) axis of the chordate body, compared with protostomes [10,14,15,56–58]. This idea goes back to the early nineteenth century when Geoffroy St Hilaire compared the anatomy of arthropods (protostomes) and vertebrates (deuterostomes). In arthropods and annelids, the central nervous system (CNS) runs ventral to the digestive tract, and therefore these groups are sometimes called Gastroneuralia [9,59]. By contrast, in vertebrates, the CNS runs dorsal to the digestive system; hence they are sometimes called Notoneuralia. That is, the D-V axis appears to be inverted between annelids and vertebrates.

Nearly 140 years later, this notion was revitalized by the discovery of genes responsible for D-V axis formation, encoding members of TGF-β family proteins, bone-morphogenic proteins (BMPs) and their antagonists, including chordin and anti-dorsalizing morphogenetic protein (Admp) [60,61]. In *Drosophila melanogaster* (arthropod), Dpp (i.e. BMP) is expressed at the dorsal side of the embryo and functions in dorsalization of the embryo, while Sog (i.e. chordin) is expressed at the ventral side of the embryo and functions in ventralization [60]. By contrast, in *Xenopus laevis* (vertebrate), BMP is expressed at the ventral side of the embryo and chordin at the dorsal side [61].

A question then arose as to when and where in deuterostome phylogeny the D-V axis inversion occurred. It is now evident that the inversion took place between non-chordate deuterostomes and chordates. In echinoderms and hemichordates, BMP is expressed on the aboral side of the embryo and chordin on the oral side [62,63]. By contrast, in cephalochordate embryos, BMP is expressed on the ventral side and chordin on the dorsal side [64]. *Saccoglossus kowalevskii* is an acorn worm in which fertilized eggs develop directly into adults without a tornaria larval stage. In this species, the oral–aboral orientation of embryos becomes a ventral–dorsal orientation in adults. Therefore, the D-V axis inversion appears to have occurred during the evolution of chordates [15,16].

However, several studies demonstrate the formation of a dorsal, neural tube-like structure in acorn worm *adults* [65–67], which is reminiscent of dorsal, neural tube formation of chordate *embryos*. It should be noted that the inversion hypothesis cannot necessarily explain the occurrence of chordate-specific structures or the notochord [18,55]. The notochord is a dorsal, midline structure, profoundly associated with the so-called 'organizer' of vertebrate embryos [68]. Therefore, the inversion hypothesis should be further refined in relation to *de novo* formation of chordate-specific, dorsal structures of the embryo.

(d) The aboral-dorsalization hypothesis

The aboral-dorsalization (A-D) hypothesis was proposed to explain developmental mechanisms involved in evolution of the chordate body plan from a deuterostome common ancestor (or ancestors) [18,55]. The A-D hypothesis stands on recent deuterostome phylogeny and emphasizes the occurrence of fish-like or tadpole-like (FT) larvae as a critical

developmental event that led to the evolution of chordates. As is seen in cephalochordates and many vertebrates, the form of FT larvae (or juveniles) might fit their developmental strategy to settle in new habitats; therefore, the morphology of larvae (or juveniles) does not change so much during metamorphosis to reconstruct the adult form, with the exceptional case of ascidians.

First, it is now the consensus view that a chordate ancestor(s) was free-living [12,17,18] and that cephalochordates retain characters of ancestral chordates. Second, when chordate features such as a notochord, a dorsal neural tube, myotomes, a postanal tail, pharyngeal slits and an endostyle are rethought in relation to their function, it appears that the first four are primarily associated with locomotion, while the last two pertain to the digestive system. Recent studies reveal the presence of genes relevant to formation of pharyngeal slits not only in chordates but also hemichordates [69,70]. The stomochord is an anterior outgrowth of the pharynx into the proboscis of acorn worms. In 1886, Bateson [3] proposed an evolutionary homology of this organ (the hemichord, i.e. 'half-chord') to the chordate notochord, crowning this animal group 'hemichordates'. A recent evo–devo study has demonstrated that *FoxE* is commonly expressed in the stomochord and the chordate endostyle, suggesting that the stomochord is evolutionarily related to the endostyle, rather than the notochord [71]. That is, the two digestive-system-associated structures evolved prior to divergence of chordates from non-chordate deuterostomes [17,18], although the system developed more complex functions in chordates.

