**Figure 1. Orchid mantis.**
A juvenile *Hymenopus coronatus*.

appears analogous to forms of pollinator deception seen in flowers. Some flowers do not provide nectar, but are able to fool pollinating insects into visiting by mimicking a co-occurring 'model' species that does provide nectar. Other non-rewarding flowers, however, are still able to attract pollinators even without a similar 'model' species. Thus, the orchid mantis' predatory strategy can be interpreted as a form of 'generalised food deception' rather than 'floral mimicry'. Whether pollinators cognitively perceive orchid mantises as flowers is difficult to tell. Using artificial models of orchid mantises, studies found that removing certain flower-like characteristics, such as symmetry and petal-like appendages, did not affect the number of pollinators that were attracted to them, confirming the idea of the orchid mantis tapping into general sensory biases of pollinators, rather than mimicking floral traits.

**How do predators perceive orchid mantises?** Evidence from visual modelling suggests that, as in many insects, predators such as birds may not be able to distinguish between the colour of orchid mantises and the colour of flowers. It is entirely possible that the orchid mantis' floral guise also functions as a protective strategy, either as a form of camouflage or masquerade.

**Where do orchid mantises come from?**
They were first described in 1972 from a specimen collected in Ambon, Indonesia. There are accounts of orchid mantises across Malaysia, Indonesia, India, Thailand, Vietnam and Southern China. Orchid mantises are rarely encountered in the wild, so there has been no systematic survey of their populations and there is little information available on their microhabitat or fine scale distributions. The orchid mantis is also a popular specimen for hobbyist insect breeders and collectors. Despite its popularity and appeal, the orchid mantis is an elusive and poorly understood animal. Whether the collection of wild-caught specimens to supply the hobbyist industry poses any threats to natural populations is unknown.

**Where can I find out more?**

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**Primer**

# Tunicates

Linda Z. Holland

Tunicates, also called urochordates, are an extremely diverse subphylum of the Chordata, a phylum that also contains the vertebrates and cephalochordates. The tunicates seem to have undergone especially rapid evolution: while remaining exclusively marine, they have radiated to occupy habitats ranging from shallow water, to near shore to the open ocean and the deep sea. Furthermore, they have evolved a variety of remarkable reproductive strategies, combining asexual and sexual modes of reproduction that allow for very rapid expansion of populations. An outstanding question is what happened to allow tunicates to evolve so much faster than their nearest relatives, cephalochordates and vertebrates.

There are three classes of tunicates, Ascidiacea, Thaliacea, and Appendicularia (Figure 1), with over 2000 species of ascidians, about 72 species of thaliaceans and about 20 of appendicularians. They are called tunicates because the zooids are encased in an extracellular sheath or tunic, which unusually for animals contains cellulose, synthesized by a cellulose synthase that was evidently acquired in an ancestral tunicate by horizontal gene transfer from a bacterium. Ascidians are the best known tunicates as they typically live in shallow water, fastened to rocks or docks or the bottoms of boats, and as some are very colorful. When touched, they contract and squirt water out their siphons — hence the common name 'sea squirt'. The other tunicates constitute a major part of the jelly plankton; they live in the open ocean and are rarely seen except when they occur in large blooms when food is especially abundant. After reproducing sexually, these pelagic tunicates may die *en masse* and wash up in the thousands on beaches.

Because of the tadpole-like larvae of some ascidians and the tadpole-larva-like adults of appendicularians (also called larvaceans), tunicates have long featured in discussions of

how vertebrates evolved from their invertebrate ancestors. In the 20th century, the simplicity of these tadpole-like forms led to the idea that tunicates are ancestral to both cephalochordates and vertebrates. However, genome sequencing has revolutionized this thinking. Phylogenetic analyses with large sets of nuclear genes now place tunicates as the sister group of vertebrates, with cephalochordates basal in the chordates. This reversal has led to the realization that tunicates not only evolve rapidly, but have become secondarily simplified in the process of evolution. They have lost a lot of what the long extinct ancestral tunicate once possessed. Today, a heated argument revolves around the question of just how vertebrate-like the ancestral tunicate was. In the absence of fossils, this argument promises to continue for a long time. In this primer I will discuss the highly derived body plans and life styles of the tunicate classes, their importance in the marine food web and their genomics. The emphasis is on the impact of their especially rapid evolutionary rates on understanding how vertebrates evolved from their invertebrate ancestors.

