Title

Evolution of apoptotic signalling pathways among metazoans: insights from lophotrochozoans

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Competing Interest Statement

The authors declare no competing interests.

Data Availability

All data needed to evaluate the conclusions in this study are present in the paper and the Supplementary Materials. Any requests can be addressed to the corresponding author GK.

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GK managed the project. GK and HRH wrote the manuscript. HRH, GK, MV, NP, and EG produced the data. All authors contributed to comment, discussion, and approved the manuscript.

Significance statement

Apoptosis, a form of programmed cell death, has been long studied in model organisms such as fly, mouse, and in humans. The restricted focus on these models has led to an overall view that the evolution of genes involved in apoptosis is highly conserved across all animals. The advent of next generation sequencing has led to a boom in the omics data available across the tree of life. Thanks to this we explored the evolution of key genes involved in apoptosis in the clade Lophotrochozoa (i.e. molluscs, annelids, flatworms), one of the three large clades that make up bilaterian animals. We found a complex evolutionary history of apoptosis genes, with multiple losses, gains, divergences and redundancies, highlighting the value of exploring gene evolution in Lophotrochozoans.

Abstract

Apoptosis is a form of regulated cell death commonly observed in metazoans. Apoptotic pathways are well characterised in nematode, fly and mammals, leading to a vision of the conservation of apoptotic pathways in metazoans. However, we recently showed that intrinsic apoptosis is in fact divergent among metazoans. In addition, extrinsic apoptosis is poorly studied in non-mammalian animals, making its evolution unclear. Consequently, our understanding of apoptotic signalling pathway evolution is a black-box which must be illuminated by extending research to emergent biological models. Lophotrochozoans are a major clade of

metazoans which, despite their considerable biological diversity and key phylogenetic position as sister group of ecdysozoans (*i.e.* fly, nematode), are poorly explored. Traditionally each apoptotic signalling pathway was considered to rely on a specific initiator Caspase, associated with an activator. To shed light on apoptosis evolution in animals, we explore the evolutionary history of initiator Caspases, Caspase activators and BCL-2 (which control mitochondrial apoptotic pathway) in lophotrochozoans using phylogenetic analysis and protein interaction predictions. We discovered a diversification of initiator Caspases in molluscs and annelids, and the loss of key extrinsic apoptosis components in platyhelminths, along with a clade specific Caspase with an ankyrin pro-domain. Taken together, our data show a specific history of apoptotic actors' evolution in lophotrochozoans, further demonstrating the emergence of distinct apoptotic signalling pathways during metazoan evolution.

Keywords

Apoptosis, Caspases, signalling pathways, BCL-2 family, lophotrochozoans, phylogeny, evolution

INTRODUCTION

Apoptosis is a form of regulated cell death that sculpts the body during embryonic development and allows the removal of obsolete tissues or supernumerary cells[1]. Present at the metazoans scale[2–9], apoptosis is defined by a conserved set of morphological features that depend on a multigenic family, the Caspases[10,11]. While Caspases are found in all animals, they have mainly been studied in nematodes, fly and vertebrates, in which apoptotic signalling pathways are well described[10,12,13]. Historically, two main apoptotic signalling pathways were described, currently known as extrinsic (formerly the death receptor pathway) and intrinsic apoptosis (formerly the mitochondrial pathway)[14]. In both cases, Caspases play a central role upstream (as initiators Caspases) and downstream (as executioner Caspases) of the apoptotic signalling patways[10,12,15]. Importantly, each apoptosis pathway is characterised by a specific initiator Caspase, itself activated by a protein complex in which a specific activator is involved (Figure 1). While all Caspases are composed of the common P10 and P20 domains, initiator Caspases also contain an additional long pro-domain, either a CARD or DED domain, specific to intrinsic and extrinsic apoptosis, respectively.

In mammals, intrinsic apoptosis is activated through mitochondrial outer membrane permeabilization (MOMP) a process controlled by BCL-2 family proteins (Figure 1) [10]. MOMP leads to the release of cytotoxic molecules such as cytochrome c in the cytosol. Cytochrome c forms the apoptosome complex with the specific adaptor protein APAF-1 (Apoptotic protease activating factor 1) and initiator Caspase-9, leading to activation of executioner Caspase-3[10,14,16]. Key components of the intrinsic pathway are also present in fly and nematodes [10,13,17–19]. However, several fundamental mechanistic differences exist between these models and mammalians. For instance, cytochrome c is crucial to trigger apoptosis in mammals, where it is dispensable in fly and nematode. Indeed, as BCL2 members BAX and BAK have not been identified in *Drosophila melanogaster* and *Caenorhabditis elegans* genomes, MOMP does not occur. Instead, Caspase activation requires apoptosome formation without the participation of cytochrome c [10,13,20]. In *D. melanogaster* the APAF-1 homologue, DARK, oligomerizes in an eightfold apoptosome complex which does not rely on cytochrome c for assembly. In addition, *D. melanogaster* apoptotic pathways depend on a removal of inhibition controlled by Grim, Reaper, and Hind[13]. These three proteins do not have homologues in other species. In *C. elegans*, the unique BCL-2 homolog CED-9 interacts with an asymmetric dimer of the APAF-1 homologue CED-4. When EGL-1 binds to CED-9, CED-4 is released and results in homotetrameric complex formation that activates the caspase, CED-3 [21]. Interestingly, we recently discovered that DRONC and CED-3, the initiator caspase of intrinsic apoptosis of fly and nematode, respectively, are homologues (here orthologous, coming from speciation) to the vertebrate Caspase-2 but not to Caspases-9[22]. Caspase-9 is deuterostome specific, while Caspase-2 is ancestral and conserved among bilaterian animals. Interestingly, Caspase-2 in mammals is implicated in apoptosis induced by DNA damage, but is not involved in the main intrinsic and extrinsic pathways (Figure 1) [23,24]. More recently, molecular characterization of the BCL-2 family in the placozoan *Trichoplax adhaerens*, identified *a bona fide* BAX-like protein which performs MOMP *in vitro* [25]. Altogether, these findings suggest that although MOMP is ancient in metazoans, intrinsic apoptosis pathways are divergent and evolved independently in most metazoan phyla.

Extrinsic apoptosis is well described only in mammals due to its absence in fly and nematode. This pathway is activated by death receptors (*i.e.* TNFr – Tumor Necrosis Factor receptors) on cell membranes, themselves able to recruit activator proteins FADD (Fas-Associated protein with Death Domain) or TRADD (Tumor necrosis factor Receptor type 1-Associated Death Domain) into a DISC (Death-Inducing Signalling

Complex), which activates Caspases-8 or 10, respectively (Figure 1) [12,14]. Despite this clear molecular cascade, extrinsic apoptosis is poorly described outside of mammals except for a few anecdotal studies such as in cnidarians or amphioxus in which FADD can induce apoptosis in cultured cells[26–28]. Consequently, its evolution is relatively unknown.

Taken together, it clearly appears that apoptotic signalling pathways' evolution is a black box that must be revisited, as the lack of knowledge outside three key model species (mammals, fly and nematode) artificially creates biased inferences on apoptotic regulation pathways' evolution theory. Thus, in bilaterian animals, our knowledge is based on intensive studies, but restricted to deuterostomes (mouse, human) and ecdysozoans (fruit fly, nematode) while the major group of the lophotrochozoans remain largely unexplored despite tremendous diversity. The gap of knowledge in lophotrochozoans creates a critical void as they are one of the two major protostome clades, along with the ecdysozoans. Consequently, a comprehensive and global understanding of apoptotic signalling pathways evolution is definitively impossible to reach without exploring lophotrochozoan animals (i.e. molluscs, annelids, brachiopods, and platyhelminths).

In this study, we explored the apoptotic network of lophotrochozoans by conducting exhaustive BLAST search followed by phylogenetic analysis to identify apoptotic actors, especially Caspases, BCL-2 family proteins and activators such as FADD or APAF-1. We next analysed sequence evolution based on mutation rate and predicted initiator Caspase/activator interactions. Our results highlight an unexpected diversity and complexity within lophotrochozoans, characterised by the loss of the key intrinsic apoptosis activator APAF-1 in molluscs and annelids, in parallel to the loss of the extrinsic apoptosis actors in the platyhelminths, notably FADD and DED-Caspases. Surprisingly, platyhelminths present Caspases with an ankyrin pro-domain, a unique feature never observed in vertebrates, fly or nematode. The differential evolution between platyhelminths and [molluscs + annelids] argues in favour of specific apoptotic pathways not yet functionally described. Our work highlights the non-linear, divergent evolution of apoptotic pathways in animals and argues in favour of the study of lophotrochozoans as emerging biological models.