Third, all four remaining features (a notochord, a dorsal neural tube, myotomes and a postanal tail) are deeply associated with the evolution of FT larvae, a new larval type that swims using a beating tail. The occurrence of FT larvae within deuterostomes (not by changing the form of dipleurula or auricularia-like larvae) is therefore critical to understanding chordate origins. It should be emphasized that all structures are formed through embryogenesis until larval formation. This suggests that an embryological comparison between non-chordate (e.g. acorn worms) and chordate deuterostomes (e.g. lancelets) might be the first step to elucidate developmental mechanisms of chordate origin, as structures corresponding to a dorsal neural tube and notochord never develop in acorn worm *embryos*. The postanal tail is likely to be associated with formation of the so-called 'tailbud', which probably functions as an organizer for tail elongation in chordate embryos [72]. Therefore, it is wiser to ask how these structures are newly formed in chordate embryos rather than to seek possible homologies with other structures of acorn worm embryos and larvae.

Embryologically, the notochord and neural tube are recognized as dorsal-midline organs that are deeply involved in the formation of chordate body plans. Viewed from the vegetal pole, the early embryo of non-chordate deuterostomes is radially symmetrical, suggesting the possibility of forming the dorsal-midline structures everywhere. However, the fact is that these structures are only formed at the dorsal side, which corresponds to the aboral side of non-chordate deuterostome embryos. That is, the A-D hypothesis speculates that the oral side is spatially limited due to formation of the mouth so that the dorsal-midline organs were allowed to form in the aboral side of ancestral chordate embryos. Thus, dorsalization of the aboral side of the ancestral

embryo may have been a key developmental event that led to formation of the basic chordate body plan. When compared with the inversion hypothesis, the A-D hypothesis emphasizes the role of dorsal-midline structure formation that superficially appears to be the D-V axis inversion.

In summary, among several scenarios on the origin and evolution of chordates, the inversion hypothesis and aboral-dorsalization hypothesis should reciprocally be refined to reach better understanding of evo–devo mechanisms underlying the evolution of the basic body plan of chordates.

5. Reclassification of chordates

On the basis of issues discussed above, we believe that the taxonomic position of chordates should be reconsidered. We propose a superphylum Chordata, composed of three phyla—Cephalochordata, Urochordata and Vertebrata (figure 1c)—as discussed below. Some modern textbooks [20,73] have similar chordate classification, but lack detailed consideration of the merits of this taxonomic ranking.

(a) Chordata as a superphylum

Using molecular phylogenetic techniques, protostomes have now been reclassified into two major, reciprocally monophyletic groups above the phylum level, the Lophotrochozoa and the Ecdysozoa [34–36]. These two are readily distinguishable by their different developmental pathways. The former is characterized by spiral cleavage, the latter by exoskeleton molting. With robust support from molecular phylogeny, the deep gap between FT (for Chordata) and dipleurula (for Ambulacraria) larval forms among deuterostomes merits a classification higher than the phylum. Thus, Lophotrochozoa (consisting of approx. 15 phyla), Ecdysozoa (approx. eight phyla), Ambulacraria (two phyla) and Chordata (three phyla) are here classified each at the superphylum level, and then the Protostomia (the first two) and the Deuterostomia (the last two) each merit infrakingdom rank. These two infrakingdoms can be united into the subkingdom Bilateria of the kingdom Animalia (figure 1c). As was emphasized in the previous section, the occurrence of FT larvae is profoundly involved in chordate origins; therefore, FT larvae may be viewed as supporting the superphylum Chordata. This is the first major reason for proposing the superphylum Chordata as well as its constituent phyla Cephalochordata, Urochordata and Vertebrata.

(b) Cephalochordata, Urochordata and Vertebrata as phyla

Metazoans are classified into approximately 34 phyla [20,74,75]. Only a few of them, however, are distinguished by a specific, diagnostic structure, such as nematocytes for Cnidaria, comb plates for Ctenophora and segmented appendages for Arthropoda. Consistent with this classification, the Urochordata and Vertebrata have their structural features, supporting their recognition as phyla. This is the second reason to support the superphylum Chordata, comprising the three phyla Cephalochordata, Urochordata and Vertebrata.

