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Agnathan brain anatomy and craniate phylogeny

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Abstract

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The central nervous system of hagfishes displays unique characteristics that are distinct from any other craniate neuroanatomic features. Whether these hagfish characters are general for all craniates, autapomorphies of hagfishes, or merely a derived state of the general cyclostome condition is still a matter of debate that relates to the question of the monophyly or paraphyly of the cyclostomes. The present cladistic study includes 123 neuroanatomical characters of nine chordate species and supports cyclostome paraphyly, in contrast to most current molecular sequence-based phylogenies, which support cyclostome monophyly. An understanding of the unique neural characters in hagfishes is critical to inspiring further comparative and developmental studies with regards to these two conflicting results and the very deep divergence between craniates and their presumed sister groups. The recent access to hagfish developmental data may provide exciting perspectives in the understanding and characterization of the basalmost craniate node and the interpretation of hagfish brain structure.

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Introduction

Hagfishes and lampreys are the only extant agnathans (jawless vertebrates), classically gathered in a taxon Cyclostomi (cyclostomes). The monophyly of agnathans and, more recently, cyclostomes has been a heatedly debated question among comparative anatomists and palaeontologists for over a century. This issue is crucial in the definition of the morphotypic vertebrate (or craniate) condition. The central nervous system in hagfishes defies detailed morphological comparison with that of other craniates, including the lampreys. Nevertheless, most recent molecular phylogenies seem to support the monophyly of the cyclostomes; that is, lampreys and hagfishes are the living sister group of gnathostomes (jawed vertebrates). Since Lovstrup's formalization of the theory of cyclostome paraphyly (or "vertebrate theory") on the basis of a few anatomical and physiological characters (Lovtrup 1977), more such characters have been pointed out in support of either this theory, or its rival, cyclostome monophyly (or "cyclostome theory"). For instance, Donoghue *et al.* (2000) have led a cladistic study on 103 morphological characters on 17 chordate taxa (mainly fossil) and found a strong support for cyclostome (and agnathan) paraphyly. A single morphological study on the oral region and 'lingual' apparatus of cyclostomes lends some support to their monophyly (Yalden 1985), but homologies are particularly difficult to assess in this anatomical territory. In contrast, the vast majority of molecular phylogenetic studies support agnathan monophyly.

Although some neuroanatomical characters have been considered in the early years of this debate, no extensive character analysis has ever been made on the basis of a large sample of reputedly primitive vertebrates, including hagfishes, lampreys and plesiomorphic representatives of the major gnathostome taxa. This is surprising, because neuroanatomical characters long had the (untested) reputation

of providing a strong phylogenetic signal (at any rate stronger than that of the venous system, for example); however, it may be explained by the fact neuroanatomists working on primitive living fishes are scarce.

We examined data from nine species, representing the major chordate and craniate groups: two 'protochordates', (1) the tunicate *Ciona intestinalis* and (2) the cephalochordate *Branchiostoma lanceolatum* (amphioxus); two agnathans, (3) the pacific hagfish *Eptatretus stoutii*, and (4) a lamprey, *Petromyzon marinus*; and five gnathostomes, (5) a chondrichthyan, the spiny dogfish *Squalus acanthias*, (6) the Senegal bichir *Polypterus senegalus*, two piscine sarcopterygians, (7) the Australian lungfish *Neoceratodus forsteri* and (8) the Indian Ocean coelacanth *Latimeria chalumnae*, and (9) one tetrapod, the tiger salamander *Ambystoma tigrinum*. The analysis aimed to test whether the characters of the central nervous system characters could provide a reliable phylogenetic pattern and contribute to the debate about cyclostome monophyly.

The issue for this study is therefore to show whether morphological neural characters, regarded as highly conserved features, can bring new insights into the striking discrepancy between morphological and molecular data.

Materials and Methods

Data source

We used data from the literature to select and describe 123 neural characters from two out-group species and seven in-group species. Several character states were checked using the neuroanatomical collection of one of the authors (RGN).

Taxon sampling

The cephalochordate *Branchiostoma lanceolatum* (the amphioxus) and the urochordate *Ciona intestinalis* were chosen as out-groups. These 'protochordates' are the best known non-craniate chordates that share some neural characters with craniates.

Among hagfishes, the Myxinidae are more derived, morphologically, than Eptatretidae (Adam and Straham 1963; Fernholm 1998). Nevertheless, a study based on the mitochondrial 16S ribosomal RNA gene in 14 species of Myxinidae (Kuo *et al*. 2003) suggests that these two families diverged very early. Although the Eptatretidae and the Myxinidae, are sister groups, it is still not clear whether the Eptatretidae are paraphyletic. We have chosen to describe neural characters in *Eptatretus stoutii*, the Pacific hagfish, mainly because the neuroanatomy of this species has been most extensively studied. Similarly, we selected the comparatively well studied *Petromyzon marinus* as the lamprey species of choice, and the spiny dogfish, *Squalus acanthias,* as the chondrichthyan. In the context of our subgroup of morphological characters, we believe that the brains in each of these species reliably represent those in the larger taxa.

There are only two species of extant coelacanths, *Latimeria chalumnae* from the Comoro Islands and *Latimeria menadoensis* from Indonesia. The brain of *Latimeria menadoensis* has not been studied, but it is likely that the two extant crossopterygians are closely similar (if not synonyms).

The genus *Polypterus* comprises numerous species, including the Senegal bichir, *Polypterus senegalus*. There are relatively more data available for *Polypterus* than for the other cladistian genus *Erpetoichthys*, and no reason to believe that they differ fundamentally in their neuroanatomy, so we considered the Senegal bichir, *Polypterus*, our Osteichthyan representative of choice.

Lungfishes, on the contrary are a more diverse group. Neuroanatomical data are available for both the African lungfish, *Protopterus* (more closely related to the South American lungfish, *Lepidosiren*), and the Australian lungfish, *Neoceratodus*. We chose the latter, which probably retains the largest number of plesiomorphic characters of any piscine sarcopterygian. Where characters differ between the lungfish taxa, these cases will be discussed separately.

Despite the considerable anatomical diversity of the tetrapods, we have only used here the urodele *Ambystoma tigrinum*, which is assumed to illustrate the general tetrapod condition. The main question addressed in this study is the monophyly or paraphyly of agnathans. The characters chosen for the dataset are therefore major fundamental characters of the central nervous system, and, in this context, any other tetrapod would have the same character coding as a salamander.

Character coding

We have used a presence/absence coding (Pleijel 1995). This choice raises theoretical problems when a taxon does not possess a structure that other taxa display in more than one condition. For instance, details about the abducens nerve nucleus were coded NA in hagfishes, because they possess no oculomotor system. Unknown character states were coded by a question mark. PAUP 4.0 (beta version) does not treat NA and '?' differently. This distinction is nevertheless retained in the data table for information.

Phylogenetic analysis

We used PAUP 4.0 (beta-version) with its default settings. No monophyly constraint was imposed on the in-group. Polytomy was chosen for the two out-groups. Bremer indices were calculated according to the usual method. We searched for rogue taxa among gnathostomes by calculating all the trees resulting from the deletion of each of the gnathostome taxa.

Results

Character description

For all the characters, 0 denotes absence and 1 presence. The coding of the characters is given in Table 1. The following anatomical references were used: Dean (1899); Kupffer (1900); Johnston (1902); Johnston (1908); Conel (1929); Jansen (1930); Damas (1944); Marinelli and Strenger (1954); Hardisty (1982); Gorbman and Tamarin (1985); Northcutt (1986); Wicht and Northcutt (1992); Fritzsch and Northcutt (1993); Northcutt and Bemis (1993); Wicht and Northcutt (1995); Janvier (1996); Piotrowski and Northcutt (1996); Nieuwenhuys (1998); Nieuwenhuys and Nicholson (1998); Ronan and Northcutt (1998); Wicht and Nieuwenhuys (1998); Wicht and Tusch (1998); Mackie and Burighel (2005); Wicht and Lacalli (2005). Data sources on more precise neuroanatomical structure are cited case by case.

1. Neurulation by fusion of neural plate folds (absent = 0, present = 1)

The neural tube of hagfish forms by invagination of the neural plate and fusion of the medullary fold at the dorsal midline. In contrast, neurulation in lampreys and teleosts involves the formation of a neural keel. Cephalochordates neurulate by folding their neural plate after its invagination under the overlying ectoderm.

