Evolutionary crossroads in developmental biology: cyclostomes (lamprey and hagfish)

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Summary

Lampreys and hagfish, which together are known as the cyclostomes or 'agnathans', are the only surviving lineages of jawless fish. They diverged early in vertebrate evolution, before the origin of the hinged jaws that are characteristic of gnathostome (jawed) vertebrates and before the evolution of paired appendages. However, they do share numerous characteristics with jawed vertebrates. Studies of cyclostome development can thus help us to understand when, and how, key aspects of the vertebrate body evolved. Here, we summarise the development of cyclostomes, highlighting the key species studied and experimental methods available. We then discuss how studies of cyclostomes have provided important insight into the evolution of fins, jaws, skeleton and neural crest.

Key words: Cyclostome, Evolution, Gnathostome, Hagfish, Lamprey

Introduction

Lampreys and hagfish are unusual animals, and you are not likely to forget them if you have seen them. Most lampreys are ectoparasites on fish, using a circular, sucker-like mouth to clamp onto their hosts. Rasping, tooth-like structures then grind into host flesh for feeding. Hagfish, by contrast, are typically deep-sea scavengers, feeding on sunken carcasses by burrowing inside via an orifice or wound. They lack clear vertebrae allowing them to tie their body in a knot, and they can produce huge quantities of slime when provoked. Together, lampreys and hagfish are usually referred to as the cyclostomes, 'agnathans' or jawless vertebrates (see Glossary, Box 1), and, as these names imply, they lack the hinged jaws characteristic of other living vertebrates. The latter are accordingly known as jawed vertebrates, or gnathostomes (see Glossary, Box 1), and several other characteristics support this separation, perhaps most notably the presence of paired fins/limbs and a mineralised skeleton in the gnathostomes and their absence in cyclostomes.

Although there is general consensus that living gnathostomes comprise a clade (Fig. 1), the precise relationship between the lamprey, hagfish and jawed vertebrate lineages has been more controversial. Three arrangements of these lineages are possible, and two have been widely championed in recent years. One places lampreys as most closely related to jawed vertebrates in the clade Vertebrata (see Glossary, Box 1), with hagfish more distantly related; all three together then comprise Craniata (see Glossary, Box 1). This phylogenetic scheme is commonly seen in text books

and is appealing because it implies a gradual assembly of vertebrate characters, and supports the hagfish and lampreys as experimental models for distinct craniate and vertebrate evolutionary grades (i.e. perceived 'stages' in evolution). However, only comparative morphology provides support for this phylogenetic hypothesis. The competing hypothesis, which unites lampreys and hagfish as sister taxa in the clade Cyclostomata, thus equally related to gnathostomes, has enjoyed unequivocal support from phylogenetic analyses of protein-coding sequence data (e.g. Delarbre et al., 2002; Furlong and Holland, 2002; Kuraku et al., 1999). Support for cyclostome theory is now overwhelming, with the recognition of novel families of non-coding microRNAs that are shared exclusively by hagfish and lampreys (Heimberg et al., 2010). Perhaps most significantly, contradictory morphological evidence has eroded, particularly through new insights into hagfish development, which have demonstrated that they have lost, not primitively lacked, many of the characteristics [e.g. vertebrae (Ota et al., 2011)] used previously to diagnose a lamprey-gnathostome clade. A monophyletic cyclostome clade thus provides two experimental models that, in contrast with gnathostomes, can reveal the nature of the ancestral vertebrate. However, this clade implies a phenotypically complex ancestral vertebrate, broadening the gulf in bodyplan organisation distinguishing vertebrates from their spineless invertebrate relatives, and making the challenge of explaining its origin in developmental evolution all the more formidable.

The immediate invertebrate relatives of vertebrates are also chordates: the Urochordata (ascidians and other tunicates; see Glossary, Box 1) and the Cephalochordata (amphioxus and allies; see Glossary, Box 1), both of which have been the subject of other articles in this Evolutionary crossroads in development biology series (Bertrand and Escriva, 2011; Lemaire, 2011). Phylogenomic (see Glossary, Box 1) data convincingly place the Urochordata, not Cephalochordata, as more closely related to Vertebrata (Delsuc et al., 2006). Hence, from the perspective of living taxa, a whole suite of characteristics unite Vertebrata, including an axial skeleton, brain complexity, cranial sensory complexity, neural crest cells (see Glossary, Box 1) and their many derivatives, and perhaps also a predatory (as opposed to a filter feeding) lifestyle. This can give the appearance of a step-wise change in complexity at this point in evolutionary history. However, the vertebrates also have a rich fossil record (Fig. 1) with many well-defined lineages, allowing the point at which certain characteristics appeared to be understood relatively well. Their study instead points to a more gradual acquisition of vertebrate complexity. This in turn has important implications for interpreting molecular and developmental studies of cyclostomes, as we discuss below.

A final characteristic that unites gnathostomes is genome duplication: all living gnathostomes appear to have evolved from an ancestral lineage that experienced two rounds of whole genome duplication, commonly known as '1R' and '2R' (see Glossary, Box 1), and this explains the relative complexity and redundancy of

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Box 1. Glossary

1R/2R. Shorthand for the whole genome duplication events that occurred early in vertebrate evolution.

Agnathan (Agnatha). Literally meaning 'lacking jaws' this refers to the jawless fish including lampreys and hagfish. It is an evolutionary grade, not a clade, although it is sometimes inappropriately used as a synonym of 'cyclostomes'.

Cephalochordata. A clade of invertebrate chordates commonly known as the 'lancelet' or 'amphioxus'. For more information, see Bertrand and Escriva (Bertrand and Escriva, 2011).