## Who are the tunicates?
### Ascidians
Although all adult ascidians are sessile and most have a tadpole larva with the characteristic chordate features of a notochord and a dorsal, hollow nerve cord, a number of features have evolved repeatedly among all three ascidian suborders, the Stolidobranchia, Aplousobranchia, and Phlebobranchiata (Figures 1 and 2A). For example, tailless larvae have evolved at least five times within the stolidobranchs. Furthermore, coloniality resulting from asexual reproduction by budding of the original sexually-produced zooid has evolved independently several times. In some instances, the asexually produced zooids remain closely adherent in a common tunic and in others, they stay attached only through narrow stolons. When colonies of the same, but not different, genotype of the stylelid ascidian *Botryllus* expand to touch each other, they fuse; however, colonies of the aplousobranch ascidian *Diplosoma listerianum* fuse regardless of genotype.



**Figure 1. Representatives of the five major groups of tunicates.**
Clockwise from top left. A solitary ascidian, *Ciona savignyi* (photo courtesy of Robert Zeller). An appendicularian, *Oikopleura dioica*; a ripe female with an ovary full of eggs inside its house (photo courtesy of Fabien Lombard). Then three thaliaceans: a small colony of a pyrosome, *Pyrosoma atlanticum*; a nurse doliolid with a stolon bearing feeding polyps (trophozooids) along the sides (the phorozooids along the midline of the stolon appear to have broken off); and a solitary salp, *Cyclosalpa affinis*, with an asexually-produced chain of aggregates. In this genus, the aggregates are organized into whorls (photos of the three thaliaceans courtesy Laurence P. Madin). Sizes are not to scale.

While tunicates are almost all filter feeders, eating phytoplankton and other small particles, there are several deep-sea species of ascidians, chiefly in the family Octacnemidae (Phlebobranchia), in which the oral or incurrent siphon is enlarged to form a mouth that can capture large prey. Because adult ascidians are fixed to the substratum, they invite predation by fish and other carnivores. As a result, they have a variety of chemical defenses, most thought to be synthesized by symbiotic bacteria, which presumably act to deter predators, and some ascidians concentrate vanadium, which has been shown to deter predatory fish.

The size range of ascidian zooids is very wide. The solitary adults of phlebobranchs and stolidobranchs usually are fairly large, up to 6–7 cm in height, while the carnivorous ones can be up to 26 cm in height. In contrast, aplousobranchs, which, except for the solitary *Ciona* spp. (Figures 1 and 2A), are colonial and usually have very small zooids, just a few millimeters high. In spite of this enormous variation in size, reproduction and mode of feeding, the basic body plan is relatively conserved. The typical body plan of a zooid is shown in Figures 1 and 2A. Feeding occurs as water enters via an oral or buccal siphon, passes through a branchial basket perforated by numerous gill slits, and exits by the excurrent siphon. The endostyle within the branchial basket secretes mucous, which traps the particles and carries them into the stomach. The nervous system is reduced to a dorsal

ganglion, but there are numerous ectodermal sensory cells. The heart is located basally near the stomach, and periodically reverses direction. The blood flows from the heart through hemocoel spaces. All ascidians are hermaphroditic; self-fertilization is avoided in at least some simultaneous hermaphrodites, such as *Ciona*, by incompatibility of genetic variants of cell-surface proteins.

Ascidian embryos undergo rapid development to a tadpole larva. Cleavage is determinant, meaning that cell fates become fixed very early in embryogenesis. If the first two cells are separated, each develops only into the cells that it would normally form in the intact egg. Cytoplasmic movements within the zygote shortly after fertilization set aside cytoplasm, known as myoplasm, that will form most of the larval tail muscle. In *Ciona*, hatching to a tailed, tadpole larva occurs within 24 hours, depending on the temperature (the total time from embryo to fertile adult is just three months). The larva swims for a few days at most until encountering a suitable substratum to which it attaches by anterior adhesive papillae. The tail is resorbed, and within about two days of settlement, the larva completes metamorphosis into a juvenile, with incurrent and excurrent siphons and two gill slits. Ascidians typically reproduce sexually at regular intervals during their life span, which can be a year or more. In colonial species, embryos are often brooded.

