microRNAs reveal the interrelationships of hagfish, lampreys, and gnathostomes and the nature of the ancestral vertebrate

Alysha M. Heimberg[a], Richard Cowper-Sal·lari[b], Marie Sémon[c], Philip C. J. Donoghue[d,1], and Kevin J. Peterson[a,1]

[a]Department of Biological Sciences, Dartmouth College, Hanover, NH 03755; [b]Department of Genetics, Dartmouth Medical School, Hanover, NH 03755; [c]Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Centre National de la Recherche Scientifique, Institut National de la Recherche Agronomique, Ecole Normale Supérieure de Lyon, 69364 Lyon Cedex 07, France; and [d]Department of Earth Sciences, University of Bristol, Bristol BS8 1RJ, United Kingdom

Edited* by J. William Schopf, University of California, Los Angeles, CA, and approved September 22, 2010 (received for review July 15, 2010)

Hagfish and lampreys are the only living representatives of the jawless vertebrates (agnathans), and compared with jawed vertebrates (gnathostomes), they provide insight into the embryology, genomics, and body plan of the ancestral vertebrate. However, this insight has been obscured by controversy over their interrelationships. Morphological cladistic analyses have identified lampreys and gnathostomes as closest relatives, whereas molecular phylogenetic studies recover a monophyletic Cyclostomata (hagfish and lampreys as closest relatives). Here, we show through deep sequencing of small RNA libraries, coupled with genomic surveys, that Cyclostomata is monophyletic: hagfish and lampreys share 4 unique microRNA families, 15 unique paralogues of more primitive microRNA families, and 22 unique substitutions to the mature gene products. Reanalysis of morphological data reveals that support for cyclostome paraphyly was based largely on incorrect character coding, and a revised dataset is not decisive on the mono- vs. paraphyly of cyclostomes. Furthermore, we show fundamental conservation of microRNA expression patterns among lamprey, hagfish, and gnathostome organs, implying that the role of microRNAs within specific organs is coincident with their appearance within the genome and is conserved through time. Together, these data support the monophyly of cyclostomes and suggest that the last common ancestor of all living vertebrates was a more complex organism than conventionally accepted by comparative morphologists and developmental biologists.

complexity | cyclostomata | evolution | organ | homology

The origin and early evolution of vertebrates have been a focus of molecular and organismal evolutionary biology because of the fundamental events that attended this formative episode of our own evolutionary history over one-half billion years ago (1). However, attempts to integrate these perspectives have been stymied by the different phylogenetic perspectives afforded by molecular and morphological datasets. Molecular datasets, incorporating protein-coding genes, ribosomal RNA genes, and/or mitochondrial genes (2–21), invariably find that the jawless hagfish and lampreys constitute a clade, Cyclostomata (Fig. 1, on the left). In contrast, morphological datasets (22–36) have supported a closer relationship between lampreys and gnathostomes, rendering Cyclostomata paraphyletic (Fig. 1, on the right) and hagfish not vertebrates but mere craniates (33).

Attempts have been made to reconcile these two views: a number of morphological characters have been identified that support the monophyly of cyclostomes (37, 38), but they have been overwhelmed by a seemingly far greater number of characters supporting cyclostome paraphyly (30, 31). Indeed, an analysis of combined morphological and molecular datasets has suggested that the signal of cyclostome paraphyly in morphological datasets is stronger than the signal for monophyly from molecular data (39). The interrelationships of hagfish, lampreys, and gnathostomes thus remain uncertain, and this has become a classic example of phylogenetic conflict between morphological and molecular data (7, 39). If morphological phylogenies are correct, hagfish provide an experimental model for investigating the evolutionary assembly of the vertebrate body plan shared by lampreys and gnathostomes. Alternatively, if the molecular phylogenies are correct, then it would indicate that the shared similarities of lampreys and gnathostomes are convergent or that these characters are absent through loss in the hagfish lineage. These would represent the most extraordinary examples of convergence or degeneracy, respectively, in vertebrate evolutionary history (18, 35).

We attempted to resolve the interrelationships of hagfish, lampreys, and gnathostomes through analysis of their microRNA (miRNA) repertoire. miRNAs are small, noncoding regulatory genes implicated in the control of cellular differentiation and homeostasis and as such, might be involved in the evolution of complexity (40–42). Because ancient miRNAs show a level of sequence conservation exceeding that of ribosomal DNA (43), it is possible to discern the evolutionary origins of miRNA families at even the deepest levels of animal phylogeny (43, 44). The rarity with which ancient miRNAs were lost within most evolutionary lineages, coupled with the continuous acquisition of miRNAs through geologic time in all metazoan lineages examined to date, makes miRNAs one of the most useful classes of characters in phylogenetics (45). Thus, miRNAs can be used to discern the interrelationships among the major vertebrate lineages and simultaneously, lend insight into the origin of vertebrate characteristics.

We constructed small RNA libraries from total RNA (*Methods*) from ammocoete larvae of the brook lamprey *Lampetra planeri*, from a single adult individual of the Atlantic hagfish *Myxine glutinosa*, from the catshark *Scyliorhinus canicula*, and for nine individually processed organs/regions (brain, gills, gut, heart, kidney, liver, mouth, muscle, and skin) from a single adult individual of the sea lamprey *Petromyzon marinus*. Using a combination of high-throughput 454 pyrosequencing and Illumina technology, we identified miRNAs from each library and found that shared gains of miRNAs support the monophyly of cyclostomes (lamprey and hagfish). We also revised, expanded, and reanalyzed an extensive morphological dataset previously found to support cyclostome paraphyly (23) and show that cyclostome monophyly is just as likely given these data. In addition, profiling

Author contributions: A.M.H., P.C.J.D., and K.J.P. designed research; A.M.H., P.C.J.D., and K.J.P. performed research; R.C.-S. and M.S. contributed new reagents/analytic tools; A.M.H., P.C.J.D., and K.J.P. analyzed data; and A.M.H., P.C.J.D., and K.J.P. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Data deposition: The data reported in this paper have been deposited in miRBase, www.mirbase.org.

[1]To whom correspondence may be addressed. E-mail: phil.donoghue@bristol.ac.uk or kevin.j.peterson@dartmouth.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1010350107/-/DCSupplemental.

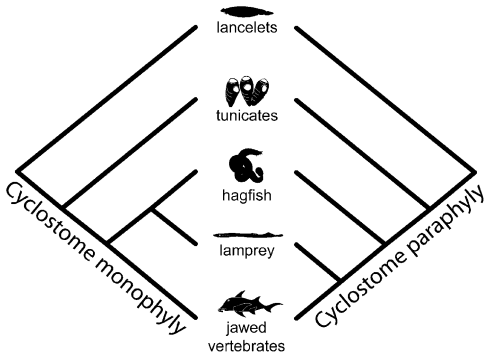

Fig. 1. The two competing hypotheses. Either lampreys are more closely related to hagfish than they are to gnathostomes, making Cyclostomata monophyletic (on the left), or lampreys are more closely related to gnathostomes than they are to hagfish, making Cyclostomata paraphyletic (on the right).

the miRNA expression within nine organs of *P. marinus* shows conservation with known expression profiles in homologous organs across vertebrates. Our data suggest that the role of miRNAs within specific organs is coincident with their appearance within the genome, and thus, miRNAs may have played a role in the acquisition of organismal complexity in vertebrates.

Results and Discussion

miRNAs Shared Between Lampreys and Hagfish Support Cyclostome Monophyly. Derivative cDNA libraries from the brook lamprey *L. planeri*, the sea lamprey *P. marinus*, and the Atlantic hagfish *M. glutinosa* were sequenced using high-throughput 454 pyrosequencing (*Methods*), yielding 422,122 (59,759 nonredundant) parsed high-quality reads. Additionally, we sequenced small RNAs from the catshark *Scyliorhinus canicula* using Illumina technology, yielding 333,294 (127,015 nonredundant) parsed high-quality reads. The resulting reads from all four taxa were then interrogated using miRMiner (43) to identify known and unknown miRNAs (Dataset S1).

Because the genome traces of the sea lamprey *P. marinus* are publicly available (http://www.ncbi.nlm.nih.gov/genomeprj?term=petromyzon), we first focused on elucidating the miRNA repertoire of this species. We identified 245 miRNA genes in *P. marinus*, including one family lost in gnathostomes (miR-315) and a second family lost in osteichthyans (mir-281) (Dataset S1). An additional 24 miRNA genes are inferred to be present in the genome of *P. marinus*, because, although the genes could not be located in the trace archives, reads of these phylogenetically conserved miRNAs were discovered in our libraries (e.g., miR-31, -34, -122, etc.) (Dataset S1). Of the 269 genes present in *P. marinus*, 202 are conserved in other animals, with 21 shared only with the brook lamprey, *L. planeri* (Dataset S1).

Lampreys lack the bilaterian miRNAs miR-71, miR-242, miR-252, and miR-278, as do urochordates and all other vertebrates examined to date. However, very few miRNA genes have been lost within the lamprey lineage itself: only a single miRNA family seems to have been lost in *P. marinus* (miR-214), because reads were detected in *L. planeri* (Dataset S1); however, reads were not detected in *P. marinus*, and the gene was not located in the trace archives. Conversely, we failed to detect transcripts of only two miRNA families in *L. planeri*—the lowly expressed miRNAs (Dataset S1) miR-202 and miR-875 (although we did not examine reads from an adult individual, and no genomic sequence for this species is currently available to confirm a true absence). Therefore, these two lamprey species share a miRNA complement of at least 200 genes and between them, have together lost no more than three miRNA families total since they last shared a common ancestor some time in the last 10–40 million y (10).

To determine the phylogenetic position of hagfish, we analyzed the conserved miRNA complement of *M. glutinosa*. Of the 46 vertebrate-specific miRNA families shared between lamprey and gnathostomes (Fig. 2), we detected all but two in our hagfish library: miR-1329 (which is expressed exclusively in the lamprey kidney) (Dataset S1) and miR-4541, an miRNA family found thus far only in the two sharks and the two lamprey species (Dataset S1). However, the hagfish shares four unique miRNA families with the lampreys that are not found or expressed in gnathostomes or in any other animal species investigated to date, miR-4542, miR-4543, miR-4544, and miR-4545 (Dataset S1 and Fig. S1), and a phylogenetic analysis based on the presence and absence of miRNA families (Dataset S2) supports the monophyly of the cyclostomes (Fig. 2 and Fig. S2).

Further evidence of cyclostome monophyly is found in the paralogy group relations within miRNA families (46). Fifteen paralogues of previously described miRNA families (Fig. 3 and Dataset S1) are shared by the hagfish and lampreys to the exclusion of gnathostomes—we did not detect a single paralogue supporting cyclostome paraphyly. Finally, we examined the mature sequences of each miRNA to ask if polarizable nucleotide substitutions had occurred that supported either cyclostome monophyly or paraphyly (or some other set of relations). We did not find any nucleotide substitutions in the mature sequence of any vertebrate miRNA that is shared between gnathostomes and lampreys to the exclusion of hagfish (or between hagfish and gnathostomes to the exclusion of lamprey). However, we did find 22 derived nucleotide substitutions in the mature sequences of 18 miRNAs exclusive to the three cyclostome taxa investigated (Fig. 3 and Dataset S1). Thus, the acquisition of miRNA families, miRNA genes, and the nucleotide substitution patterns of conserved miRNA genes all support cyclostome monophyly.