Conserved noncoding elements (CNEs). Regions of a genome that show atypically high levels of sequence similarity, implying selective constraint, and that do not encode protein.

Craniate (Craniata). The chordate clade, named after the cranium or skeletal braincase, that includes hagfishes, lampreys and gnathostomes. Assuming cyclostome monophyly, it is equivalent to the vertebrate clade and is, therefore, defunct.

Crown group. A clade circumscribed by its living members and their last common ancestor, including all the extinct species that also share this same common ancestor.

Cyclostome (Cyclostomata). Literally meaning 'circular mouth', this refers to the clade of jawless hagfishes and lampreys, but not the extinct skeletonised jawless vertebrates that are more related to gnathostomes.

Gnathostome (Gnathostomata). The clade encompassing living jawed vertebrates. Importantly, it might not include all jawed vertebrates because the extinct placoderms are considered by some to be a sister clade to Gnathostomata.

Neural crest. A population of cells that emerge from the neural tube and migrate to the periphery where they differentiate into one or more of a characteristic suite of cell types, including neurons, glia, pigment cells, osteocytes, chondrocytes, odontocytes and connective tissue.

Osteostracans. An extinct clade of jawless fishes, more closely related to jawed vertebrates than any other such clade.

Pharyngeal. Pertaining to the pharynx, which is a chamber at the anterior end of the digestive tract.

Phylogenomic. Assessment of phylogenetic relationships using large quantities of sequence data.

Placoderms. An extinct clade or grade of jawed vertebrates, considered either to be the sister lineage of living jawed vertebrates or to include the members of the ancestral lineages of living jawed vertebrates plus chondrichthyans and osteichthyans.

Somites. Embryonic paired segmental masses of mesoderm that occur lateral to the neural tube and that differentiate into sclerotome (forming vertebrae), dermatome (dermis) and myotome (skeletal muscle).

Stem group/lineage. Extinct members of an extant evolutionary lineage that fall outside the crown group to which they are more closely related.

Stem-gnathostomes. Extinct vertebrates more closely related to living jawed vertebrates than to living cyclostomes.

Urochordata. A clade of invertebrate chordates commonly known as the 'tunicates' or 'sea squirts'. For more information, see Lemaire (Lemaire, 2011).

Vertebrata. The chordate clade comprising cyclostomes, gnathostomes, their last common ancestor and all of its descendents, living and extinct.

has it that 2R occurred within the gnathostome stem lineage (see Glossary, Box 1) after cyclostome divergence; however, molecular phylogenetic analyses of individual gene families are often unclear on this (Kuraku et al., 2009). Thus, it remains possible that both 1R and 2R occurred in the vertebrate stem lineage, or that extra cyclostome genes are derived from independent duplications, or a mixture of shared and independent duplications.

As we discuss below, despite recent advances neither lampreys nor hagfish are easy systems to work with compared with model vertebrates. Their attraction stems from where they branch in animal phylogeny. As the only surviving lineages from a once diverse and disparate evolutionary grade of jawless fishes, they provide an experimental window into the developmental biology and genomic constitution of the ancestral vertebrate. It is important to note, however, that neither lampreys nor hagfish can be taken as literal proxies for the ancestral vertebrate: both lineages have acquired characteristics specific to cyclostomes, and both have transformed or lost ancestral vertebrate characters. However, in comparison to vertebrate outgroups, such as the urochordates, and ingroups, such as sharks and bony fishes, lampreys and hagfish can provide insights into the molecular and genomic changes that underlie the assembly and subsequent evolution of the vertebrate and gnathostome bodyplans.

Model species and life cycles

Neither lampreys nor hagfish are speciose taxa, with 38 and 60 species defined, respectively (Hardisty, 2006). Both have habitats or life cycles that render them relatively difficult model systems for developmental biology, though notable progress has been made on this front in recent years. The stages in typical lamprey and hagfish life cycles are shown in Fig. 2. Two lamprey species, *Petromyzon marinus* and *Lethenteron japonicum*, account for the majority of published lamprey studies, although other species such as *Lampetra fluviatilis* and *Lampetra planeri* are also studied. Adult lampreys are usually ectoparasites and many are marine. However, when ready to breed they enter river systems and swim upriver, spawning on the gravel bottom of relatively fast-flowing river sections. Here, they clear stones using their sucker-like mouth, creating a small depression into which eggs are deposited and males add sperm. The embryos develop in the gravel, initially in a chorion, before hatching and continuing to develop until they reach a feeding larval stage known as an ammocoete, which is similar to the adult but lacks the characteristic feeding apparatus and some other structures. The ammocoete buries itself in mud on the river bottom and lives as a filter feeder for several years before undergoing metamorphosis into the adult. Adults usually migrate to the sea, although in some species, such as *L. planeri*, they may proceed directly to reproduction without further feeding, and notably in the Great Lakes in North America the invasive population of *P. marinus* spends its adult phase in the fresh water of the Lakes.