The anterior neural plate of hagfishes closes before the posterior neural plate, a sequence sometimes referred to as unique among craniates. Nevertheless, this chronology seems to be dependant on developmental speeds and is highly variable from one craniate species to another. For instance, in amphibians, the tube closes simultaneously on its whole length.

Interestingly, secondary caudal neurulation is not described in hagfishes.

2. Neural crest (absent = 0, present = 1)

In embryos of the tunicate *Ecteinascidia turbinata*, dorsal neural crest-like cells migrate out of the neural tube and form pigment cells (Jeffery *et al*. 2004). There is no such cell population in cephalochordates. Neural crest derivatives are present in all craniates.

Interestingly, *Eptatretus* embryos present dorsal and ventral neural ridges already observed by Kupffer (1900). The presence of a ventral neural crest has also been noted by Wicht and Tusch (1998). Recent data (Ota *et al*. 2007) tend nevertheless to show that the ventral ridges are not equivalent to a ventral neural crest.

3. Ectodermal nasal placode (absent = 0, present = 1)

The stomodeal invagination of *Amphioxus*, termed Hatschek's pit, is topographically comparable to Rathke's pouch in vertebrates and expresses *Pitx*, a pituitary-specific gene (Gorbman 1999; Yasui *et al*. 2000). Nevertheless, it does not produce any of the hypophyseal hormones.

The nasal placode of hagfishes could be endodermal and the nasohypophyseal complex would therefore be entirely endodermal in these animals. In lampreys and gnathostomes, the nasohypophyseal complex derives from an ectodermal placode and from Rathke's pouch.

Since Hatschek's pit is a derivative of the endodermal embryonic foregut, and since the urochordate territory that expresses *Pitx* is ectodermal, cephalochordates and hagfishes may be the only chordates to have an endodermal hypophyseal placode.

4. Nasohypophyseal duct (absent = 0, present = 1)

The nasohypophyseal opening of hagfishes is terminal, while it is dorsal in lampreys. The nasohypophyseal duct is posteriorly closed in lampreys, but opens into the pharynx in hagfishes and is involved in branchial respiration.

5. Paired nasal placode (absent = 0, present = 1)

The olfactory organ of agnathans is either unpaired (hagfishes), or consists of confluent nasal sacs (lampreys). In gnathostomes, this organ is paired with separate nasal sacs.

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)

Choanae and tear ducts are a tetrapod feature, and homology of the intrabuccal posterior nostrils of lungfishes with the tetrapod choanae has been long debated, essentially because of their lateral position relative to the maxillary branches of the trigeminal nerve.

8. Olfactory bulbs (absent = 0, present = 1)

9. Pedunculated olfactory bulbs (absent = 0, present = 1)

Hagfishes have paired, five-layered, sessile olfactory bulbs. Pedunculated olfactory bulbs have been acquired independently in several species such as *Squalus* and *Latimeria*. *Neoceratodus*, contrary to lepidosirenid lungfishes, also shows pedunculated olfactory bulbs. This character can be considered as primitive in lungfish.

10. Five concentric cellular layers in the olfactory bulbs (absent = 0, present = 1)

In all craniates, the olfactory bulbs have five layers. Dipnoans have a supplementary layer of unknown function, the subependymal fibre plexus, between the internal cellular layer and the ependyma.

11. Terminal nerve (absent = 0, present = 1)

The terminal nerve is a ganglionated cranial nerve with peripheral processes that enter the nasal cavity, and centrally directed processes towards the forebrain. The terminal nerve demonstrates immunoreactivity to FMRFamide and gonadotrophin-releasing hormone (GnRH). Lampreys and hagfishes lack a terminal nerve (Eisthen and Northcutt 1996), as also does *Latimeria*.

12. Single, main olfactory organ (absent = 0, present = 1)

In most amphibians, the olfactory organ is divided into a dorsal main olfactory epithelium and into a ventral sensory epithelium termed Jacobson's or vomeronasal organ. In all non-tetrapod vertebrates, there is no equivalent of an accessory olfactory organ.

Table 1 Character coding

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Amphioxus	0	0	0	0	0	0	–	0	–	–
Ciona	1	1	1	0	0	0	–	0	–	–
Hagfish	1	1	0	1	0	1	–	1	0	1
Lamprey	0	1	1	1	1	1	–	1	0	1
Dogfish	1	1	1	0	1	1	1	1	1	1
Bichir	0	1	1	0	1	1	1	1	0	1
Lungfish	1	1	1	0	1	1	1	1	1	0
Coelacanth	1	1	1	0	1	1	1	1	1	1
Salamander	1	1	1	0	1	1	0	1	1	1
	11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Amphioxus	–	–	0	1	0	0	–	0	–	–
Ciona	–	–	0	0	0	0	–	0	–	–
Hagfish	0	1	1	1	0	1	0	1	0	0
Lamprey	0	1	1	1	1	1	1	1	1	1
Dogfish	1	1	1	1	1	1	1	1	1	1
Bichir	1	1	1	1	1	1	1	1	1	1
Lungfish	1	1	1	1	1	1	1	1	1	1
Coelacanth	1	1	1	1	1	1	1	1	1	1
Salamander	0	0	1	1	1	1	1	1	1	1
	21	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Amphioxus	0	–	–	0	0	–	–	–	–	0
Ciona	0	–	–	1	0	–	–	–	–	0
Hagfish	1	0	0	0	0	0	0	–	0	0
Lamprey	1	1	1	0	1	0	1	0	1	1
Dogfish	1	1	0	0	1	1	1	1	1	0
Bichir	1	1	0	1	1	1	1	1	1	0
Lungfish	1	1	1	1	1	1	1	1	1	1
Coelacanth	1	1	0	0	1	1	1	1	1	1
Salamander	1	1	0	0	1	1	1	1	1	1
	31	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
Amphioxus	–	–	–	0	–	–	–	0	0	0
Ciona	–	–	–	0	–	–	–	0	0	0
Hagfish	–	0	1	1	0	–	0	0	0	1
Lamprey	1	0	1	1	1	0	1	1	1	1
Dogfish	1	1	1	1	1	1	1	1	1	1
Bichir	0	1	1	1	1	1	1	1	1	1
Lungfish	0	1	0	1	1	1	1	1	1	1
Coelacanth	0	1	1	1	1	1	1	1	1	1
Salamander	1	1	0	1	1	1	1	1	1	1
	41	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**
Amphioxus	–	–	–	–	–	–	–	–	0	–
Ciona	–	–	–	–	–	–	–	–	0	–
Hagfish	?	0	0	0	0	1	0	0	1	0
Lamprey	0	1	?	0	1	1	1	0	1	0
Dogfish	1	1	1	1	1	0	1	1	1	1
Bichir	1	1	1	1	1	0	1	1	1	1
Lungfish	1	1	1	1	1	1	1	1	1	1
Coelacanth	1	1	1	1	1	0	0	1	1	1
Salamander	1	1	1	1	1	0	1	1	1	1
	51	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
Amphioxus	–	1	–	0	–	0	–	0	–	0
Ciona	–	1	–	0	–	0	–	0	–	1
Hagfish	1	1	0	1	0	1	1	1	0	0
Lamprey	1	1	1	1	1	1	1	1	1	1
Dogfish	0	0	1	1	1	1	1	1	1	1
Bichir	1	0	1	1	1	1	1	1	1	1
Lungfish	1	0	1	1	1	1	1	1	1	1
Coelacanth	1	0	1	1	1	1	0	1	1	1
Salamander	1	0	1	1	1	1	0	1	1	1
	61	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**
Amphioxus	0	–	–	–	0	–	0	–	–	0
Ciona	0	–	–	–	0	–	0	–	–	0
Hagfish	0	–	–	–	0	–	0	0	0	0
Lamprey	1	0	0	0	0	–	?	0	0	1