Phenotypic Cladistic Data Do Not Distinguish Between Cyclostome Monophyly vs. Paraphyly. The phylogenetic distribution of vertebrate miRNAs corroborates molecular sequence data in supporting cyclostome monophyly (2–21), contradicting what has been considered an equally strong signal from phenotypic datasets supporting cyclostome paraphyly (22–36). To determine the source of this discordance, we augmented a phenotypic dataset based on the nervous system (23), with characters representative of other organ systems recoded from observations and the primary literature rather than recycled from previous analyses (*SI Text* and Dataset S3). In so doing, we considered all characters that have been marshaled previously in support of cyclostome monophyly or paraphyly. We find that, although the revised dataset (*SI Text*) marginally favors cyclostome paraphyly (monophyly is one step longer in a tree of 237 steps) (Fig. S3), Templeton (47), Kishino–Hasegawa (48), and approximate two-tailed Shimodaira–Hasegawa (49) tests reveal that the dataset is indecisive on this question (Templeton: $P = 0.8415$; K–H: $P = 0.8421$; approximate S–H is one-half P of K–H) (49). This is because much of the evidence traditionally interpreted as supporting cyclostome paraphyly has been based on spurious character design. For example, many of the characters are inapplicable to the outgroup, making it impossible to discriminate between the primary or secondary absence in hagfish of characters otherwise found only in lampreys and gnathostomes (e.g., the proximity of the atrium and ventricle of the heart, radial muscles, and retinal synaptic ribbons). In addition, some characters have been coded as absent in hagfish when data have merely been lacking (e.g., heart response to catecholamines, pituitary control of gametogenesis, and sexual dimorphism). Finally, the uncritical recycling of characters and their codings between generations of analyses has resulted in the repeated use of obsolete data (50). For instance, similarities in the immune system of lampreys and gnathostomes have been exploited to draw a distinction from hagfish (30–35, 51). However, it has been long established that lampreys

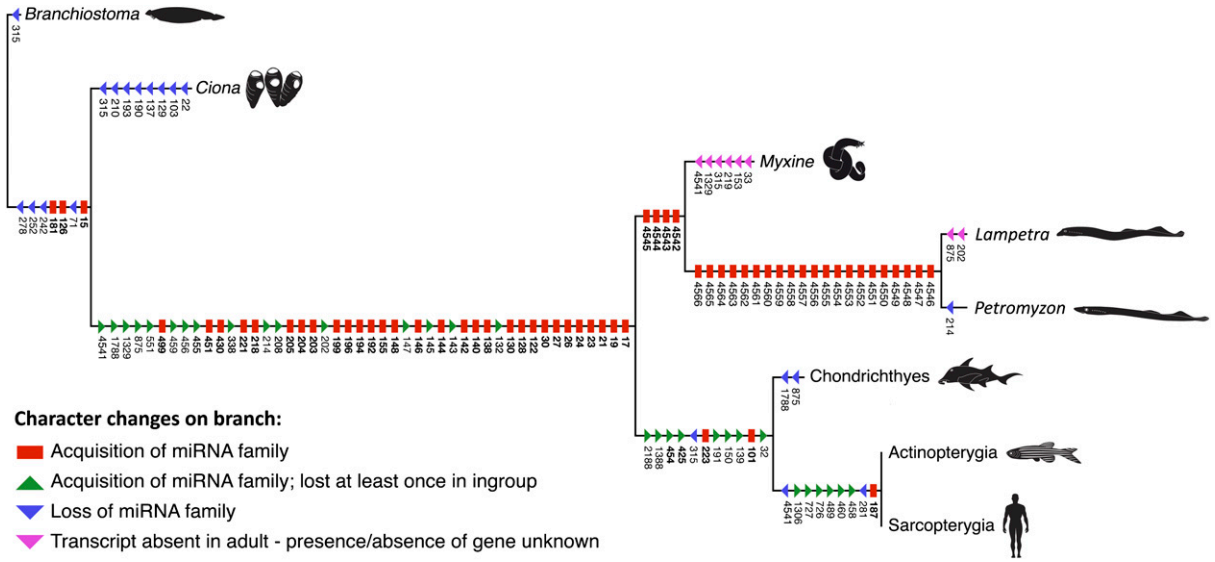

Fig. 2. Phylogenetic distribution of all miRNA families analyzed in chordates (see Dataset S2 for data matrix and Fig. S2 for complete phylogenetic analysis). Cyclostomes share four miRNA families not found in any other animal species investigated to date, and a maximum parsimony analysis supports the monophyly of Cyclostomata. Note that miRNA families specific to a single species are not indicated, but losses of more primitive families are indicated. Of particular interest is the number of miRNA families acquired in the stem lineage leading to the vertebrate crown group.

and hagfish share a distinct type of adaptive immune system based on variable lymphocyte receptors, rather than the Ig-based T and B antigen receptors that characterize the lymphocytes of jawed vertebrates (52), and thus, similarities in the immune system of lampreys and jawed vertebrates are convergent.

miRNA Expression Profiles Are Conserved Across Vertebrates. The origin of vertebrates occurred in association with a very high rate of miRNA family innovation, and it has been proposed that this is a causal association, because where expression data are available, vertebrate miRNAs are often expressed in tissues and organs that are unique to vertebrates (41). This hypothesis predicts that the organ-specific expression of vertebrate-specific miRNAs is highly conserved, such that data from the zebrafish (*Danio*) and the mouse (*Mus*) are representative not only of

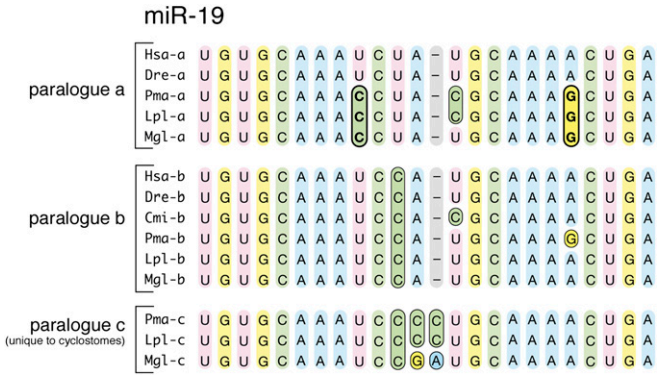

Fig. 3. The presence of paralogues of more primitive miRNA families and conserved nucleotide substitutions both support the monophyly of Cyclostomata. Shown is miR-19 as an example of a group of miRNAs that shows both conserved nucleotide substitutions (19a; *Top*, bold) with respect to the other paralogue(s) (19b and 19c; *Middle* and *Bottom*) and the possession of a paralogue (miR-19c) not present in any known gnathostome (Dataset S1 has the complete description of both paralogues and nucleotide substitutions supporting cyclostome monophyly). Cmi, *Callorhinchus milii;* Dre, *Danio rerio*; Hsa, *Homo sapiens*; Lpl, *Lampetra planeri*; Mgl, *Myxine glutinosa*; Pma, *Petromyzon marinus*.

osteichthyans (the clade that they circumscribe) but of vertebrates more generally. Our phylogenetic results indicate that a comparison of existing data with lampreys will provide an adequate test of the hypothesis, because together, these taxa circumscribe the clade of all living vertebrates (Fig. 2). Expression data for seven different *P. marinus* organs are shown in Fig. 4. Similar to *Danio* (53, 54) and *Mus* (55), each lamprey organ expresses a specific suite of miRNAs that gives the organ a unique miRNA expression profile. For example, ignoring the ubiquitously expressed let-7, the four highest expressed miRNA genes in the lamprey brain are miR-9a, miR-338a, miR-138a, and miR-125a, whereas the four highest expressed miRNA genes in the lamprey gut are miR-194, miR-192, miR-200a, and miR-429 (Fig. 4 and Dataset S4). Furthermore, similar to mouse (56), the lamprey brain is the most complex of the organs queried, and the gut and liver are the least, at least in terms of the number of different miRNAs expressed (Dataset S4). With just one exception (the heart), the miRNA with the highest expression in each of the lamprey organs is also expressed in that same organ in both *Danio* (Fig. 4 *Insets*) and hagfish (Fig. S4). Thus, homologous organs in vertebrates more often than not (57) express homologous miRNAs, consistent with the hypothesis that miRNAs (e.g., miR-30 and miR-122) were instrumental in the evolutionary origin of vertebrate-specific organs (e.g., kidney and liver, respectively) (41).

Conclusions

Hinging on debate over the interrelationships of living jawless and jawed vertebrates has been the nature of the ancestral vertebrate and the pattern and sequence of organismal and genomic evolution, on which hypotheses of developmental evolution are based. We conclude that cyclostomes are monophyletic, and thus, characters reconstructed as lamprey and gnathostome synapomorphies are actually shared primitive characters of all vertebrates, with hagfish anatomy having degenerated to a remarkable degree (18, 36). Cyclostome paraphyly (22) and a hierarchical distinction between craniates and vertebrates (33) afforded insight into the assembly of vertebrate characters (58). With the recognition of cyclostome monophyly, however, that taxonomic distinction and evolutionary insight are lost. Evidently, the crown ancestor of vertebrates was more complex, phenotypically and

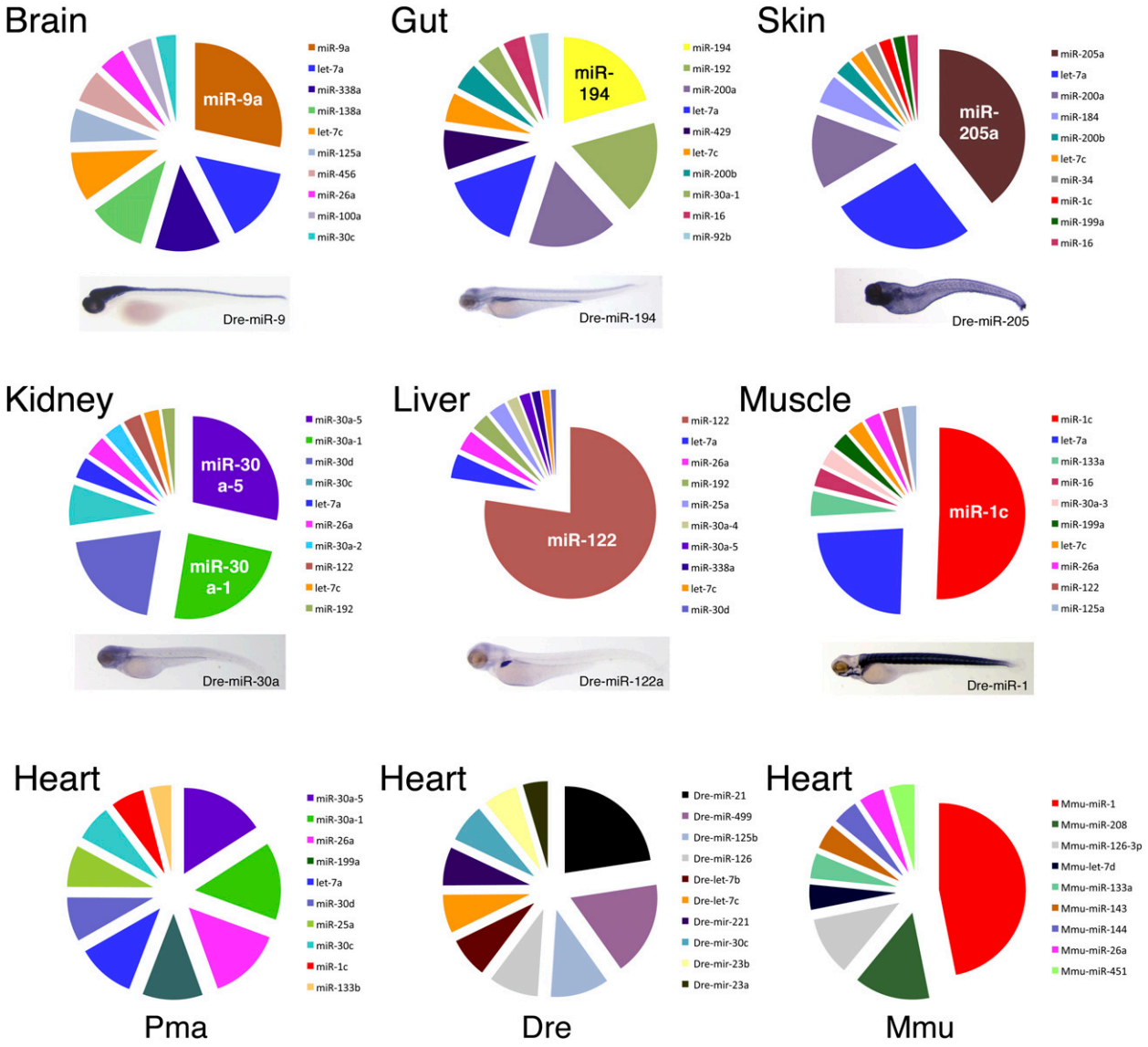

Fig. 4. miRNA expression profile of seven different lamprey organs. Only the top 10 highest expressed miRNAs (Dataset S4) are shown, and each specific miRNA is given a distinct color for all pie charts. Below each pie chart is the expression pattern of the highest expressed gene in the lamprey library in the zebrafish (54)—note the concordance between the lamprey and zebrafish for all organs queried except for the heart (*Bottom*). Pma, *Petromyzon marinus*; Dre, *Danio rerio*; Mmu, *Mus musculus*.

developmentally, than has been perceived hitherto (58), making attempts to explain mechanistically the distinction between vertebrates and invertebrates even more formidable. Nonetheless, in reconciling phylogenies grounded in genotype and phenotype, we provide a holistic framework for uncovering the formative events in the evolutionary emergence of vertebrates. We predict that the renaissance in hagfish embryology (59) will further show the loss of vertebrate characters, but with the recognition of cyclostome monophyly, attempts to dissect the assembly of the vertebrate body plan can be focused on comparative analysis of lamprey development and genomics. The prolific origin of miRNA families in the vertebrate stem-lineage and their expression in vertebrate-specific tissues and organs supports the idea that miRNAs played a pivotal role, as part of a broader gene regulatory landscape, in the assembly of the vertebrate body plan (41).