The ability of ascidians to regenerate all or part of their body was noted over 100 years ago. Interest in this has grown of late because of the potential of pluripotent stem cells for treating various human maladies such as spinal cord injuries. Regeneration is likely related to asexual reproduction by budding. Even so, solitary ascidians that don't bud, such as *Ciona*, can also regenerate parts of the body. This is particularly useful because they cannot escape predators. Thus, if a fish bites off part of a siphon or even the neural complex, they can regrow the missing parts. A particularly critical experiment was done in 1964 by G. Freeman, who showed that gamma irradiation inhibited cell division and budding in a colonial ascidian, and that injection of blood cells from an unirradiated individual of the same species into the irradiated one restored budding. These pluripotent blood cells can, therefore, give rise to all the body parts in the right position; just how they can do this is a hot topic for research.

### Appendicularians

Appendicularians are in some ways the simplest tunicates. Most are very small, about 2 mm long, and as adults they resemble the tadpole larva of ascidians, with a trunk, motile tail, notochord and nerve cord; hence their alternative name, Larvacea (Figures 1 and 2B). But they have also evolved some extremely bizarre novel traits, including secretion of a 'house' around themselves which serves in filter feeding and provides buoyancy (Figure 1). This house has meshes of varying sizes that concentrate particles and filter out ones too large to fit in the mouth. The beat of the tail draws water through the house and into the mouth. When the house becomes clogged with particles, the animal swims out of the house and inflates another, which has already been secreted and lies deflated around the trunk until needed. The structure of the house is very intricate, with meshes of different sizes. Moreover, the morphology of the house differs among the various genera. Particularly large appendicularians with trunks 0.5–1.0 cm long, such as *Bathychordaeus* and *Mesochordaeus* from Monterey Bay, California, have houses up to 30 cm in diameter.

It is still a bit of a mystery how such small, simple animals can make such elaborate houses. The ectoderm cells that secrete the house, termed the 'oikoplast', are polyploid; the glycoproteins that these cells secrete, known as 'oikosins', are unique to appendicularians. Specific regions of the oikoplast secrete different oikosins, but how they form the different mesh sizes is a major unanswered question.

Not only do appendicularians have simple, tadpole-like bodies (Figure 1B), their reproductive strategy is also streamlined. They all reproduce exclusively sexually and, with the exception of *Oikopleura dioica*, they are hermaphroditic, producing eggs and sperm sequentially, thereby avoiding self-fertilization. Their life spans are very short: that of *O. dioica*, the only appendicularian that has been cultured in the laboratory, is 10 days or less, depending on temperature. When reproducing, they swim out of the house towards the water surface. In the wild, over a thousand individuals per liter have been documented. Fertilization is external, and after shedding the gametes, the adults die.

In *O. dioica*, development to adult takes just 24 hours. Gametogenesis is rapid, with oocyte nuclei and polyploid nurse nuclei in a syncytium termed the coenocyst. At the end of oogenesis, the oocytes are individualized and begin to undergo the meiotic divisions. Fertilization occurs at first meiotic metaphase. There are cytoplasmic movements after fertilization, and it seems likely that they may also segregate the myoplasm, though this aspect of embryogenesis has not been studied in appendicularians. Gastrulation in *O. dioica* occurs one cell division earlier than in *Ciona*. At the end of development, the tadpole hatches and its tail rotates 90° and begins to beat and inflate the first house.

### Thaliaceans: salps, doliolids and pyrosomes

Although thaliaceans appear to be monophyletic, they are quite divergent from one another, as well as from ascidians and appendicularians. They have all lost the dorsal, hollow nerve cord and notochord, except for a rudimentary one in some doliolid larvae. Their reproductive strategies are particularly strange, with very complex alternation of asexual and sexual generations (Figure 2C–E). The selective advantage of asexual reproduction in thaliaceans is presumably that it allows them to exploit blooms of phytoplankton to rapidly expand the population. Because thaliaceans live in the open ocean, they are difficult to maintain in the laboratory and have been much less studied than ascidians or even *O. dioica*.

Salps are usually very scarce in the ocean, but when there are blooms of phytoplankton, the populations can explode. Net tows have documented over 1,000 individuals of *Salpa thompsoni* per 1,000 cubic meters in Antarctic waters. Together with appendicularians and doliolids, they are major players in the marine food web, cycling energy from shallow waters deep into the water column by eating

phytoplankton and excreting carbon-rich fecal pellets, which together with their dead carcasses sink down into the water. The carcasses are colonized by bacteria, which in turn are colonized by viruses. Together with the discarded houses of appendicularians, the bodies of dead pelagic tunicates make up a substantial proportion of the so-called 'marine snow'. Salps are parasitized by the amphipod *Phronema*, which eats the insides of the zooids and uses the tunic as a house as it moves through the ocean.

