Evolutionary Genomics of Tunicates

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Abstract: Tunicates are the closest living relatives of vertebrates and are a group comprised exclusively of marine animals. In the current review, I aim to present some of the most interesting aspects of tunicate genomics within an evolutionary context to a non-specialized scientific audience. Tunicates are of scientific interest for many reasons. First, their phylogenetic position, as well as their internal evolutionary relationships, are heavily debated. Second, multiple species have been studied as developmental biology and evolutionary models. Third, some tunicate species play crucial ecological roles and functions. Lastly, tunicates have become an interesting field of study in evolutionary genomics since the beginning of the new millennium. Tunicate genomes are atypical within chordates, bearing many hints of divergence and differentiation. In addition, their genomes are fast-evolving and highly plastic, with functional genetic units uncharacteristically often organized in operons. It is likely that continued research efforts surrounding tunicates will continue to challenge current understanding of the mechanisms driving molecular adaption as well as evolutionary genomic processes.

Keywords: Tunicates, Chordates, Genomics, Operons, Genome Plasticity.

Tunicates and the Evolution of Chordates.

The phylum Chordata includes three subphyla, namely Craniata (or Vertebrata, which includes humans), and two non-vertebrate subphyla, Cephalochordates and Urochordates (or tunicates) (Maisey 1986; Cameron et al., 2000; Delsuc et al., 2006; Delsuc et al., 2008; Stach, 2008; Zhong et al., 2009). The primary feature shared by all chordates is the presence of a notochord within a tadpole-like body plan developed at the end of embryogenesis, hence the source of the phylum's name (Lemaire, 2011). Notwithstanding the long-known close evolutionary relationships between chordates (Turbeville et al., 1994; Cameron et al., 2000), as well as their monophyly (Turbeville et al., 1994; Cameron et al., 2000; Bourlat et al., 2006; Stach, 2008; Satoh et al., 2014), the exact evolutionary relationships between the three subphyla have been long-debated, and a consensus regarding this issue has yet to be reached.

There are three main hypotheses that attempt to explain the evolutionary relationships of chordates (**Figure 1**).

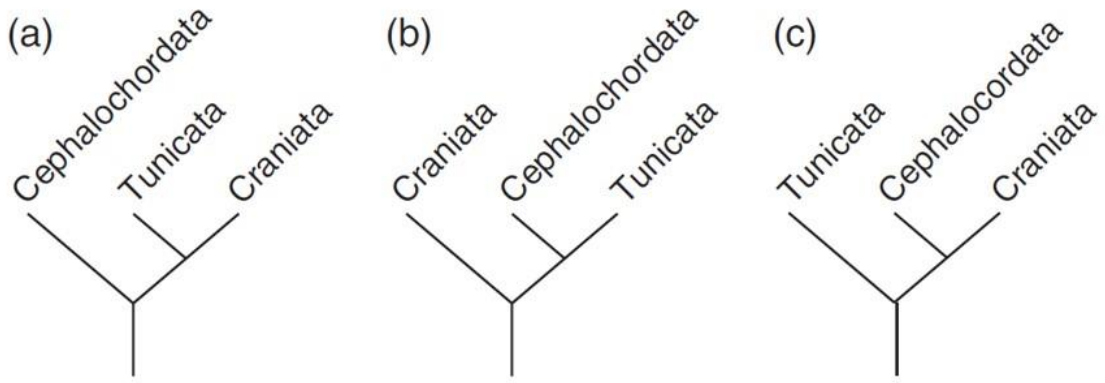

Figure 1: *The three hypotheses on chordates evolutionary relationships: (a) the 'Ofactores hypothesis', (b) the Atriozoa hypothesis', and (c) Notochordata hypothesis'. Modified from Stach, 2008.*

Notably in each, the relative location of tunicates within the Chordata evolutionary tree varies. In the first hypothesis, named the '*Atriozoa hypothesis*', tunicates are considered a sister taxon to the Cephalochordata. In the second hypothesis, called the '*Olfactores hypothesis*', Tunicata is placed as a sister taxon to Craniata. Finally, the '*Notochordata hypothesis*' postulates that Craniata and Cephalochordata are sister taxa (Stach, 2008). The Notochordata hypothesis has been widely accepted by the scientific community for more than a century, while the Atriozoa hypothesis is of mere historical interest, having been rejected decades ago (Stach, 2008). The Olfactores hypothesis is also an older hypothesis, and was principally invoked only due to the controversial interpretation of unusual fossil records (Jefferies, 1986).