Methods

Total RNA Extraction, Northern Analysis, and Small RNA Library Construction. Embryonic brook lampreys (*L. planeri)* were collected from Highland Water, upstream of Millyford Bridge, New Forest National Park (United Kingdom) and allowed to develop in captivity at 16 °C in filtered river water until hatching. Adult sea lamprey (*P. marinus*) were collected from Lake Champlain (Vermont), and a single individual was dissected to isolate the brain, gut, gills, heart, kidney, liver, mouth and tongue, muscle, and skin. Atlantic hagfish (*M. glutinosa)* were collected at Kristineberg Marine Station, Gullmarsfjord, Sweden and purchased from Gulf of Maine Inc. (Pembroke, ME). RNA was extracted from 20 combined larval *L. planeri*, from each dissected tissue and organ derived from a single adult *P. marinus*, and from a single adult *M. glutinosa*. From these animals, small RNA libraries were constructed individually and sequenced with a unique barcode using 454 DNA pyrosequencing (Branford, CT) as described previously (43). The resulting reads were then analyzed with miRMiner to identify known and unknown miRNAs (43), with additional filters for transfer and ribosomal RNAs written with custom shell scripts.

RNA was also extracted from the brain, gut, heart, kidney, liver, muscle, and skin derived from a single adult *M. glutinosa*, and northern analyses using Starfire probes (IDT) designed against the mature miRNA sequence (sequences available on request) were performed as previously described (43). Catshark (*S. canicula*) embryos were obtained from commercial sources, and RNA was extracted from five embryos near hatching. *S. canicula* RNA was sequenced

for small RNAs using the Illumina sequencing platform and analyzed using miRMiner as described (43). All genomic inquiries for miRNAs in *P. marinus* and *Callorhinchus milii* (elephant shark) were made through National Center for Biotechnology Information using the available genomic traces. All alignments and sequence analyses were performed using MacVector (v. 10.0.2). Secondary structures of precursor miRNA transcripts were predicted using mFold (60).

Morphological Analysis. The phenotypic dataset was coded directly from the primary literature and from direct observation of anatomy (*SI Text*). We designed and coded characters using a contingent coding strategy, because it is the only approach that is theoretically and operationally valid in instances, as here, where many of the characters are inapplicable to the outgroup (61). We restricted our analyses to a parsimony-based approach, because phenotypic support for hagfish–lamprey–gnathostome relationships has always been debated using this method of phylogenetic inference. The cladistic parsimony analyses, Bremer support index calculations, and Templeton and Kishino–Hasegawa tests were performed in PAUP*4.0b10 running on Mac OS9 within a Sheepshaver 2.3 emulator on an Intel MacBook.

ACKNOWLEDGMENTS. We thank E. Marsden, S. Shimeld, and M. Thorndyke for access to materials and J. Mallatt, E. Sperling, D. Pisani, and M. Schubert for comments on a previous draft. P.C.J.D. is supported by the Biotechnology and Biological Sciences Research Council, European Commission Seventh Framework Programme EU FP7, The Leverhulme Trust, Natural Environment Research Council, and National Endowment for Science Technology and the Arts (NESTA); K.J.P. is supported by the National Aeronautics and Space Administration/Ames and National Science Foundation. A.M.H. was supported by Award Number T32GM008704 from the National Institute of General Medical Sciences of the National Institutes of Health.

1. Shimeld SM, Holland PWH (2000) Vertebrate innovations. *Proc Natl Acad Sci USA* 97: 4449–4452.
2. Stock DW, Whitt GS (1992) Evidence from 18S ribosomal RNA sequences that lampreys and hagfishes form a natural group. *Science* 257:787–789.
3. Delsuc F, Tsagkogeorga G, Lartillot N, Philippe H (2008) Additional molecular support for the new chordate phylogeny. *Genesis* 46:592–604.
4. Lartillot N, Philippe H (2008) Improvement of molecular phylogenetic inference and the phylogeny of Bilateria. *Philos Trans R Soc Lond B Biol Sci* 363:1463–1472.
5. Blair JE, Hedges SB (2005) Molecular phylogeny and divergence times of deuterostome animals. *Mol Biol Evol* 22:2275–2284.
6. Yu SY, et al. (2008) Phylogenetic analysis of 48 gene families revealing relationships between hagfishes, lampreys, and Gnathostomata. *J Genet Genomics* 35:285–290.
7. Goodman M, Miyamoto MM, Czelusniak J (1987) Pattern and processes in vertebrate phylogeny revealed by coevolution of molecules and morphologies. *Molecules and Morphology in Evolution: Conflict or Compromise?* ed Patterson C (Cambridge University Press, Cambridge, UK), pp 141–176.
8. Delarbre C, et al. (2000) The complete nucleotide sequence of the mitochondrial DNA of the agnathan *Lampetra fluviatilis*: Bearings on the phylogeny of cyclostomes. *Mol Biol Evol* 17:519–529.
9. Kuraku S, Hoshiyama D, Katoh K, Suga H, Miyata T (1999) Monophyly of lampreys and hagfishes supported by nuclear DNA-coded genes. *J Mol Evol* 49:729–735.
10. Kuraku S, Kuratani S (2006) Time scale for cyclostome evolution inferred with a phylogenetic diagnosis of hagfish and lamprey cDNA sequences. *Zoolog Sci* 23:1053–1064.
11. Delsuc F, Brinkmann H, Chourrout D, Philippe H (2006) Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* 439:965–968.
12. Lanfranchi G, Pallavicini A, Laveder P, Valle G (1994) Ancestral hemoglobin switching in lampreys. *Dev Biol* 164:402–408.
13. Turbeville JM, Schulz JR, Raff RA (1994) Deuterostome phylogeny and the sister group of the chordates: Evidence from molecules and morphology. *Mol Biol Evol* 11:648–655.
14. Lipscomb DL, Farris JS, Källersjo M, Tehler A (1998) Support, ribosomal sequences and the phylogeny of the eukaryotes. *Cladistics* 14:303–338.
15. Winchell CJ, Sullivan J, Cameron CB, Swalla BJ, Mallatt J (2002) Evaluating hypotheses of deuterostome phylogeny and chordate evolution with new LSU and SSU ribosomal DNA data. *Mol Biol Evol* 19:762–776.
16. Mallatt J, Sullivan J, Winchell CJ (2001) The relationship of lampreys to hagfishes: A spectral analysis of ribosomal DNA sequences. *Major Events in Early Vertebrate Evolution: Palaeontology, Phylogeny, Genetics and Development*, ed Ahlberg PE (Taylor & Francis, London), pp 106–118.
17. Mallatt J, Winchell CJ (2007) Ribosomal RNA genes and deuterostome phylogeny revisited: More cyclostomes, elasmobranchs, reptiles, and a brittle star. *Mol Phylogenet Evol* 43:1005–1022.
18. Delarbre C, Gallut C, Barriel V, Janvier P, Gachelin G (2002) Complete mitochondrial DNA of the hagfish, *Eptatretus burgeri*: The comparative analysis of mitochondrial DNA sequences strongly supports the cyclostome monophyly. *Mol Phylogenet Evol* 22:184–192.
19. Mallatt J, Sullivan J (1998) 28S and 18S rDNA sequences support the monophyly of lampreys and hagfishes. *Mol Biol Evol* 15:1706–1718.
20. Furlong RF, Holland PWH (2002) Bayesian phylogenetic analysis supports monophyly of ambulacraria and of cyclostomes. *Zoolog Sci* 19:593–599.
21. Takezaki N, Figueroa F, Zaleska-Rutczynska Z, Klein J (2003) Molecular phylogeny of early vertebrates: Monophyly of the agnathans as revealed by sequences of 35 genes. *Mol Biol Evol* 20:287–292.
22. Løvtrup S (1977) *The Phylogeny of the Vertebrata* (Wiley, New York), p 330.
23. Khonsari RH, Li B, Vernier P, Northcutt RG, Janvier P (2009) Agnathan brain anatomy and craniate phylogeny. *Acta Zool* 90:52–68.
24. Jefferies RPS (1986) *The Ancestry of the Vertebrates* (British Museum Natural History, London).
25. Forey PL, Janvier P (1993) Agnathans and the origin of jawed vertebrates. *Nature* 361: 129–134.
26. Forey PL (1984) Yet more reflections on agnathan-gnathostome relationships. *J Vertebr Paleontol* 4:330–343.
27. Janvier P (1996) The dawn of the vertebrates: Characters versus common ascent in the rise of current vertebrate phylogenies. *Palaeontology* 39:259–287.
28. Janvier P (1996) *Early Vertebrates* (Oxford University Press, Oxford).
29. Donoghue PCJ, Smith MP (2001) The anatomy of *Turinia pagei* (Powrie) and the phylogenetic status of the Thelodonti. *Trans R Soc Edinb Earth Sci* 92:15–37.
30. Donoghue PCJ, Forey PL, Aldridge RJ (2000) Conodont affinity and chordate phylogeny. *Biol Rev Camb Philos Soc* 75:191–251.
31. Gess RW, Coates MI, Rubidge BS (2006) A lamprey from the Devonian period of South Africa. *Nature* 443:981–984.
32. Forey PL (1995) Agnathans recent and fossil, and the origin of jawed vertebrates. *Rev Fish Biol Fish* 5:267–303.
33. Janvier P (1981) The phylogeny of the Craniata, with particular reference to the significance of fossil "agnathans". *J Vertebr Paleontol* 12:121–159.
34. Hardisty MW (1979) *The Biology of Cyclostomes* (Chapman & Hall, London).
35. Hardisty MW (1982) *Lampreys and Hagfishes: Analysis of Cyclostome Relationships. The Biology of Lampreys* (Academic, London).
36. Hardisty MW (2006) *Lampreys: Life Without Jaws* (Forrest Text, Tresaith-Ceredigion-SA43 2JG-UK).
37. Yalden DW (1985) Feeding mechanisms as evidence of cyclostome monophyly. *Zool J Linn Soc* 84:291–300.
38. Mallatt J (1997) Crossing a major morphological boundary: The origin of jaws in vertebrates. *Zoology* 100:128–140.
39. Near TJ (2009) Conflict and resolution between phylogenies inferred from molecular and phenotypic data sets for hagfish, lampreys, and gnathostomes. *J Exp Zool B Mol Dev Evol* 312:749–761.
40. Christodoulou F, et al. (2010) Ancient animal microRNAs and the evolution of tissue identity. *Nature* 463:1084–1088.
41. Heimberg AM, Sempere LF, Moy VN, Donoghue PCJ, Peterson KJ (2008) MicroRNAs and the advent of vertebrate morphological complexity. *Proc Natl Acad Sci USA* 105: 2946–2950.
42. Peterson KJ, Dietrich MR, McPeek MA (2009) MicroRNAs and metazoan macroevolution: Insights into canalization, complexity, and the Cambrian explosion. *Bioessays* 31:736–747.
43. Wheeler BM, et al. (2009) The deep evolution of metazoan microRNAs. *Evol Dev* 11: 50–68.
44. Grimson A, et al. (2008) Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature* 455:1193–1197.
45. Sperling EA, Peterson KJ (2009) microRNAs and metazoan phylogeny: Big trees from little genes. *Animal Evolution–Genomes, Trees and Fossils*, eds Telford MJ, Littlewood DTJ (Oxford University Press, Oxford), pp 157–170.
46. Goodman M, Czelusniak J, Moore GW, Romero-Herrera AE, Matsuda G (1979) Fitting the gene lineage into its species lineage, a parsimony strategy illustrated by cladograms constructed from globin sequences. *Syst Zool* 28:132–163.
47. Templeton A (1983) Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution* 37:221–224.
48. Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. *J Mol Evol* 29:170–179.
49. Goldman N, Anderson JP, Rodrigo AG (2000) Likelihood-based tests of topologies in phylogenetics. *Syst Biol* 49:652–670.
50. Jenner RA (2001) Bilaterian phylogeny and uncritical recycling of morphological data sets. *Syst Biol* 50:730–742.
51. Nicholls H (2009) Evolution: Mouth to mouth. *Nature* 461:164–166.
52. Pancer Z, Cooper MD (2006) The evolution of adaptive immunity. *Annu Rev Immunol* 24:497–518.
53. Soares AR, et al. (2009) Parallel DNA pyrosequencing unveils new zebrafish microRNAs. *BMC Genomics* 10:195.
54. Wienholds E, et al. (2005) MicroRNA expression in zebrafish embryonic development. *Science* 309:310–311.
55. Landgraf P, et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 129:1401–1414.
56. Strauss WM, Chen C, Lee C-T, Ridzon D (2006) Nonrestrictive developmental regulation of microRNA gene expression. *Mamm Genome* 17:833–840.
57. Ason B, et al. (2006) Differences in vertebrate microRNA expression. *Proc Natl Acad Sci USA* 103:14385–14389.
58. Janvier P (2001) Ostracoderms and the shaping of the gnathostome characters. *Major Events in Early Vertebrate Evolution: Palaeontology, Phylogeny, Genetics and Development*, ed Ahlberg PE (Taylor and Francis, London), pp 172–186.
59. Ota KG, Kuraku S, Kuratani S (2007) Hagfish embryology with reference to the evolution of the neural crest. *Nature* 446:672–675.
60. Néron B, et al. (2009) Mobyle: A new full web bioinformatics framework. *Bioinformatics* 25:3005–3011.
61. Strong EE, Lipscomb D (1999) Character coding and inapplicable data. *Cladistics* 15: 363–371.