Salps can be quite large: while individuals of a nearshore species, *Thalia democratica*, are only 1–2 cm in length, those other species range up to about 10 cm long. They typically undergo a diurnal migration, going as deep as several hundred meters during the day and coming up to the surface at night, where reproduction, both sexual and asexual, occurs.

Salps move through the water by rhythmic contractions of muscles that nearly (but not quite) encircle the zooid and pump water in through the mouth and out the cloaca. The individual zooid produced by union of egg and sperm is termed the solitary form (Figures 1 and 2D); it periodically buds off a chain of up to 200 asexually generated individuals, termed aggregates, each of which contains an oocyte (egg) and a testis. As the chain is extruded, the individuals at the tip of the chain inflate and begin to pump water. Inflation of zooids progresses from the tip to the base of the chain. Each aggregate typically produces a single egg (rarely two), which is fertilized internally as soon as the zooid begins to pump; the embryo is brooded. Thus, a given chain has zooids with embryos at successive stages of development.

Typically, after release from a solitary zooid, the chain breaks up into individuals. The embryos grow into mature solitaries and swim out of the cloaca of the parent. Then the testis of the aggregate sheds sperm, after which the zooid dies. The sperm, which are drawn into the branchial chamber of the newly inflated aggregates, find their way to where the 'oviduct', a column of cells with no lumen, meets the branchial chamber. Oddly, the corkscrew-shaped sperm burrow through the centers of the cells of the oviduct to find the egg.
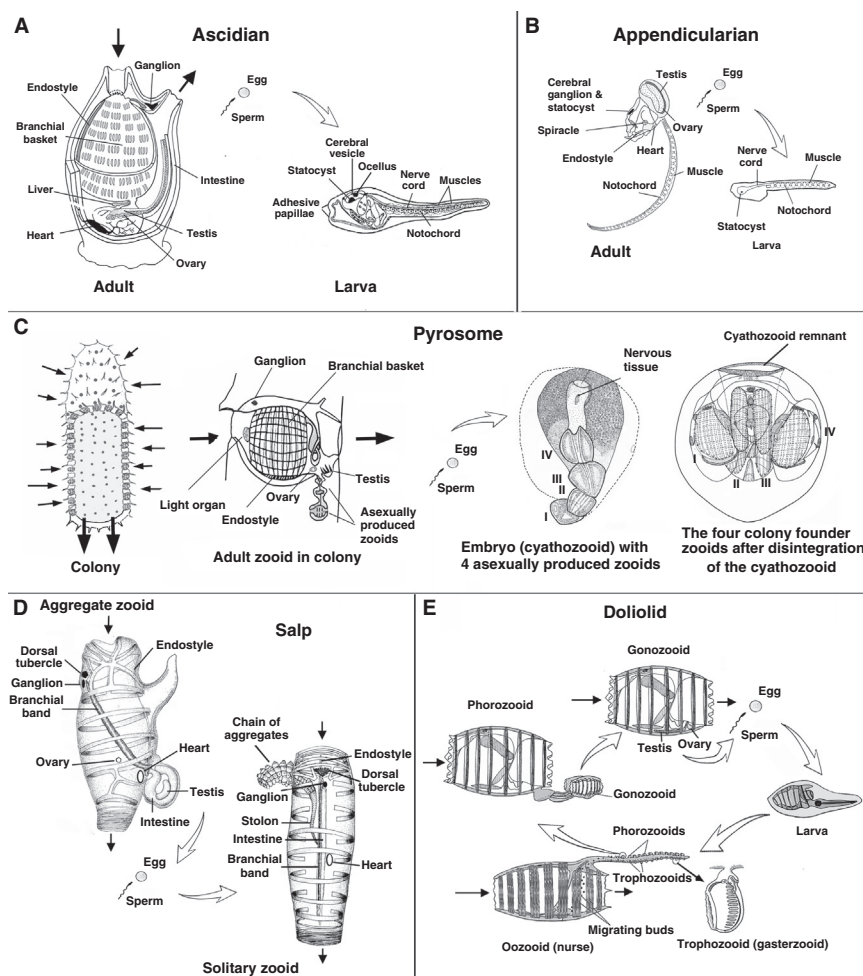