Table 1 *Continued*

Dogfish	1	0	1	1	1	1	1	1	1	1
Bichir	1	0	1	1	1	1	1	1	1	1
Lungfish	1	0	1	1	1	1	1	1	0	1
Coelacanth	1	1	1	1	1	0	1	0	0	1
Salamander	1	1	1	1	1	0	1	1	0	1
	71	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**
Amphioxus	–	0	–	–	0	–	–	–	–	–
Ciona	–	0	–	–	0	–	–	–	–	–
Hagfish	–	1	0	1	1	0	0	1	0	–
Lamprey	0	1	1	0	1	1	0	0	0	0
Dogfish	1	1	1	1	1	1	1	1	0	0
Bichir	1	1	1	1	1	1	1	1	0	1
Lungfish	1	1	1	1	1	1	1	1	1	1
Coelacanth	1	1	1	1	1	1	1	1	1	1
Salamander	1	1	1	1	1	1	1	1	0	1
	81	**82**	**83**	**84**	**85**	**86**	**87**	**88**	**89**	**90**
Amphioxus	0	–	0	–	–	–	–	–	0	0
Ciona	0	–	0	–	–	–	–	–	1	0
Hagfish	1	1	1	0	–	0	–	1	0	1
Lamprey	1	0	1	?	?	1	–	0	0	1
Dogfish	1	0	1	1	1	1	0	1	1	1
Bichir	1	0	1	1	1	1	0	1	1	1
Lungfish	1	1	1	1	1	1	1	1	0	1
Coelacanth	1	1	1	1	0	1	1	1	1	1
Salamander	1	0	1	1	0	1	0	1	0	1
	91	**92**	**93**	**94**	**95**	**96**	**97**	**98**	**99**	**100**
Amphioxus	–	0	–	1	0	–	–	0	0	–
Ciona	–	0	–	1	0	–	–	0	0	–
Hagfish	0	1	0	0	1	0	1	0	1	0
Lamprey	1	1	1	1	1	1	1	0	1	1
Dogfish	1	1	0	1	1	1	1	0	1	1
Bichir	1	1	1	1	1	1	0	1	1	1
Lungfish	1	1	1	1	1	1	1	1	1	1
Coelacanth	1	1	1	1	1	1	1	1	1	1
Salamander	1	1	1	1	1	1	1	0	1	1
	101	**102**	**103**	**104**	**105**	**106**	**107**	**108**	**109**	**110**
Amphioxus	–	0	–	–	–	–	0	–	–	–
Ciona	–	0	–	–	–	–	0	–	–	–
Hagfish	0	1	0	–	0	–	1	1	1	1
Lamprey	1	1	1	1	1	0	1	1	0	1
Dogfish	1	1	1	1	1	1	1	0	1	1
Bichir	1	1	1	0	1	1	1	0	1	1
Lungfish	1	1	1	1	1	1	1	0	1	1
Coelacanth	1	1	1	1	1	1	1	0	1	0
Salamander	1	1	1	1	1	1	1	0	1	0
	111	**112**	**113**	**114**	**115**	**116**	**117**	**118**	**119**	**120**
Amphioxus	–	1	–	0	1	?	0	0	0	1
Ciona	–	?	–	0	?	–	0	0	0	0
Hagfish	0	0	0	0	0	–	0	0	0	1
Lamprey	1	1	1	0	0	1	1	0	1	1
Dogfish	1	1	1	1	0	1	1	1	1	1
Bichir	1	1	1	0	0	1	1	1	1	1
Lungfish	1	1	1	0	0	0	1	1	1	1
Coelacanth	1	1	1	1	0	1	1	1	1	1
Salamander	1	1	1	0	0	1	1	1	1	1
	121	**122**	**123**							
Amphioxus	1	0	0							
Ciona	?	?	0							
Hagfish	0	1	1							
Lamprey	0	0	0							
Dogfish	1	1	1							
Lungfish	1	1	1							
Coelacanth	1	1	1							
Salamander	1	1	1							

13. Two eyes (absent = 0, present = 1)
Both urochordates (tunicates) and cephalochordates have a photosensory region that can be homologized to the craniate eye. Nevertheless, this region is single and median in both groups.

14. Layers of photoreceptors in the visual organ (absent = 0, present = 1)
The lamellar organ of *Amphioxus* comprises photoreceptors that are organized in a way which resembles that of the craniate retina. There is no such organ in urochordates.

15. Lens (absent = 0, present = 1)
Although a lens placode is described in *Eptatretus* embryos (Price 1896; Stockard 1907; Fernholm and Holmgren 1975) there is no lens in adult hagfishes. Even such gnathostomes, as caecilians (Wake 1985), mole rats (Cooper *et al*. 1993) and cavefishes (Jeffery 2001), which possess small, subcutaneous eyes like *Eptatretus*, almost always retain lenses. Caecelians only loose their lens in the most extreme cases of eye reduction. On the contrary, the eyes of *Eptatretus* are highly differentiated and the absence of lens in this animal may not be secondary (Locket and Jørgensen 1998).

16. Chiasm (absent = 0, present = 1)

17. Externally visible optic chiasm (absent = 0, present = 1)
The optic chiasm of hagfishes is inside the brain (Wicht *et al*. 1998).

18. Retina (absent = 0, present = 1)

19. Retinal epithelium pigmentation (absent = 0, present = 1)
The retina of hagfishes is remarkable by its total absence of epithelial pigmentation (Holmgren and Öhman 1976).

20. Synaptic ribbons in retina (absent = 0, present = 1)
No synaptic ribbons are reported in the hagfish retina, contrary to lampreys (Holmgren and Öhman 1976) and other gnathostomes. Points of synaptic contact are only marked by membrane densities with clusters of 'synaptic bodies' (Locket and Jørgensen 1998).

21. Lateral line system (absent = 0, present = 1)

22. Six lateral line placodes – anterodorsal, anteroventral and supratemporal lateral line nerves (absent = 0, present = 1)
Most gnathostomes and lampreys have six lateral line placodes (epibranchial placodes), while eptatretid hagfishes only display three. One-to-one homologies between these placodes are not straightforward but it is likely that *Eptatretus* lateral line placodes are homologous to the anterodorsal lateral line placode, the anteroventral lateral line placode and the supratemporal lateral line placode of other craniates. Interestingly, *Myxine* has no lateral line system.

23. Recurrent ramus of the anterior lateral line nerve (absent = 0, present = 1)
A recurrent ramus of the anterior lateral line nerve which passes along with fibres of the posterior lateral line nerve occurs in dipnoans, lampreys and gymnotoid teleosts.

24. Spiracular organs (absent = 0, present = 1)

25. Mechanoreceptors with stair-stepped microvilli (absent = 0, present = 1)
In dipnoans, cladistians, chondrosteans and non-teleost neopterygians, paired diverticulae of the hyoid pouches, termed spiracular organs, are innervated by the otic lateral line nerve and may have a mechanoreceptor function. Neuromasts are homologous in all craniates with the possible exception of hagfishes. Typical craniate neuromasts display a long kinocilium and an array of stair-stepped microvilli. The so-called neuromasts of hagfishes display a long central kinocilium surrounded by a crown of much shorter microvilli (Braun and Northcutt 1998).

Cephalochordates have sensory cells with a centrally located apical cilium surrounded by a crown of microvilli (Bone and Ryan 1978). These cells are either primary or secondary sensory neurones, probably involved in mechanoreception or chemoreception. When secondary, they are termed 'type II epithelial sensory cells' (Lacalli and Hou 1999) and may be homologous to the hagfish 'neuromasts'. The tunicate *Botryllus schlosseri* has similar sensory cells grouped in organs termed the 'coronal organs', which may be homologous to the spiracular organs (Burighel *et al*. 2003).

26. Neuromast cupulae (absent = 0, present = 1)
Hagfishes and lampreys neuromasts have no cupulae (Braun and Northcutt 1997).

27. Internal taste buds (absent = 0, present = 1)

28. Facialis innervation of taste buds (absent = 0, present = 1)
Internal (oropharyngeal) taste buds are present in all craniate groups except hagfish. They are innervated by the facial, glossopharyngeal and vagal nerves, except in lampreys, where the facial innervation is lacking.

In some non-teleost species, such as sturgeon, bowfin and gar, external taste buds occur (Norris 1925). *Protopterus* also possesses external taste buds (Fahrenholz 1929). Some teleosts display external taste buds on specialized parts of their body such as the lips and the barbels. Catfish are an exception among teleosts as they have thousands of external taste buds scattered over the entire body (Atema 1971). External taste buds are always innervated by a ramus of the facial nerve (Finger 1978).