(i) Cephalochordata
Cephalochordates or lancelets comprise only approximately 35 species of small (approx. 5 cm), fish-like creatures that burrow

in sand. They are often called 'acraniates' in comparison with vertebrates ('craniates'), because their CNS consists of a neural tube with a small anterior vesicle that does not develop into the tri-partitioned brain seen in urochordate larvae and vertebrates [76–78]. Although they have no structures corresponding to well-organized eyes or a heart, as seen in vertebrates, they develop a well-organized feeding and digestive system as ciliary-mucous suspension feeders with a wheel organ, a Hatchek's pit, an endostyle and a pharynx with gill slits. In addition, vertebrate-like myotomes developed from larval somites facilitate very rapid, fish-like locomotion.

The morphological similarity of extant lancelets is striking. This may not be attributable to their recent diversification, because the divergence time of the last common ancestor of the three extant genera is estimated to be 162 million years ago (Ma) [79] and that of the genus *Asymmetron* around 100 Ma [80]. These extant forms are reminiscent of some fossils with similar body plans, including *Cathaymyrus* [81] and *Pikaia* [82] dating back to earlier than 500 Ma. Furthermore, the similarity can be explained as morphological stasis, rather than genetic piracy [83]. The presence of extensive allelic variation (3.7% single nucleotide polymorphism, plus 6.8% polymorphic insertion/deletion) revealed by the decoded genome of an individual *Branchiostoma floridae* may support this notion (electronic supplementary material, table S1) [45]. The lancelet genome appears to be basic among chordates.

Relevant to chordate origins, the mode of lancelet embryogenesis appears intermediate between that of the non-chordate deuterostome clade and the urochordate–vertebrate clade [18,77,78]. For example, soon after hatching, larvae start movement with cilia, a trait typical of non-chordate deuterostomes (but also seen in *Xenopus*). After a while, however, ciliate locomotion is replaced by twitching of the muscular tail. The lancelet notochord is formed by pouching-off from the dorsal region of the archenteron (also seen in the notochord formation in some urodele amphibians), and it displays muscular properties that are not found in other chordate groups [84,85].

Thus, cephalochordates show many features seen in extant descendants of chordate ancestors. However, morphological, physiological and genomic characteristics are unique; hence they should be recognized as a phylum.

(ii) Urochordata (Tunicata)

Urochordates comprise three classes of approximately 3000 extant species—the Ascidiacea (ascidians; sessile), the Appendicularia (larvaceans; planktonic, tadpole-like juveniles) and Thaliacea (salps; planktonic, barrel shaped). Two orders of ascidians include the Enterogona (those with unpaired gonads, including *Ciona*) and the Pleurogona (those with paired gonads, including *Styela*). They appear to have evolved as filter-feeding specialists [55]. Owing to their great variety of lifestyles, their evolutionary relationships remain controversial [14,55,86]. Recent molecular phylogeny suggests that thaliacians are included in the Enterogona clade [87]. The phylogenic position of larvaceans is still enigmatic. Some authors insist upon a basic position among urochordates while others place it within the Pleurogona clade [55,88,89].

A distinctive feature that characterizes urochordates as a phylum is that they are the only animal group that can directly synthesize cellulose, a biological function normally associated only with bacteria and plants, but not metazoans. As was noticed

in the early nineteenth century [24], the entire adult urochordate body is invested with a thick covering, the tunic (or test); hence the common name 'tunicates'. A major constituent of the tunic is tunicin, a type of cellulose (electronic supplementary material, figure S1*a,b*). The tunic may function as an outer protective structure, like a mollusc shell, and has undoubtedly influenced the evolution of various lifestyles in this group. The Early Cambrian fossil tunicates from southern China, such as *Shankouclava*, exhibit an outer morphology similar to extant ascidians, suggesting that the first ascidians, at approximately 520 Ma, also had a tunic [90].

Cellulose is synthesized by a large multimeric protein complex in the plasma membrane, called the terminal complex. Two key enzymes for cellulose biosynthesis are cellulose synthase (CesA) and cellulase. The *Ciona* genome contains a single copy of CesA (*Ci-CesA*) [91,92]. Molecular phylogeny indicates that *Ci-CesA* is included within a clade of *Streptomyces* CesA, suggesting that the bacterial *CesA* gene probably jumped horizontally into the genome of a tunicate ancestor earlier than 550 Ma. Interestingly, *Ci-CesA* encodes a protein with a CesA domain and a cellulase domain. *Ci-CesA* is expressed in larval and adult epidermis (electronic supplementary material, figure S1*c*). Its inevitable function in cellulose biosynthesis became evident from a mutant called *swimming juvenile* (*sj*), in which the enhancer element of *Ci-CesA* is a transposon-mediated mutation and thereby lacks cellulose biosynthetic activity (electronic supplementary material, figure S1*d*) [93].