Despite its surrounding controversy, the Olfactores hypothesis has become the main competitor of the Notochordata hypothesis since the beginning of the new millennium, mainly due to novel genomics insights via technical advances in genome sequencing. The most obvious reason for supporting the Notochordata hypothesis was the high resemblance of a Craniata body plan. However, large datasets reflective of genomics and phylogenomic analyses have since challenged this notion and spurred the recent turn towards the long-neglected Olfactores hypothesis (Delsuc et al., 2006; Delsuc et al., 2008; Dunn et al., 2008; Swalla & Smith, 2008). The Olfactores hypothesis has now become the most widely accepted hypothesis.

Deuterostomia is a major evolutionary group which includes three clades (Echinodermata, Hemichordata, and Chordata). More recently (Satoh et al., 2014), it has been suggested that a general revision of Deuterostomia is required to fully describe the evolutionary relationships between higher animals. According to the proposed revision, Bilateria, the Echinodermata and Hemichordata phyla will form a new superphylum (Ambulacraria), while Cephalacordata, Urochordata and Vertebrata (the last two jointly forming the Ofactoria group) will be recognized as proper phyla, with Chordata categorized as the other superphylum within Deuterostomia.

Phylogeny of tunicates.

Tunicates are clearly pivotal in higher animal evolution despite the uncertainty surrounding their phylogenetic position in the animal tree of life. The understanding of phylogenetic affinities within tunicates is also a matter of debate, but new molecular data is working to provide further insights.

Tunicates are commonly divided into three classes, namely Ascidiacea (or ascidians), Appendicularia (or larvaceans) and Thaliacea (or thaliaceans) (Lemaire, 2011). Each of the three classes bears distinctive life-history traits and developmental modes. The number of species included in all the

three classes is highly uncertain, since molecular techniques are revealing a higher-than-expected number of cryptic, morphologically hard-to-distinguish, species (Lopez-Legentil and Turon, 2006; Caputi et al., 2007; Pérez-Portela et al., 2013; Plessy et al., 2024). The most biodiverse class are the ascidians, with more than 2,500 species known and commonly classified into three orders (Phlebobranchia, Aplousobranchia and Stolidobranchia) based on branchial sac structure and molecular data (Stach and Turbeville, 2002; Lemaire, 2011). The most evident developmental and lifestyle characteristic of the ascidians is that all species live as free-swimming tadpole-like larvae that later undergo metamorphosis resulting in a sac-like sessile solitary or colonial adult life form (Lemaire, 2011). From a historical point of view, benthic species capable of colonizing ports and other areas in the proximity of densely populated human settlements such as *Ciona intestinalis* and *C. robusta* have become, thanks also to the easiness of finding,, ideal model species for evolutionary and developmental studies. Appendicularia and Thaliacea (or thaliaceans) consist of exclusively planktonic species. Like the ascidians, thaliaceans also undergo metamorphosis to form an adult organism (Lemaire, 2011). However, unlike ascidians, the species belonging to this class retain larval-like morphology for their entire lifespan (Bone, 1998). Consequently, Appendicularia is of interest from an evolutionary development point of view (Delsuc et al., 2006).

The most recent studies on the evolutionary relationships between tunicates have challenged the canonical three-class partition. A molecular phylogeny based on a large dataset found at least four major clades (Tsagkogeorga et al., 2009; Kocot et al., 2018; Delsuc et al., 2018). The first class was determined to consist of only Appendicularia and the Thaliacea and Ascidiacea classes were not confirmed. Rather, the second class included all Thaliacea, plus ascidians Phlebobranchia and Aplousobranchia, while Molgulide alone formed the third class and Styelidae with Pyuridae the fourth (**Figure 2**).