RESULTS

Differential gains and losses of initiator Caspases between molluscs and platyhelminths

Initiation of apoptotic signalling pathways rely on specific initiator Caspases upstream of the pathway, which next activate the executioner Caspases downstream of the regulation[10–12].

First of all, we conducted BLAST search of Caspases, in all available genomes of lophotrochozoans representing molluscs (pink), annelids (blue), and platyhelminths (orange) (Figure 2, Supplementary Table 1). Queries were human, nematode, *D. melanogaster*, and *Crassostrea gigas* Caspases. In all cases we performed reciprocal BLAST searches, and extended the query in accordance to the results (*i.e.* newly identified Caspases were added to the query). We first conducted a phylogenetic analysis on all Caspases (both initiator and executioner Caspases) from a selection of species' representative of lophotrochozoan diversity (in this study: platyhelminths, molluscs, annelids) to understand their evolution within this main lineage (Figure 2). This strategy allowed inclusion of all types of Caspases at the lophotrochozoan scale without over saturating the sequence number which usually leads to poorly resolved topologies when working on short alignment[22]. Globally, the topology is characterised by a polyphyletic distribution of Caspases with a CARD pro-domain (CARD-Caspases), most of them being close to the root which is a *Reticulomyxa filosa* (foraminifera) Caspase-like sequence, already successfully used to run metazoan Caspases phylogenies[22,29]. Most Caspases with DED pro-domains (DED-Caspases) form a strongly supported group (PP=0.98) composed of mollusc, annelid, and brachiopod sequences, but devoid of platyhelminths. We also identified several Caspases presenting an ankyrin pro-domain (ANK-Caspases), an unexpected feature which has not previously been observed in either deuterostomes or ecdysozoans. ANK-Caspases form a maximum supported clade (PP=1.00) specifically restricted to platyhelminths, and branching as a sister group to *Haliotis diversicolor* sequences and are close to DED-Caspases. Globally, it appears that executioner Caspases form a coherent, maximally supported clade (PP=1.00).

Due to their pivotal role, we next decided to focus more specifically on initiator Caspases (Supplementary Table 2). Regarding the short length of Caspase sequences, this strategy allowed us to reduce the sequence number per species and secondarily to increase the sampling to a maximum of lophotrochozoan species to conduct a more in-depth analysis with higher resolution. We reconstructed their phylogenetic relationships and four monophyletic groups were identified: Caspase-Y, ANK-Caspases, DED-Caspases, and Caspase-2 (Figure 3). Both Caspase-2 and Y possess a CARD pro-domain and consequently belong to the CARD-Caspase type. Caspase-Y is a strongly supported (PP=0.97) group that we already identified in a

previous study, which here is strictly composed of sequences from molluscs and annelids, showing specific diversification [22]. The Caspase-2 encompasses sequences from molluscs and annelids, grouped together with Caspase-2 from platyhelminths. All lophotrochoazoan animals possess a Caspase-2, which is consistent with our previous work showing that this Caspase is conserved and ancestral in bilaterians. *Crassostrea gigas* Caspase-Y1 (dotted black arrow) is grouped close to Caspase-2, likely a result of Long Branch attraction artefact as we had previously identified this sequence as a Caspase-Y homologue [22]. ANK-Caspases and DED-Caspases are sister-groups and together form a well-defined, maximally supported (PP=1.00) clade. The ANK-Caspase group exhibits maximum support (PP=1.00) and is specifically restricted to parasitic platyhelminths. In contrast, the DED-Caspase group is composed of mollusc and annelid sequences, together with two non-parasitic, early diverging platyhelminths, *Dugesia japonica* and *Schmidtea mediterranea*. Interestingly, platyhelminths which have DED-Caspases do not have ANK-Caspases and *vice versa*. In addition to ANK-Caspases, we discovered two specific Caspases with unexpectedly long pro-domains that have not been identified in mammals, fruit fly or nematode (Figure 4). Annelid *Platynereis dumerilli* has a Caspase with a z-binding pro-domain which is a paralogue of the *Platynereis* Caspase-2 (Figure 3, white arrow). Platyhelminth *Macrostomum lignano* presents a Caspase with a protein kinase like pro-domain, positioned as sister to the ANK-Caspase group (Figure 3, black arrow).

To explore the history of Caspase pro-domains and potentially discover early emerging initiator Caspase types, we artificially built three topologies which correspond to three potential scenarios of evolution, firstly with ANK-Caspases as sister to [DED-Caspases + CARD-Caspases], secondly DED-Caspases as sister to [ANK-Caspases + CARD-Caspases], and thirdly CARD-Caspases as sister to [DED-Caspases + ANK-Caspases] (Figure 5). Next, we conducted topology tests to evaluate the most likely scenario[30–33]. Interestingly, regardless of the statistical test used, there is no topology that appears to be the most likely, making it difficult to hypothesise on an ancestral type of Caspase, arguing instead for a complex evolutionary model, but also confirming the ancestral presence of both DED-Caspases and CARD-Caspases among lophotrochozoans. (Figure 5).

Taken together, our topology indicates that Caspase-Y are a group of CARD-Caspases specific to [molluscs + annelids], ANK-Caspases are specific to parasitic platyhelminths, and parasitic platyhelminths have

lost DED-Caspases. The proximity between ANK-Caspases and DED-Caspases could indicate a specific transformation of platyhelminths ancestral DED domain during evolution.

Losses and gains of activators is consistent with initiator Caspases inventory

Initiator Caspases are activated by specific platforms that depend on an activator which possesses a proteomic domain similar to their corresponding pro-domain. Thus, the CARD domain of APAF-1 binds to the CARD pro-domain of Caspase-9 within the apoptosome in mammals, or of Caspase-2 in fruit flies and nematode[10,34,35]. Mammalian Caspase-2 is activated by CARD domain containing PIDD (CRADD, also known as RAIDD) [36–38]. PYCARD is known to activate Caspase-1 in mammals, as both have a CARD domain[39]. DED domain containing activators FADD and TRADD activate initiator Caspase-8 and 10 in the DISC complex, respectively[12,40].

We conducted an exhaustive inventory of Caspase activators within lophotrochozoan groups (Figure 6A). Interestingly, we found a FADD homologue in the [molluscs + annelids + brachiopod] group, which is consistent with the presence of DED-Caspases in these taxa (Figure 6A1, Supplementary Table 3). However, FADD was not detected in platyhelminths. Our FADD phylogenetic analysis gave rise to a topology that is globally consistent with the species tree with molluscs grouped into a maximum supported monophyletic clade (PP=1.00) and split into gastropods and bivalves. Annelids and brachiopods branch first and appear as sister group to molluscs. Topology consistency between sequences and species suggest gene conservation in these groups. Conversely, APAF-1 was detected in platyhelminths, but, and consistently with previous work including our study [22], we did not identify any APAF-1 in [molluscs + annelids + brachiopod] which confirms the loss of this activator in these taxa (Figure 6A2, Supplementary Table 4). The APAF-1 topology is also congruent with the species tree and all clusters are maximally supported (PP=1.00), with monophyly of cestode platyhelminths branching with the sister trematodes. At the basis of the topology are the first branching triclads platyhelminths. Next, we investigated the possibility of activators for [molluscs + annelids + brachiopod] CARD-Caspases and discovered that these species possess a CRADD homologue, which is absent in platyhelminths (Figure 6A3, Supplementary Table 5). Phylogenetic analysis of CRADD genes also indicates a conservation of the gene, as the topology is in accordance with species relationships: molluscs are monophyletic (PP=0.53) and encompass gastropods (PP=0.76) and bivalves (PP=1.00), with [brachiopods + annelids] at the “basis” of the tree

(PP=0.99). Lastly, we were unable to detect any PIDD, PYCARD or TRADD (which activate Caspase-2, Caspase-1, and Caspase-10 in mammals, respectively) despite an exhaustive search in all available genomes/transcriptomes/proteomes of lophotrochozoan species.