**Figure 2. Diagrams of the five major groups of tunicates.**
(A) A solitary ascidian. Coloniality has evolved several times in ascidians. Adults are sessile. Arrows indicate water flow into the incurrent and out of the excurrent siphon. They are simultaneous hermaphrodites, but self-sterile due to gamete surface protein incompatibility. The larva undergoes a short pelagic phase before attaching to the substratum by the adhesive papillae; the tail is then resorbed and the nervous system remodeled. (B) Appendicularians, most only a few millimeters long, are exclusively pelagic. They secrete a series of houses for buoyancy and filter feeding. None is colonial. All except *Oikopleura dioica* are hermaphroditic. Larval development is direct. Tunicates have hearts that periodically reverse the direction of the blood flow. (C–E) The three groups of thaliaceans. (C) Pyrosomes form colonies up to several meters long, which move slowly by jet propulsion. Each zooid in the colony reproduces asexually, enlarging the colony, and sexually, producing an embryo termed a cyathozooid brooded within the colony. Each cyathozooid is resorbed after budding four zooids; this colony is expelled from the parent colony to start a new one. (D) Salps have a biphasic life cycle; the solitary zooid repeatedly buds off chains of zooids, called aggregates, each of which typically has just one egg, fertilized as soon as the zooid expands and begins pumping water. The eggs develop directly into small solitary zooids and are then expelled. Sperm are produced about the time the embryo matures. (E) Doliolids have a very complex life cycle. The larva develops into a non-feeding oozoid or nurse, which then produces a stolon that bears two types of zooids, trophozooids, which only feed and stay attached to the stolon, and phorozooids, which break off the stolon. The phorozooids then bud off gonozooids, each of which develops an ovary and a testis. They are distinguished from salps by muscle bands that are continuous around the body. (A) Adult image ©BIODIDAC; both adapted by Jon Houseman, Wikimedia Commons. (B) Adult image adapted with permission from SAHFOS. (C) Colony and Adult zooid images adapted with permission from Anne and Wilfried Bay-Nouailhat; cyathozooids after Godeaux, J. (1987); (D) after Kim *et al.* (2012); (E) adapted from Braconnot (1971).

Early development in the ovary is quite bizarre. After the third division of the embryo, the blastomeres separate and non-germinal follicle cells termed calymnocytes move in between them. Development must, therefore,
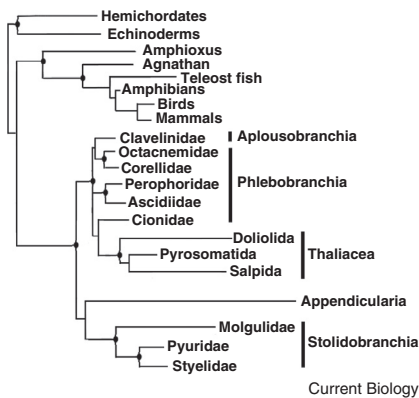
**Figure 3. Tunicate phylogeny based on 18S rRNA (ascidians are not monophyletic).**
Thaliacea (Doliolida, Pyrosomatida and Salpida) are most closely related to a group of six families of ascidians (Clavelinidae, Octacnemidae, Corelidae, Perophoridae, Ascidiidae and Cionidae). In this phylogenetic tree, appendicularians are most closely related to Molgulidae, Pyuridae and Styelidae, but the branch length is very long. In trees constructed from concatenated nuclear genes and fewer species of tunicates, appendicularians are basal in the tunicates and amphioxus basal to a group of vertebrates plus tunicates. Bayesian majority-rule consensus tree. The dots indicate nodes that are supported by four different methods. Adapted from Tsagkogeorga *et al.* (2009).

be determinant. The calymnocytes apparently function to nourish the blastomeres, which eventually come back together to form an embryo. Salps can live for months to a year or more.

Doliolids are also major players in the ocean food web, though they are generally smaller than salps, being rarely longer than 4–5 cm. They are preyed upon by copepods. High densities have been documented, for example, up to 550 individuals per cubic meter of *Doliolum dentaculatum* in the Yellow Sea. Doliolids are distinguished from salps by having muscles that entirely encircle the body. Instead of pumping water through the body to filter feed and move through the water like salps, doliolids glide through the water propelled by the action of cilia on the branchial basket. At intervals, their muscles contract and they jerk ahead.