All craniate species possess another chemosensory cell type, the solitary chemosensory cells. The relations between these solitary cells and taste buds are not clear but they are considered homologous among all craniate species (Braun 1998).

29. Chemosensory organs innervated only by cranial nerves (absent = 0, present = 1)
Hagfishes have a unique chemosensory system formed by Schreiner organs. These organs are innervated indiscriminately by any somatic nerve that innervates their epithelial location, and not exclusively by cranial nerves (Braun 1998).

30. Electroreceptors (absent = 0, present = 1)
Electroreceptors are present in lampreys, chondrichthyans, chondrosteans, lungfishes and *Latimeria* as well as in some amphibians. They are absent in hagfishes (Gibbs 2004).

31. Numerous electroreceptive regions on epithelium (absent = 0, present = 1)

The primitive condition of electroreceptor organization in craniates is believed to be the Lorenzini ampullae of chondrichthyans. The topology of electroreceptors of lampreys differs from this primitive type, In lampreys, the sensory cells are located in terminal buds. Among gnathostomes, *Latimeria* possesses a specific electroreceptive organ called the rostral organ, and *Neoceratodus* shows invaginated epithelial sacs in the snout, innervated by the anterior lateral line nerve. The histology of these organs is poorly known but they may be homologous to the rostral organ of *Latimeria*.

Interestingly, some teleost fish – siluriformes, gymnotiformes and mormyriformes – have acquired electroreception independently and display a variety of teleost-type ampullae (Gibbs 2004).

32. External opening of the endolymphatic duct (absent = 0, present = 1)

33. Rudimentary endolymphatic duct (absent = 0, present = 1)

The endolymphatic duct of gnathostomes has an external opening. This duct is generally rudimentary, except in amphibians and in lepidosirenid lungfishes, where it expands to fill most of the neurocranium. These endolymphatic sacs do not occur in *Neoceratodus* and their absence is probably primitive among lungfishes.

The fact that the endolymphatic sacs of amphibian type never open to the exterior, as opposed to the externally opening endolymphatic ducts of osteostracans, placoderms and certain living elasmobranchs, may indicate that these two structures are not homologous.

34. Semi-circular canals (absent = 0, present = 1)

35. Vertical semicircular canals (absent = 0, present = 1)

36. Vertical semi-circular 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)

All gnathostomes have three semicircular canals and at least two maculae in their inner ear. Lampreys have two semicircular canals and a single, well-differentiated macula that can be readily divided into three parts, probably homologous to the utriculus, sacculus and lagena of gnathostomes (Lowenstein *et al*. 1968).

Hagfishes only have a single semicircular canal and a single macula that is not as well differentiated as the macula of lampreys (Jørgensen 1998).

38. Hair cells with stereociliae only (absent = 0, present = 1)

39. Hair cell cupulae (absent = 0, present = 1)

Hagfishes have not only hair cells with the typical kinocilium and stereovilli organization but also hair cells with a central long kinocilium surrounded by a crown of much shorter microvilli. This organization is unique in craniates. Furthermore, hagfish crista hair cells have no cupulae (Jørgensen 1998).

40. Trigeminal nerve (absent = 0, present = 1)

41. Trigeminal motor nucleus in r2 or/and r3 (absent = 0, present = 1)

The trigeminal motor nucleus of lampreys arises at the junction between rhombomeres (r) r1 and r2, whereas in gnathostomes, the trigeminal motor nucleus largely originates from r2 and r3. The caudal limit of the trigeminal motor nucleus of lampreys extends into r4, as opposed to gnathostomes, where it reaches the r3–r4 border (Gilland and Baker 2005).

42. Trigeminal ramification limited to the profundal, external and velobuccal branches (absent = 0, present = 1)

Three main rami of the trigeminal nerve are present in all craniates: deep ophthalmic (profundal), maxillary (external) and mandibular (velobuccal, dental) rami. An additional palatine nerve innervating the roof of the mouth cavity, which also innervates the Schreiner organs, is specific to hagfishes (Lindström 1949).

43. Ventral ophthalmic projections in the trigeminal sensory nucleus (absent = 0, present = 1)

The projection of the ophthalmic ramus into the trigeminal sensory nucleus is located ventrally in gnathostomes but dorsomedially in hagfishes (Ronan 1988).

44. Mesencephalic trigeminal nucleus (absent = 0, present = 1)

Both lampreys and hagfishes lack a mesencephalic trigeminal nucleus: there is no ascending primary trigeminal tract in these animals.

45. Trigemino-spinal projections (absent = 0, present = 1)

The descending trigeminal tract does not reach spinal levels in hagfishes (Ronan 1989).

46. Fusion of the profundal nerve ganglion with the trigeminal ganglion (absent = 0, present = 1)

The deep ophthalmic (profundus) ramus is a somatosensory nerve that innervates the dorsal rostral part of the head. In *Latimeria*, its territory comprises the skin of the snout and the membranes of the tubes in the rostral organ. The ganglion of the profundal nerve fuses with the trigeminal ganglion in hagfishes, lampreys and lungfishes.

47. Superficial ophthalmic 'trigeminal' ramus (absent = 0, present = 1)

The dorsal ramus of the anterodorsal lateral line nerve is often referred to as the superficial ophthalmic ramus of the trigeminal nerve as it is located dorsally to the profundal trigeminal ramus. This superficial ramus is lacking in both hagfishes and *Latimeria*.

48. Fusion of the maxillary trigeminal ramus with the buccal ramus of the anterolateral lateral line nerve (absent = 0, present = 1)

In all craniates except agnathans, the maxillary trigeminal ramus fuses with the ventral ramus of the anterodorsal lateral line nerve termed the buccal ramus. This nerve bundle is called the bucco-maxillary complex.

49. Facial nerve(absent = 0, present = 1)

50. Division of the facial nerve into pharyngeal, pre- and post-trematic branches (absent = 0, present = 1)

The facial nerve of gnathostomes is divided into three branches: pharyngeal, pre- and post-trematic branches. This division is not found in agnathans.

51. Distinct facial, glossopharyngeal and vagal nuclei (absent = 0, present = 1)

In chondrichthyans, the branchiomeric motor nuclei consist of a separate rostral trigeminal nucleus and of a single caudal complex formed by the fusion of the facial, glossopharyngeal and vagal nuclei (Smeets *et al.* 1983).

52. Hypodermal and dermal nerve fibre plexi (absent = 0, present = 1)

The hypodermal and dermal nerve fibre plexus of hagfishes contains a great number of neuronal cell bodies. The nerves enter the hypodermal slime glands and are probably involved in triggering the secretion of the slime. No other craniate has such a specialization of its peripheral nervous system. This plexus is innervated by spinal nerves (Bone 1963). Subcutaneous neurones are described in lampreys, which do not have slime glands. However, 'protochordates' also have such peripheral plexi.

53. More than one postotic cranial nerve (absent = 0, present = 1)

Hagfishes have a single postotic branchiomeric nerve called the glossopharyngeal/vagal nerve (Matsuda *et al.* 1991). All other craniates have separate IX and X nerves.

54. Viscero-sensory nerves (absent = 0, present = 1)

55. Periventricular viscerosensory rhombencephalic zone (absent = 0, present = 1)

In all craniates, except for hagfishes, the rhombencephalic viscerosensory zone is located close to the fourth ventricle and forms a U-shaped structure embracing the calamus scriptorius. In hagfishes, the viscerosensory nuclei have migrated toward the periphery of the rhombencephalon.

56. Glossopharyngeal nerve (absent = 0, present = 1)

57. Single glossopharyngeal ganglion (absent = 0, present = 1)

Medial and lateral vagal ganglia are present in all gnathostomes except for ratfish, whereas lateral and medial glossopharyngeal ganglia are a tetrapod feature; yet also present in *Latimeria*.

58. Vagal nerve (absent = 0, present = 1)

59. Division of vagal nerve into pharyngeal, pre- and post-trematic branches (absent = 0, present = 1)

Hagfishes are the only craniates that lack division of the vagal nerve into pharyngeal, pre- and post-trematic branches.