Next, we explored the theoretical initiator Caspase/activator binding interactions that could trigger their activation (Figure 6B). To do so, we evaluated interaction scores (S-score) using PSOPIA software, specifically designed to predict protein-protein interactions. Firstly, we calculated the S-score between DED-Caspases and FADD for all species which had both proteins. Consistent with the literature, our results suggest that mollusc and annelid FADD could activate the corresponding DED-Caspases (Figure 6B1). We found the same result with the APAF-1 activator and CARD-Caspase from platyhelminth species (Figure 6B2). Interestingly, the S-scores for CRADD and CARD-Caspases of [molluscs + annelids] also indicate a high binding probability, with a preference for Caspase-2 rather than Caspase-Y (Figure 6B3).

Our results indicate a consistent relationship between the inventory of initiator Caspases and potential interactors allowing the proposition of activation pairs (Figure 6C), according to specific loss within each group of lophotrochozoans that we focused on.

BCL-2 family is conserved with non-linear evolution within lophotrochozoans

Finally, we focused on the BCL-2 family which are crucial regulators of MOMP[41]. Within their primary structure, BCL-2 proteins possess one or more conserved sequences, called BCL-2 homology or BH motifs. Based on their number, they are divided into multi-motif and “BH3-only”. Multimotif BCL-2 can be either pro-apoptotic including BAX and BAK and BOK which allow cytochrome c release following MOMP in mammals[16,42,43] or anti-apoptotic such as BCL-2, BCL-XL and MCL-1. Notably, multimotif pro- and anti-apoptotic BCL-2 members have conserved 3D structure, suggesting a common evolutionary origin. On the other hand, BH3-only proteins represent a polyphyletic group with pro-apoptotic activities [44]. Thus, BCL-2 family evolution is complex, with phylogenetic analyses usually producing unclear topologies [22]. Importantly, the short length of BCL-2 proteins restricts the number of informative sites to run the phylogenetic analyses. To circumvent this, we conducted phylogenetic analysis on the BCL-2 family by incorporating only two molluscs (*Biomphalaria glabrata* and *Crassostrea gigas*), one annelid (*Platynereis dumerilii*), one Brachiopod (*Lingula anatina*), and three platyhelminths (*Schistosoma mansoni*, *Taenia asiatica*, and *Echinococcus granulosus*)

(Figure 7, Supplementary Table 6), to represent the diversity of lophotrochozoans without increasing the sequence number. Despite these precautions, the topology obtained by Bayesian inference shows a low-resolution level with numerous unresolved nodes, in line with what we previously observed [22]. Conversely, the topology arising from maximum likelihood method allows the identification of BCL-2 family relationships (Figure 7). The topology shows three main pro-apoptotic clades corresponding to BAK, BAX, and BOK. Molluscs, annelids and brachiopods possess all of them, in addition to numerous specific duplications (i.e. Crassostrea BAX or Lingula BAK). Conversely, platyhelminths only have the BAK pro-apoptotic, suggesting a loss of BAX and BOK. Four anti-apoptotic clades are identified, one composed of the MCL1 restricted to molluscs and close relatives, and three BCL-2-like groups. We prioritised BCL-2 like names as evolutionary relationships with BCL-2 are variable and difficult to establish, as we already experienced [22]. The relatively clear separation between pro- and anti-apoptotic genes clarified which species have a complete BCL-2 inventory (both functions represented). All species from our sampling have both pro- and anti-apoptotic BCL-2, but the number is variable. More specifically, the two parasitic platyhelminths only have three BCL-2 representative, while the diversity is high in molluscs and close relatives. As such the composition of the ancestral BCL-2 repertoire remains elusive.

DISCUSSION

Molluscs present specific apoptotic network architecture resulting from differential evolution

Molluscs and close relatives', annelids and brachiopods, present an apoptotic network divergent to mammals and ecdysozoans. Molluscs possess a large diversity of BCL-2 which likely leads to MOMP (conversely to ecdysozoans), as cytochrome c was observed in *Helix pomatia* and *Crassostrea gigas*[45,46]. The loss of APAF-1, which is the shared component of intrinsic apoptosis, suggests a specific modality of CARD-Caspase activation. Conversely to ecdysozoans, Caspase-2 (and also Caspase-Y) in molluscs may be activated by CRADD similarly to what occurs in mammals, despite being more phylogenetically distant[37,38]. This makes these animals the only ones currently known to have a potential intrinsic apoptosis which is not based on an apoptosome, but resulting potentially from Caspase-2 activation by CRADD and cytochrome c.

Caspase-Y is restricted to the clade [molluscs + annelids + brachiopods], indicating a diversification of CARD-Caspases in this clade. Interestingly, CARD-Caspase diversification in mammals led to functional

specialisation between apoptosis and immunity. Caspase-1 is involved in the inflammatory response, while apoptosis is regulated by other CARD-Caspases such as Caspase-2 or Caspase-9[47]. Also, it has been shown that Caspase-2 of *Crassostrea angulate* is expressed during the apoptotic event of larvae metamorphosis, suggesting a primordial role of this Caspase in apoptosis[48]. Importantly, Caspases have been reported to be fundamental in immune response to various stress and inflammation in several mollusc species[6,49–51]. Consequently, we may hypothesise that CARD-Caspase diversification in molluscs and close relatives may have induced separate functions as well. Caspase-2 could be involved in apoptosis, and Caspase-Y in immunity. However, both Caspases seem to be activated by CRADD, the only activator with a CARD domain in molluscs and close relatives, while mammalian inflammatory Caspases are activated by specific activators such as PYCARD. The presence of only one CARD domain containing activator in these animals certainly induced an evolutionary constraint leading to the conservation of CRADD. The loss of this unique activator may lead to the inability to trigger apoptosis and could be deleterious for development and homeostasis. The dN/dS is a ratio usually used to evaluate selection pressure on genes during evolution. A ratio less than 1 indicates a prevalence of synonymous mutations (without impact on protein sequence, and likely protein functions) resulting from a strong selection pressure. Conversely, a ratio higher than 1 corresponds to an accumulation of non-synonymous mutations which likely altered the amino acid composition of proteins, that may indicate low selection pressure. The ratio we found for lophotrochozoan CRADD less than 1 (dN/dS=0.91) confirming strong selection pressure on these genes (Table 1).

Molluscs, along with annelids and brachiopods, possess both FADD and DED-Caspases, two actors known to trigger extrinsic apoptosis in mammals (Figure 8). The theoretical binding affinity between both strongly suggests that extrinsic apoptosis could be activated with similar modality as that which is already known in mammals. However, the evolutionary relationships of DED-Caspases at the metazoan scale is as yet unclear, as the phylogenies performed so far are difficult to analyse and interpret[52,53]. Indeed, the topology of DED-Caspase sequences are significantly different to the species tree topology, and the branching of animal clades is not consistent with the current knowledge on metazoan relationships. Importantly, the presence of a potential extrinsic apoptosis in molluscs indicates that its absence in fly and nematode could be a secondary loss that does not reflect the commonality of non-deuterostome animals. Finally, DED-Caspases and FADD are the only two apoptotic actors identified here to present a low selection pressure (dN/dS>1), a selection relaxation

that could be due to the presence of both intrinsic and extrinsic apoptosis-like pathways, leading to functional redundancy (Table 1).

Taken together, pioneering experimental works on lophotrochozoans[48,51,54,55] coupled with our study suggest that molluscs and close relatives present original apoptotic pathways which are different to what is known in mammals, fly and nematode.

Platyhelminths conserve intrinsic apoptotic actors but have lost extrinsic apoptosis networks

DED-Caspase history in platyhelminths is particularly interesting, as early branching platyhelminths possess one DED-Caspase while the parasitic species appear to have lost this gene. In addition, the DED-Caspase activator FADD was not detected in platyhelminth genomes despite our investigations (Figure 8). The loss of one of these actors possibly leads to the lack of mobilisation of the other one, inducing a mutual relaxation of selection pressure leading to their parallel loss during evolution. Gene losses are frequent in animals with parasitic life styles due to genome and morphological simplification [56–58], which could explain absence of some apoptotic actors in platyhelminths. This absence of extrinsic apoptosis in platyhelminths is shared with ecdysozoans, but is likely the result of convergent evolution, as extrinsic apoptotic actors are documented at a large evolutionary scale and are notably present in molluscs, annelids, and brachiopods (this study, [52,59,60]).