Doliolids have an incredibly complex life cycle, the most complex of any tunicate (Figure 2E). The sexually produced individual, called the nurse, deriving from union of egg and sperm, does not directly feed, but produces

a stolon which bears three rows of zooids (Figures 1 and 2D). Those in the center are called phorozooids, while those on either side are trophozooids or gastrozooids. The latter remain attached to the stolon and filter feed, nourishing the nurse and the growing phorozooids. The phorozooids detach from the stolon and bud off gonozooids, which are hermaphroditic. The gonozooids become sexually mature and typically release a few eggs, which are fertilized either within the branchial cavity of the doliolid or after being shed into the sea water. Then the zooids release sperm. Fertilization has not been observed. At 20° C, the entire life cycle can take about 20 days. Development is semi-direct. *Doliolum* embryos develop a tail, in some respects like that in ascidians, which has a notochord flanked by muscle cells but lacks a nervous system. The tailed larva does not hatch, and it is likely that tail movements, if any, are rather weak. The anterior portion of the embryo develops directly into a young adult, and the tail resorbs.

Pyrosomes are never common. Although the individual zooids are very small, like those in colonial ascidians, the colonies, consisting of thousands of asexually-produced zooids, can become very large, up to about 20 m long (Figures 1 and 2C). The colony is closed at one end, and open at the other; the opening can be up to nearly 2 m in diameter. A dead penguin trapped inside a pyrosome colony has been reported, and there are photos of divers with their heads inside pyrosomes.

Each small pyrosome zooid is oriented with the mouth pointing to the outside of the colony and the cloaca pointing inward. Water moves into the mouth and out through the cloaca and is expelled through the opening of the colony, driving it forward by slow jet-propulsion. Colonies of pyrosomes are most abundant in subtropical and tropical waters where they are often eaten by sea turtles. They are sometimes pink, and fishermen have called them 'silk stockings'. Each zooid has light organs, which luminesce brilliantly when the colony is stimulated mechanically or by light. It is unclear whether this luminescence is due to symbiotic bacteria or not. A bacterial-

type luciferase has been isolated from pyrosomes; bacteria have been seen in the light organ, but none have been cultured.

Pyrosomes have always been thought to be fairly closely related to ascidians, and recent phylogenetic analyses have placed them together with the other thaliaceans as sister group to the stolidobranch ascidians (Molgulidae, Pyuridae and Styeledae). They are hermaphroditic, reproducing both sexually and asexually, with internal fertilization. The embryo cleaves into a group of cells termed the cyathozooid, which, before it is released into the sea water, buds off four zooids. Budding continues after release from the parent until the colony becomes quite large.

**Evolution and phylogeny of tunicates**
The question of where tunicates fit in the animal kingdom has been a bone of contention ever since they were first described, around 350 BC, by Aristotle, who thought they were molluscs. In contrast, Lamarck, who coined the name Tuniciers (Tunicates) in the early 19th century, thought they were closer to holothurians among the echinoderms. With the discovery of tailed ascidian larvae with a notochord, dorsal nerve cord and tail muscle by Kowalevsky (1866), their chordate affinities were recognized, though von Baer (1873) persisted in classifying tunicates as molluscs.

By the late 1800s, the evolution of tunicates and their relationship to vertebrates had become even more hotly contested. For example, Brooks (1893), like most authors of the time, regarded appendicularians with their motile tails as adults, as akin to the ancestral tunicate. He also argued that salps, pelagic but tailless, had evolved from a sessile ascidian-like ancestor. Similarly, Willey (1894) argued that the proximate vertebrate ancestor was free-swimming and intermediate between an ascidian tadpole larva and a cephalochordate. This idea prevailed until Walter Garstang (1894) proposed that chordates evolved from an adult ancestor similar to the larva of a hemichordate or echinoderm. He later modified this theory to include a sessile, tentaculate ancestor rather like a pterobrach hemichordate or a solitary ascidian with tentacles,

arguing that subsequently the larva of this animal acquired a muscular tail and, by paedomorphosis (neoteny), became a prematurely sexual, vertebrate-like adult. As ascidian larvae are very simple, with relatively few cells, Garstang assumed that such a larva preceded the more complex body plans of chordates. He further proposed that the sessile adult form evolved into modern ascidians which then gave rise to doliolids, which in turn gave rise to appendicularians.