Therefore, together with other features (such as pharyngeal remodification) that characterize urochordates, the ability of cellulose biosynthesis to form a distinct tunic structure supports phylum-level classification of urochordates.

(iii) Vertebrata

It is well accepted that vertebrates have distinctive features that are not found in other metazoans [8,94,95]. These include a neural crest, an endoskeleton, an adaptive immune system, a genome constitution, a placode and others (figure 2*a*) [11,99,100]. We discuss here the first four.

Neural crest. A recent view of chordate evolution, mentioned above, suggests that vertebrates evolved from a lancelet-like ancestor by developing a head and jaws, which fostered the transition from filter feeding to active predation in ancestral vertebrates. The neural crest is a key vertebrate character deeply involved in development of the head and jaws [11,101]. It is an embryonic cell population that emerges from the neural plate border. These cells migrate extensively and give rise to diverse cell lineages, including craniofacial cartilage and bone, peripheral and enteric neurons and glia, smooth muscle, and melanocytes. The gene regulatory network (GRN) underlying neural crest formation appears to be highly conserved as a vertebrate innovation (figure 2*b*) [97,102]. Border induction signals (BMP and Fgf) from ventral ectoderm and underlying mesendoderm pattern dorsal ectoderm, inducing expression of neural border specifiers (*Zic* and *Dlx*). These inductive signals then work with neural border specifiers to upregulate expression of neural crest specifiers (*SoxE, Snail* and *Twist*). Neural crest specifiers cross-regulate and activate various effector genes (*RhoB* and *Cadherins*), each of which mediates a different aspect of the neural crest phenotype, including cartilage (*Col2a*), pigment cells (*Mitf*) and peripheral neurons (*cRet*) (figure 2*b*).