To our knowledge, lampreys have never been taken through a complete life cycle in captivity, and developmental studies are based on wild-caught specimens. Fertilised eggs can be collected from spawning sites and easily cultured. Gravid adults can also be collected and held for some time in cool fresh water, before strip-spawning and in vitro fertilisation. For a methodological description, see Nikitina et al. (Nikitina et al., 2009). In vitro development has allowed the establishment of staging series, including one for *P. marinus* (Piavis, 1971) and *Lampetra reissneri* (Tahara, 1988), although as there are few differences between species, these stagings are often used for other lamprey species.

many developmental gene families in vertebrate model species compared with invertebrate models, such as *Drosophila*. The 1R ploidy event demonstrably post-dated the divergence of the vertebrate, amphioxus and urochordate lineages (Dehal and Boore, 2005), but the timing of the 2R ploidy event with respect to cyclostome-gnathostome divergence is less clear. Received wisdom
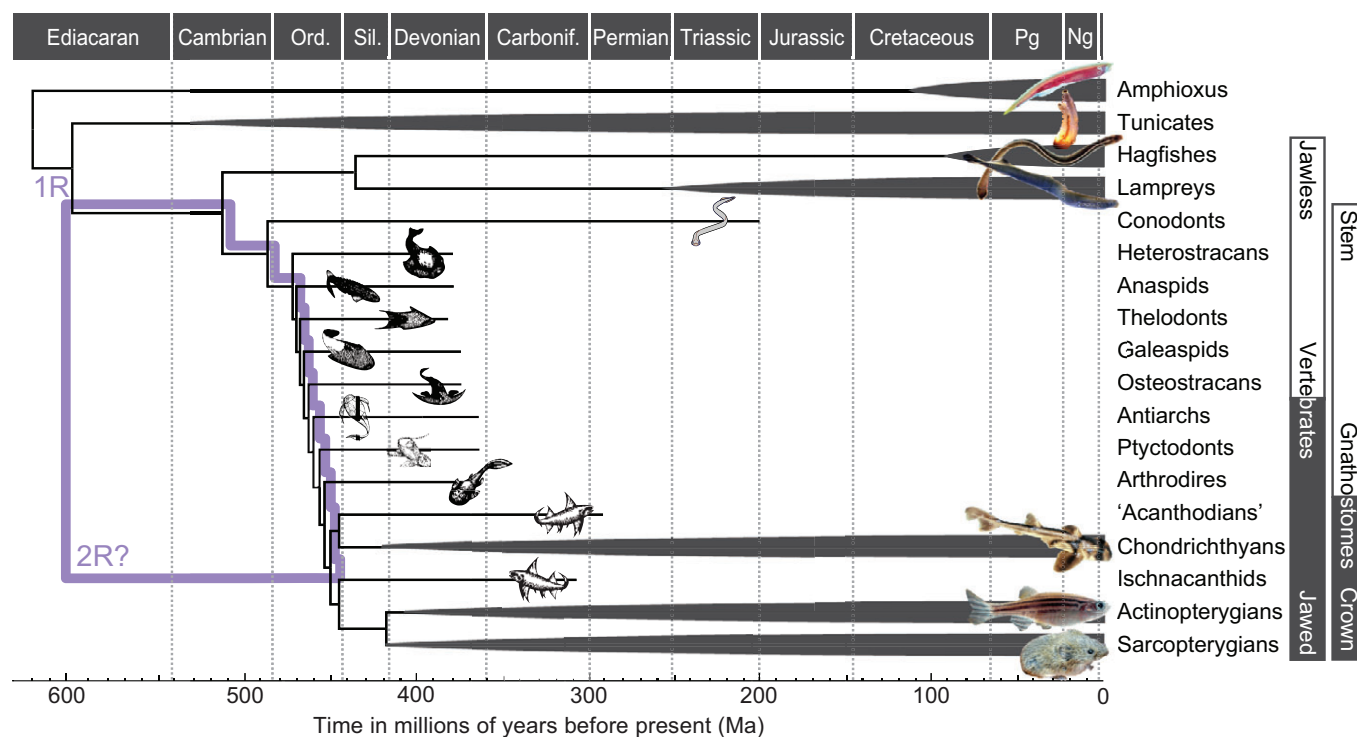
Fig. 1. Phylogeny of living and a representative selection of extinct chordates within the context of geological time. The 1R whole genome duplication event occurred in the vertebrate stem lineage, but it is unclear whether the 2R event occurred in the vertebrate or gnathostome stem lineages. Relationships are based on Delsuc et al. (Delsuc et al., 2006), Donoghue et al. (Donoghue et al., 2000) and Brazeau (Brazeau, 2009). The spindles (dark grey) on the living lineages reflect the timing of origin of their respective crown groups (see Glossary, Box 1): amphioxus (Nohara et al., 2004); tunicates (Swalla and Smith, 2008); hagfishes and lampreys (Kuraku and Kuratani, 2006); chondrichthyans (Inoue et al., 2010); actinopterygians (Inoue et al., 2003). Geological timescale from Ogg et al. (Ogg et al., 2008). Ord., Ordovician; Sil., Silurian; Carbonif., Carboniferous; Pg, Paleogene; Ng, Neogene.

Lampreys have proven to be tricky developmental models, but hagfish are harder still. Finding eggs in slime is relatively common, although they are invariably unfertilised; where and how hagfish fertilise then lay their eggs in the wild is unknown (Gorbman, 1997). The only sizeable collections of hagfish embryos are those of the Pacific hagfish *Eptatretus stoutii* which were made in the late 19th and early 20th centuries (Conel, 1931; Dean, 1898; Doflein, 1898; Price, 1896). The eggs were recovered by fishermen in Monterey Bay, California, captured in the slime extruded by the animals. The Atlantic hagfish *Myxine glutinosa* is known from studies of only three embryos (Fernholm, 1969; Holmgren, 1946). In all instances, these early studies of hagfish embryology were limited to morphology and classical histology.

Recently, however, research groups in Japan and Taiwan have succeeded in getting captive hagfish of the species *Eptatretus burgeri* to produce fertilised eggs in aquaria (Ota et al., 2007). This has allowed the application of molecular developmental methods for the first time to hagfish and promises to re-invigorate research in this area (Ota and Kuratani, 2007; Ota and Kuratani, 2008).