However, all parasitic species display an ANK-Caspase, a type of Caspase firstly identified in *Schistosoma*[61], which is unique to platyhelminths as there are no Caspases with the same pro-domain in other currently available genomes from metazoans. Interestingly, ankyrin domains are known to be involved in protein-protein interactions and recognition[62]. In addition, proteins with ankyrin domains are able to interact with Caspases[63,64]. As ANK-Caspases are close to DED-Caspases, we may hypothesize that acquisitions of ankyrin pro-domains could be the result of a significant modification of the DED pro-domain of the ancestral DED-Caspases during evolution, as the recruitment of a new, but not yet identified, activator with an ankyrin domain was likely positively selected during the establishment of parasitism in these species. These specific Caspases suggest a pathway, different to what is currently known, that is of primary interest to investigate.

Platyhelminths also present an apoptotic network composed of a unique CARD-Caspase, Caspase-2, and the activator APAF-1, similarly to fly and nematode. Conversely to ecdysozoans, platyhelminths BCL-2 members seems able to induce MOMP and consequently the subsequent cytochrome c release, at least in

Schmidtea mediterranea[18]. The intrinsic apoptosis of platyhelminths appears to be one underlaid by CARD domain interactions, implicating the common APAF-1 activator, Caspase-2 (like fruit fly and nematode) and cytochrome c release (like mammals). The resulting hypothetical apoptosome may share similarities with, or diverge from, apoptosomes from mammals and ecdysozoans, making it a new type of activation platform (APAF-1 + Caspase-2 + cytochrome c). The presence of a single pathway type likely induced high selection pressure for conservation of these actors as a deleterious modification or loss would lead to dysregulation of apoptosis (dN/dS<1) (Table 1).

Apoptotic signalling pathways evolve in a divergent way between metazoan clades

The current understanding of apoptotic signalling pathway evolution has benefited from decades of studies conducted primarily on nematode, fly and mammals[10,13,17,65]. Ecdysozoans are characterised by the loss of numerous secondary genes and an absence of extrinsic apoptosis, while in mammals there is an abundance of various apoptotic actors; this has led to the false conclusion that apoptotic pathways are simple in "invertebrates". In this study we discovered a large diversity of apoptotic network components in lophotrochozoans, along with clade-specific differences, suggesting complex evolution marked by several losses, acquisitions, and diversifications (Figure 8).

Phylogenetic evidence suggests that the central initiator of intrinsic apoptosis, Caspase-9, is deuterostome specific, while Caspase-2 is conserved among metazoans, and triggers apoptosis in fly and nematode[22]. Consequently, it is not surprising that the only shared initiator Caspase we identified between all lophotrochozoan groups studied here is Caspase-2. Functional evidence shows that Caspase-2 in mammals, and its orthologues Dronc and Ced-3 in fly and nematode, respectively, are involved in myriad processes, including cell cycle regulation, tumour suppression, oxidative stress, aging and cell death, and DNA repair [23,24,37,66,67]. In addition, Caspase-2 has the possibility to activate both intrinsic and extrinsic apoptosis, in addition to DNA damage pathway apoptosis in mammals[66,68–70]. This employment capacity is highlighted by the large range of proteins able to interact with Caspase-2, such as APAF-1 in ecdysozoans and CRADD or PIDD in mammals[36,38]. This ancestral multifunctionality suggests that a mutation/loss of Caspase-2 would lead to destabilisation of various signalling cascades, increasing the probability of deleterious consequences, as such conservation of this initiator among bilaterian animals including lophotrochozoans is vital.

Our data suggest the presence of a possible apoptotic pathway in molluscs based on Caspase-2/CRADD interaction, and another one in platyhelminths triggered by the Caspase-2/APAF-1 complex, with both cases relying on cytochrome c release. Cytochrome c release is likely an ancient trait of apoptosis regulation which is conserved in metazoans[18,41,42], suggesting that fly and nematode intrinsic apoptosis reflect an accumulation of derivative features concerning the implication of BCL-2 and mitochondria. Conservation of both anti- and pro-apoptotic BCL-2 in lophotrochozoans certainly results in their ability to play on mitochondria and apoptosis, but also on their multiple, ancestral and pleiotropic functions making them crucial in various molecular pathways[25,71–73].

Finally, our data indicate that apoptotic signalling pathways accumulated taxon-specific features across evolution, making these pathways divergent among metazoans and potentially convergent to the ultimate conserved trait: cell death by apoptosis execution regardless of the molecular pathways involved.

MATERIAL AND METHODS

Sequence dataset construction

Putative lophotrochozoans genes were identified using tBLASTn and BLASTp searches using genes from human, fly, nematode, cnidarians, and oysters as queries. Reciprocal BLAST was performed on all genes, including newly identified lophotrochozoan sequences. BLAST searching was conducted using NCBI databases for most species, and on PdumBase in addition to our recent transcriptome for annelid *Platynereis dumerilii*[74,75]. After identification of genes in target species, proteins were analysed with ScanProsite (ExPaSy)[76] to verify the presence of specific domains such as P20 and P10 for Caspases, CARD and Death Domains for CRADD, CARD and NB-ARC for APAF-1, and DED and Death domains for FADD. Sampling was conducted on all available lophotrochozoan genomes/transcriptomes, and for each species all Caspases and Caspase activators were explored.

Caspase family proteins are short (containing the large common P20 and the small P10 domains) with a high number of genes per species, which rapidly limits the relevance of the phylogenetic analyses. To reduce artefact branching and unreadable topologies, and to maximize phylogenetic diversity across lophotrochozoans, the dataset constructed for phylogenetic analysis with all types of Caspases was built with restricted number of representative lophotrochozoan species which recapitulate lophotrochozoan diversity. Secondly, analysis of

Caspases with long pro-domains only was conducted with all species possible (for which unambiguous Caspase identifications were made). The BCL2 phylogeny was also conducted on a representative species subset to overcome the same aforementioned artefacts.

FADD, CRADD, and APAF-1 analyses were conducted with all sequences identified at the lophotrochozoan scale which had the two specific domains.

Multiple sequence alignments of protein sequences were generated using MAFFT version 7[77] with default parameters, and Clustal Omega[78], to verify the congruence of the different alignments. All sequences were then manually checked in BioEdit 7.2 software to verify the presence of specific domains previously identified. Gblocks version 0.91b[79] was used to remove vacancies and blur sites. Final alignments comprise of 379, 214, 212, 560, 199, and 145 amino acids for the following alignments; Caspases with long pro-domain alignment, BCL-2 alignment, Apaf-1, CRADD alignment, and FADD alignment, respectively.

Sampling strategy

Species sampling was conducted to include the highest diversity of lophotrochozoans possible in our analysis. When sampling we looked for caspases and potential activators in all currently available genomes of lophotrochozoan species. Regarding the APAF-1, FADD, CRADD, and initiator Caspases sampling, we collected all sequences we detected in all genomes available on NCBI. The PSOPIA score was calculated for all species were CARD-caspases plus APAF-1 and/or CRADD were identified, and same for DED-caspases plus FADD. Species sampled in one of our phylogenetic analysis but which is not included in the PSOPIA score analysis are species for which we did not detected caspases activator candidates. Similarly, species represented in initiator Caspase analysis but not in APAF-1, CRADD, or FADD analyses, are species for which we did not detected caspase activator candidates. Regarding the analysis on all type of Caspases and BCL-2, we had to change the strategy to run the phylogenetic analysis in accordance with alignment length, in other word, the quantity of phylogenetic information. Caspase proteins are short, which makes it challenging to conduct phylogenetic analyses with a large number of species, and consequently high level of sequences (especially when including all of the multigene family), without sacrificing reliability. For these reasons, model animals and taxa were restricted, but animals with functional or well-studied genomic data were prioritised, in addition to be representative of the lophotrochozoans diversity.

BCL-2 alignment is very short, and poor in phylogenetic information, leading to challenges in the analysis. To maintain readability and methodology efficiency, the sampling was minimised as much as possible, while ensuring representation of metazoan diversity.

Phylogenetic analysis

Phylogenetic analyses were carried out from the amino-acid alignment with Maximum-Likelihood (ML) method using PhyML 3.1[80]. The best amino-acid evolution models to conduct analyses were determined using MEGA11[81] and appeared to be WAG for the Caspase alignments, Apaf-1 alignment, CRADD alignment, FADD alignment, and Cprev for the BCL-2 alignment. Node robustness was evaluated by 500 bootstrap replicates sampling.