60. Cardiac innervation (absent = 0, present = 1)

Vagal innervation of the heart is totally absent in hagfishes, as opposed to all other craniates. Cardiac innervation is also absent in cephalochordates. Interestingly, in *Ciona*, tyrosine hydroxylase (TH)-positive neurones have been observed in close contact with the heart and could correspond to a neural cardioregulatory system (P. Vernier, personal observation).

61. Oculomotor system (absent = 0, present = 1)

Hagfishes lack extrinsic eye and oculomotor tracts. In contrast, the oculomotor system of lampreys and gnathostomes is globally similar and homologous (Fritzsch *et al.* 1990).

62. Abducent nerve with retractor bulbi innervation (absent = 0, present = 1)

Lampreys have an accessory abducent nucleus that innervates the caudal rectus muscle (Fritzsch *et al.* 1990). This pattern of innervation may be homologous to the abducent innervation of the retractor bulbi of tetrapods (Fritzsch 1998) and *Latimeria*.

63. Caudal (r5–r7) localization of abducent motoneurones (absent = 0, present = 1)

In lampreys, abducent motoneurones are as rostral as caudal r4 but they are shifted to more caudal locations in gnathostomes (r5–r7) (Gilland and Baker 2005).

64. Ventral trochlear nucleus (absent = 0, present = 1)

In lampreys, the trochlear axons leave the brain dorsally, as in gnathostomes, but the IVth nucleus is located dorsally in the alar plate, whereas it has a ventral position in all gnathostomes.

65. Ciliary ganglion (absent = 0, present = 1)

66. Extracranial ciliary ganglion (absent = 0, present = 1)

A ciliary ganglion is found in all gnathostomes and is located intracranially in *Latimeria* and tetrapods.

67. Hypobranchial nerve (absent = 0, present = 1)

The organization of the nerves that issue caudal to the vagal nerve is variable. In hagfishes, the first two nerves caudal to the vagal nerve are termed 'spino-occipital' (Worthington 1906). They possess sensory ganglia and their ventral rami do not fuse to form a hypobranchial nerve. In lampreys, the hypobranchial nerve is not formed by the rostralmost spinal nerves but by the fusion of parts of the ventral rami of spinal nerves 4 to 12.

68. Ventral branch of spinal ganglionated nerves contribution to the hypobranchial nerve (absent = 0, present = 1)

69. Hypobranchial nerve formed by the fusion of the ventral branches of intracranial non-ganglionated postvagal nerves *and* of the ventral branches of spinal ganglionated nerves (absent = 0, present = 1)

In *Squalus*, three nerves devoid of sensory ganglia issue caudally to the vagal nerve from the medulla (Norris and Hughes 1920). The second and the third of these occipital nerves fuse with the first two spinal nerves, which possess a sensory ganglion, to form the hypobranchial nerve.

In most of the actinopterygian fishes, the hypobranchial nerve is formed by (1) two or more non-ganglionated occipital intracranial nerves and (2) at least one more caudal spinal ganglionated nerve. *Polypterus* is an exception, because only one non-ganglionated nerve and one more caudal ganglionated nerve contribute to the hypobranchial nerve.

Lungfish (*Protopterus*) and amphibians show a pattern that is close to that observed in actinopterygians fishes (Pinkus 1895).

In *Latimeria*, only the first two of the three intracranial non-ganglionated occipital nerves contribute to the hypobranchial nerve.

The number of postvagal non-ganglionated nerves, and their contribution to the hypobranchial nerve therefore seems closely related to the extension of the postotic part of the skull (Piotrowski and Northcutt 1996).

70. Cerebellar primordia (absent = 0, present = 1)

71. Corpus cerebelli (absent = 0, present = 1)

All gnathostomes have a cerebellum. Hagfishes do not display any structure that could be a cerebellar homologue. Nor do they show any cerebellar primordia during their development.

In lampreys, the structure commonly called the cerebellum cannot be considered as homologous to the gnathostome cerebellum. It lacks the central corpus as well as Purkinje cells (Weigle and Northcutt 1998). The lamprey cerebellum appears to correspond to the eminentia granularis of the lateral line lobes of gnathostomes (Ronan and Northcutt 1990).

The absence of a red nucleus in hagfishes may be correlated with the absence of a cerebellum.

72. Hypothalamus (absent = 0, present = 1)

73. Multiple hypothalamic nuclei (absent = 0, present = 1)

The hypothalamus of hagfishes is limited to a poorly differentiated periventricular sheet of cells and to a lateral nucleus infundibularis (Wicht and Northcutt 1992).

In all other craniates, several cell groups can be identified. The hypothalamus of lampreys is for instance subdivided into two dorsal nuclei, the nucleus commissurae postopticae and the nucleus dorsalis hypothalami, and into a ventral zone formed by the preinfundibularis, the ventralis hypothalami and the postinfundibularis nuclei.

74. GnRH-positive cell groups in the caudal diencephalon or the mesencephalon (absent = 0, present = 1)

A GnRH-like system including the infundibular hypothalamus and the preoptic area in found in hagfishes (Braun *et al.* 1995).

All gnathostomes display a GnRH cell group in the caudal diencephalon or the mesencephalon, and some of the gnathostomes also have a GnRH-positive nervus terminalis.

There is no caudal GnRH-positive cell group in lampreys.

75. Hypophysis (absent = 0, present = 1)

76. Adenohypophysis differentiated in a pars intermedia and a pars distalis (absent = 0, present = 1)

77. Median eminence (absent = 0, present = 1)

All craniates have a pituitary gland.

In hagfishes, the adenohypophysis is poorly developed and has no pars intermedia. The adenohypophysis of lampreys is differentiated into a pars intermedia and a pars distalis.

In both hagfishes and lampreys, the vascular connections between the neuro- and adenohypophysis are poor and there is no median eminence.

78. Serotoninergic median raphe (absent = 0, present = 1)

Both hagfishes and lampreys display a catecholaminergic nucleus in the isthmic tegmentum that can be homologized to locus coeruleus (Pierre *et al.* 1994).

A serotoninergic median raphe is present in hagfishes and gnathostomes but lacking in lampreys (Pierre *et al.* 1992).

79. Superficial isthmic nucleus (absent = 0, present = 1)

A rostral superficial isthmic nucleus occurs in *Latimeria* and in lungfishes. Its only homologues would be, in teleosts, the tegmental optic nucleus, located at the level of the oculomotor nucleus, and the nucleus isthmi, situated in the caudal mesencephalon; the distinctive topography of these homologues indicates that the superficial isthmic nucleus may be a synapomorphy of *Latimeria* and lungfishes.

80. Preoptic area with magno- and parvocellular parts (absent = 0, present = 1)

The preoptic area of lampreys and chondrichthyans does not show any differentiation into a magno- and parvocellular parts, as opposed to all other gnathostomes.

Hagfishes have a highly differentiated preoptic area with four nuclei. Two of these nuclei consist of small-sized neurones and one is characterized by larger neurones, but the homology with the preoptic area of other craniates is unclear.

81. Tectum (absent = 0, present = 1)

82. Less than five tectal laminae (absent = 0, present = 1)

The optic tectum has a laminated structure of alternating cellular and fibrous layers. Tectal lamination displays a peculiar pattern of variation and is probably related to the relative importance of visual, olfactory and auditory stimuli. The bichir has an unsually large number of tectal laminae (Northcutt 2002).

83. Thalamus (absent = 0, present = 1)

84. Dorsal and ventral thalami (absent = 0, present = 1)

85. Overlap of the areas with tectal and retinal projections in the dorsal thalamus (absent = 0, present = 1)

Dorsal and ventral thalami can be clearly defined in all gnathostomes. In the classical conception of the thalamus, mainly based on amphibian data, the dorsal thalamus processes information from several sensory modalities and relays this information to the telencephalon, whereas the ventral thalamus is connected with the subpallium of the telencephalon and various mesencephalic centres and is involved in motor control.

The dorsal thalamus is schematically divided into an anterior lemnothalamus involved in visual pathways without tectal relays and into a posterior collothalamus integrated in visual and auditory pathways with midbrain relays. This division does not hold for actinopterygians, as the anterior dorsal thalami of *Polypterus* (Holmes and Northcutt 2003) and *Cyprinus* (Northcutt 2006) do not show pallial connections.

These connections are also absent in *Squalus* and lungfishes (R.G. Northcutt, unpublished observations).