Supporting Information

Heimberg et al. 10.1073/pnas.1010350107

SI Text

Contents. This text contains the descriptions of characters used in the phenotypic cladistic data matrix. The characters are based on those used previously in morphological cladistic analyses or otherwise highlighted as potential synapomorphies of lampreys and gnathostomes, lampreys and hagfishes, or hagfishes and gnathostomes. We did not include characters used solely to resolve the phylogenetic position of the extinct jawless ostracoderms unless they have a bearing on the interrelationships of living jawless and jawed vertebrates. Practically, the character matrix was based on the recent revision of nervous system and neural characters compiled by Khonsari et al. (1), and further description of the characters can be found there; our modifications to character definition and coding are noted and justified. The remaining characters were identified through review of previous phylogenetic analyses (2–20) but recoded from the primary literature and through primary observation.

We deleted or refined characters for the following reasons.

No Persistent Pronephros in Adult. It is identified as a character supporting cyclostome paraphyly by Løvtrup (2) and Janvier (19) but rejected as a character by Forey (13) and Schaeffer and Thomson (10), because the alternative presence state is a hagfish autapomorphy and therefore, is not phylogenetically informative.

No Accessory Hearts. It is identified as a character supporting cyclostome paraphyly by Løvtrup (2) and Janvier (19) but rejected as a character by Forey (13) and Schaeffer and Thomson (10), because the alternative presence state is a hagfish autapomorphy and therefore, is not phylogenetically informative.

Single Cuvierian Duct. It is identified as a character supporting cyclostome monophyly (18), contrasting with the paired Cuvierian ducts in extant gnathostomes. However, the conditions in hagfish and lampreys differ, with only a vestige of the right duct in the adult hagfish (21) and the left duct atrophying at metamorphosis in the lamprey (22).

Absence of a Unique Dermatan Sulfate. It is identified as a character supporting cyclostome paraphyly by Løvtrup (2) and Janvier (19) but rejected as a character by Forey (13) and Schaeffer and Thomson (10), because the alternative presence state is a hagfish autapomorphy and therefore, is not phylogenetically informative.

No Naso-Pharyngeal Duct. It is identified as a character supporting cyclostome paraphyly by Løvtrup (2) and Janvier (19) but rejected as a character by Forey (13) and Schaeffer and Thomson (10), because the alternative presence state is a hagfish autapomorphy and therefore, is not phylogenetically informative.

Blood Volume That Had Been Used in a Number of Previous Analyses of Hagfish, Lamprey, and Gnathostome Relations. This character has typically been expressed as blood volume relative to body volume (3–5). However, blood volume is always normalized to body weight. We could find no data for *Polypterus* or *Coelacanth*, but hagfish (23) are 13.8 mL 100 g^{-1}, lampreys (24) are 10.9–6.5 mL 100 g^{-1}, sharks (25, 26) are in the range 5–7 mL 100 g^{-1}, *Neoceratodus* (27) is 5.1 ± 0.3 mL 100 g^{-1}, and actinopterygians are in the range from 3.8 to 1.8 mL 100 g^{-1}, with nonteleost actinopterygians (28) (which we take as a proxy for *Polypterus*) range 3.0–3.8 mL 100 g^{-1}. Although there seems to be a general trend, there is no clear division in the range that could be used as character states useful in this context.

Haemoglobins with Low O_2 Affinity and Significant Bohr Effect (Absent = 0, Present = 1). The point of this character has been that hagfish hemoglobin has low affinity for O_2 and therefore, shows little Bohr effect (29), whereas all other vertebrates, including lampreys, exhibit a strong Bohr effect (29–33). However, very little is known concerning hemoglobin in cephalochordates (34) or tunicates (35), and furthermore, although lampreys exhibit a Bohr effect comparable with gnathostomes, they achieve this, like hagfish, through pH-driven aggregation of their hemoglobin monomers into dimers or tetramers rather than through proton binding of oxygen to stable hemoglobin tetramers, which is the condition in gnathostomes (30, 36). Hence, it seems that the protein structure of the hemoglobins in vertebrates supports monophyly, rather than paraphyly, of cyclostomes—although this remains contingent on a dearth of knowledge of the hemoglobins in the invertebrate chordates.

Sexual Dimorphism (Absent = 0, Present = 1). This is not as clear cut a character as it has been portrayed. To be sure, lampreys and gnathostomes exhibit sexual dimorphism, and sexual dimorphism is also apparent in cephalochordates (37); its absence in solitary tunicates reflects the fact that they produce both male and female gametes, and although some are self-sterile, as in *Ciona intestinalis*, most are hermaphroditic, as in *C. savignyi* (38). So little is known concerning reproduction in hagfishes (39, 40) that it is not appropriate to conclude that they do not exhibit sexual dimorphism. Indeed, there is some evidence that, at sexual maturity, females are slightly longer than males (41).

Gonads (Paired = 0, Unpaired = 1). The general condition in chordates is paired gonads, seen in *Branchiostoma* and the vast majority of vertebrates. Hagfish and lampreys possess just a single gonad, and hence, this character has been proposed as a cyclostome synapomorphy (18). However, this condition is achieved through fusion of gonads in lampreys and failed development of a gonad in hagfishes, and furthermore, this condition is achieved in many other vertebrates by the same means.

High Metabolic Rate (Absent = 0, Present = 1). In terms of oxygen consumption, hagfishes (42), lungfishes (43), and *Latimeria* (44) have a significantly lower metabolic rate than *Branchiostoma* (45), *Ciona* (46), lampreys (47), dogfish (48), and *Ambystoma* (49) (there seems to be no data for *Polypterus*). However, the nature of comparison is dubious considering the fact that the comparisons are made among organisms that differ in mass by several orders of magnitude. It is perhaps for this reason that there is no phylogenetic signal in the data, and for this reason, we exclude the character.

Condensed and Discrete Pancreas (Absent = 0, Present = 1). This character has previously been used to draw a distinction between the condensed vs. diffuse endocrine pancreas in lampreys and hagfish, respectively (3–5, 12, 19). The distinction between hagfishes and lampreys is one of degree (13), and indeed, lampreys are polymorphic for this character, both ontogenetically and systematically (50). Rather, hagfishes and lampreys are more similar to one another than to gnathostomes in that there is a complete separation of their endocrine pancreas (islet organ) and their exocrine pancreases. The endocrine and exocrine pancreas are coassociated in crown gnathostomes (50). In *Branchiostoma* and *Ciona*, there is no diverticulum, as there is in hagfishes, lampreys, and gnathostomes, only dispersed insulin-secreting cells in the

walls of the gastrointestinal tract (51, 52). This constitutes the basis for a replacement character.

High Proportion of Serine and Theronine Collagen (Absent = 0, Present = 1). This character was originally codified by Hardisty (18), who drew a distinction between hagfishes vs. lampreys and gnathostomes on similarities in collagen proteins inferred from the proportion of proline vs. serine and threonine; it was perpetuated by Janvier (19), Forey (13), Donoghue et al. (4), and Gess et al. (3). However, there is no longer a need to infer the similarities and differences between hagfish, lamprey, and gnathostome collagen proteins, because these can be determined directly from their amino acid sequences. These show that hagfish, like lampreys and gnathostomes, possess members of both the Col1 and Col2 paralogy groups with Clade A of fibrillar collagens, and furthermore, within these paralogy groups, hagfish and lamprey proteins comprise a clade to the exclusion of gnathostome proteins (53). Higher proportions of proline in lampreys and gnathostomes than in hagfish must, therefore, represent convergence or secondary reversal, although, of course, the original comparison always lacked outgroup constraint. This vertebrate synapomorphy has become a molecular character, and therefore, we exclude the character on this basis.

Lactate Dehydrogenase 5 (Absent = 0, Present = 1). Hardisty (18) drew a distinction between lampreys vs. hagfishes and gnathostomes on the premise that lampreys have just a single lactate dehydrogenase (LDH) isozyme, whereas hagfishes have two, compatible with the gnathostome condition in which there are two gene loci coding for the A and B chains of LDH (18). Donoghue et al. (4) incorrectly coded LDH-5 as absent in hagfish and present in lampreys, and this coding error was perpetuated by Gess et al. (3). However, paralogy relations between LDHA and LDHB clearly indicate duplication from a single LDH gene after the divergence of tunicates from vertebrates (54). Thus, it is clear that lampreys have secondarily lost an LDHA-encoding gene, and thus, this character has no discriminatory power in resolving the relationships among hagfish, lampreys, and gnathostomes. Because of the problem of coding LDH5 as present in lampreys, albeit secondarily lost, we have excluded this vertebrate/craniate synapomorphy a priori.

Typhlosole in Intestine. This character was originally codified as the presence of a spiral intestine by Løvtrup (2) and developed as spiral intestine (or spiral fold intestine) by Janvier (12) and subsequently, typhlosole in intestine by Forey (19), Janvier (9), Donoghue et al. (4), and Gess et al. (3) The character is based on the premise of homology between the spiral valve in the intestine of many gnathostomes and the typhlosole of lampreys. This proposition of homology has been criticized, because the spiral turns of the spiral valve result from torsion of the entire gut in gnathostomes, whereas the typhlosole of lampreys is confined to the mucosa (55); furthermore, hagfish also possess a typhlosole manifest as permanent zig-zag ridges in the mucosa (56). The presence of a typhlosole in *Ciona* (57) indicates that this is not a cyclostome synapomorphy. It is, thus, phylogenetically uninformative, and the character is excluded a priori for this reason.

Inner Ear Hair Cells with Stereocilia Only. Khonsari et al. (1) codified this character to draw out the distinction between lampreys and gnathostomes, which have only stereocilia in their inner ears, and hagfish, which have both stereocilia and a second class of hair cells where there is a long central cilium surrounded by much shorter cilia. However, the presence of this second class of hair cells in hagfish is clearly an autapomorphy and not, therefore, of phylogenetic significance. Given that hagfish, lampreys, and gnathostomes all possess inner ears that contain stereociliae and the outgroup lacks both inner ears and stereocilia, the character is uninformative concerning the phylogenetic relations of hagfish, lampreys, and gnathostomes, and therefore, we exclude it on that basis.