It has been shown that amphioxus lacks most neural crest specifiers and the effector subcircuit controlling neural crest
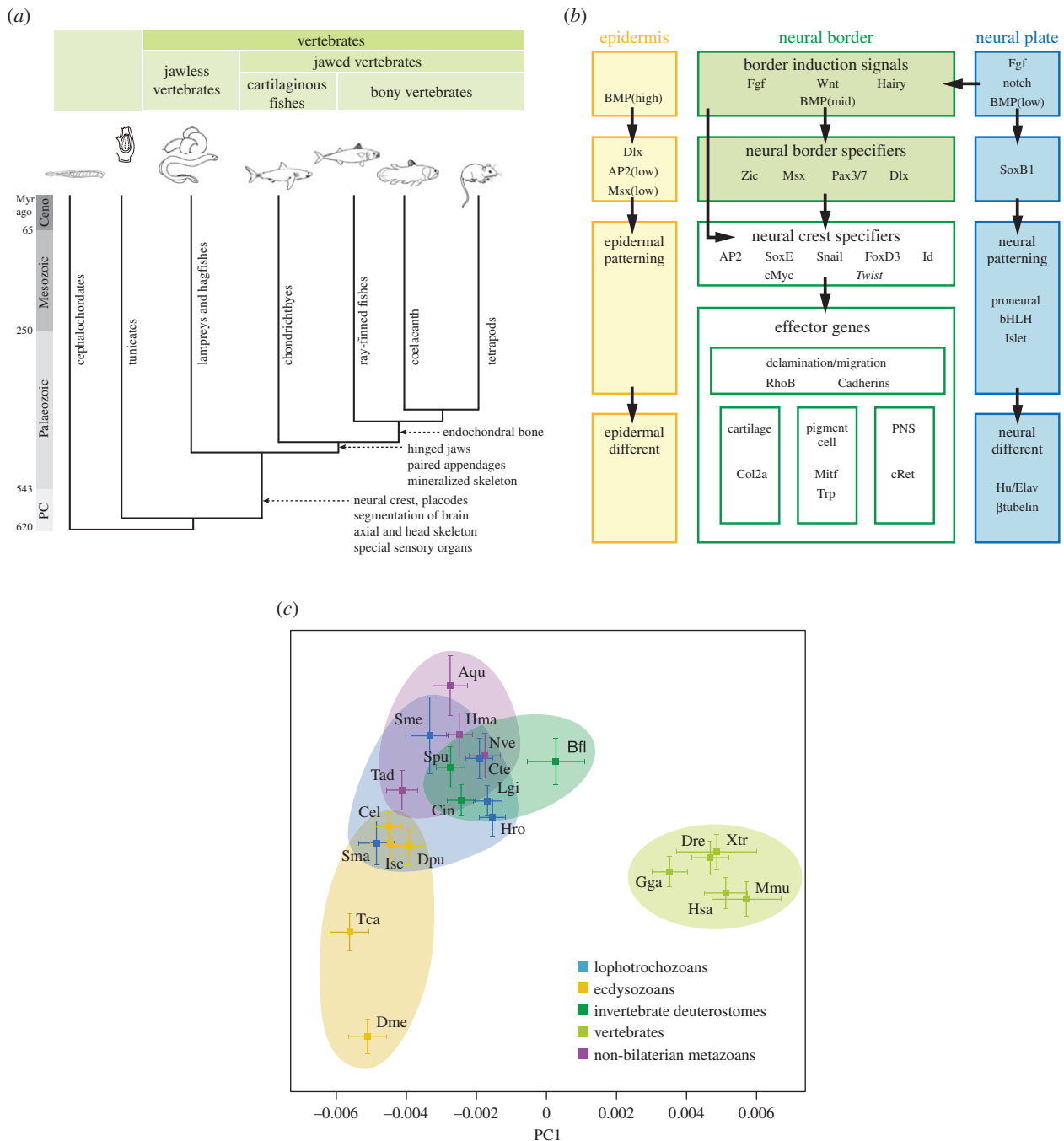
Figure 2. Features that characterize the Vertebrata as a phylum. (*a*) Major shared features of various vertebrate taxa. Lampreys and hagfishes (cyclostomes) lack mineralized tissues. By contrast, cartilaginous fishes produce extensive dermal bone, such as teeth, dermal denticle and fin spine. However, they lack the ability to make endochondral bone, which is unique to bony vertebrates (adapted from Venkatesh *et al.* [96]). (*b*) The neural crest GRN in vertebrates. Black arrows indicate empirically verified regulatory interactions. Shaded areas represent the conserved subcircuits of the respective GRNs between vertebrates and cephalochordates; the amphioxus genes are not used for the circuit of neural crest specifiers and the effector subcircuit controlling neural crest delamination and migration (adapted from Yu [97]). (*c*) Clustering of metazoan genomes in a multi-dimensional space of molecular functions. The first two principal components are displayed, accounting for 20% and 15% of variation, respectively. At least three clusters are evident, including a vertebrate cluster (red circle), a non-bilaterian metazoan, invertebrate deuterostome or spiralian cluster (green circle), and an ecdysozoan group (yellow circle). *Drosophila* and *Tribolium* (lower left) are outliers. Aqu, *Amphimedon queenslandica* (demosponge); Bfl, *Branchiostoma floridae* (amphioxus); Cel, *Caenorhabditis elegans*; Cin, *Ciona intestinalis* (sea squirt); Cte, *Capitella teleta* (polychaete); Dme, *Drosophila melanogaster*; Dpu, *Daphnia pulex* (water flea); Dre, *Danio rerio* (zebrafish); Gga, *Gallus gallus* (chicken); Hma, *Hydra magnipapillata*; Hro, *Helobdella robusta* (leech); Hsa, *Homo sapiens* (human); Isc, *Ixodes scapularis* (tick); Lgi, *Lottia gigantea* (limpet); Mmu, *Mus musculus* (mouse); Nve, *Nematostella vectensis* (sea anemone); Sma, *Schistosoma mansoni*; Sme, *Schmidtea mediterranea* (planarian); Spu, *Strongylocentrotus purpuratus* (sea urchin); Tad, *Trichoplax adhaerens* (placozoan); Tca, *Tribolium castaneum* (flour beetle); Xtr, *Xenopus tropicalis* (clawed frog) (adapted from [98]).

delamination and migration (figure 2*b*) [97,102]. Although the presence of a neural crest in ascidians has been debated [103], a recent study of *Ciona* embryos demonstrates that the neural crest melanocyte regulatory network pre-dated the divergence of tunicates and vertebrates, but the cooption of mesenchyme determinants, such as *Twist*, into neural plate ectoderm is absent (figure 2*b*) [104]. That is, the neural crest evolved as a vertebrate-specific GRN innovation.