In agnathans, such a division is not straightforward. Lampreys seem to have two cell groups that may correspond to ventral and dorsal thalami. Nevertheless, these two groups are connected to the pallium. Furthermore, both the anterior and posterior parts of the dorsal thalamus have pallial connections, and the thalamic areas with retinal projections overlap those with tectal projections (Wicht and Northcutt 1998). The classical distinction between a collo- and a lemnothalamus does not hold for these taxa.

86. Periventricular thalamus (absent = 0, present = 1)

If the thalamus of lampreys can be considered as bipartite, mainly on the basis of topological considerations, the situation is much less evident in hagfishes. Two nuclei contain most of the cells projecting to the telencephalon, and would functionally correspond to a dorsal thalamus.

Nevertheless, in all non-amniote craniates, except for hagfishes, the thalamus is composed of periventricular nuclei. Hagfishes on the other hand have extensive diencephalic neuronal migration and their migrated 'thalamic' nuclei may not be homologous to any other craniate structure. A massive group of nuclei, termed the central prosencephalic complex, may also contain thalamic elements, even though it seems to be of mixed pallial and diencephalic origins.

Based on connectional data, subnuclei of the central prosencephalic nucleus may in fact be homologous to the thalamic eminence, the ventral thalamus and the medial pallium (Krug *et al*. 1993; Amemiya and Northcutt 1996). But their disposition, per se, represents a hagfish autapomorphy.

87. Protrusion of the dorsal thalamus in the third ventricle (absent = 0, present = 1)

Lungfish and *Latimeria* share a striking and unique protrusion of the dorsal thalamus into the third ventricle.

88. Tuberculo-pallial tracts (absent = 0, present = 1)

The posterior tubercle of all investigated gnathostomes projects to the telencephalon. Similarly, tuberculo-pallial tracts are described in hagfishes (Wicht and Northcutt 1998) but are not found in lampreys (Polenova and Vesselkin 1993; Northcutt and Wicht 1997).

Interestingly, a TH-positive cell group is located in the posterior tubercular area and in the hypothalamic infundibulum of lampreys. A similar cell group is found in hagfishes. This nucleus may be the homologue of the substantia nigra of amniotes.

89. Saccus vasculosus (absent = 0, present = 1)

The saccus vasculosus is a thin evagination of the ependyma of the caudal wall of the posterior tubercule. It is heavily vascularized and may have hormonal functions. The saccus vasculosus occurs in most chondrichthyans and actinopterygians, as well as in *Latimeria*, but is lost in lungfishes and amphibians. The coronet cells of the sensory vesicles in the larvacean urochordates are generally considered homologous to the saccus vasculosus.

90. Pretectum (absent = 0, present = 1)

91. Neuronal migration in the pretectum (absent = 0, present = 1)

The pretectal area of gnathostomes is formed by a periventricular nucleus and two migrated nuclei, the central and the superficial nuclei (Fite 1985). Lampreys show some amount of neuronal migration and their pretectum is divided into periventricular and superficial areas (Puzdrowski and Northcutt 1989). The pretectum of hagfishes is formed by a single periventricular nucleus situated dorsolaterally to the posterior commissure (Jansen 1930).

92. Epithalamus (absent = 0, present = 1)

93. Paraphysis (absent = 0, present = 1)

The epithalamus is formed by the habenular nucleus and two diverticula – a left paraphysis (or parietal) and a right epiphysis (or pineal). Lampreys display both neural diverticula but chondrichthyans have retained only the right, pineal one, whereas actinopterygians and sarcopterygians exhibit both organs.

Surprisingly, hagfishes do not show any trace of a pineal organ, although a topological homologue of the habenular nucleus is described.

The left habenular nucleus and left fasciculus retroflexus are larger than the right ones in all craniates.

94. Extra-ocular photoreceptor region expressing the pineal opsins (absent = 0, present = 1)

The pineal gland is a source of melanin for most of the craniates. It also contains photoreceptors. Amphioxus and tunicates both present a dorsal region rich in photoreceptors in the anterior neural tube, which expresses pineal opsins, but this region contains no melatonin in both groups.

95. Telencephalon (absent = 0, present = 1)

96. Telencephalic evagination including the medial pallium (absent = 0, present = 1)

Evagination in the rostral end of the neural tube creates the hemispheres and the ventricles. In gnathostomes, the hemispheres comprise the olfactory bulbs, the pallium, the septum and most of the striatum. In hagfishes, numerous structures border the collapsed ventricles: the olfactory bulbs, the striatum, the pallium and part of the central prosencephalic nucleus.

Evagination in lampreys is much more limited: the main part of the striatum, the dorsal and medial pallia and the septum remain in the flanks of the third ventricle in continuity with diencephalic structures.

97. Evaginated telencephalon (absent = 0, present = 1)

Telencephalic development in actinopterygians occurs by a unique process of eversion. In all other craniates, the telencephalon develops by evagination. The very peculiar shape of the telencephalon of *Latimeria* is the result of an unusual evagination, but not of an eversion as in actinopterygians.

98. Extensive median septum ependymale (absent = 0, present = 1)

Latimeria, lungfishes and actinopterygians share an extensive non-neural median septum ependymale (Nieuwenhuys and Hickey 1965).

99. Subpallium (absent = 0, present = 1)
100. Subpallial septum expressing acetylcholinesterase (absent = 0, present = 1)
Rostrally, the gnathostome subpallium is divided into a medial septum and a lateral striatum. More caudally, it is formed by one or more divisions of the amygdala. The rostral subpallial differentiation is not clear in hagfishes and so contradicts such classical conception of an anteriority of subpallial versus pallial specializations. In fact, the septum of hagfishes does not show the typical chemoarchitectural characteristics, such as acetylcholinesterase (Wicht and Northcutt 1994), even though it is innervated by the medial olfactory tract (Wicht and Northcutt 1993).
101. Subpallial striatum in contact with the septum (absent = 0, present = 1)
The area defined as a striatum in hagfishes is topographically very different from the striatum of other craniates. It is characterized by numerous acetylcholinesterase-positive neurones and receives TH- and dopamine-positive fibres from the infundibular/posterior tubercle TH-positive cell group but has no contact with the septum.
102. Pallium (absent = 0, present = 1)
103. Pallium with mainly migrated neurones (absent = 0, present = 1)
104. Migrated pallium with three subdivisions (absent = 0, present = 1)
The pallium of all craniates, except for hagfishes, is constituted by telencephalic cell groups, which have migrated from a periventricular to a subpial position.

The pallium of hagfishes consists of two subdivisions that are not possible to homologize with any other craniate structure: superficial strikingly laminated cell groups and deep ganglia. The laminated pallium displays five layers and spans the entire telencephalon. Connectional data indicate that some of the nuclei of the central prosencephalic complex are also of pallial origin.

The pallium of most gnathostomes and lampreys is subdivided into three areas whereas that of *Polypterus* has only two subdivisions. *Polypterus* has a dorsomedial pallium, receiving olfactory input, and a dorsolateral pallium, receiving diencephalic input. When all the subdivisions are taken together, the pallium of lampreys and of all gnathostomes are homologous.
105. Partial pallial olfactory projections (absent = 0, present = 1)
106. Olfactory projections restricted to the lateral pallia (absent = 0, present = 1)
The secondary olfactory systems of craniates are bilateral and consist of three tracts: a medial tract directed towards the septum, a lateral pallial tract and a ventral tract innervating the basal diencephalon. Hagfishes display a massive olfactory projection covering the whole pallium (Wicht and Northcutt 1993). In gnathostomes, this projection is schematically restricted to the lateral pallium. Lampreys display secondary dorsal and partial medial pallial olfactory projections (Northcutt and Puzdrowski 1988).
107. Spinal chord with spinal nerves and spinal nerve roots (absent = 0, present = 1)
108. Ribbon-shaped spinal chord (absent = 0, present = 1)
109. Blood supply in the spinal chord (absent = 0, present = 1)
110. Regularly decreasing spinal diameter along the anteroposterior axis (absent = 0, present = 1)
111. Dorsal and ventral roots of spinal nerves on the same side of the intersegmental artery (absent = 0, present = 1)
The spinal cord of both agnathan taxa is ribbon shaped, but that of lampreys shows no blood supply.