Techniques for investigating lamprey and hagfish development

The methods used to investigate lamprey and hagfish development are summarised in Table 1. For hagfish, these methods are currently limited to descriptive studies of gene expression. The study of lampreys is more advanced and includes numerous gene expression studies on a range of species and several methods of embryo manipulation. Perhaps the most

effective of these has been the injection of antisense morpholino oligonucleotides for gene knockdown in *P. marinus* (McCauley and Bronner-Fraser, 2006), which has been used for the detailed dissection of the neural crest gene regulatory network (GRN), as discussed in detail below. Despite these advances, however, the long life cycle and difficult culture of these species means that genetic and germ line transgenesis methods are unlikely to be successful.

Key findings and impact on the field

The study of lamprey and hagfish development is motivated principally by the phylogenetic position of these organisms; they provide a window into understanding the developmental processes present in early vertebrates and, hence, a key to understanding what has changed during the evolution of novel structures, such as fins and jaws. This is not to say that either lampreys or hagfish are directly representative of the common ancestor; both lineages have their own suites of specialisations and indeed the living cyclostome and gnathostome lineages have been diverging for exactly the same length of time. However, the cyclostomes are an outgroup to the gnathostomes. Outgroups are fundamentally important for phylogenetic inference in comparative biology and, in this case, comparison between cyclostomes and gnathostomes can reveal what is shared between them, and hence ancestral, and therefore also what is derived. Below, we review a selection of recent advances in this vein. We also discuss recent advances in our understanding of the genome-level similarities and differences between cyclostome and gnathostome development.
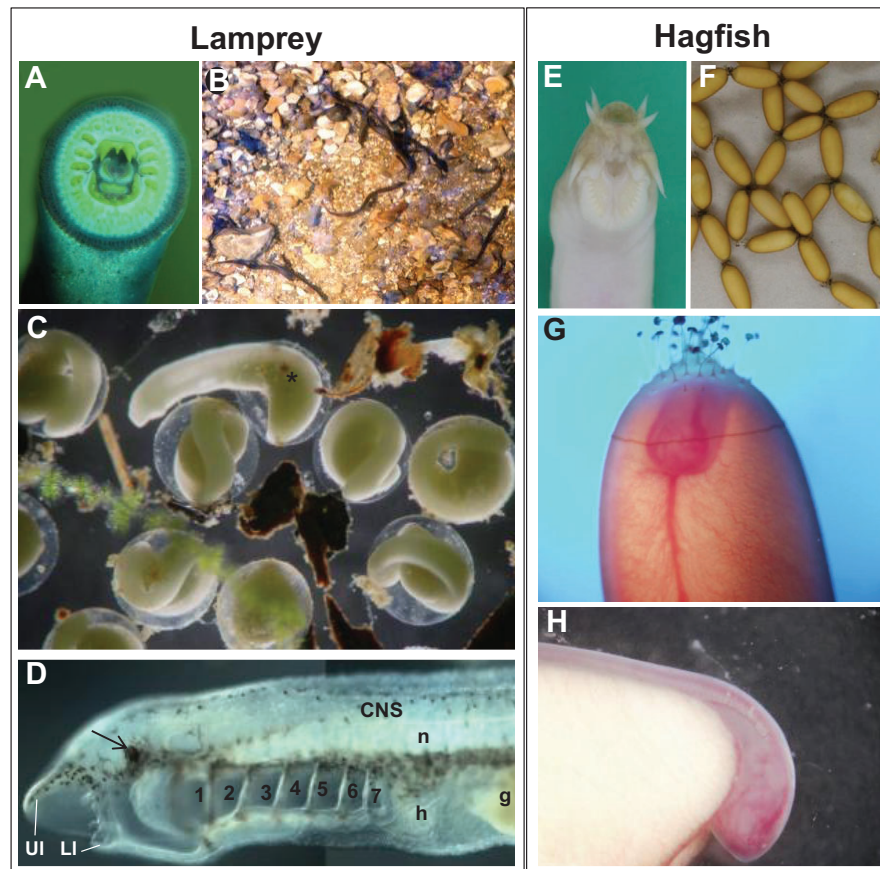
DEVELOPMENT

Fig. 2. Lamprey and hagfish adults and embryos. (**A**) Ventral view of the head of an adult lamprey, *Petromyzon marinus*. Note the circular mouth and arrangement of tooth-like structures. (**B**) Spawning behaviour in the brook lamprey *Lampetra planeri* in a stream in southern England. The adults clear a small area of gravel, creating a pit-like nest into which the eggs are laid. (**C**) Developing embryos of *L. planeri* collected from a nest. At this stage, equivalent to about stage 24-25 of Tahara (Tahara, 1988), the embryo head has extended away from the yolk. Most embryos are still within their chorion; however, the one marked with an asterisk is in the process of hatching. (**D**) Very early ammocoete larva of *L. planeri* in lateral view with anterior to the left. The pigmented eye is visible (arrow), as are numerous pigmented melanocytes (brown). The upper lip (Ul) and lower lip (Ll) are marked, and posterior to these come the seven pharyngeal slits (numbered), the heart (h) and the gut (g), which contains the remaining yolk. A prominent notochord (n) serves as an axial skeleton, above which is the CNS. (**E**) Ventral view of the head of an adult hagfish, *Eptatretus burgeri*, showing the circular mouth and anterior tentacles. (**F**) Eggs of *E. burgeri*. Note the small terminal hooks, which act to connect eggs into strings. (**G**) Close up of an *E. burgeri* fertilised egg showing the developing embryo and associated vasculature, plus the terminal hooks. (**H**) Head of the developing embryo of *E. burgeri*, with anterior to the right. Note the very large quantity of yolk. For more details on hagfish embryology and anatomy the reader is referred to a recent review on the subject (Ota and Kuratani, 2008). The image in A was reproduced with kind permission from Lyman Thorsteinson, courtesy of USGS (United States Geological Survey); C was reproduced with kind permission from Ricardo Lara-Ramirez; E-H were reproduced with kind permission from Kinya G. Ota.