Endoskeleton. Vertebrate cartilage and bone are used for protection, predation and endoskeletal support. As there are no similar tissues in cephalochordates and urochordates, these tissues represent a major leap in vertebrate evolution

[95]. It appears that these mineralized tissues were obtained gradually during vertebrate evolution because extant jawless vertebrates (lamprey and hugfish) have no mineralized tissues (figure 2a). The earliest mineralized tissue was found in the feeding apparatus of extinct jawless fishes, the conodonts. Cartilaginous fish produce calcified cartilage and dermal bone, including teeth, dermal denticles and fin spines, but their cartilage is not replaced with endochondral bone (figure 2a). Endochondral ossification is established by a highly complex process unique to bony vertebrates. Recent decoding of the elephant shark genome suggests that the lack of genes encoding secreted calcium-binding phosphoproteins in cartilaginous fishes explains the absence of bone in their endoskeleton [96].

Adaptive immune system. All metazoans protect themselves against pathogens using sophisticated immune systems. Immune responses of invertebrates are innate and usually stereotyped. On the other hand, vertebrates adopted an additional system or adaptive immunity using immunoglobulins, T-cell receptors and major histochompatibility complex (MHC) molecules [105]. The adaptive immune system enables more rapid and efficient response upon repeated encounters with a given pathogen. Surveys of cephalochordate and urochordate genomes failed to detect genes encoding immunoglobulins, T-cell receptors or MHC molecules, indicating that the adaptive immune system is another vertebrate innovation [106,107]. The recent discoveries of alternative antigen receptor systems in jawless vertebrates suggest that the cellular and molecular changes involved in evolution of the vertebrate adaptive immune system are more complex than previously thought [108,109].

Genome constitution. It has been revealed that a high grade of synteny is conserved between cephalochordate and vertebrate genomes [45]. The vertebrate genome has experienced both quantitative and qualitative alterations during evolution, clearly distinguishing vertebrates from invertebrates, including lancelets and tunicates. Quantitatively, it has been argued that two rounds of genome-wide gene duplication (2RGD) occurred in the lineage leading to vertebrates [110,111]. Indeed, numerous gene families, including those encoding transcription factors (Hox, ParaHox, En, Otx, Msx, Pax, Dlx, HNF3, bHLH), signalling molecules (hh, IGF, BMP) and others (dystrophin, cholinesterase, actin, keratin) were expanded by gene duplication in the vertebrate stem lineage [111]. This yielded an increase in genetic complexity, which is one of the key events underlying increased morphological complexity under developmental control. Recent decoding of the lamprey genomes suggests that duplication occurred in the early phase of vertebrate divergence [96,112] (electronic

supplementary material, table S1). However, the mechanism and exact timing of 2RGD still remain to be elucidated.

The qualitative uniqueness of vertebrate genomes becomes evident from principal component analysis of genome-wide functional diversification of genes across metazoan genomes [98] (figure 2c). This phenetic approach groups the newly sequenced mollusc and annelid genomes with those of invertebrate deuterostomes (amphioxus, sea urchins and sea squirts) and non-bilaterian metazoan phyla (cnidarians, placozoans and demosponges). Given that this grouping includes both bilaterians and non-bilaterian metazoans, cladistic logic implies that these genomes approximate the ancestral bilaterian (and metazoan) genomic repertoires. By contrast, vertebrate genomes form a distinct cluster, and are thus functionally derived relative to this ancestral bilaterian state (figure 2c). Although this analysis may be skewed by the more complete functional annotation of vertebrates, the clear separation of vertebrates from other metazoan genomes is evident.

6. Conclusion

This review discusses evolutionary relationships among deuterostomes and proposes a reclassification of chordate groups, namely with the Chordata as a superphylum together with another superphylum, the Ambulacraria of the infrakingdom Deuterostomia. The Cephalochordata, Urochordata and Vertebrata each merit phylum rank. This proposal is reasonable based on recent discoveries in this field and is also acceptable in view of historic studies of chordates.

The occurrence of FT larvae during deutrostome diversification is highly likely to have led to the origin of chordates. The hollow, dorsal neural tube and dorsal notochord are hallmarks of the chordate body plan, which is closely associated with the organizer for chordate body formation. The dorso-ventral axis inversion hypothesis and aboral-dorsalization hypothesis should be extended by examining GRNs responsible for the evolutionary origin of the organizer. The present reclassification of chordate groups provides better representation of their evolutionary relationships, which is beneficial for future studies of this long-standing question of chordate and vertebrate origins.

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