Character Descriptions

1. Neurulation by fusion of neural plate folds (absent = 0, present = 1).
2. Neural crest (absent = 0, present = 1).
3. Ectodermal nasal placode (absent = 0, present = 1).
4. Nasohypophyseal duct (absent = 0, present = 1).
5. Paired nasal placode (absent = 0, present = 1). Khonsari et al. (1). incorrectly coded lampreys as possessing paired nasal placodes (58, 59). The character is applicable to the outgroup, because both *Branchiostoma* (60) and *Ciona* (61) are considered to possess an olfactory placode.
6. Posterior nostrils (absent = 0, present = 1).
7. Extrabuccal posterior nostrils or posterior nostril lateral to the maxillary ramus of the trigeminal nerve (absent = 0, present = 1).
8. Olfactory bulbs (absent = 0, present = 1).
9. Pedunculated olfactory bulbs (absent = 0, present = 1).
10. Five concentric cellular layers in the olfactory bulbs (absent = 0, present = 1).
11. Terminal nerve (absent = 0, present = 1).
12. Single main olfactory organ (absent = 0, present = 1).
13. Two eyes (absent = 0, present = 1).
14. Layers of photoreceptors in the visual organ (absent = 0, present = 1).
15. Lens (absent = 0, present = 1). Hagfish lack a lens, but a lens placode occurs in development (62, 63).
16. Optic chiasma (absent = 0, present = 1). This character is inapplicable for *Branchiostoma* and *Ciona*, because the chiasma can only be present when there are paired eyes.
17. Optic chiasma externally visible (absent = 0, present = 1).
18. Retina (absent = 0, present = 1).
19. Retinal epithelium pigmentation (absent = 0, present = 1). The absence of pigmentation of retinal epithelium in hagfish has been related to the lens placode that does not develop a lens (character 15) (62).
20. Synaptic ribbons in retina (absent = 0, present = 1).
21. Lateral line system (absent = 0, present = 1).
22. Six lateral line placodes—anterodorsal, anteroventral, and supratemporal lateral line nerves (absent = 0, present = 1).
23. Recurrent ramus of the anterior lateral line nerve (absent = 0, present = 1).
24. Spiracular organs (absent = 0, present = 1).
25. Lateral line mechanoreceptors with stair-stepped microvilli (absent = 0, present = 1).
26. Lateral line neuromast cupulae (absent = 0, present = 1).
27. Internal taste buds (absent = 0, present = 1).
28. Facialis innervation of taste buds (absent = 0,present = 1).
29. Chemosensory organs innervated only by cranial nerves (absent = 0, present = 1).
30. Electroreceptors (absent = 0, present = 1). Incorrectly coded in Khonsari et al. (1, 10, 13, 64).
31. Numerous electroreceptive regions on epithelium (absent = 0, present = 1).
32. External opening of the endolymphatic duct (absent = 0, present = 1).
33. Blind-ended endolymphatic duct (absent = 0, present = 1).
34. Semicircular canals (absent = 0, present = 1).
35. Vertical semicircular canals (absent = 0, present = 1). The coding for hagfish has been debated extensively, and there remains a view that, because there are two sensory ampullae in hagfish, their single semicircular canal is an evolutionary rudiment of two that have coalesced (10, 13, 64).

36. Vertical semicircular canals forming distinct loops, well-separated from the saccular part of the labyrinth (absent = 0, present = 1).
37. Three macular partitions (absent = 0, present = 1).
38. Statoliths composed of calcium phosphate (absent = 0, present = 1).
39. Hair cells in the inner ear with cupulae (absent = 0, present = 1).
40. Trigeminal nerve (absent = 0, present = 1).
41. Trigeminal motor nucleus in rhombomere 2 and/or rhombomere 3 (absent = 0, present = 1).
42. Trigeminal ramification limited to the profundal, external, and velobuccal branches (absent = 0, present = 1).
43. Ventral ophthalmic projections in the trigeminal sensory nucleus (absent = 0, present = 1).
44. Mesencephalic trigeminal nucleus (absent = 0, present = 1).
45. Trigemino-spinal projections (absent = 0, present = 1).
46. Fusion of the profundal nerve ganglion with the trigeminal ganglion (absent = 0, present = 1).
47. Superficial ophthalmic trigeminal ramus (absent = 0, present = 1).
48. Fusion of the maxillary trigeminal ramus with the buccal ramus of the anterolateral lateral line nerve (absent = 0, present = 1).
49. Facial nerve (absent = 0, present = 1).
50. Division of the facial nerve into pharyngeal, pre-, and posttrematic branches (absent = 0, present = 1).
51. Distinct facial, glossopharyngeal, and vagal nuclei (absent = 0, present = 1).
52. Hypodermal and dermal nerve plexi (absent = 0, present = 1).
53. More than one postotic cranial nerve (absent = 0, present = 1).
54. Viscero-sensory nerves (absent = 0, present = 1).
55. Periventricular viscerosensory rhombencephalic zone (absent = 0, present = 1).
56. Glossopharyngeal nerve (absent = 0, present = 1).
57. Single glossopharyngeal ganglion (absent = 0, present = 1).
58. Vagal nerve (absent = 0, present = 1).
59. Division of vagal nerve into pharyngeal, pre-, and posttrematic branches (absent = 0, present = 1).
60. Cardiac innervation (absent = 0, present = 1).
61. Oculomotor system (absent = 0, present = 1). This character is contingent on the presence of eyes and is, therefore, inapplicable to the outgroup.
62. Abducent nerve with retractor bulbi innervation (absent = 0, present = 1).
63. Caudal (rhombomeres 5–7) localization of abducent motoneurons (absent = 0, present = 1).
64. Ventral trochlear nucleus (absent = 0, present = 1).
65. Ciliary ganglion (absent = 0, present = 1).
66. Extracranial ciliary ganglion (absent = 0, present = 1).
67. Hypobranchial nerve (absent = 0, present = 1).
68. Ventral branch of spinal ganglionated nerves contributes to the hypobranchial nerve (absent = 0, present = 1).
69. Hypobranchial nerve formed by the fusion of the ventral branches of intracranial nonganglionated postvagal nerves and the ventral branches of spinal ganglionated nerves (absent = 0, present = 1).
70. Cerebellar primordia (absent = 0, present = 1).
71. Corpus cerebelli (absent = 0, present = 1).
72. Hypothalamus (absent = 0, present = 1).
73. Multiple hypothalamic nuclei (absent = 0, present = 1).
74. gonadotropin-releasing hormone (GnRH)-positive cell groups in the caudal diencephalon or the mesencephalon (absent = 0, present = 1).
75. Hypophysis (absent = 0, present = 1).
76. Adenohypophysis: develops from a combined nasohypophyseal placode = 0, develops from a discrete hypophyseal placode = 1. This character is inapplicable to *Branchiostoma* and *Ciona*, because they lack a hypophysis.
77. Adenohypophysis differentiated in a pars intermedia and a pars distalis (absent = 0, present = 1).
78. Median eminence (absent = 0, present = 1).
79. Serotoninergic median raphe (absent = 0, present = 1).
80. Superficial isthmic nucleus (absent = 0, present = 1).
81. Preoptic area with magno- and parvocellular parts (absent = 0, present = 1).
82. Tectum (absent = 0, present = 1).
83. Less than five tectal laminae (absent = 0, present = 1).
84. Thalamus (absent = 0, present = 1).
85. Dorsal and ventral thalami (absent = 0, present = 1).
86. Overlap of the areas with tectal and retinal projections in the dorsal thalamus (absent = 0, present = 1).
87. Periventricular thalamus (absent = 0, present = 1).
88. Protrusion of the dorsal thalamus into the third ventricle (absent = 0, present = 1).
89. Tuberculo-pallial tracts (absent = 0, present = 1).
90. Saccus vasculosus (absent = 0, present = 1).
91. Pretectum (absent = 0, present = 1).
92. Neuronal migration in the pretectum (absent = 0, present = 1).
93. Epithalamus (absent = 0, present = 1).
94. Paraphysis (absent = 0, present = 1).
95. Extraocular photoreceptor region expressing the pineal opsins (absent = 0, present = 1).
96. Telencephalon (absent = 0, present = 1).
97. Telencephalic evagination including the medial pallium (absent = 0, present = 1).
98. Evaginated telencephalon (absent = 0, present = 1).
99. Extensive median septum ependyma (absent = 0, present = 1).
100. Subpallium (absent = 0, present = 1).
101. Subpallial septum expressing acetylcholinesterase (absent = 0, present = 1).
102. Subpallial striatum in contact with the septum (absent = 0, present = 1).
103. Pallium (absent = 0, present = 1).
104. Pallium with mainly migrated neurons (absent = 0, present = 1).
105. Migrated pallium with three subdivisions (absent = 0, present = 1).
106. Partial pallial olfactory projections (absent = 0, present = 1).
107. Olfactory projections restricted to the lateral pallia (absent = 0, present = 1).
108. Spinal cord with spinal nerves and spinal nerve roots (absent = 0, present = 1).
109. Dorsal and ventral roots of the spinal nerves (remain unjoined = 0, join outside the nerve cord = 1). *Ciona* lacks homologs of vertebrate spinal nerves (65), rendering the character inapplicable to this taxon.
110. Ribbon-shaped spinal cord (absent = 0, present = 1).
111. Blood supply in the spinal cord (absent = 0, present = 1).
112. Regularly decreasing spinal cord diameter along the anteroposterior axis (absent = 0, present = 1). Khonsari et al. (1) provided no outgroup coding for this character; this condition obtained in *Branchiostoma* but not in *Ciona*.
113. Dorsal and ventral roots of spinal nerves on the same side of the intersegmental artery (absent = 0, present = 1).
114. Rohon–Béard cells (absent = 0, present = 1). No data are available for tunicates.
115. Lemnothalamic tracts (absent = 0, present = 1). *Branchiostoma* and *Ciona* lack a thalamus (character 83), and therefore, this character is inapplicable for these taxa.
116. Müller cells (absent = 0, present = 1).
117. Mauthner cells (absent = 0, present = 1). This character was incorrectly coded by Khonsari et al. (1), who coded presence for the invertebrate chordates and absence in vertebrates. Mauthner cells are absent from invertebrate

chordates and hagfish but present in lampreys and gnathostomes (66).
118. Mauthner cell axons ensheathed in an isolated fiber (absent = 0, present = 1).
119. Dorsal arcualia (absent = 0, present = 1). Ota and Kuratani (64) have provided a preliminary report of the presence of vertebrae in the embryos of hagfishes.
120. Ventral arcualia (absent = 0, present = 1).
121. Choroid plexi (absent = 0, present = 1).
122. Reissner's fiber (absent = 0, present = 1).
123. Oligodendrocytes (absent = 0, present = 1).
124. Astroglia (absent = 0, present = 1).
125. Anastomotic capillary network in the brain (absent = 0, present = 1).

Additional Characters Augmenting Khonsari et al. (1)

Feeding and Respiration.

126. Pouch-shaped gills (absent = 0, present = 1).
127. Elongate branchial series (more than 10 gill pouches/slits = 0, fewer than 10 = 1).
128. Opercular flaps associated with gill openings (absent = 0, present = 1).
129. Endodermal gill lamellae (absent = 0, present = 1).
130. Gill lamellae with filaments (absent = 0, present = 1).
131. Velum (absent = 0, present = 1).
132. Distinct stomach within digestive tract (absent = 0, present = 1).

Circulatory System.

133. Relative position of atrium and ventricle of heart (well-separated = 0, close to each other = 1). This character is inapplicable to the outgroup, because they lack structures that can be compared with either the atrium or ventricle of vertebrate hearts (67, 68).
134. Closed pericardium (absent = 0, present = 1). This character is inapplicable to Branchiostoma, because it lacks a distinct heart and therefore, lacks a distinct pericardial region that may be compared with the pericardium of vertebrates (67). However, Ciona is interpreted to possess a direct homolog of the vertebrate heart, and this is enclosed in a discrete pericardial cavity (67).
135. Open blood system (absent = 0, present = 1).
136. Paired dorsal aortae (absent = 0, present = 1).
137. Large lateral head vein (absent = 0, present = 1).
138. Pulmonary vein (absent = 0, present = 1).
139. Lymphocyte-based recombinatorial system of anticipatory immunity (absent = 0, present = 1).
140. Lymphocytes with variable lymphocyte receptor (VLR) antigen receptors = 0, true lymphocytes with T and B antigen receptors = 1. Lampreys and hagfishes have conventionally been coded as possessing and lacking true lymphocytes, respectively (3–5). In fact, both lampreys and hagfish possess lymphocyte-like cells (69, 70), but these do not possess the diverse repertoire of T and B antigen receptors characteristic of the true lymphocytes, which are restricted to living jawed vertebrates (71).

Fins and Fin-Folds.

141. Dorsal fin: separate dorsal fin (absent = 0, present = 1).
142. Anal fin separate (absent = 0, present = 1).
143. Fin ray supports (absent = 0, present = 1).
144. Radial muscles (absent = 0, present = 1).
145. Pectoral fins (absent = 0, present = 1).
146. Pelvic fins (absent = 0, present = 1).
147. Monobasal paired fins (absent = 0, present = 1). This character is inapplicable to taxa that lack paired fins and also salamander.
148. Tail shape (no distinct lobes developed = 0, ventral lobe much larger than dorsal = 1, dorsal lobe much larger than ventral = 2, dorsal and ventral lobes almost equally developed = 3).
149. Chordal disposition relative to tail development (isochordal = 0, hypochordal = 1, hyperchordal = 2). Hagfish are conventionally coded as isochordal, (3). However, Janvier (72) has shown that they are hypochordal.

