MOUTH TO MOUTH

Hagfish and lampreys are the only surviving fish without jaws. And they could solve an evolutionary mystery, finds **Henry Nicholls**.

In the basement of the National Museum of Natural History in Paris, two men come to a standstill in the long, gloomy corridor nicknamed 'the submarine'. Philippe Janvier, a senior palaeontologist at the museum, unlocks a door, flicks on the light and leads the way into the 'salle poissons', the room that houses the museum's impressive collection of fossil fish. His visitor, Shigeru Kuratani, is a developmental biologist at Okayama University, Japan, who usually studies the lamprey — one of only two groups of jawless fish with living members. But today, he has come to see some of its long-extinct cousins.

As Kuratani peers at the vivid impression of a jawless fish etched into rock around 400 million years ago, the two get talking. Janvier suggests that Kuratani try to get his hands on an embryo from a hagfish, the only other group of jawless fish that still survives. Few researchers have been able to do it; if Kuratani could, it might resolve a taxonomic dispute that has troubled scientists for more than a century.

For several years after that encounter in 2000, Kuratani mulled it over. Then, in 2004, he took on Kinya Ota as a postdoc at his lab at the RIKEN Center for Developmental Biology in Kobe, and set him the task of succeeding where dozens had failed. "If you get embryos,"

Kuratani assured him, "just one or two, it will make a very important paper."

Kuratani and Janvier are not alone in their obsession with hagfish and lampreys. To a dedicated group of biologists these 'living fossils' are highly prized for what they promise to reveal about some of the earliest events in vertebrate evolution. And advances in developmental biology and molecular genetics are starting to fulfil that promise.

Hagfish and lampreys take researchers back around 500 million years to a time when the first jawed vertebrates, or gnathostomes,

evolved along with a truly 'vertebrate' body plan. The gnathostomes eventually dominated; apart from the hagfish and lampreys, the jawless 'agnathans' went extinct. The question is how exactly the split occurred between the hagfish, lampreys

and gnathostomes (pictured above, left to right), and the conflict between researchers' answers has been described as "one of the most vexing problems in vertebrate phylogenetics"¹. "We are struggling with this discrepancy at the very base of the vertebrate tree and we can't get out of it right now," says Janvier. "We have to find more and different kinds of data."

It is a problem with a history. In 1806, French zoologist André Duméril decided that the striking but similar mouthparts of hagfish and

lampreys meant that they should be grouped together (see 'Two trees') and called cyclostomi, or 'round mouths'. But from the 1970s onwards, morphologists began to have their doubts. Looking beyond the mouth, they found that adult lampreys boast a suite of characteristics that hagfish don't have, including elements of a vertebral column, an ability to control water content by osmoregulation, and the presence of true lymphocytes, a type of white blood cell. This suggested a tree in which lampreys were more closely related to gnathostomes than to the more primitive hagfish lineage.

That might have been the end of it, were it not for molecular biology. From the first trickle of sequence data to today's bioinformatics deluge, just about every molecular analysis suggests that Duméril was right after all: hagfish and lampreys are more closely related to each other than either is to gnathostomes. In this case, the last common ancestor of the two had a vertebral column and other characteristics, and these were secondarily lost by hagfish.

Only one of these trees can be right. It is rather important which one, as the precise route that these branches took has a profound effect on what can be inferred about the evolution of early vertebrates. For many researchers, the morphologists' tree is rather more alluring, as it would allow them to map out the events on the evolutionary path from headless invertebrates through hagfish with heads

"We are struggling with this discrepancy at the very base of the vertebrate tree."

— Philippe Janvier

K. Ota; M. Roggo/NaturePL.com; Stephen Frink Collection/Alamy

but no vertebrae, to lampreys with vertebrae but no jaws, to jawed gnathostomes (see 'Fossil finds'). But morphologists and molecular biologists — each of whom are staking out their own arrangements — seem unlikely to come to any kind of consensus. To Janvier, the idea of plugging these different types of data into a combined analysis doesn't make much sense.