Spinal enlargement associated with paired fins is only observed in *Latimeria* and tetrapods. The dorsal and ventral roots of the spinal nerves are on the same side of the intersegmental artery in all craniates, except for hagfishes, where they are located on each side of the artery.
112. Rohon–Béard cells (absent = 0, present = 1)
Large dorsal sensory touch- and pressure-sensitive cells in the spinal chord are termed *Rohon–Béard cells* in amphibians and fishes. Amphioxus has homologues of the Rohon–Béard cells, the *Retzius bipolar cell* (Bone 1961), as opposed to hagfishes and all amniotes (Fritzsch and Northcutt 1993). Rohon–Béard cells are supposed to derive from the neural crest, and Amphioxus has not yet been proven to have neural crest. This character requires further investigation.
113. Lemnothalamic tracts (absent = 0, present = 1)
The spinal lemniscal tracts of hagfishes do not project to the thalamus and are predominantly uncrossed.
114. Müller cells (absent = 0, present = 1)
115. Mauthner cells (absent = 0, present = 1)
116. Mauthner cells axons entsheathed in an isolated fibre (absent = 0, present = 1)
Surprisingly, both Müller and Mauthner cells do not seem to occur in the reticular formation of hagfishes. *Latimeria* also lacks Mauthner cells. The Mauthner cells of lungfishes are peculiar, because their axons are initially unmyelinated and do not run in an isolated bundle, but are ensheathed in a complex including several axons from other reticular neurones. Mauthner cell homologues also occur in Amphioxus.
117. Dorsal arcualia (absent = 0, present = 1)
118. Ventral arcualia (absent = 0, present = 1)
The spinal chord of lampreys is flanked by arcualia, as in the gnathostomes. The position of the lamprey arcualia relative to the spinal nerve roots and the segmental blood vessels indicates that they are homologous to the basidorsals and the interdorsals of the gnathostomes. There is no trace of vertebrae or vertebral elements in hagfishes.
119. Choroid plexi (absent = 0, present = 1)
Hagfishes are the only craniates lacking choroid plexi, and share this feature with protochordates.

120. Reissner's fibre (absent = 0, present = 1)
The only chordates lacking the Reissner's fibre are the urochordates (tunicates).
121. Oligodendrocytes (absent = 0, present = 1)
122. Astroglia (absent = 0, present = 1)
Glia is not well studied in tunicates. However, because the nervous system of *Ciona* contains about 300 cells and only 100 neurones, the glia has probably a major biological role in these animals. Interestingly, among other non-neural cellular types, Amphioxus is believed to possess oligodendrocyte homologues, but no trace of myelin has been found in this group. Myelin is in fact only present in gnathostomes (Bullock *et al*. 1984).

Astroglia-like cells are found in great number in the brain of hagfishes (Wicht *et al*. 1994) whereas the glial population of the central nervous system of lampreys and cephalochordates is largely dominated by ependymal cells.
123. Anastomic capillary network in the brain (absent = 0, present = 1)
Capillary networks occur in the brains of all craniates but lampreys do not display anastomotic capillaries. The brain of Amphioxus has no vascularization.

Phylogenetic analysis

Out of the 123 total characters used for this analysis, 50 were parsimony-uninformative and 73 were parsimony-informative (i.e. shared by two taxa or more). Parsimony analysis resulted in five most parsimonious trees (153 steps) after exhaustive search for the minimal length trees among 135 135 trees.

The five trees were

1. ((((Coelacanth, Salamander), (((Dogfish, Bichir), Lungfish)), Lamprey), Hagfish), Ciona, Amphioxus),
2. (((((((Dogfish, Bichir), Lungfish), Salamander), Coelacanth), Lamprey), Hagfish), Ciona, Amphioxus),
3. ((((Coelacanth, Salamander), (Dogfish, (Bichir, Lungfish)), Lamprey), Hagfish), Ciona, Amphioxus),
4. (((((((Bichir, Lungfish), Dogfish), Salamander), Coelacanth), Lamprey), Hagfish), Ciona, Amphioxus),
5. (((((((Lungfish, Coelacanth), Bichir), Dogfish), Salamander), Lamprey), Hagfish), Ciona, Amphioxus).

A strict consensus was calculated from the five best trees (Fig. 1). The homoplasy index for this consensus tree was 0.1961 (0.2913 excluding uninformative characters) and its consistency index was 0.8039 (0.7087 excluding uninformative characters). The rescaled consistency index was 0.5698.

The Bremer index for the gnathostomes is 11 – there were seventeen 164-step best trees with paraphyletic gnathostomes. The consensus of these 17 trees left the craniates unresolved.

The Bremer index for the clade grouping lampreys and gnathostomes was 7 – there were six 160-step trees with monophyletic agnathans and the consensus was then ((Gnathostomes *unsolved*), (Hagfish, Lamprey), Ciona, Amphioxus)). The gnathostomes and the craniates were in this case monophyletic but agnathans did not appear as a natural group.

The one-by-one deletion of each of the gnathostome taxa did not influence agnathan paraphyly. The results with the deleted gnathostome taxa were the following:

1. The deletion of the salamander from the ingroup resulted in one 143-step tree: ((Dogfish, (Bichir, (Lungfish, Coelacanth)))).
2. The deletion of the coelacanth from the ingroup resulted in five 144-step tree, and the strict consensus was: (gnathostomes *unresolved*).
3. The deletion of the lungfish from the ingroup resulted in three 143-step tree, and the strict consensus was: (Dogfish, Bichir, (Coelacanth, Salamander)).
4. The deletion of Bichir from the ingroup resulted in four 146-step trees, and the strict consensus was (gnathostomes *unresolved*).
5. The deletion of the dogfish from the ingroup resulted in five 147-step trees, and the strict consensus was (gnathostomes *unresolved*).

No single rogue taxon therefore appears, as the non-resolution of the gnathostomes requires the simultaneous presence of the lungfish and the salamander.

Discussion

Discrepancy between morphological and molecular studies

Despite the almost general consensus about cyclostome paraphyly among morphologists, only a few molecular phylogenies provide clear conclusions in favour of this pattern. On the contrary, cyclostome monophyly is strongly supported by molecular data. Two points have to be considered to interpret these results from a morphologist's point of view.

1. The choice of the agnathan species included in the molecular studies (for instance *Lampetra* versus *Petromyzon*, or *Eptatretus* versus *Myxine*) is probably not misleading. In fact, even if the mitochondrial DNA sequences are not identical between species of the same clade, for the protein coding regions the percentage of identity is 85.8% between *Lampetra* and *Petromyzon* (Delarbre *et al*. 2000) and 84% between *Eptatretus* and *Myxine* (Delarbre *et al*. 2002). Nevertheless, the control regions of the mitochondrial DNA of *Eptatretus* and *Myxine* cannot be aligned, whereas the same regions show a similarity of 90% in *Lampetra* and *Petromyzon*. In any case, the results of the molecular phylogenies do not seem to be influenced by the choice of the species.

2. The analysis of data from very different sources (RNA, DNA, proteins) by using maximum parsimony and maximum likelihood, or other statistical methods, always shows the same overall result: agnathan monophyly. Nevertheless, the very distant out-groups often chosen in