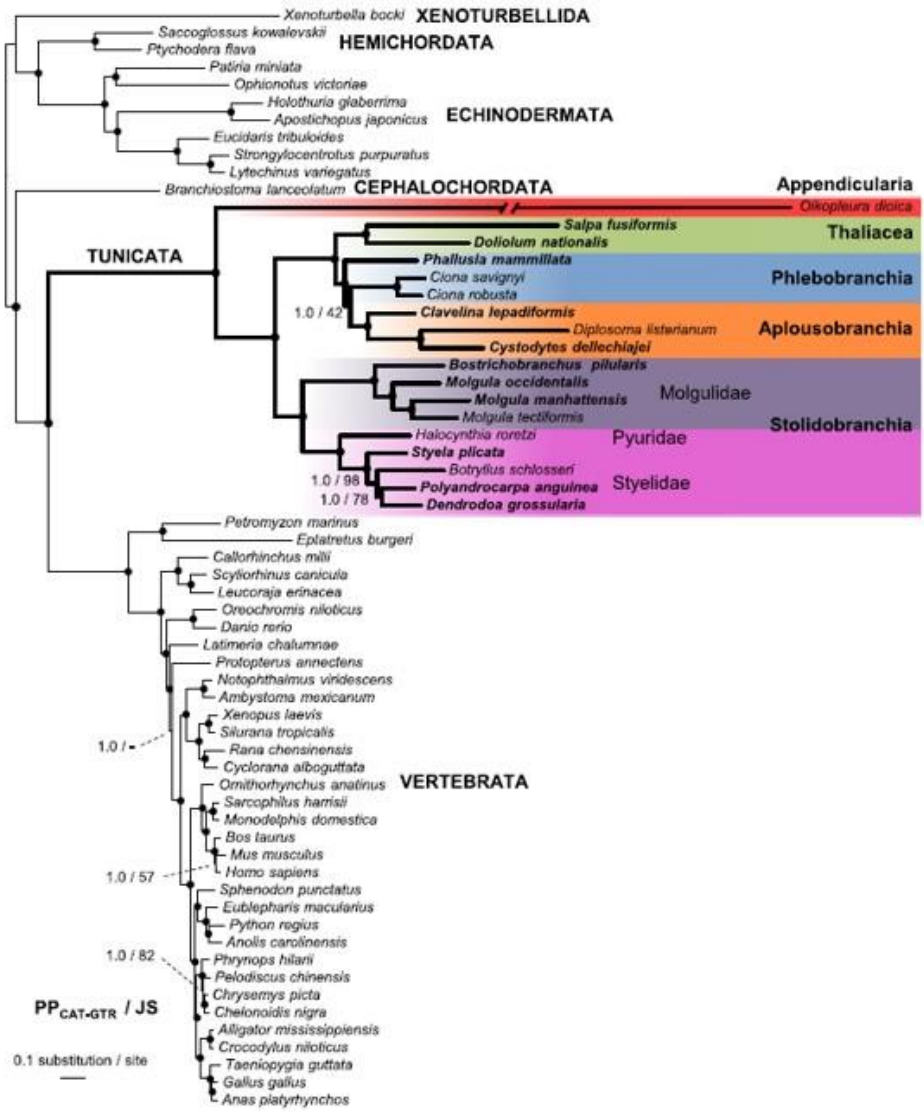

Figure 2: *Phylogenetic relationships between major clades of tunicates. Modified from Delsuc et al., 2018.*

Genomic Features of tunicate genomes.

Tunicates show a remarkable number of unique genomics features within chordates. From the presence of operons to unusually fast evolutionary rates and highly packed genomes to a disrupted Hox genes cluster, tunicates display divergent characteristics not evident in other chordates.

Fast-evolving genomes.

Tunicate species are among the fastest evolving chordates in terms of molecular evolution. Species belonging to the *Ciona* genus such as *C. intestinalis* and *C. robusta*, show evolutionary rates approximately 50% faster than other chordates, while *O. dioica* show evolutionary rates of up to three time faster (Berna and Alvarez-Valin, 2014). Two processes are thought to be involved. First, in some species (for instance, *O. dioica*) the absence of many genes related to DNA repair has been noted, albeit not definitive (Berna and Alvarez-Valin, 2014). The second suspected process involves high lineage-specific selective pressure acting on tunicate genome coding regions (Berna and Alvarez-Valin, 2014). One study (Yokomori et al., 2014.) reported that the *C. intestinals* proteome showed high intraspecific diversity, efficient purifying selection, and a substantial percentage of adaptive amino acid substitutions, resulting in a two to six times higher per-year mutation rate compared to Vertebrata and

Cephalochordata. Research on the evolutionary dynamics of *Wnt* genes in chordates reinforced the notion of tunicates diverging from cephalochordates and vertebrates (Martí-Solans et al., 2021). The authors interestingly opposed the "extraordinary genomic stasis in cephalochordates" to the "liberal and dynamic evolutionary patterns of gene loss and duplication in urochordate genome" (Martí-Solans et al., 2021). Apart from the most well-known and studied case, the *Hox* genes cluster (see below), other examples of extraordinary gene gain, loss and amplification are seen in the evolution of Metallothioneins, a family of small proteins binding metals such as cadmium, zinc, copper, iron and mercury (Calatayud et al., 2021), or by the evolution of gene networks related to the development of olfactory organs, eyes, hair cells and motoneurons (Fritzsch & Glover, 2024).