The evolution of paired appendages

All living gnathostomes (excluding lineages in which secondary loss has occurred, such as snakes and eels) have paired pelvic and pectoral appendages. These form fins in cartilaginous and bony fish, and are modified into limbs in tetrapods. Both hagfish and lampreys lack paired appendages and so it is clear that these structures evolved in the gnathostome lineage after it separated from cyclostomes. Paired bodywall outgrowths were widespread among extinct stem-gnathostomes (see Glossary, Box 1) (Wilson et al., 2007), but unequivocal homologues are first manifested in the pectoral position in the jawless osteostracans (see Glossary, Box 1). Pelvic appendages are first encountered in the earliest jawed vertebrates, the placoderms (see Glossary, Box 1) (Young, 2010).

Appendage development has been studied intensively in model vertebrates for many years and, more recently, some authors have begun to explore the molecular control of appendage development in cartilaginous fish (Dahn et al., 2007; Freitas et al., 2006).

Comparisons of model vertebrates and cartilaginous fish have helped to reveal the likely ground plan for gnathostome appendage development, which includes a role for Hox genes, retinoic acid (RA), Hedgehog (Hh) and fibroblast growth factor (FGF) signalling, T-box (Tbx) genes, and a mode of muscularisation (Freitas et al., 2006; Gillis et al., 2009; Neyt et al., 2000).

As cyclostomes lack paired appendages, how can they be studied experimentally to understand appendage evolution? Freitas et al. (Freitas et al., 2006) took the approach of trying to define the origin of the molecular circuitry controlling the development of paired appendages. They showed that features of paired and median fin development, including Hox and Tbx expression, are shared in the catshark. Lampreys also develop a continuous medial fin, and Freitas et al. (Freitas et al., 2006) showed that the development of this fin was also marked by Hox and Tbx gene expression. This suggests that aspects of paired appendage developmental control were co-opted from the medial fin, which is a primitive feature of chordates. Other

Table 1. Summary of experimental methods applied to lamprey and hagfish embryos

Method	Species	Summary	Selected references
mRNA visualisation	Lampreys: *P. marinus* *L. japonica* *L. fluviatillis* *L. planeri* Hagfish: *E. burgeri*	Standard whole-mount in situ hybridisation techniques work well, though can be difficult on early developmental stages. This method has also been adapted for gene expression in the adult lamprey spinal cord	(Boorman and Shimeld, 2002; Derobert et al., 2002; Murakami et al., 2001; Neidert et al., 2001; Ogasawara et al., 2000; Ota et al., 2007; Swain et al., 1994)
microRNA visualisation	Lamprey: *P. marinus* Hagfish: *Myxine glutinosa*	Analysis via locked nucleic acid probes as for microRNA visualisation in other species	(Pierce et al., 2008)
Experimental embryology	*P. marinus* *L. japonica*	Methods reported include lineage tracing via injection of markers dyes, and ablation experiments	(Langille and Hall, 1988; McCauley and Bronner-Fraser, 2006; Shigetani et al., 2002)
Gene knockdown	*P. marinus*	Morpholino oligonucleotide methods are well described and very effective at early developmental stages	(McCauley and Bronner-Fraser, 2006; Sauka-Spengler et al., 2007)
Transgenesis	*L. japonica*	Expression of plasmid-based reporter genes used to express GFP from a strong promoter (CMV) and gene-specific promoters	(Kusakabe et al., 2003)
Pharmacological methods	*L. japonica*	Use of cyclopamine to inhibit Hh signalling and SU5402 to inhibit FGF signalling. Retinoic acid has also been applied	(Murakami et al., 2004; Sugahara et al., 2011)

aspects of paired appendage development in gnathostomes have not been identified in lamprey median fin development, including Hh signalling and a role for RA. However, Hh and RA signalling occurs in gnathostome gill arches (Gillis et al., 2009), structures also found in cyclostomes. The development of gill arches has not been well studied in cyclostomes, but it is known that the lamprey pharynx is affected by RA exposure (Kuratani et al., 1998) and that expression of the Hh receptor *Patched* indicates that Hh-mediated developmental regulation of these gill arches is likely (Hammond et al., 2009). Overall, this suggests that the evolution of paired appendages occurred via co-option of the molecular circuitry regulating primitive chordate structures, prior to the divergence of cyclostomes and gnathostomes (Fig. 3A). Dissection of the regulatory interactions between key genes in cyclostome development would clarify this further.

The origin of articulated jaws

Articulated jaws are a diagnostic characteristic of gnathostomes and are often postulated to have allowed a more active predatory life history (Gans and Northcutt, 1983). It has also been considered that the evolution of jaws allowed jawed vertebrates to outcompete jawless fish, leaving just the relict lineages of lampreys and hagfish, although the timing of jawless fish lineage extinction shows that, in reality, their extinction was much more complex than this (Purnell, 2001).