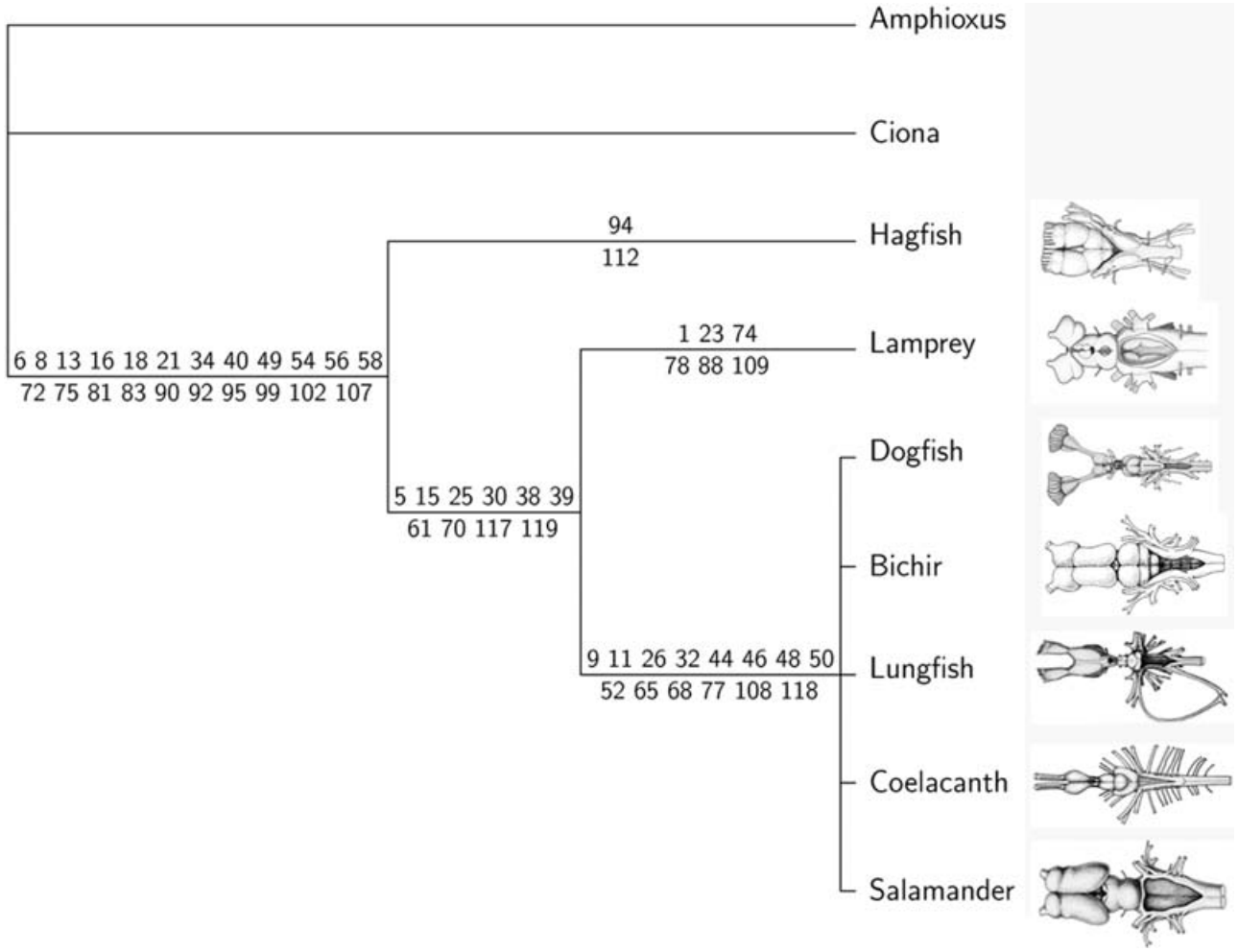

Fig. 1—Strict consensus of five most parsimonious trees resulting from an exhaustive search of the dataset with 153 imposed steps. Non-ambiguous synapomorphies and autapomorphies are provided for selected groups.

such studies – such as insects or echinoderms – may perturb the segregation (phylogenetic signal) of the most closely related species. More suitable out-groups, such as 'proto-chordates' (cephalochordates and tunicates), cannot be considered as ideal either, but may be closer to craniate than the other deuterostomes.

Agnathan monophyly versus agnathan paraphyly

The evolutionary implication of agnathan monophyly is that all the characters shared by lampreys and gnathostomes are homoplasic. This would be a unique, though not impossible, evolutionary curiosity. The middle ear of mammals is now believed to have appeared independently three times, but was once interpreted as the strongest synapomorphy among mammals (Martin and Luo 2005). The problem with the hagfish brain is that it contains a great number of examples of such unexpectedly complicated evolutionary histories, such as the disappearance of the pineal gland (characters 93; 1 step; Ci 0.500; Ri 0.667; Hi 0.500), the absence of any oculomotor system (character 61; 1 step; Ci 1.000; Ri 1.000; Hi 0.000), or the original laminated structure of the pallium (character 103; 1 step; Ci 1.000; Ri 1.000; Hi 0.000). The paraphyly of agnathans on the other hand suggests that at least some of the character states found in hagfishes may correspond to the ancestral craniate condition, especially those shared with lampreys but not with gnathostomes, such as the nasohypophyseal duct (character 4; 1 step; Ci 0.500; Ri 0.000; Hi 0.500) and the ribbon shape of the spinal chord (character 108; 1 step; Ci 0.500; Ri 0.000; HI 0.500).

Gnathostome phylogeny

Gnathostome monophyly is strongly supported (Br 11) but the relationships of osteichthyans, chondrichthyans and sarcopterygians are unresolved, nor can the monophyly and

interrelationships of sarcopterygians be resolved. This result parallels the current debate about the position of tetrapods relative to osteichthyans (Noack *et al.* 1996). Neural characters alone are in any case not sufficient to definitely answer these issues (Northcutt 1986). The central nervous system is therefore not a source of highly conserved characters, as it is often believed to be by many morphologists.

The origin of the pituitary

This highly controversial question cannot be solved with the current outdated data on hagfish development. In fact, our study suggests that the primitive origin of the nasohypophyseal complex is endodermal and that its ectodermal origin has been acquired independently by tunicates and the group including lampreys and gnathostomes (character 3; 1 step; Ci 0.500; Ri 0.000; Hi 0.500). Nevertheless, the homology between Hatschek's pit and the pituitary is not strictly established (Gorbman 1999). Furthermore, the expression territories of such genes as *Pitx* have not yet been described in hagfish embryos.

Oculomotricity in early vertebrates

According to our study, the most inclusive extant chordates that possess an oculomotor system are lampreys (character 61; 1 step; Ci 1.000; Ri 1.000; Hi 0.000). In mice, several mutations that interfere with the formation of the oculomotor nuclei are known, such as the combined mutation of Hoxa3 and Hoxb3 that disturbs the formation of motoneurones in the abducens nucleus (Gaufo *et al*. 2003). Human malformation syndromes can involve oculomotor nuclei. For instance, in Duane retraction syndrome type I, motoneurones of the sixth (abducent) nerve nucleus are absent (Traboulsi 2004). Since there is no trace of any oculomotor system element in hagfish, these animals may be good models for tracking the genes responsible for oculomotor nuclei development disorders.

The primitive structure of neuromasts

Neuromasts with stair-stepped microvilli are the rule among craniates, except in hagfishes (character 25; 1 step; Ci 1.000; Ri 1.000; Hi 0.000). Some sensory cells in cephalochordates closely resemble hagfish neuromasts, whereas coronet cells in tunicates seem to be closer to the neuromasts of lampreys and gnathostomes. The ancestral lateral line receptor morphology could be represented by hagfish type, but the homology between hagfish and craniate neuromasts is not yet reliably supported.

Pineal gland formation

Hagfish completely lack the pineal gland (character 94; 1 step; Ci 0.500; Ri 0.667; Hi 0.500). This anatomical peculiarity is particularly surprising in an animal with nearly non-functional eyes, in which the pineal gland would expectedly play a major role in light-regulated processes such as spawning. Several genes are known in the pineal gland development (Mano and Fukada 2007) and some major developmental factor such as HOXD13 and SOX4 are over-expressed in human pineal tumours (Fèvre-Montagne *et al*. 2006). The homologues of these genes should be looked for in the hagfish genome.

Neuronal migration in hagfishes

A striking feature of hagfish neuroanatomy is the unique pattern of diencephalic and telencephalic neuronal migration. For instance, the thalamus in these animals presents several migrated nuclei, while it is mainly periventricular in all other craniate (character 86; 1 step; Ci 1.000; Ri 1.000; Hi 0.000). Furthermore, the pretectum of hagfishes shows a very limited proportion of migrated neurones (character 91; 1 step; Ci 1.000; Ri 1.000; Hi 0.000), whereas their pallium is a mixed structure made up by deep nuclei and a superficial laminated 'cortex' (character 104; 1 step; Ci 1.000; Ri 1.000; Hi 0.000). The question of the di- or telencephalic nature of the central prosencephalic nucleus makes the situation even more intricate. Gene expression territories in hagfish embryos will bring definitive answers to several of these homology problems and clarify the evolutionary history of the craniate forebrain.

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