Bayesian analyses were performed using MrBayes (v3.2.6)[82] under mixed model. For each analysis, one fourth of the topologies were discarded as burn-in values, while the remaining ones were used to calculate posterior probability. The run for the Caspase alignment was carried out for 500,000 generations with 15 randomly started simultaneous Markov chains (1 cold chain, 14 heated chains) and sampled every 100 generations. The runs for APAF-1, CRADD, and FADD alignments were carried out for 500,000 generations with 15 randomly started simultaneous Markov chains (1 cold chain, 4 heated chains) and sampled every 100 generations. For the BCL-2 5,000,000 generations with 20 randomly started simultaneous Markov chains (1 cold chain, 19 heated chains) was conducted and sampled every 100 generations. ML bootstrap values higher than 50% and Bayesian posterior probabilities are indicated on the Bayesian tree. The outgroup for the all Caspase phylogeny is *Reticulomyxa filosa* (ETO10778.1), a sequence recognizes to be sister of metazoan Caspases[83]. For the BCL2 phylogeny, the outgroup used is a BCL-2-like from Porifera *Amphimedon queenslandica* (XP_003387574.1). Outgroups for APAF-1, CRADD, and FADD phylogenies are their human relatives.

Topology tests were evaluated using IQ-TREE[30]. We conducted Kishino-Hasegawa test[31], Shimodaira-Hasegawa test[33], and approximately unbiased test[32].

Protein-protein binding score prediction

Theoretical protein-protein binding scores were calculated using PSOPIA[84]. Binding scores range from 0 (no affinity) to 1 (maximum binding probability estimation). For each category of measures (CARD-

Caspase/APAF-1; CARD-Caspase/CRADD; FADD/DED-Caspase) we included positive controls (the relevant paired vertebrate proteins according to the literature) and negative controls (a Caspase plus a randomly selected protein which should not interact). Negative controls for DED-Caspase/FADD binding (Figure 6B1) are Negative-1: *Crassostrea gigas* FADD (NP_001295786.1) plus *Phodopus campbelli* insulin-like growth factor 2 (AFQ62592.1); Negative-2: *Mus musculus* Caspase-8 (AAH49955.1) plus *Hydra vulgaris* rhamnose-binding lectin (XP_002166914.1). Positive controls for DED-Caspase/FADD binding (Figure 6B1) are Positive-1: *Homo sapiens* Caspase-8 (AAD24962.1) plus FADD (CAG33019.1); Positive-2: *Mus musculus* Caspase-8 (AAH49955.1) plus FADD (AAA97876.1). Negative controls for CARD-Caspase/APAF-1 binding (Figure 6B2) are Negative-1: *Macrostomum lignano* CARD-Caspase (PAA59014.1) plus *Columba livia* Xrcc4 (PKK31261.1); Negative-2: *Drosophila melanogaster* Dronc (NP_524017.1) plus *Hydra vulgaris* rhamnose-binding lectin (XP_002166914.1). Positive controls for CARD-Caspase/APAF-1 binding (Figure 6B2) are Positive-1: *Homo sapiens* Caspase-9 (BAA82697.1) plus APAF-1 (NP_863651.1); Positive-2: *Caenorhabditis elegans* Ced-3 (AAG42045.1) plus Ced-4 (CAA48781.1). Negative control for CARD-Caspase/CRADD binding (Figure 6B3) is Negative-1: *Macrostomum lignano* CARD-Caspase (PAA59014.1) plus *Columba livia* Xrcc4 (PKK31261.1). Positive control for CARD-Caspase/CRADD binding (Figure 6B3) is Positive-1: *Homo sapiens* Caspase-2 (KAI4016188.1) plus CRADD (CAG28571.1).

3D proteins models

3D structures were predicated using Phyre[85] and the resultant PDB files were imported to Chimera X[86] for model construction. Functional domains were detected using ScanProsite (ExPaSy).

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FIGURES AND LEGENDS

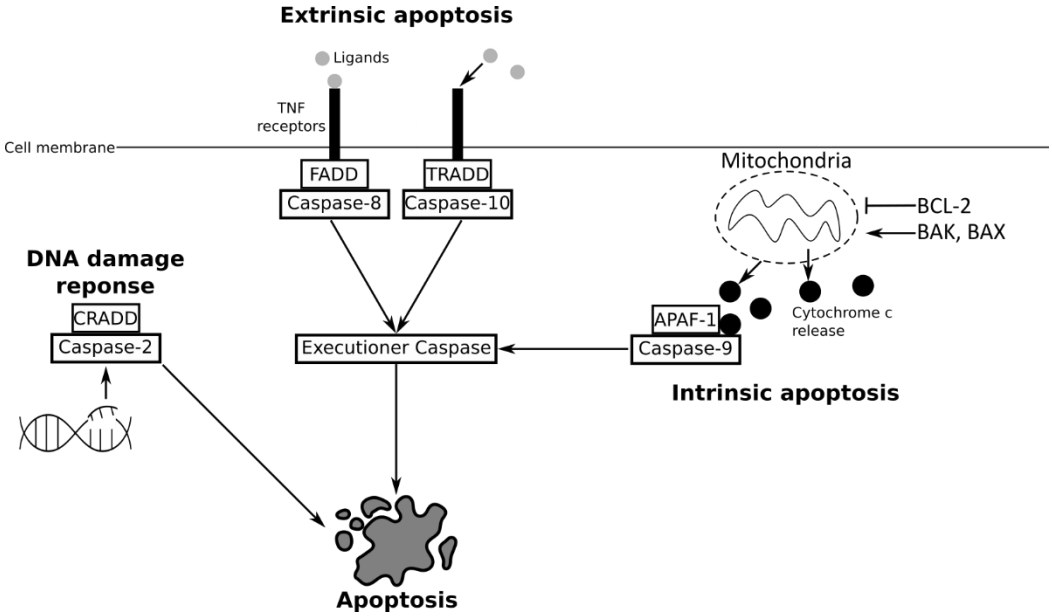
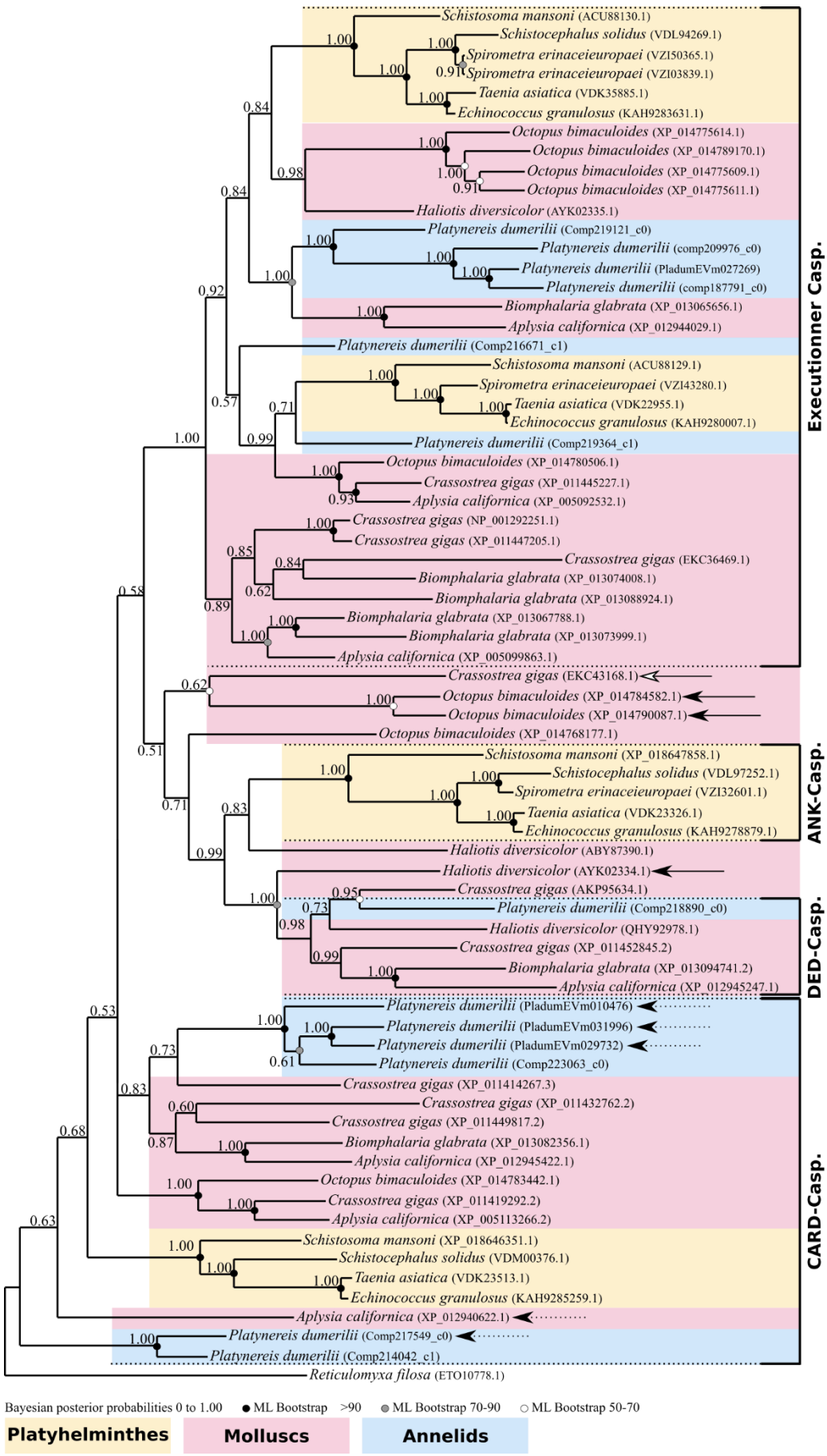