A study earlier this year did combine them, and in doing so it illustrated the depth of the divide. Thomas Near, a molecular systematist at Yale University, was the first person to force morphological and molecular data sets into a single analysis¹. With molecular data pulled together from 4,638 ribosomal RNA sites and more than 10,000 amino acids, hagfish and lampreys emerge as undisputed sister groups. But the addition of just 115 morphological characteristics (from the skeleton and from the sensory, nervous and circulatory systems, for example) re-roots the tree, suggesting instead that lampreys are more closely related to gnathostomes. Near says that it is probably the molecular data that are giving the misleading result, because of difficulties in using DNA and protein sequences to shed light on events that occurred over a very short timescale — hagfish, lampreys and gnathostomes all diverged within a few million years — relative to the hundreds of millions of years that have passed since then. The findings give reason, the paper concludes, "to view the strong support for cyclostome monophyly inferred from molecular data sets with a measured degree of skepticism"¹. So how to resolve the problem?

Start at the beginning

That's where Kuratani's embryos come in. One way of working out evolutionary relationships is to look for a common developmental trajectory in the shape and growth of embryos — a field called 'evo-devo'. "As a general rule there is a danger of looking at an adult and assuming homology between different structures," Kuratani says. "Embryology cuts through that problem."

What researchers want to do is line up the embryos of hagfish, lampreys and a descendant of an early jawed vertebrate — such as the tropical brown-banded bamboo shark

Fossil finds

Palaeontologists have been trying to resolve early events in vertebrate evolution by attempting to illuminate the journey from a jawless to a jawed existence. A pressing challenge has been to make sense of the masses of extinct jawed fish that seem to shoal around this evolutionary transition and place the acquisition of anatomical features onto a timeline. And some fossil finds raise questions about the validity of groups such as the heavily armoured placoderms and the spiny-shark-like acanthodians.

The discovery earlier this year of *Guiyu oneiros* (pictured), an ancient example of a bony fish, has certainly shaken things up⁵. The appearance of this really complex fish around 419 million years ago suggests that most major events in the evolution of modern vertebrates occurred much earlier than was thought. The remains of cartilaginous, lobe-finned and ray-finned fish should populate the fossil

record prior to *G. oneiros*, but nobody has found them.

Where are they? A very real possibility is that some of their remains — isolated teeth, scales and spines — have been unearthed but classified wrongly, says Michael Coates, a palaeontologist at the University of Chicago in Illinois. "Instead of discovering early sharks, we identify these fragments as placoderms or acanthodians," he says. "We miss the opportunity to track the early evidence of how sharks diverged from bony fishes."

A recent report lends support to this view. Earlier this year, Martin Brazeau, then a PhD student at Uppsala University in Sweden,

published his analysis of an overlooked acanthodian braincase that boasted a combination of characteristics of several different groups⁶. Brazeau's work suggests that neither placoderms nor acanthodians are bona fide biological entities, bringing the groups one step closer to disintegration, says Coates. An artificial grouping could be an exciting opportunity for palaeontologists. These miscellaneous groups could contain a wealth of information that will resolve the order and timing at which key characteristics such as jaws, teeth, paired fins and internal fertilization were acquired during early vertebrate evolution. **H.N.**

(*Chiloscyllium punctatum*) — and compare not only their morphological development but also their patterns of gene expression. But getting hold of embryos from hagfish, lampreys or a species representative of early gnathostomes has proven extremely tricky.

For many years, lampreys have been the only cyclostome that evo-devo biologists have had to work with. These slender animals spend most of their lives as mud-dwelling, filter-feeding larvae before metamorphosing into toothy adults that often latch onto fish, rasping them with their tongue until they make enough of a wound to suck blood. The embryos are available for only a few weeks a year, so are difficult to obtain. For several years, members of Marianne

Bronner-Fraser's lab at the California Institute of Technology in Pasadena, for example, collected adults in the field, massaged the gametes from them, then performed *in vitro* fertilization and rudimentary investigations of lamprey development on the spot. Then, Bronner-Fraser says, "we realized the adults could be FedExed", and have since worked out how to extend their reproductive period in the lab.

Hagfish embryos have been even more challenging. The natural habitat of the few dozen described species is in the sludge at the bottom of the ocean. So elusive are hagfish that in the 1860s, the Danish Royal Academy of Sciences and Letters in Copenhagen offered a reward for the first person to work out the reproductive and developmental secrets of the Atlantic hagfish (*Myxine glutinosa*). Almost a century and a half later, the prize is still unclaimed.