Nearly a decade ago, a number of studies (e.g. Horigome et al., 1999; Kuratani et al., 1999; Neidert et al., 2001; Shigetani et al., 2002) started to address jaw evolution at the level of gene expression. These studies revealed a high level of similarity between lamprey and gnathostome pharyngeal (see Glossary, Box 1) patterning in terms of the expression of transcription factor genes, such as those of the distal-less (Dlx), muscle segment homeobox (Msx) and Hand families (Fig. 3B), and the position and

function of signalling pathways. These data, coupled with analyses of neural crest cell migration, led Shigetani et al. (Shigetani et al., 2002) to propose that changes in the interaction between neural crest populations and pharyngeal tissues might underlie jaw evolution. More recently, these studies have been extended and, although still confirming much similarity in gene expression, have revealed what might be a key difference between lampreys and gnathostomes. The lamprey first arch lacks the focal expression of the homebox-containing transcription factor *Bapx1* (*Nkx3.2*) and the Transforming growth factor (TGF)-β signalling molecule *Gdf5/6/7* that, in gnathostomes, pattern the jaw joint (Cerny et al., 2010; Kuraku et al., 2010). These results suggest a model (Fig. 3B) in which the extensive existing pattern in the first arch of the vertebrate ancestor has been subtly adapted in the gnathostome lineage by acquisition of a focused patterning system specifying a joint, a requirement for hinged jaws. How this might have occurred is unknown and other explanations remain possible.

Somites and skeletons

The somites (see Glossary, Box 1) of jawed vertebrates are patterned into compartments that give rise to distinct tissues: the sclerotome, dermatome and myotome, which form axial skeleton, dermis and muscle, respectively. In model vertebrates, the mechanisms controlling this are quite well understood and involve initial signalling from the notochord, neural tube and more lateral mesoderm to subdivide the somite (Bothe et al., 2007). Lamprey somites appear to be essentially the same as this, and a small sclerotome forms the axial cartilaginous nodules that are homologous to vertebrae (Tretjakoff, 1926). Hagfish have been considered historically to lack similar structures. Recently, however, it has become clear that they do develop a sclerotome compartment that gives rise to small axial cartilaginous elements (Ota et al., 2011).
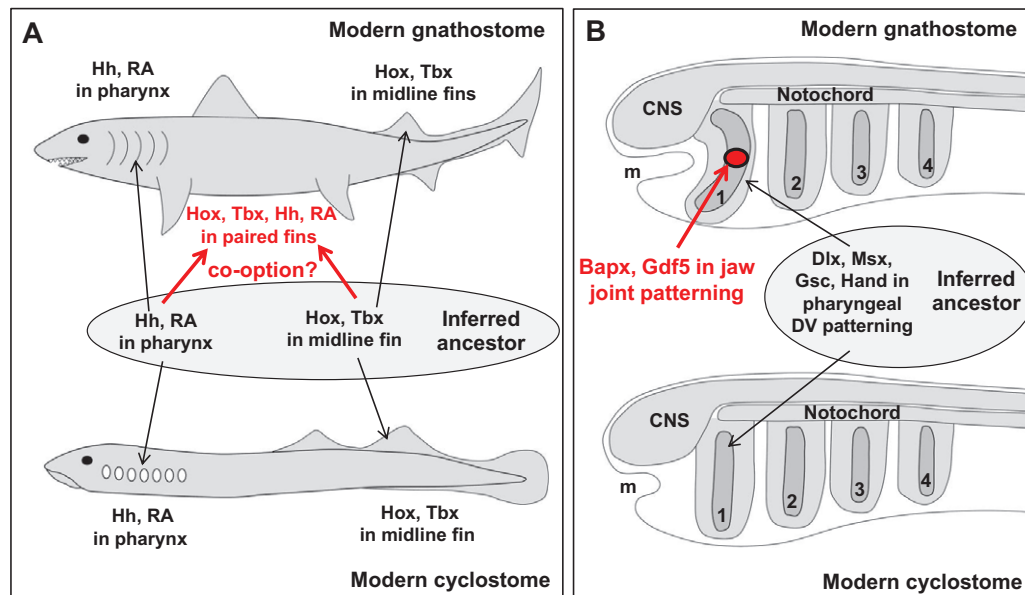
Fig. 3. Models of vertebrate jaw and paired fin evolution. (**A**) Modern gnathostomes (top, represented by a shark) and cyclostomes (bottom, represented by a lamprey) both use Hedgehog (Hh) and retinoic acid (RA) in patterning the pharynx, and Hox and Tbx in patterning midline fins. This allows us to infer that both were present in the common ancestor (centre). A hypothesis for paired fin evolution in the jawed vertebrates is shown in red, and involves co-option of these patterning mechanisms to new sites of fin development on the flank. Many other changes would also have been required, including the evolution of muscularisation and associated innervation. (**B**) Modern gnathostomes and cyclostomes [both represented by stylised diagrams, with the anterior and mouth (m) to the left] use similar suites of genes to pattern the dorsoventral (DV) axis of the pharyngeal arches (numbered), including Dlx, Msx, Gsc and Hand genes (Cerny et al., 2010; Kuraku et al., 2010). A difference, shown in red, is the use of *Bapx* and *Gdf5/6/7* by gnathostomes to pattern a joint in the skeletal elements of the first arch. This is hypothesised to have been required for forming hinged jaws.

Gnathostomes also develop an extensive dermal skeleton, which is usually mineralised and is derived primarily from neural crest cells (Donoghue et al., 2008). This is generally confined to the head in amniotes; however, non-tetrapod vertebrates and, in particular, fossil data show that extensive dermal armour evolved early in the gnathostome lineage after its separation from cyclostomes. It is also clear from the paleontological record that the dermal mineralised skeleton significantly pre-dates the origin of a mineralised vertebral skeleton, which is first encountered in the earliest jawed vertebrates (Donoghue and Sansom, 2002).