Figure 1: Main apoptotic pathways described in mammals. Initiator Caspases rely on a specific activator and are specific to each pathway. Extrinsic apoptosis depends on Caspase-8 or Caspase-10 and their activators, FADD and TRADD, respectively. Intrinsic apoptosis is controlled by the BCL-2 family (*i.e.* anti-apoptotic BCL-2, pro-apoptotic BAK or BAX) which can induce mitochondrial outer membrane permeabilisation, leading to cytochrome c release. Cytochrome c binds to initiator Caspase-9 and APAF-1 forming an activation complex, the apoptosome. Initiator Caspases induce activation of the common executionner Caspases downstream of the regulation pathways. Caspase-2, activated by CRADD, is involved in apoptosis induced by DNA damage.


Schistosoma mansoni (ACU88130.1)
Schistocephalus solidus (VDL94269.1)
Spirometra erinaceieuropaei (VZI50365.1)
Spirometra erinaceieuropaei (VZI03839.1)
Taenia asiatica (VDK35885.1)
Echinococcus granulosus (KAH9283631.1)
Octopus bimaculoides (XP_014775614.1)
Octopus bimaculoides (XP_014789170.1)
Octopus bimaculoides (XP_014775609.1)
Octopus bimaculoides (XP_014775611.1)
Haliotis diversicolor (AYK02335.1)
Platynereis dumerilii (Comp219121_c0)
Platynereis dumerilii (comp209976_c0)
Platynereis dumerilii (PladumEVm027269)
Platynereis dumerilii (comp187791_c0)
Biomphalaria glabrata (XP_013065656.1)
Aplysia californica (XP_012944029.1)
Platynereis dumerilii (Comp216671_c1)
Schistosoma mansoni (ACU88129.1)
Spirometra erinaceieuropaei (VZI43280.1)
Taenia asiatica (VDK22955.1)
Echinococcus granulosus (KAH9280007.1)
Platynereis dumerilii (Comp219364_c1)
Octopus bimaculoides (XP_014780506.1)
Crassostrea gigas (XP_011445227.1)
Aplysia californica (XP_005092532.1)
Crassostrea gigas (NP_001292251.1)
Crassostrea gigas (XP_011447205.1)
Crassostrea gigas (EKC36469.1)
Biomphalaria glabrata (XP_013074008.1)
Biomphalaria glabrata (XP_013088924.1)
Biomphalaria glabrata (XP_013067788.1)
Biomphalaria glabrata (XP_013073999.1)
Aplysia californica (XP_005099863.1)
Crassostrea gigas (EKC43168.1)
Octopus bimaculoides (XP_014784582.1)
Octopus bimaculoides (XP_014790087.1)
Octopus bimaculoides (XP_014768177.1)
Schistosoma mansoni (XP_018647858.1)
Schistocephalus solidus (VDL97252.1)
Spirometra erinaceieuropaei (VZI32601.1)
Taenia asiatica (VDK23326.1)
Echinococcus granulosus (KAH9278879.1)
Haliotis diversicolor (ABY87390.1)
Haliotis diversicolor (AYK02334.1)
Crassostrea gigas (AKP95634.1)
Platynereis dumerilii (Comp218890_c0)
Haliotis diversicolor (QHY92978.1)
Crassostrea gigas (XP_011452845.2)
Biomphalaria glabrata (XP_013094741.2)
Aplysia californica (XP_012945247.1)
Platynereis dumerilii (PladumEVm010476)
Platynereis dumerilii (PladumEVm031996)
Platynereis dumerilii (PladumEVm029732)
Platynereis dumerilii (Comp223063_c0)
Crassostrea gigas (XP_011414267.3)
Crassostrea gigas (XP_011432762.2)
Crassostrea gigas (XP_011449817.2)
Biomphalaria glabrata (XP_013082356.1)
Aplysia californica (XP_012945422.1)
Octopus bimaculoides (XP_014783442.1)
Crassostrea gigas (XP_011419292.2)
Aplysia californica (XP_005113266.2)
Schistosoma mansoni (XP_018646351.1)
Schistocephalus solidus (VDM00376.1)
Taenia asiatica (VDK23513.1)
Echinococcus granulosus (KAH9285259.1)
Aplysia californica (XP_012940622.1)
Platynereis dumerilii (Comp217549_c0)
Platynereis dumerilii (Comp214042_c1)
Reticulomyxa filosa (ETO10778.1)
Executionner Casp.
ANK-Casp.
DED-Casp.
CARD-Casp.
Bayesian posterior probabilities 0 to 1.00
ML Bootstrap >90
ML Bootstrap 70-90
ML Bootstrap 50-70
Platyhelminthes
Molluscs
Annelids

661

Figure 2: Topology of phylogenetic analysis of Caspases at the lophotrochozoan scale made by Bayesian inference method. Caspases with a CARD pro-domain (CARD-Caspases) present a paraphyletic distribution at the base of the tree. Among them, five sequences do not have a CARD domain (dotted black arrow). Caspases with a DED pro-domain (DED-Caspases) are restricted to molluscs and annelids, devoid of platyhelminth representatives. DED-Caspases form a monophyletic group, with only one from *Crassostrea gigas* out of the cluster (white arrow). Caspases with an ankyrin pro-domain (ANK-Caspases) form a monophyletic group specific to platyhelminths. Three CARD-Caspases (black arrow) are close to DED-Caspases and ANK-Caspases. Executioner Caspases are grouped into a maximally supported clade composed of sequences from molluscs, annelids, and flatworms. Outgroup is a Caspase-like of the foraminifera *Reticulomyxa filosa*. Node robustness correspond to posterior probabilities. Bootstrap correspond to robustness from maximum likelihood phylogeny which gave similar topology.