Compact genomes.

Tunicate genomes are generally considered to be compact, with patterns of extensive gene loss (Berna and Alvarez-Valin, 2014). Rapid variations in genome size occur mainly through a phenomenon known as whole-genome duplications, or bursts in the activity of transposable elements (TEs) (Piegu et al., 2006). *O. dioica*, whose genome is particularly small for even a tunicate, notably lacks elements found in the most ancient families of animal retrotransposons (Denoeud et al., 2010). Recently, a study examined the genome size of various Appendicularea and found that genome size increased with body length, and that, although no evidence was found for whole-genome duplications, the global amount of TEs strongly correlated with genome size (Neville et al., 2019). Non-autonomous TEs, and particularly short interspersed nuclear elements (SINEs), explained as much as 83% of interspecific genome size variation.

Genomic rearrangements.

Due to their fast evolutionary rates, tunicate genomes have also undergone extensive genomic rearrangements (Denoeud et al., 2010; Aase-Remedios & Ferrier, 2021). A striking example of the level and frequency of this is the comparison of the chromosomal architecture of the two closely related, conspecific, and morphological cryptic species *C. intestinalis* and *C. robusta*. These two species live in partial sympatry in the English Channel (Caputi et al., 2007), where hybrids can be found. Chromosomal alignments between the two *Ciona* revealed the existence of numerous chromosomal inversions, which likely contributed to genetic isolation and speciation (Satou et al., 2021). Patterns of chromosomal rearrangements are even more extreme in cryptic species belonging to the genus *Oikopleura* (Plessy et al., 2024).

Conserved non-coding elements in Tunicates.

Another interesting characteristic of the tunicate genome is the so-called '*Olfactores conserved non-coding elements*' (Sanges et al., 2013; Ambrosino et al., 2019). These elements, while significantly associated with transcription factors showing specific functions fundamental to animal development, such as multicellular organism development and sequence-specific DNA binding, are highly syntenic within vertebrates. However, synteny is not preserved between tunicates and vertebrates, again showing that tunicate genomes are highly divergent within chordates.

Disrupted Hox gene cluster and chromosomal rearrangements in tunicates.

Hox genes are determinants of the body plan along the antero-posterior axis (Lewis, 1978). In many species along the Bilateria tree of life, they are clustered such that the order of the genes along the chromosome corresponds with the order of their expression along the body (collinearity - Lewis, 1978). Collinearity is also referred to as 'spatial collinearity', while 'temporal collinearity' refers to the timing of Hox gene expression and the subsequent formation of structures in the developing body plan. In **Figure 3**, Hox gene arrangements in the chromosomes of key species across the Deuterostomia tree of life are shown.