Skeletal.

150. Ability to synthesize creatine phosphatase (absent = 0, present = 1).
151. Visceral arches fused to the neurocranium (absent = 0, present = 1).
152. Relative position of the pharyngeal skeleton to the gills and associated vasculature (lateral = 0, medial = 1). This character has been proposed as a general feature or synapomorphy of cyclostomes by numerous authors (8, 12, 13, 18). However, it may be a primitive character for chordates (73, 74), if not deuterostomes.
153. Keratinous teeth (absent = 0, present = 1).
154. Trematic rings (absent = 0, present = 1).
155. Piston cartilage and apical plate (absent = 0, present = 1).
156. Midline retractor muscle, dorsal to piston cartilage, and paired retractor muscles (absent = 0, present = 1).
157. Longitudinally aligned tooth rows providing transverse bite (absent = 0, present = 1).
158. Jaws (absent = 0, present = 1).
159. Braincase with lateral walls (absent = 0, present = 1).
160. Neurocranium entirely closed dorsally and covering the brain (absent = 0, present = 1).
161. Occiput enclosing vagus and glossopharyngeal. Enclosure of cranial nerves IX and X and glossopharyngeal and vagus nerves (absent = 0, present = 1).
162. Perichondral bone (absent = 0, present = 1).
163. Calcified cartilage (absent = 0, present = 1). The report of calcified cartilage in a lamprey is unconvincing given that it is based solely on the observation that the cartilage attenuates X-rays (75).
164. Cellular dermal bone (absent = 0, present = 1).
165. Dentine (absent = 0, present = 1).
166. Enamel/oid (absent = 0, present = 1).
167. Sclerotic ossicles (absent = 0, present = 1). Using information reported by de Beer (74) and Franz-Odendaal and Hall (76), the condition in *Ambystoma* is unknown, but de Beer (74) records only one species of salamander with sclerotic ossicles.
168. Ossified endoskeletal sclera encapsulating the eye (absent = 0, present = 1). Using information reported by de Beer (74) and Franz-Odendaal and Hall (76), the condition in *Ambystoma* is unknown.

Physiological.

169. Hemoglobins (exist as monomers when oxygenated and form complex dimeric or tetrameric aggregates when deoxygenated = 0, exist as stable tetramers = 1). It has been argued that oxygen transport is a role to which globins were co-opted during the transition to multicellularity (77), and therefore, it should be possible to polarize the character states encountered in hagfish, lampreys, and gnathostomes. However, Hoffmann et al. (36) have recently argued that cyclostomes and gnathostomes have co-opted distinct phylogenetically

globins to the role of oxygen transport. Structural and functional data are lacking for invertebrate chordate globins, although hemoglobins are known from *Branchiostoma* (34).

170. Heart response to catecholamines (absent = 0, present = 1). Hagfish have traditionally been coded absent for heart response to catecholamines, although it has long been established that a variety of catecholamines have a significant effect on the heart rate of the multifarious hearts of hagfishes (78–80). Tunicates and amphioxus have a cardiac pump that is in some sense homologous to vertebrate heart (67, 68), and one of these in *Branchiostoma*, the endostyle, is associated with catecholamine-containing cells. However, it has not been shown that there is a cardiovascular response to catecholamines in either clade of invertebrate chordates, and thus, they are coded as unknown. No data are available for *Latimeria* and *Polypterus*.
171. High blood pressure (absent = 0, present = 1).
172. Hyperosmoregulation (absent = 0, present = 1). Hagfishes, like the outgroup, are essentially isoosmotic with seawater, whereas lampreys and gnathostomes are hyperosmotic. Schaeffer and Thompson (10) argued that lampreys and gnathostomes achieved this condition by different means, but, whereas gnathostomes (primitively, at least) emphasize urea synthesis and retention, lampreys seem to adopt the same gill-based mechanisms of osmoregulation as marine teleosts (81). That said, these same mechanisms are apparent in the mitochondria-rich cells in the gill epithelia of hagfishes (82), although they remain incapable of establishing an ionic gradient across their gills (81).
173. Pituitary control of gametogenesis (absent = 0, present = 1). In contrast to lampreys and gnathostomes, hagfish have previously been coded as lacking pituitary control of gametogenesis (3–5, 12), but for some time, circumstantial evidence has indicated that this view is incorrect. Specifically, it had been shown that gonadotrophins are expressed not only in the pituitary of hagfishes with developing gonads (83–85) but also in their gonads and that the glycoprotein hormone synthesized in the hagfish adenohypophysis is capable of inducing sex steroids in vitro (86). This character is contingent on character 75 (hypophysis), and therefore, it is coded as inapplicable for *Branchiostoma* and *Ciona*. Attempts have also been made to draw a distinction between lampreys and gnathostomes vs. hagfish on the grounds that (*i*) hagfish show no evidence of pituitary control of melanophores, because hagfishes apparently show no color change (12)—although this is based on a paucity of data on their reproductive biology and regardless, the hagfish pituitary contains proopiomelanocortin-like secreting cells (87) (the joint precursor protein of melanotropins and corticotopin in gnathostomes not found in lampreys)—and (*ii*) gonadial secretions responsible for secondary sexual characteristics (12) assume that there are no secondary sexual differences in hagfishes, a view that may be incorrect (41). In *Branchiostoma*, there is evidence of gonadotropin-based hormonal control of gametogenesis (88), and Candiani et al. (89) have argued for homology between the adenohypophysis of vertebrates and Hatschek's Pit in *Branchiostoma*; however, no link has yet been made between the Hatschek's Pit and gametogenesis. In *Ciona*, it has been shown that gonadotopin receptors are expressed in the neural gland (90), but no evidence has been presented linking their activity to gametogenesis.
174. Ion transport in gills (absent = 0, present = 1). Hagfishes have invariably been coded as lacking ion transport in gills (3–5), and it is true that they do not establish an ionic gradient across their gills (81). However, ion transport does occur, and its presence is now well-documented in both *Myxine glutinosa* (91, 92) and *Eptatretus stouti* (82). Furthermore, although hagfish cannot regulate their osmolality, they can regulate the ionic concentration of Ca and Mg ions (93). There are no data for *Branchiostoma* or *Ciona*, and although it is likely that they are iso-osmotic with seawater, as with hagfish, this does not preclude the possibility that their gills are incapable of ion transport. There are few data on ionoregulation in *Latimeria* (94), *Polypterus*, and lungfishes (95). However, both *Polypterus* and lungfishes are freshwater species, and therefore, their gills must be capable of ionoregulation.

Miscellaneous.

175. Spleen (absent = 0, present = 1). Neither hagfish nor lampreys possess what might be considered a discrete and condensed spleen. Hagfish possess dispersed lymphoid tissue within the submucosa of the intestine (96) associated with the portal vein (97), whereas lymphoid tissue is associated with the typhlosole portion of the intestine in lampreys (96). Amemiya et al. (98) have called into question the traditional histological similarities between the lympho-hematopoietic structures in lampreys and gnathostomes, given that they are now known to possess distinct adaptive immune systems.
176. Collecting tubules in kidneys (absent = 0, present = 1).
177. Separate endocrine and exocrine pancreas (absent = 0, present = 1). Hagfishes and lampreys are unique in the complete separation of their endocrine pancreas (islet organ) and their exocrine pancreas (50). The endocrine and exocrine pancreas are coassociated in crown gnathostomes (50). In *Branchiostoma* and *Ciona*, there is no diverticulum as there is in hagfishes, lampreys, and gnathostomes, only dispersed insulin-secreting cells in the walls of the gastrointestinal tract (51, 52).
178. A cells (absent = 0, present = 1). Lampreys were coded as possessing pancreatic A cells in their endocrine pancreas in a number of previous cladistic analyses (3–5). However, both lampreys and hagfishes lack A cells, which are exclusive to living jawed vertebrates (50, 99).
179. B cells (absent = 0, present = 1). B cells are found in the endocrine pancreas of all vertebrates (50, 99).
180. Corpus luteum (absent = 0, present = 1). The corpus luteum is a transient endocrine gland that develops from the postovulatory or atretic follicles and secretes progesterone; it is thought to be related to egg retention. A corpus luteum is known in hagfish (100), *Squalus* (101), and salamander (100) but not lamprey (102). There are no data for *Branchiostoma* and *Ciona*, and given that they are multiple spawners, there is no a priori reason to conclude that they lack a corpus luteum; thus, they are coded as unknown.
181. Male gametes shed directly through the coelom (absent = 0, present= 1). In both *Branchiostoma* and *Ciona*, the gametes are shed into the atrium (which has no homologs in vertebrates) rather than into the coelom (103). Thus, there is no a priori justification for interpreting this character as general to craniates/vertebrates or *contra* (13, 19).
182. Forward migration of postotic myomeres (absent = 0, present = 1).
183. Larval phase (absent = 0, present = 1).
184. Horizontal septum in trunk myomeres (absent = 0, present = 1).

ACKNOWLEDGMENTS. We thank the following for providing help with the codification of characters and their coding: Keith Choe, Michael I. Coates, David Evans, Peter L. Forey, Martin Grosell, Philippe Janvier, Federico Hoffmann, Roman Hussein Khonsari, Trevor Lamb, Jon Mallatt, R. Glenn Northcutt, and Roy E. Weber.