Berrill and Romer popularized this theory, which made its way into many textbooks of the 20th century. But the theory of appendicularians as descended from ascidians began to lose credence in the late 1980s, when transmission electron microscopy revealed that even though ascidian gametes have lost structures present in the gametes of most marine invertebrates — cortical granules in the egg and an acrosomal granule in the sperm — appedicularians have them. So if appendicularians evolved from an ascidian larva they would have had to reinvent these structures, which seems highly unlikely. This led to a revival of the idea that appendicularians were basal in the tunicates and that the ancestral chordate was a pelagic free-swimming adult.

Because both mitochondrial and nuclear genomes of tunicates are evolving exceptionally rapidly, a broad consensus as to the phylogenetic relations within tunicates and between tunicates and vertebrates has been rather elusive. Given their simple tadpole larva, morphology-based trees had placed tunicates basal in the chordates. Phylogenetic trees constructed with sequences of 18s rRNA concurred, placing appendicularians as the sister group of either ascidians plus thaliaceans or of the stolidobranch ascidians (Figure 3). However, this branch is very long and not well-supported, and as discussed below, it is most likely that appendicularians are basal in the tunicates, but are simply evolving far more rapidly than other tunicates.

The breakthrough in understanding the phylogenetic relations within the chordates came from analyses with concatenated sets of nuclear genes, which evolve more slowly than mitochondrial genes and have,

therefore, proven to provide a more accurate estimation of phylogenetic relations at the phylum and subphylum levels. These analyses, in agreement with gamete morphology, placed appendicularians basal in the tunicates, albeit with a long branch, and tunicates as sister group to vertebrates, with cephalochordates basal in the phylum.

### Genes, genomes and development
To date, the only tunicate genomes sequenced are those of the ascidians *Ciona intestinalis* and *Ciona savignyi* and of the appendicularian *Oikopleura dioica*. Although a PhD dissertation on sequencing the genome of *Salpa thompsoni* was submitted in 2014, the genome sequence is not publically available. Analyses with nuclear genes show that tunicate genomes are evolving much, much faster than those of any other chordate (Figure 4). As a result of the loss of both intergenic regions and genes themselves, tunicate genomes are very small: those of *Ciona* sp. are 160–190 Mb, and that of *O. dioica* is only about 70 Mb, compared to genome sizes of about 520 Mb in cephalochordates, 814 Mb in a sea urchin and 3 Gb in humans.

Features that contribute to genome contraction in tunicates include having a high percentage of genes in operons, where several genes are transcribed into a single mRNA, and a leader sequence is *trans*-spliced in at the 5′ ends of the individual mRNAs to create monocistronic mRNAs, which are transported to the cytoplasm. This is uncommon among bilaterians, but is also found in nematodes and in some platyhelminths, which have similarly reduced genomes. Surprisingly, gene loss in tunicates includes a number of developmental genes that are essential in cephalochordates and vertebrates. For example, while both *O. dioica* and *C. intestinalis* have discarded *Hox7* and *Hox8*, *O. dioica* has also lost *Hox3*, *Hox5*, and *Hox6*, while *C. intestinalis* has lost *Hox9* and *Hox11*. Even so, the number of genes is still reasonably high at ~18,000 in *O. dioica* and ~15,000 in *C. intestinalis.* In part this is due to independent gene duplications; for example, in *O. dioica* and *C intestinalis*, there are two *Pax2/5/8* genes compared to one in the cephalochordates, indicating an independent duplication in an ancestral tunicate.
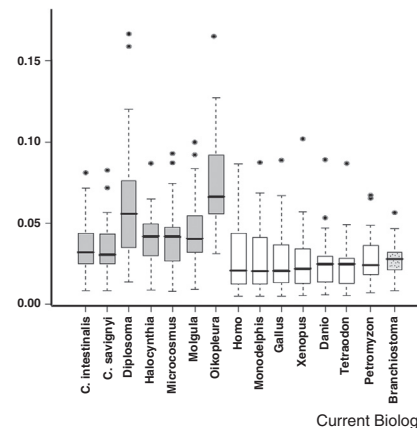


**Figure 4. Evolutionary rates of tunicates are much faster than those of other chordates.** The evolutionary rates are based on branch lengths of trees for 35 proteins. Among the tunicates, the appendicularian *Oikopleura dioica* is evolving fastest. The horizontal bar indicates the median of rate distributions. The boxes indicate the quartiles. The dotted lines indicate 1.5 X the interquartile range. Circles indicate outliers. Tunicates are indicated by grey bars and amphioxus (*Branchiostoma*) by a dotted bar. (Adapted from Tsagkogeorga *et al.* (2010) with permission of Springer.)