How and when did skeletal tissues and their mineralisation evolve? As lampreys and hagfish have cranial neural crest-derived and axial sclerotome-derived skeletons, both these pre-date the radiation of living vertebrates. Studies in lampreys, hagfish and amphioxus have suggested that cartilage in all three taxa is molecularly similar to that of jawed vertebrates, both with respect to the proteins forming the cartilaginous matrix itself and to the transcription factor genes that mark their differentiation, such as the Runt-related (*Runx*) and Sry-related (*SoxD* and *SoxE*) transcription factors (Cattell et al., 2011; Hecht et al., 2008; Kaneto and Wada, 2011; McCauley and Bronner-Fraser, 2006; Ohtani et al., 2008; Wada, 2010; Zhang and Cohn, 2006; Zhang et al., 2006). However, a recent detailed study of lamprey cartilage has suggested that such generalisations should be treated with caution: lampreys are known to have several structurally distinct cartilages, and Cattell et al. (Cattell et al., 2011) found that these cartilage types display considerable diversity in cartilage gene expression. Based on this, they proposed a complex multistep model for the evolution of cartilage diversity in vertebrates. Testing this model will require deconstruction of regulatory interactions in at least jawed vertebrates and lampreys, and possibly also in amphioxus.

The development of the neural crest

The neural crest is an enigmatic tissue, attracting attention from evolutionary biologists because of its often-hypothesised specificity to vertebrates and significant contribution to the cranial complexity that separates vertebrates from other animals. Lampreys have a neural crest that is very similar to that of gnathostomes, including an ability to differentiate into a wide range of tissues, and hagfish, although less well studied, are likely to be similar (Ota et al., 2007). There are also data suggesting that neural crest might pre-date vertebrate origins (Donoghue et al., 2008) as some urochordates possess migratory cells with similar properties (Jeffery et al., 2004).

Recent studies have used gene knockdown strategies to dissect carefully the gene regulatory interactions controlling lamprey neural crest (Nikitina et al., 2008; Sauka-Spengler et al., 2007). Such experiments can be hard to interpret in gnathostome models, in which developmental speed could mask direct and indirect interactions. However, in lampreys, experiments of this nature can be conducted with high fidelity, as the very slow pace of development allows detailed dissection of both the relative timing of normal gene expression and the impact of gene knockdown on putative target genes. The combined data from these experiments has allowed the construction of a detailed GRN model of lamprey neural crest development, defining genes at different tiers in the progression from dorsoventral epidermal patterning and neural plate specification to the cell biology of neural crest migration (Sauka-Spengler and Bronner-Fraser, 2008; Sauka-Spengler et al., 2007).

In turn, this has allowed the inference of key differences between vertebrates and invertebrate chordates, particularly amphioxus (Yu et al., 2008). Some aspects of development, including neural dorsoventral patterning, appear to be conserved between these

groups. However, a key difference is the absence of expression in amphioxus of some genes involved in specifying the neural-epidermal border region (where the crest will arise) and of genes responsible for delamination of crest cells and their subsequent migration (Yu et al., 2008). These data suggest that the neural crest evolved by adding a new regulatory tier under pre-existing mechanisms for dorsoventral neural patterning.

Genome-level insights into vertebrate development and evolution

Many genes classically considered to be 'developmental', such as those encoding transcription factors and proteins involved in cell signalling, are duplicated in jawed vertebrates, in comparison to amphioxus and urochordates. These so-called 'transdev' genes are often multi-copy in lampreys too. As discussed above, the additional paralogues in jawed vertebrates are usually derived from whole genome duplications, and a simple explanation for this is that cyclostomes and jawed vertebrates share these genome duplications. Molecular phylogenetics have, however, been unclear on this issue, often showing different topologies for different gene families when shared ancestry of genome duplication would predict shared topologies (Kuraku et al., 2009). A possible explanation for this is that genome duplication occurred shortly before the separation of the cyclostome and jawed vertebrate lineages, as this could allow insufficient time for resolution of paralagous genes and, hence, confused molecular phylogenies. A good cyclostome genome assembly would probably resolve this. Alternatively, incongruous topologies among gene families might reflect the dramatic editing that occurs in the genome of somatic cell lineages in lampreys and, perhaps, hagfishes (Smith et al., 2009). This is a potential problem because the lamprey genome sequencing project is based on somatic cells.

Various authors have also linked gene and genome duplication to the evolution of morphological complexity, with the underlying assumption that duplicate genes provide extra genetic material that is freed from purifying selection by redundancy and is, hence, free to evolve new functions that guide evolutionary innovation. There are a few good examples of this, including the functional diversification of globin proteins, which emerged as paralogues from 1R and 2R whole genome duplication events in early vertebrate evolution, to perform specialised roles in oxidative metabolism (Hoffmann et al., 2011). However, many of these examples describe neofunctionalisation or instances of complementary degeneracy within derived vertebrate clades. There is little evidence of whole genome duplications having effected vertebrate, or indeed, gnathostome, innovations. The gradual accumulation of vertebrate and gnathostome characteristics over considerable evolutionary time, evidenced by the fossil record (Donoghue and Purnell, 2005), coupled with the frequent re-use of existing genetic circuitry in vertebrate evolution discussed above, argue against such an interpretation. It is likely that the evolutionary consequences of whole genome duplication are more intricate and are realised over a more prolonged period than has been suggested.