After Ota accepted Kuratani's challenge, his first stop was the local fishermen. One of them agreed to supply some adult Japanese inshore hagfish (*Eptatretus burgeri*). Ota put them in a large tank back at Kuratani's laboratory, placed oyster shells and plastic drainpipes in the bottom to give the hagfish somewhere to hide, then regularly hauled the hideaway out on a

TWO TREES

Lampreys could be grouped with hagfish (left), or more closely related than hagfish to gnathostomes.

rope to check for eggs. Finally, Ota found what he was looking for: a cluster of eggs deposited on the fine-grained sand². A year later, the embryos became visible; a *Nature* paper followed soon after³.

The researchers did not resolve the phylogenetic debate, though. The paper showed that in hagfish, development of the embryonic structure called the neural crest and expression of the genes there are very similar to what is seen in both lampreys and jawed vertebrates. Since then, further embryos have been forthcoming. "We are trying to identify the basic design of vertebrates," says Kuratani. "If we can resolve this phylogenetic relationship between lamprey, hagfish and shark, then we can nail what kind of shape would have been there in the latest common ancestor of vertebrates," he says.

Head to head

For now, he and Ota are concentrating on comparing the heads of lampreys and hagfish. The head is a highly specialized structure that "defines the vertebrates", Kuratani says, because building features such as nostrils and a mouth opening required specific and "elaborate" developmental changes during evolutionary history. The researchers are comparing the first pharyngeal arch, for example — a nub of tissue that appears early in the life of vertebrate embryos and gives rise to the jaw and other head structures. This could show whether, as they suspect, the patterns

of gene expression seen in the developing lamprey more closely resemble those observed in gnathostomes.

While some researchers focus on embryos, others are concentrating on genetic sequences. With genome sequencing for the hagfish pencilled in by the US National Human Genome Research Institute in

Bethesda, Maryland, the sea lamprey already sequenced to 6× coverage and a draft genome assembled for the elephant shark (a jawed reference point), there is already a mass of genetic evidence to bring to the problem.

But as Near found in his analysis, standard sequence data may not be enough. So some researchers are now looking to other molecular data, in particular micro RNAs (miRNAs) — the snippets of RNA that are not translated into proteins but perform important regulatory functions. miRNAs are continually added to the genomes of complex eukaryotes such as vertebrates and, once they find a use in a genetic network, they are highly conserved by evolution and rarely lost. This means that if researchers can identify which miRNAs are present — much as a morphologist would score the presence or absence of a physical characteristic — they can potentially reveal more about when the two lineages split than they can by comparing in detail

Lamprey embryos (green) and hagfish (eggs shown, brown) could reveal similarities in development.

other genetic sequences, which requires complex statistics. "There's no other set of molecular data like it," says Kevin Peterson, a palaeobiologist at Dartmouth College in Hanover, New Hampshire. "Unlike other molecular data, it's treated as a set of binary characters," he says. "The morphologists can deal with these data."

A couple of years ago, Peterson compared the miRNA sequences of numerous organisms, including invertebrates such as sea urchins, and vertebrates such as sharks. He unearthed an extraordinary pulse of miRNA acquisition somewhere between 550 million and 505 million years ago — at around the same time that complex vertebrate features such as the head, gills, kidneys and thymus evolved⁴. "Something really amazing was happening to the vertebrate genome at that time," says Peterson. He says that acquisition of these miRNAs could have allowed cells to adopt more complex regulatory systems and to develop new and diverse cell functions. "It's those miRNAs that I would argue allow you to get novel cell types," he says.

But can this help solve the hagfish–lamprey problem? Peterson has been working with palaeobiologist Philip Donoghue of Bristol University, UK, to produce a library of the miRNAs present in hagfish, lampreys and some living gnathostomes — elephant shark, zebrafish and human. "We can use their presence or absence to finally resolve after 150 years or so the relationships between hagfish, lampreys and gnathostomes to work out the pattern of assembly of the body plan of jawed vertebrates," says Donoghue. The libraries have been sequenced and analysed, although neither Peterson nor Donoghue is giving away the result — yet.

On that cliffhanger, the story now rests. Whichever phylogenetic tree Peterson's results favour, he is hoping that it will be something that morphologists and molecular biologists can mull over together. "Our data clearly indicate that one answer is right," teases Peterson. "They unequivocally resolve the debate."

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In pursuit of hagfish embryos, Kinya Ota (front) approached local fishermen to obtain adult fish.