1. Khonsari RH, Li B, Vernier P, Northcutt RG, Janvier P (2009) Agnathan brain anatomy and craniate phylogeny. *Acta Zool* 90:52–68.
2. Løvtrup S (1977) *The Phylogeny of the Vertebrata* (Wiley, New York).
3. Gess RW, Coates MI, Rubidge BS (2006) A lamprey from the Devonian period of South Africa. *Nature* 443:981–984.
4. Donoghue PCJ, Forey PL, Aldridge RJ (2000) Conodont affinity and chordate phylogeny. *Biol Rev Camb Philos Soc* 75:191–251.
5. Donoghue PCJ, Smith MP (2001) The anatomy of *Turinia pagei* (Powrie) and the phylogenetic status of the Thelodonti. *Trans R Soc Edinb Earth Sci* 92:15–37.
6. Donoghue PCJ, Purnell MA, Aldridge RJ (1998) Conodont anatomy, chordate phylogeny and vertebrate classification. *Lethaia* 31:211–219.
7. Near TJ (2009) Conflict and resolution between phylogenies inferred from molecular and phenotypic data sets for hagfish, lampreys, and gnathostomes. *J Exp Zoolog B Mol Dev Evol* 312:749–761.
8. Yalden DW (1985) Feeding mechanisms as evidence of cyclostome monophyly. *Zool J Linn Soc* 84:291–300.
9. Janvier P (1996) The dawn of the vertebrates: characters versus common ascent in the rise of current vertebrate phylogenies. *Palaeontology* 39:259–287.
10. Schaeffer B, Thomson KS (1980) Reflections on agnathan-gnathostome relationships. *Aspects of Vertebrate History: Essays in Honor of Edwin Harris Colbert*, ed Jacobs LL (Museum of Northern Arizona Press, Flagstaff, AZ), pp 19–33.
11. Maisey JG (1986) Head and tails: A chordate phylogeny. *Cladistics* 2:201–256.
12. Forey PL (1995) Agnathans recent and fossil, and the origin of jawed vertebrates. *Rev Fish Biol Fish* 5:267–303.
13. Forey PL (1984) Yet more reflections on agnathan-gnathostome relationships. *J Vertebr Paleontol* 4:330–343.
14. Halstead LB (1982) Evolutionary trends and the phylogeny of the Agnatha. *Problems of Phylogenetic Reconstruction. Systematics Association Special*, eds Joysey KA, Friday AE (Academic, London), Vol 21, pp 159–196.
15. Stensiö EA (1968) The cyclostomes, with special reference to the diphyletic origin of the Petromyzontida and Myxinoidea. *Current Problems in Lower Vertebrate Phylogeny, Nobel Symposium 4*, ed Ørvig T (Almquist & Wiksell, Stockholm), pp 13–71.
16. Jarvik E (1980) *Basic Structure and Evolution of Vertebrates* (Academic, London).
17. Hardisty MW (1979) *The Biology of Cyclostomes* (Chapman & Hall, London).
18. Hardisty MW (1982) *Lampreys and Hagfishes: Analysis of Cyclostome Relationships. The Biology of Lampreys* (Academic Press, London), pp 165–259.
19. Janvier P (1981) The phylogeny of the Craniata, with particular reference to the significance of fossil "agnathans". *J Vertebr Paleontol* 1:121–159.
20. Jefferies RPS (1986) *The Ancestry of the Vertebrates* (British Museum Natural History, London).
21. Johansen K (1963) The cardiovascular system of *Myxine glutinosa* L. *The Biology of Myxine*, eds Brodal A, Fänge R (Universitetsforlaget, Oslo), pp 289–316.
22. Epple A, Hilliard RW, Potter IC (1985) The cardiovascular chromafin cell system of the Southern Hemisphere lamprey, *Geotria australis* Gray. *J Morphol* 183:225–231.
23. Forster ME, Russell MJ, Hambleton DC, Olson KR (2001) Blood and extracellular fluid volume in whole body and tissues of the Pacific hagfish, *Eptatretus stouti*. *Physiol Biochem Zool* 74:750–756.
24. Thorson TB (1959) Partitioning of body water in Sea Lamprey. *Science* 130:99–100.
25. Good JP, Wells A, Hazon N (2008) Measurement of blood volume in the elasmobranch fish *Scyliorhinus canicula* following acute and long-term salinity transfers. *J Fish Biol* 73:1301–1313.
26. Conte FP, Wagner HH, Harris TO (1963) Measurement of blood volume in the fish (*Salmo gairdneri gairdneri*). *Am J Physiol* 205:533–540.
27. Sawyer WH, Blair-West JR, Simpson PA, Sawyer MK (1976) Renal responses of Australian lungfish to vasotocin, angiotensin II, and NaCl infusion. *Am J Physiol* 231:593–602.
28. Conte FP, Wagner HH, Harris TO (1963) Measurement of blood volume in the fish (*Salmo gairdneri gairdneri*). *Am J Physiol* 205:533–540.
29. Müller G, Fago A, Weber RE (2003) Water regulates oxygen binding in hagfish (*Myxine glutinosa*) hemoglobin. *J Exp Biol* 206:1389–1395.
30. Weber RE, Fago A (2004) Functional adaptation and its molecular basis in vertebrate hemoglobins, neuroglobins and cytoglobins. *Respir Physiol Neurobiol* 144:141–159.
31. Hughes GM, Itazawa Y (1972) The effect of temperature on the respiratory function of coelacanth blood. *Experientia* 28:1247.
32. Vokac Z, El Din Ahmed N, Magid AMA (1972) The *in vitro* respiratory properties of blood of the primitive fish *Polypterus senegalus*. *J Exp Biol* 57:461–469.
33. Pinder A, Burggren W (1983) Respiration during chronic hypoxia and hyperoxia in larval and adult bullfrogs (Rana catesbeiana) II. Changes in respiratory properties of whole blood. *J Exp Biol* 105:205–213.
34. Bishop JJ, Vandergon TL, Green DB, Doeller JE, Kraus DW (1998) A high-affinity hemoglobin is expressed in the notochord of amphioxus, Branchiostoma californiense. *Biol Bull* 195:255–259.
35. Weber RE, Vinogradov SN (2001) Nonvertebrate hemoglobins: Functions and molecular adaptations. *Physiol Rev* 81:569–628.
36. Hoffmann FG, Opazo JC, Storz JF (2010) Gene cooption and convergent evolution of oxygen transport hemoglobins in jawed and jawless vertebrates. *Proc Natl Acad Sci USA* 107:14274–14279.
37. Garcia-Fernàndez J, Benito-Gutiérrez É (2009) It's a long way from amphioxus: Descendants of the earliest chordate. *Bioessays* 31:665–675.
38. Jiang D, Smith WC (2005) Self- and cross-fertilization in the solitary ascidian *Ciona savignyi*. *Biol Bull* 209:107–112.
39. Gorbman A (1997) Hagfish development. *Zoolog Sci* 14:375–390.
40. Gorbman A (1990) Sex differentiation in the hagfish Eptatretus stouti. *Gen Comp Endocrinol* 77:309–323.
41. Nozaki M, Ichikawa T, Tsuneki K, Kobayashi H (2000) Seasonal development of gonads of the hagfish, Eptatretus burgeri, correlated with their seasonal migration. *Zoolog Sci* 17:225–232.
42. Forster ME (1990) Confirmation of the low metabolic rate of hagfish. *Comp Biochem Physiol A Comp Physiol* 96:113–116.
43. Seifert AW, Chapman LJ (2006) Respiratory allocation and standard rate of metabolism in the African lungfish, *Protopterus aethiopicus*. *Comp Biochem Physiol A Mol Integr Physiol* 143:142–148.
44. Fricke H, Hissmann K (2000) Feeding ecology and evolutionary survival of the living coelacanth Latimeria chalumnae. *Mar Biol* 136:379–386.
45. Schmitz A, Gemmel M, Perry SF (2000) Morphometric partitioning of respiratory surfaces in amphioxus (*Branchiostoma lanceolatum* Pallas). *J Exp Biol* 203:3381–3390.
46. Shumway SE (1978) Respiration, pumping activity and heart rate in *Ciona intestinalis* exposed to fluctuating salinities. *Mar Biol* 48:235–242.
47. Potter IC, Macey DJ, Roberts AR (1996) Oxygen consumption by adults of the southern hemisphere lamprey *Geotria australis* in air. *J Exp Zool* 276:254–261.
48. Sims DW (1996) The effect of body size on the standard metabolic rate of the lesser spotted dogfish. *J Fish Biol* 48:542–544.
49. Ryan TJ, Hopkins WA (2000) Interaction of sex and size and the standard metabolic rate of paedomorphic Ambystoma talpoideum: Size does matter. *Copeia* 3: 808–812.
50. Youson JH, Al-Mahrouki AA (1999) Ontogenetic and phylogenetic development of the endocrine pancreas (islet organ) in fish. *Gen Comp Endocrinol* 116:303–335.
51. Chan SJ, Cao QP, Steiner DF (1990) Evolution of the insulin superfamily: Cloning of a hybrid insulin/insulin-like growth factor cDNA from amphioxus. *Proc Natl Acad Sci USA* 87:9319–9323.
52. Reinecke M, Betzler D, Drakenberg K, Falkmer S, Sara VR (1993) Occurrence of members of the insulin superfamily in central nervous system and digestive tract of protochordates. *Histochemistry* 99:277–285.
53. Zhang G, Cohn MJ (2006) Hagfish and lancelet fibrillar collagens reveal that type II collagen-based cartilage evolved in stem vertebrates. *Proc Natl Acad Sci USA* 103: 16829–16833.
54. Li YJ, Tsoi SCM, Mannen H, Shoei-lung Li S (2002) Phylogenetic analysis of vertebrate lactate dehydrogenase (LDH) multigene families. *J Mol Evol* 54:614–624.
55. Youson JH (1981) The alimentary canal. *The Biology of Lampreys*, eds Hardisty MW, Potter IC (Academic, London), Vol 3, pp 95–189.
56. Adam H (1963) Structure and histochemistry of the alimentary canal. *The Biology of Myxine*, eds Brodal A, Fänge R (Universitetsforlaget, Oslo), pp 256–288.
57. Berrill NJ (1947) The development and growth of *Ciona*. *J Mar Biol Assoc UK* 26: 616–625.
58. Gorbman A, Tamarin A (1985) Early development of oral, olfactory and adenohypophyseal structures of agnathans and its evolutionary implications. *Evolutionary Biology of Primitive Fishes*, eds Foreman RE, Gorbman A, Dodd JM, Olsson R (Plenum, New York), pp 165–185.
59. Uchida K, Murakami Y, Kuraku S, Hirano S, Kuratani S (2003) Development of the adenohypophysis in the lamprey: Evolution of epigenetic patterning programs in organogenesis. *J Exp Zoolog B Mol Dev Evol* 300:32–47.
60. Holland LZ, Holland ND (2001) Evolution of neural crest and placodes: Amphioxus as a model for the ancestral vertebrate? *J Anat* 199:85–98.
61. Mazet F, et al. (2005) Molecular evidence from Ciona intestinalis for the evolutionary origin of vertebrate sensory placodes. *Dev Biol* 282:494–508.
62. Wicht H, Northcutt RG (1995) Ontogeny of the head of the Pacific hagfish (*Eptatretus stouti*, Myxinoidea): Development of the lateral line system. *Philos Trans R Soc Lond B Biol Sci* 349:119–134.
63. Wicht H, Tusch U (1998) Ontogeny of the head and nervous system of myxinoids. *The Biology of Hagfishes*, eds Jørgensen JM, Lomholt JP, Weber RE, Malte H (Chapman & Hall, London), pp 431–451.
64. Ota KG, Kuratani S (2009) Phylogeny of early vertebrates based on evidence from developmental study of hagfish. *Integr Comp Biol* 49:e128.
65. Fritzsch B, Northcutt RG (1993) Cranial and spinal nerve organization in amphioxus and lampreys: Evidence for an ancestral craniate pattern. *Acta Anat (Basel)* 148: 96–109.
66. Bierman HS, Zottoli SJ, Hale ME (2009) Evolution of the Mauthner axon cap. *Brain Behav Evol* 73:174–187.
67. Simões-Costa MS, et al. (2005) The evolutionary origin of cardiac chambers. *Dev Biol* 277:1–15.
68. Xavier-Neto J, et al. (2007) Parallel avenues in the evolution of hearts and pumping organs. *Cell Mol Life Sci* 64:719–734.
69. Pancer Z, et al. (2005) Variable lymphocyte receptors in hagfish. *Proc Natl Acad Sci USA* 102:9224–9229.
70. Alder MN, et al. (2005) Diversity and function of adaptive immune receptors in a jawless vertebrate. *Science* 310:1970–1973.
71. Pancer Z, Cooper MD (2006) The evolution of adaptive immunity. *Annu Rev Immunol* 24:497–518.
72. Janvier P (1998) Les vertébrés avant le Silurien. *Geobios* 30:931–950.
73. Rychel AL, Smith SE, Shimamoto HT, Swalla BJ (2006) Evolution and development of the chordates: Collagen and pharyngeal cartilage. *Mol Biol Evol* 23:541–549.
74. de Beer GR (1937) *The Vertebrate Skull* (Oxford University Press, Oxford).