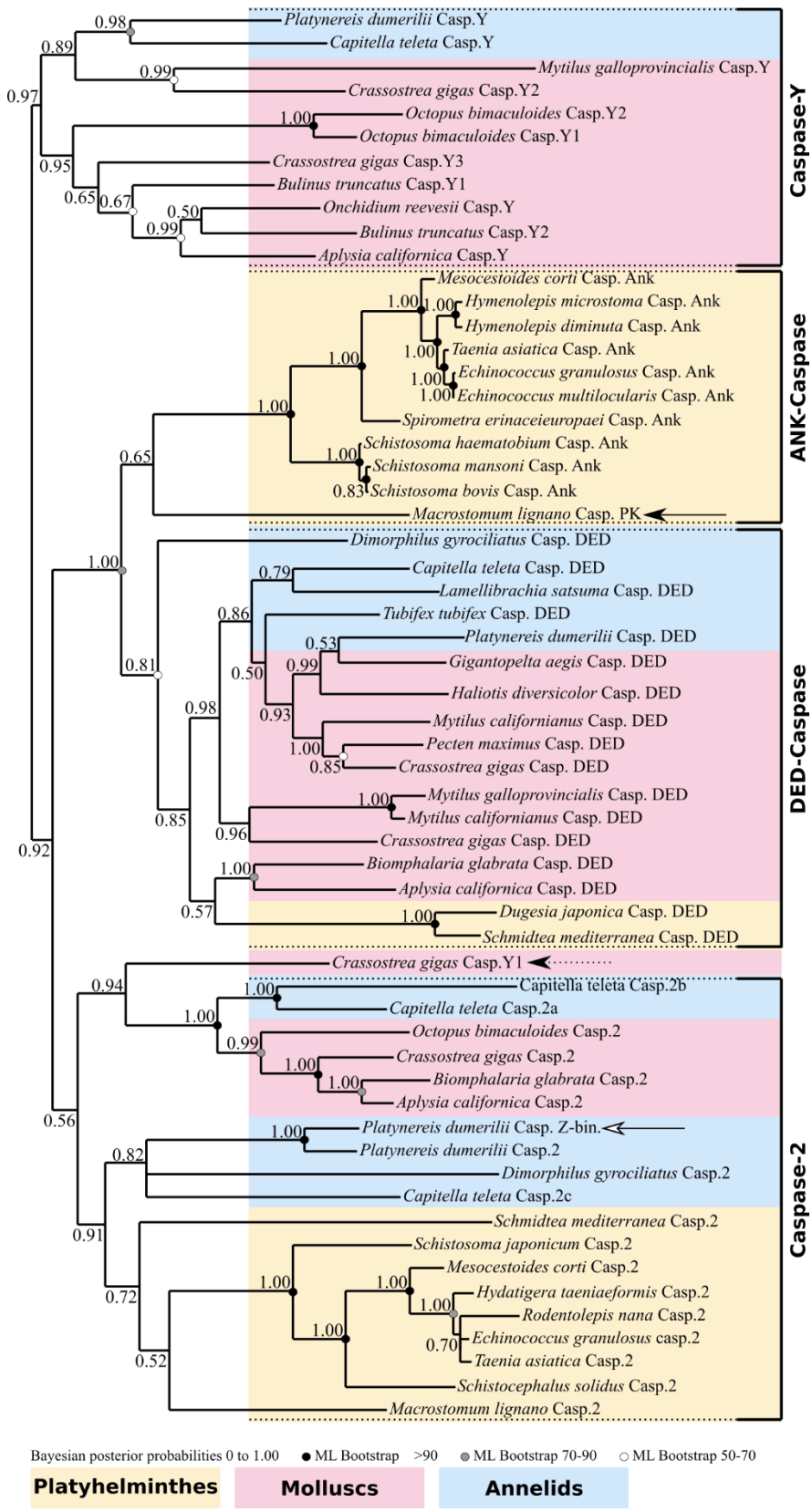


Figure 3: Unrooted phylogenetic analysis of initiator Caspases made by Bayesian inference method. Four distinct clades are present. The CARD-Caspase-Y clade diverges first and is specific to molluscs, annelids and brachiopods, devoid of platyhelminth sequences. The second major group of CARD-Caspases is the Caspase-2

clade composed of one representative for each lophotrochozoan species. Position of *Crassostrea* Caspase-Y1 (dotted black arrow) likely result of long branch attraction artefact. The sister group of *Platynereis* Caspase-2 is an unusual Caspase characterised by a Z-Repeat-Binding pro-domain (Casp. Z-bind, white arrow). DED-Caspases are monophyletic and composed of molluscs, annelids and two non-parasitic platyhelminths. ANK-Caspases are monophyletic and branch with a *Macrostomum lignano* Caspase having a protein kinase domain (Casp. PK, black arrow), forming a clade sister to the DED-Caspases and are restricted to parasitic platyhelminths. Node robustness correspond to posterior probabilities. Bootstrap correspond to robustness from maximum likelihood phylogeny which gave similar topology.

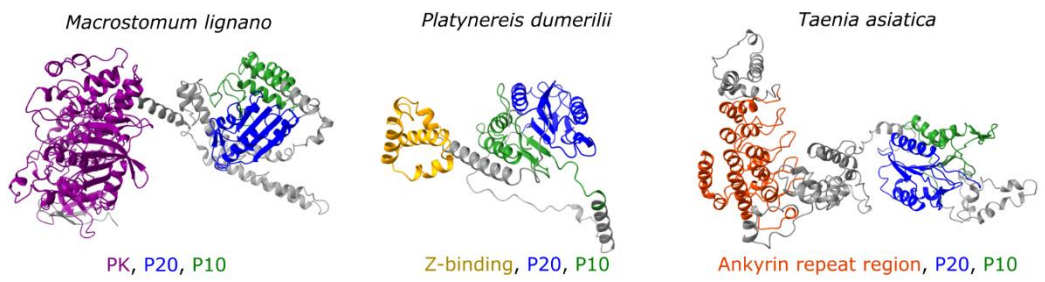
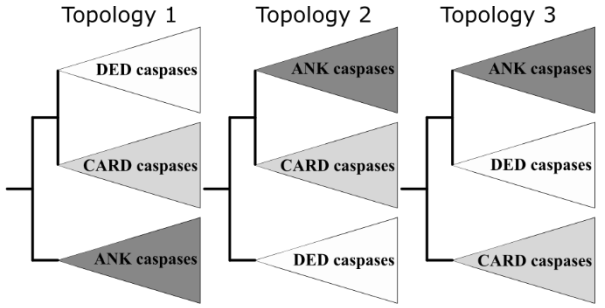


Figure 4: Specific structures of unusual initiator Caspases in lophotrochozoans. 3D representations of the Caspases of *Macrostomum lignano* (PAA54824.1), *Platynereis dumerilii* (comp217549), and *Taenia asiatica* (VDK23326.1) characterised by a protein kinase domain (PK), a Z-binding domain, and successive ankyrin repeats, respectively. All have the common Caspase specific P20 and P10 domains. *Taenia asiatica* ANK-Caspase is representative of platyhelminth ANK-Caspases.



Topology	logL	deltaL	bp-RELL	p-KH	p-SH	p-WKH	p-WSH	c-ELW	p-AU
1	-64497.69811	5.5494e-06	0.254	0.51	0.72	0.51	0.72	0.333	0.476
2	-64497.69811	0	0.316	0.496	1	0.496	0.719	0.333	0.48
3	-64497.69811	4.9262e-06	0.430	0.504	0.561	0.504	0.561	0.333	0.5

Figure 5: Topology tests conducted with IQ-TREE between initiator caspase evolution models. Three artificial topologies were built composed of three clades containing all DED-Caspases, CARD-Caspases, and ANK-Caspases, respectively. Topology tests do not allow identification of a single most-likely scenario, preventing the formation of a hypothesis on the emergence of an ancestral pro-domain within the Caspase family. Tests are noted as p-KH for Kishino-Hasegawa test p-value (Likelihood Method), p-SH for Shimodaira-Hasegawa test p-value, and p-AU for approximately unbiased test p-value. deltaL: logL difference from the maximal logl in the set. bp-RELL: bootstrap proportion using RELL method. c-ELW: Expected Likelihood Weight. p-WKH: p-value of weighted KH test; p-WSH: p-value of weighted SH test. A tree is rejected if its p-value < 0.05. Tests p-values do not allow to discriminate a more likelihood topology between the three scenarios.