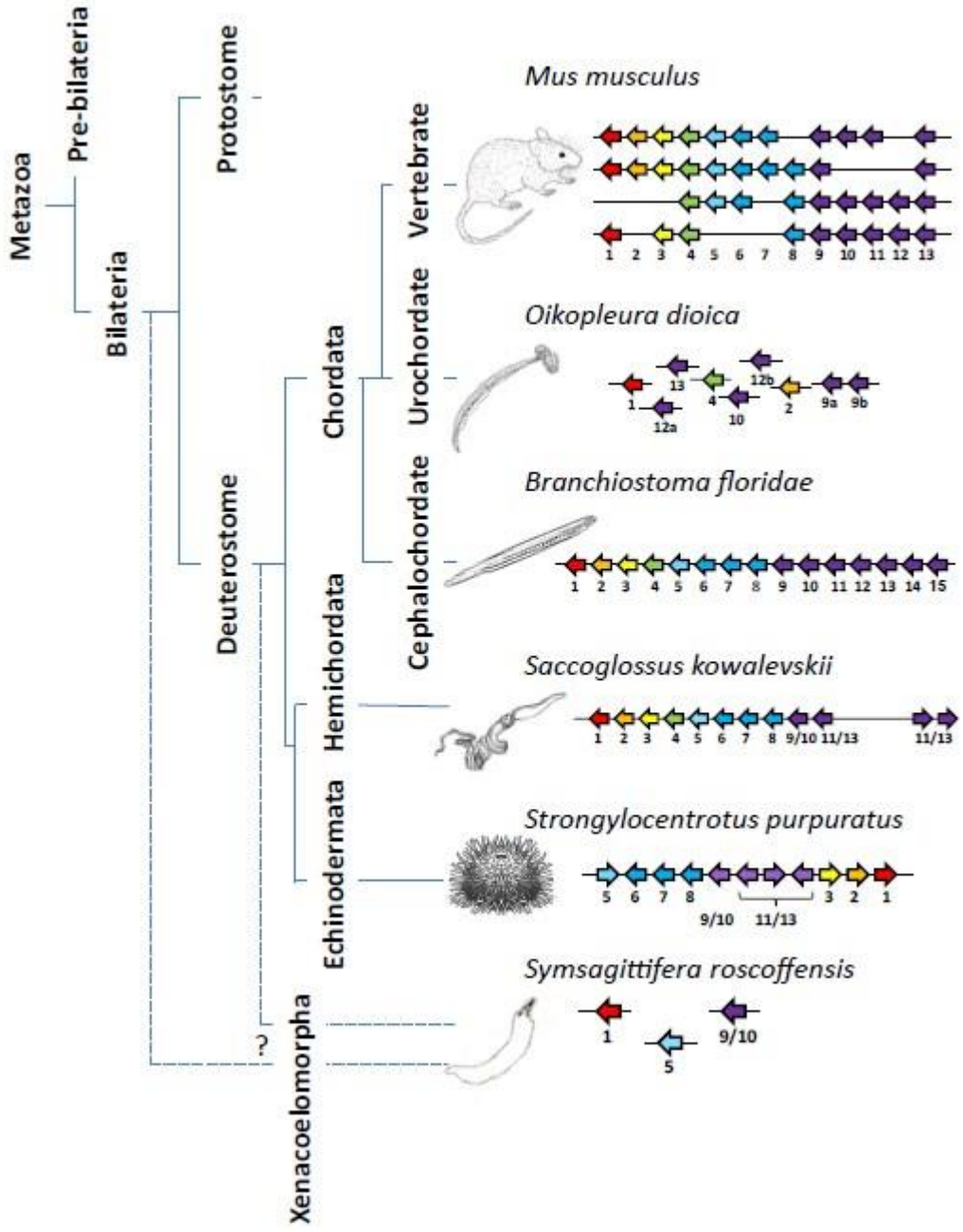

Figure 3: *Hox gene arrangements in deuterostomes. Modified from Gaunt et al., 2018.*

Of note among the tunicate species presented, *O. dioica* represents a unicum within chordates, having a disrupted cluster of Hox genes via the absence of central Hox genes (Gaunt et al., 2018). Notwithstanding the cluster disruption, Hox genes display a mode of expression which is 'spatially collinear' (Seo *et al.*, 2004). Duboule describes this as 'trans-collinearity' (Duboule, 2007). In the C. *intestinalis* genome, Hox genes are only partially dispersed (Pascual-Anaya *et al.*, 2013, Sasakura and Hozumi, 2018). *Ciona* shows residual spatial collinearity in the developing larval nervous system and in the juvenile gut during metamorphosis (Ikuta *et al.*, 2004, Nakayama *et al.*, 2016). Experimental knock-downs of *Ciona* Hox genes have demonstrated that they play minor roles in larval development but major roles during metamorphosis (Ikuta *et al.*, 2010, Sasa-

kura and Hozumi, 2018). Neither of the above urochordate species displays obvious temporal collinearity in Hox gene expression (Seo *et al.*, 2004).

The re-emergence of Operons in higher Metazoa.

Operons are defined as clusters of co-regulated genes with related functions (Osbourn and Field, 2019). Operons were classically considered as a common feature of prokaryote genomes; however, recent work has described functional gene clustering in many eukaryotes, from yeast to animals. At the beginning of the 1990s, the first genomic structures similar to classical prokaryotic operons were found in the genome of *Caenorhabditis elegans*, a nematode worm (Zorio et al., 1994). Contrary to prokaryotic operons, *C. elegans* operons produce polycistronic mRNA which is then trans-spliced into individually translated monocistronic mRNAs (Osbourn and Field, 2019). Moreover, genes within *C. elegans* operons are not typically related by sequence or function.