A better case can perhaps be made for the role of the non-coding trans-acting regulatory microRNAs in early vertebrate evolution. A combination of genome resources and small RNA library sequencing has revealed a fundamental episode of microRNA innovation in the lineage leading to vertebrates after its separation from the tunicate lineage. The rate of innovation of novel microRNA families is higher in this interval of vertebrate evolution than in any other interval of animal evolution. This phenomenon

pre-dated, and is not a consequence of, whole genome duplication, as lampreys possess multiple paralogues within these microRNA families (Heimberg et al., 2008). Furthermore, as lampreys share duplicated microRNAs with mouse and human, this evidence implies that both the 1R and 2R whole genome duplication events occurred before living vertebrates diverged. Deep sequencing of organ-specific small RNA libraries from lamprey has revealed that vertebrate-specific microRNAs are expressed in vertebrate innovations and elaborations. As lampreys exhibit expression profiles in these organs that are comparable to both fish and mouse, it appears that these were established in the last common ancestor of vertebrates and were subsequently conserved (Heimberg et al., 2010). However, analysis of the role of microRNAs in cyclostome development has not yet extended beyond expression analyses using RNA probes (e.g. Pierce et al., 2008).

The development of numerous vertebrate genome sequences has also allowed the evolution of other non-coding sequences to be assessed. Jawed vertebrate transdev genes are associated with an exceptional density of conserved non-coding elements (CNEs; see Glossary, Box 1), which, where studied, are usually regulatory (Woolfe et al., 2005). Extension of these studies to lamprey and amphioxus data reveals a surprising pattern. Although the drop off in the number and length of CNEs observed with increasing phylogenetic distance is to be expected, the degree of change is not. Lampreys have a reasonable number of conserved sequences, at least as far as has been assessed to date (McEwen et al., 2009). However, amphioxus shares only a handful of such sequences with vertebrates (Putnam et al., 2008). One interpretation of these data is that early vertebrate evolution saw considerable flexibility in gene regulatory interactions, followed by a 'locking-in' effect, such that many regulatory sequences came under purifying selection, fixing them as the CNEs we observe when comparing the genomes of living gnathostomes.

Evolution of the vertebrate adaptive immune system(s)

As recently as 2009, the immune system was marshalled as evidence for the ultimately fallacious hypothesis that lampreys are closer relatives of gnathostomes than are hagfish (Nicholls, 2009). Hagfish have long been considered to lack lymphocytes and the adaptive immune system shared by lampreys and gnathostomes. However, over the past decade it has emerged gradually that the adaptive immune system of lampreys is distinct from the immunoglobulin, T cell receptor (TCR)-, antibody- and recombination activating gene (RAG)-based system of gnathostomes, and instead it appears to be based on variable lymphocyte receptors (VLRs) (Pancer et al., 2004). Furthermore, hagfish share this VLR-based system all the way down to the VLR-encoding paralogues (Pancer et al., 2005; Rogozin et al., 2007) that, in lampreys, exhibit expression patterns specific to distinct cell lineages that might be equivalent to gnathostome T and B cells (Guo et al., 2009). VLRs, like TCRs, are assembled by somatic rearrangement of distinct gene segments, notably the leucine-rich repeat regions that bestow the physical diversity of the encoded proteins. However, this is achieved not by RAG, which is absent in cyclostomes, but, at least in part, by VLR-specific cytosine deaminases (CDAs) (Rogozin et al., 2007) at sites specific to the T-like and B-like lymphocytes (Bajoghli et al., 2011). VLRB- and CDA2-expressing B-like lymphocytes occur and, therefore, appear to develop in the typhlosole and kidneys. Lampreys have long been considered to lack a thymus, the site of T lymphocyte development in gnathostomes. However, VLRA- and CDA1-expressing T-like lymphocytes have been identified in, and therefore appear to develop in, the tissues comprising the tips of gill filaments, which have now

been dubbed 'thymoids' (Bajoghli et al., 2011). Thus, distinct T and B lymphocytes might be primitive to the vertebrate common ancestor, but adaptive immunity with a comparable repertoire of immune responses appears to have evolved independently in the cyclostome and gnathostome lineages. As such, cyclostomes provide a unique perspective on the evolution of the adaptive immune system, and auto immunity, that is enjoyed by all other vertebrates.

Limitations and future directions

The major limitation for studying both lampreys and hagfish is most likely to be the availability of embryos, coupled with the difficulty of long-term culture. Although some populations of lampreys are amenable to study, these are geographically confined and long distance transportation of adults to host laboratories is usually necessary. Breeding seasons are also limited. The prolonged larval stage prior to metamorphosis in lampreys pushes many features of evolutionary interest that are confined to the adult out of experimental reach. For hagfish, the severe restriction of embryo availability will probably continue, confining the study of these species to specialised laboratories. This will also hinder the development of embryo manipulation methods.

Despite this, we can predict advances on several fronts over the next few years. The current lack of comprehensive genome data for lampreys and hagfish is unlikely to be long term. Well-assembled genomes should address questions concerning the timing of 2R and help define orthological relationships between cyclostome and gnathostome genes more fully. This, in turn, will aid interpretation of comparative gene expression and function. It will also allow more comprehensive analysis of the non-coding genome: microRNAs, other non-coding RNAs, and regulatory elements, for example.

The successful adaptation of morpholino-based gene knockdown and other gene manipulation methods to lampreys means that gene regulatory interactions can be dissected in these species. Most studies of cyclostomes to date have been descriptive and, although these yield significant insight, understanding developmental mechanisms and hence the mechanistic basis of evolutionary change often requires functional analysis. Such studies have the potential to reveal how key vertebrate specific characteristics evolved.

Competing interests statement
The authors declare no competing financial interests.

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