This fast evolution of tunicate genomes is also shown by the initial failure of genome alignments to reveal any conserved non-coding sequences (potential gene regulatory elements) between tunicates and other chordates, even though such alignments between vertebrates and cephalochordates, or between cephalochordates and hemichordates, have revealed hundreds to thousands of such conserved non-coding sequences. Comparisons between the closely related *Ciona intestinalis* and *Ciona savignyi* revealed some conserved non-coding sequences, which also proved to be conserved with vertebrates, but these are located in different places in vertebrate genomes. Nine of these also matched sequences in *O. dioica* and 13 matched sequences in amphioxus; three that were tested were verified as gene regulatory elements.

### What was the common ancestor of tunicates and vertebrates like?
Because tunicates are the closest relatives of vertebrates yet are far simpler, there have been many attempts to look in tunicates for clues to the evolutionary origins of vertebrate-specific structures.

The most common method has been to compare patterns of embryonic gene expression. For example, expression of the *Brachyury* gene in the notochords of tunicates, amphioxus and vertebrates indicates that these notochords are probably homologous. The same is true of *Pax1/9* expression in the developing pharynx of all three organisms. However, the main interest has been in finding homologies between specific neural structures in tunicates and vertebrates. This has proven to be very difficult, as the *Ciona* larval nervous system, with only about 100 neurons, compared to millions in vertebrates and thousands in cephalochordates, has evidently lost a lot of cells over the course of its evolution.

These problems are illustrated by attempts to find in tunicates the evolutionary origins of neural crest and of specific regions of the vertebrate nerve cord. Neural crest, which is only found in vertebrates, comprises pluripotent cells that migrate from the edges of the vertebrate neural plate and differentiate into numerous cell types including pigment cells. It was first proposed that cells migrating from the vicinity of the larval neural tube of one tunicate, some of which develop into pigment cells, may be related to neural crest, although they migrate much later in development than vertebrate neural crest cells. Later it was shown that misexpression of *Twist*, a marker of migratory and post-migratory neural crest in vertebrates, in the pigment cell lineage of *Ciona*, induces some cells to migrate away from the tunicate neural tube. Both lines of evidence for neural crest precursors in tunicates are suggestive, but are simply not enough to conclude that the ancestral tunicate really did have neural crest.

Nearly as tenuous is the evidence for tunicate equivalents of the vertebrate forebrain, midbrain and hindbrain, including the vertebrate midbrain/hindbrain boundary. The *Ciona* larval nerve cord consists of an anterior sensory vesicle containing two pigment cells, a statocyst and a photoreceptor or ocellus; posterior to that are the neck region, the visceral ganglion, which contains motor neurons and the tail nerve cord. Based on similarities in gene expression it was proposed that

the sensory vesicle is largely equivalent to the vertebrate forebrain, while the neck region and visceral ganglion are equivalent to the midbrain plus the vertebrate midbrain/hindbrain boundary and anterior hindbrain. Maybe so, but as there are many differences both in gene expression and structure between the tunicate and vertebrate nerve cords, it may never be certain what the nervous system in the common ancestor of tunicates and vertebrates was like. Tunicates have simply lost too many genes, too many cells and too many structures for an accurate reconstruction of this ancestor.

## Conclusions

Tunicates are extremely diverse and have radiated to occupy most marine habitats from the deep sea to littoral zone to the open ocean. At least those for which genome sequences are available — ascidians and appendicularians — are evolving much more quickly than other chordates. Thaliaceans, and some ascidians, have complex alternation of sexual and asexual generations. Tunicates have reduced genomes compared to those of other chordates, with many genes in operons. Unlike other deuterostomes, tunicates have determinate development, with cell fates decided very early. It is very tempting to propose that such early determination of cell fates has facilitated divergence at later developmental stages, resulting in the great diversity of body plans in tunicates. But although there is also such a correlation between determinate development and rapid evolution in some other bilaterians like nematodes, a cause-and-effect relationship has not been clearly demonstrated. The questions remain: which came first in evolution, determinate development or rapid evolution, and did the first to evolve drive the second? Tunicates show what evolution *can* do, but this raises the question why they are evolving so quickly while their closest relatives, the cephalochordates and vertebrates, are evolving particularly slowly.

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