The origin of trans-splicing is debated and appears to be an unevenly distributed process across the animal kingdom. Trans-spliced operons have been found in insects such as the fruit fly (*Drosophila melanogaster*), chaetognaths (*Spadella cephaloptera*) and, among chordates, exclusively in tunicates. In *C. intestinalis*, approximately 20% of its genes reside in operons containing a high proportion of single-exon genes (Berna and Alvarez-Valin, 2014). In the extremely compact genome of the Appendicularia *O. dioica*, about 27% of the entire genome is organized in operons, again primarily consisting of single-exon genes (Ganot et al., 2004). Importantly, recent studies on the *O. dioica* genome found that, in this highly fragmented and scrambled genome (Seo et al., 2001), operon structure is not preserved between cryptic, anatomically identical species. This strongly suggests the absence of selective evolutionary pressure in maintaining their functionality and very existence, further obscuring the evolution of operons in tunicates. (Plessy et al., 2024). Ultimately, there is a need for greater understanding of the evolutionary forces that caused a reemergence of operons in tunicates.

Genomics and adaptation in tunicates.

Genomic diversity is a key component that dictates the ability of tunicates to adapt to new environments, making some species invasive pests (Micael et al., 2020; Santos et al., 2023). The high evolutionary rates of tunicate genomes is reflected by the high level of within-species diversity of genome proteomes, as well as the high percentage of adaptive amino acid substitutions (Tsagkogeorga et al., 2012). This has led to lineage-, genus- and species-specific adaptive mechanisms that ensure the success and persistence of tunicates in modern-day oceans.

Pelagic tunicates, such as Oikopleuridae, are known to quickly respond during phytoplankton oceanic blooms, mainly due to their fast life cycle (Sordino et al, 2020). A recent study on salps (sea squirts, *Thalia* spp.) (Castellano et al., 2023) used genome comparative analyses to reveal an abundance of repeats and G-quadruplex (G4) motifs, a feature typical of tunicates capable of alternate sexual and asexual reproduction. This may allow salps to be capable of asexual reproduction at birth, enabling bloom formation in optimal conditions. The sessile, invasive, and colonial species *Botryllus schlosseri* shows clear genetic and epigenetic differentiation in its global population (Gao et al., 2022) presumably due to variations caused by their local environment. Another salp species, *Styela clava*, has been found to have an expanded genome (compared to *Ciona* spp.), possibly due to an increased number of transposons (Wei et al., 2020). Specifically in this species, the heat-shock protein 70 family repertoire is expanded, likely from horizontal gene transfer from bacteria (Wei et al., 2020), possibly playing a role in the high degree of adaptability of *S. clava* to new environments.

The Oikopleura genome: challenging basic biological intuitions.

Appendicularian tunicates are one of the major components of ocean zooplankton (Hopcroft and Roff, 1998) and are among the fastest heterotrophic responders to phytoplanktonic ocean blooms (Sordino et al., 2020). The best studied Appendiculararea genus is *Oikopleura*, characterized by its very short life cycle of four days. As stated above, many genomic features including transposon diversity, developmental gene repertoire, physical gene order, and intron-exon organization are shattered in this genus and among cryptic, morphologically similar species. Chromosome arms and sex-specific regions appear to be the primary

unit of macrosynteny conservation. Regarding microsynteny, scrambling did not preserve operon structures; however, this happens without any apparent loss of functionality, suggesting the absence of selective pressure to maintain operon structure even between closely related species (Denoeud et al., 2010). The Oikopleura genome seems to challenge some basic biological assumptions. Indeed, the fact that similar, almost indistinguishable morphologies may be based on largely divergent genomes (and vice versa) is not an intuitive notion. As a corollary, this also means that genetic distances cannot be used to predict morphological similarities between species, and vice-versa (Plessy et al., 2024). Another interesting consequence of the unique features of the *Oikopleura* genome is that the basic chordate body plan, morphology, and its development is essentially preserved, strongly suggesting that global similarities of genome architecture in Metazoa are not crucial for the preservation of ancestral morphologies (Denoeud et al., 2010).

Conclusion.

Tunicates are fascinating animals. The aim of the present review was to present several of their unique and interesting characteristics to an unfamiliar audience. First, tunicates are pivotal in understanding higher animal evolution and the transition from invertebrates to vertebrates. This is not a simple task, partially because they are highly divergent and derivative at the molecular and genomic level, notwithstanding the fact that they are these closest living relative of vertebrates. Second, tunicates exhibit astonishing features. The possess an operon-like organization of many genes, despite operons not being positively selected and being a trait of genome organization that disappeared early on in evolutionary history. Tunicates also have a partial or total disruption of the Hox gene cluster that does not seem to affect their development. Tunicate genomes may showcase genome re-shuffling between extremely closely related species, but observed large differences in genomic architecture does not appear alter their morphology. For the above reasons, research on tunicates has challenged our knowledge of organismal evolution and genome function and future studies are likely to continue to unveil or run contrary to current understanding of molecular evolution.

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