75. Bardack D, Zangerl R (1971) Lampreys in the fossil record. *The Biology of Lampreys*, eds Hardisty MW, Potter IC (Academic, London), Vol 1, pp 67–84.
76. Franz-Odendaal TA, Hall BK (2006) Skeletal elements within teleost eyes and a discussion of their homology. *J Morphol* 267:1326–1337.
77. Vinogradov SN, et al. (2006) A phylogenomic profile of globins. *BMC Evol Biol* 6:31.
78. Axelsson M, Farrell AP, Nilsson S (1990) Effects of hypoxia and drugs on the cardiovascular dynamics of the Atlantic hagfish *Myxine glutinosa*. *J Exp Biol* 151:297–316.
79. Johnsson M, Axelsson M (1996) Control of the systemic heart and the portal heart of Myxine glutinosa. *J Exp Biol* 199:1429–1434.
80. Johnsson M, Axelsson M, Davison W, Forster ME, Nilsson S (1996) Effects of preload and afterload on the performance of the *in situ* perfused portal heart of the New Zealand hagfish *Eptatretus cirrhatus*. *J Exp Biol* 199:401–405.
81. Evans DH, Piermarini PM, Choe KP (2005) The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol Rev* 85:97–177.
82. Tresguerres M, Parks SK, Goss GG (2006) V-H(+)-ATPase, Na(+)/K(+)-ATPase and NHE2 immunoreactivity in the gill epithelium of the Pacific hagfish (Epatretus stoutii). *Comp Biochem Physiol A Mol Integr Physiol* 145:312–321.
83. Nozaki M, Shimotani T, Uchida K (2007) Gonadotropin-like and adrenocorticotropin-like cells in the pituitary gland of hagfish, Paramyxine atami; immunohistochemistry in combination with lectin histochemistry. *Cell Tissue Res* 328:563–572.
84. Nozaki M, Oshima Y, Shimotani T, Sower SA (2003) Immunohistochemical detection of gonadotropin-like material in the hagfish pituitary, correlated with their gonadal conditions. 85–87.
85. Miki M, Shimotani T, Uchida K, Hirano S, Nozaki M (2006) Immunohistochemical detection of gonadotropin-like material in the pituitary of brown hagfish (*Paramyxine atami*) correlated with their gonadal functions and effect of estrogen treatment. *Gen Comp Endocrinol* 148:15–21.
86. Uchida K, et al. (2010) Evolutionary origin of a functional gonadotropin in the pituitary of the most primitive vertebrate, hagfish. *Proc Natl Acad Sci USA* 107: 15832–15837.
87. Nozaki M, et al. (2005) Distribution of immunoreactive adenohypophysial cell types in the pituitaries of the Atlantic and the Pacific hagfish, Myxine glutinosa and Eptatretus burgeri. *Gen Comp Endocrinol* 143:142–150.
88. Chambery A, Parente A, Topo E, Garcia-Fernàndez J, D'Aniello S (2009) Characterization and putative role of a type I gonadotropin-releasing hormone in the cephalochordate amphioxus. *Endocrinology* 150:812–820.
89. Candiani S, Holland ND, Oliveri D, Parodi M, Pestarino M (2007) *Expression of the Amphioxus Pit-1 Gene (AmphiPOU1F1/Pit-1) Exclusively in the Developing Preoral Organ, a Putative Homolog of the Vertebrate Adenohypophysis* (Pergamon, Oxford), pp 324–330.
90. Kusakabe T, Mishima S, Shimada I, Kitajima Y, Tsuda M (2003) Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian Ciona intestinalis. *Gene* 322:77–84.
91. Bartels H (1996) *The Gills of Hagfishes*, eds Jorgensen JM, Lomholt JP, Weber RE, Malte H (Chapman & Hall, London), pp 205–222.
92. Choe KP, Edwards S, Morrison-Shetlar AI, Toop T, Claiborne JB (1999) Immunolocalization of Na+/K(+)-ATPase in mitochondrion-rich cells of the atlantic hagfish (*Myxine glutinosa*) gill. *Comp Biochem Physiol A Mol Integr Physiol* 124:161–168.
93. Sardella BA, Baker DW, Brauner CJ (2009) The effects of variable water salinity and ionic composition on the plasma status of the Pacific Hagfish (*Eptatretus stoutii*). *J Comp Physiol B* 179:721–728.
94. 2Marshall WS, Grosell M (2005) Ion transport, osmoregulation, and acid-base balance. *The Physiology of Fishes*, eds Evans DH, Claiborne JB (CRC, Boca Raton, FL), pp 177–230.
95. Wilkie MP, et al. (2007) The African lungfish (*Protopterus dolloi*): Ionoregulation and osmoregulation in a fish out of water. *Physiol Biochem Zool* 80:99–112.
96. Riviere HB, Cooper EL, Reddy AL, Hildemann WH (1975) In search of the hagfish thymus. *Am Zool* 15:39–49.
97. Satchell GH (1991) *Physiology and Form of Fish Circulation* (Cambridge University Press, Cambridge, UK).
98. Amemiya CT, Saha NR, Zapata A (2007) Evolution and development of immunological structures in the lamprey. *Curr Opin Immunol* 19:535–541.
99. Madsen OD (2007) Pancreas phylogeny and ontogeny in relation to a 'pancreatic stem cell'. *C R Biol* 330:534–537.
100. Powell ML, Kavanaugh S, Sower SA (2006) Identification of a functional corpus luteum in the Atlantic hagfish, *Myxine glutinosa*. *Gen Comp Endocrinol* 148:95–101.
101. Tsang P, Callard IP (1987) Luteal progesterone production and regulation in the vivaparous dogfish, *Squalus acanthias*. *J Exp Zool* 241:377–382.
102. Chieffi G, Chieffi Baccari G (1998) Corpora lutea of nonmammalian species. *Encyclopedia of Reproduction*, eds Knobil E, Neill JD (Academic, San Diego), Vol 1.
103. Wickstead JH (1975) Chordata: Acrania (Cephalochordata). *Reproduction of Marine Invertebrates. Vol. II. Entoprocta and Less Coelomates*, eds Giese AC, Pearse JS (Academic, New York), pp 283–319.

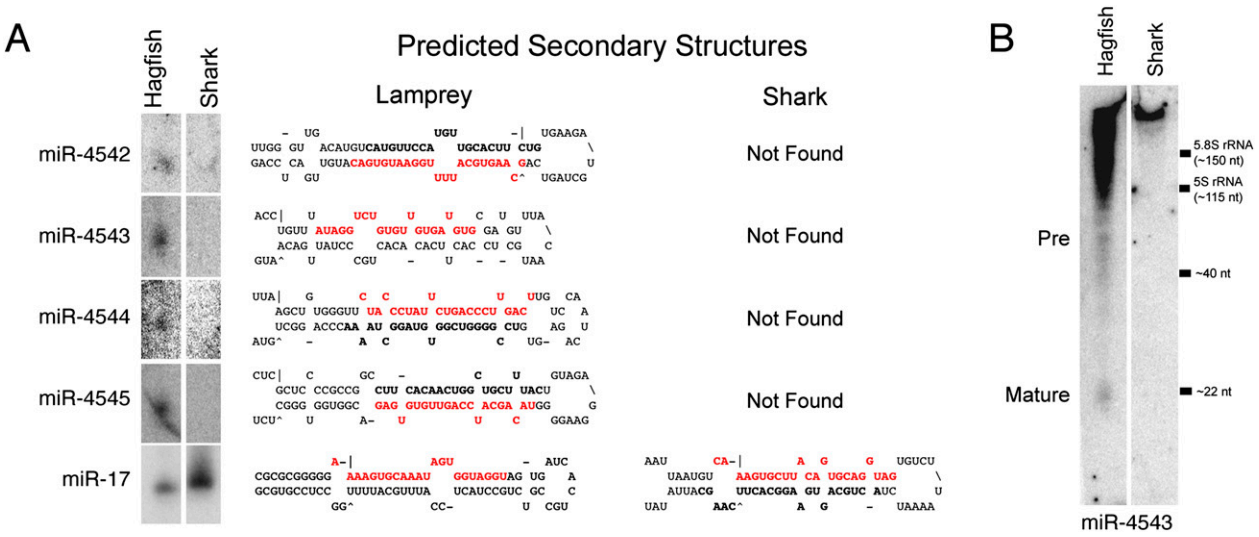

Fig. S1. Northern detection and bioinformatic results of the four cyclostome microRNAs (miRNAs) in hagfish, lamprey, and shark. (*A*) Detection of the four cyclostome miRNAs in lamprey, hagfish lamprey, and hagfish, but not sharks. All four miRNAs are detected in Atlantic hagfish *M. glutinosa* total RNA preparations, and all are found in the genomic traces of the sea lamprey *Petromyzon marinus* (in addition to being present in total RNA preparations of both lamprey species) (Dataset S1); however, none of them are detected in total RNA preparations from the catshark *Scyliorhinus canicula* or found in the genomic traces of the elephant shark *Callorhinchus milii*. In contrast, a Northern analysis against the vertebrate-specific miRNA, miR-17, is both detected by Northern analysis and found in the genomic traces of cyclostomes and sharks. The mature sequence detected in our miRNA libraraies (Dataset S1) is shown in red; star sequences are shown in bold. (*B*) Detection of the mature sequence of miR-4543 as a ~22mer and the presequence as a ~70mer in the Atlantic hagfish *M. glutinosa*. Note that neither are detected in a total RNA preparation from the catshark *S. canicula*.

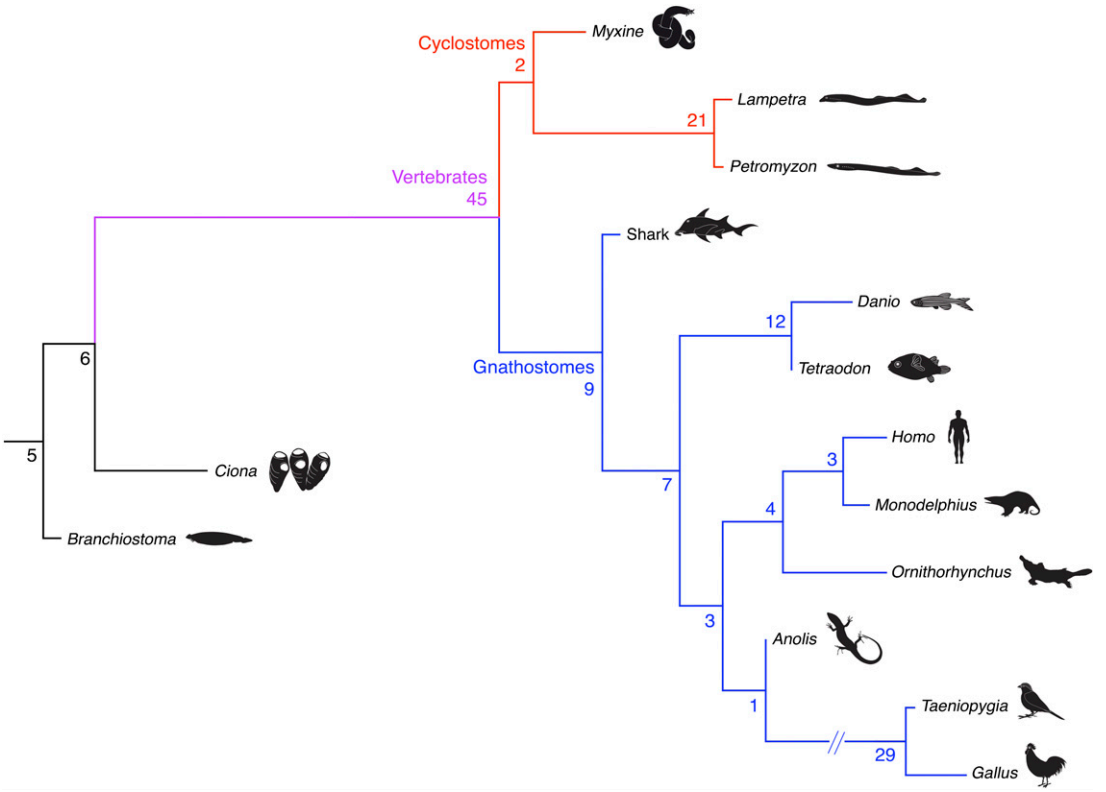

Fig. S2. Maximum parsimony analysis of miRNA families in all chordate taxa investigated (complete matrix in Dataset S2). Numbers at each of the nodes represent the Bremer Support Indexes. Note that branch lengths are scaled according to the number of changes that have occurred (Fig. 2), but the branch leading to birds has been artificially shortened. Note the monophyly of Cyclostomata (red) and the recovery of all known clades including gnathostomes (blue) and vertebrates (magenta).

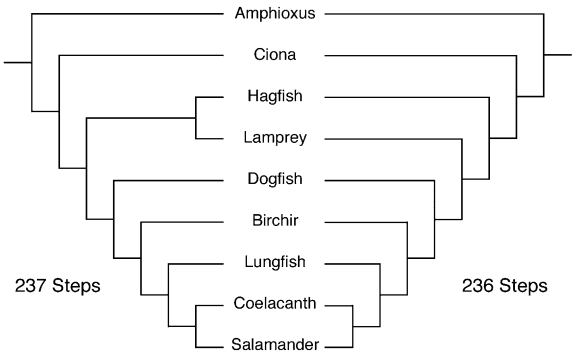

Fig. S3. Maximum parsimony analysis of the morphological data matrix (Dataset S3). The tree supporting cyclostome monophyly (left side) is only one step longer than the shortest tree that supports cyclostome paraphyly (right side).

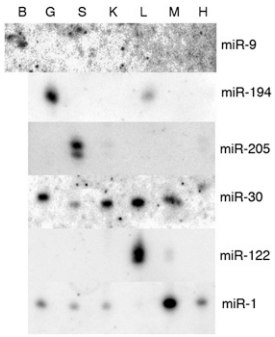

Fig. S4. Northern detection of miRNAs in Atlantic hagfish *M. glutinosa.* The highest expressed miRNA from each lamprey organ (Fig. 4 and Dataset S4) is also expressed in the same organ of the hagfish. B, brain; G, gut; S, skin; K, kidney; L, liver; M, muscle; H, heart.

Dataset S1. Expression levels (number of copies sequenced) and alignments of all sequences identified in this study, including secondary structures of all genes identified in the genomic traces of the lamprey *P. marinus*

Dataset S1 (XLS)

Dataset S2. miRNA matrix for parsimony analysis (nexus file format)

Dataset S2 (TXT)

Dataset S3. Morphology matrix for parsimony analysis (nexus file format)

Dataset S3 (TXT)

Dataset S4. Number of mature miRNA reads from *Lampetra planeri* larva and *P. marinus* adult tissues and organs sorted by expression level

Dataset S4 (XLS)