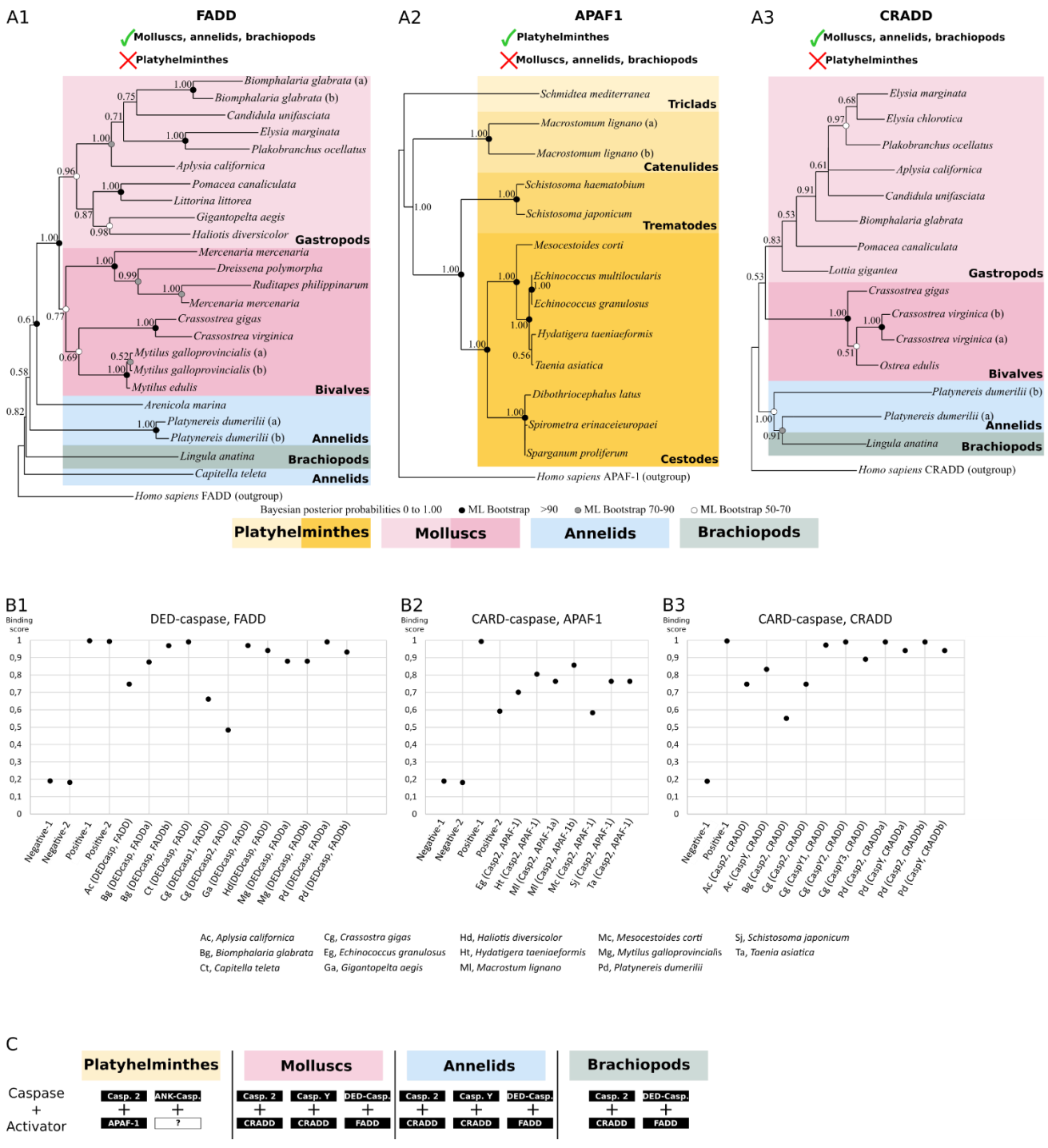


Figure 6: Analysis of initiator Caspase activators. A, phylogenetic analysis of initiator Caspase activators at the lophotrochozoan scale made by Bayesian inference method with human sequences as outgroup for FADD (A1), APAF-1 (A2), and CRADD (A3). FADD (containing a DED domain) presents a topology consistent with species relationship. Molluscs are monophyletic and split between gastropods and bivalves. Annelids and brachiopods branch early. No FADD was detected in platyhelminths. APAF-1 is absent in molluscs, annelids and brachiopods but was detected in platyhelminths. The sequence topology is similar to platyhelminth species

relationships, indicating a conservation of this gene within the group. CRADD in absent in platyhelminths but present in molluscs, annelids and brachiopods, and shows a topology consistent with the species tree. Node robustness correspond to posterior probabilities. Bootstrap correspond to robustness from maximum likelihood phylogeny which gave similar topology. **B**, *in silico* test of binding affinities made with PSOPIA software between DED-Caspases with FADD (B1), CARD-Caspases with APAF-1 (B2), and CARD-Caspases with CRADD (B3). DED domains from FADD and DED-Caspases are predicted to bind, suggesting an activation of extrinsic apoptosis. CRADD can theoretically interact with all CARD-Caspases of these species including Caspase-2 and Caspase-Y. Predictions indicate that APAF-1 interacts with platyhelminths Caspase-2. **C**, theoretical couples of initiator Caspase/activator identified in lophotrochozoans resulting from analysis conducted in **A** and **B**.

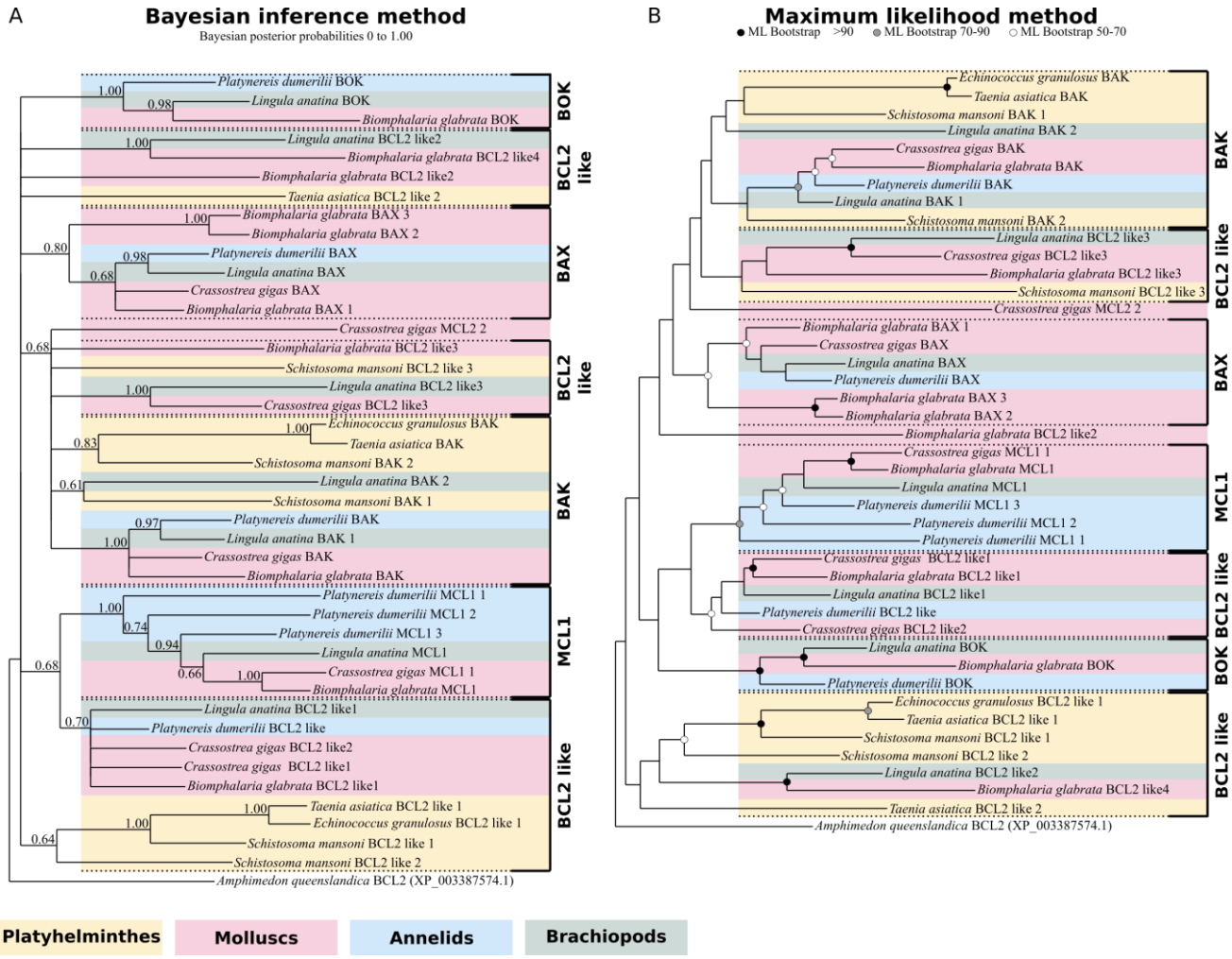


Figure 7: Topologies of the BCL2 family at the lophotrochozoan scale obtained by Bayesian inference (BI) and maximum likelihood (ML) methods. Node robustness corresponds to 500 bootstraps and posterior probabilities in ML and BI analyses, respectively. A, the BI tree exhibits numerous unresolved nodes, but the topology allows confirmation of the evolutionary relationships of several groups including BAX or MCL1, among others. **B**, the ML tree is well resolved, and sequences group into several monophyletic groups composed of either pro-apoptotic or anti-apoptotic BCL2 members. Interestingly, the platyhelminth network is very limited, composed of a unique proapoptotic (BAK), and a small number of BCL2 sequences, likely antiapoptotic. Conversely, molluscs, brachiopods and annelids have the three classical pro-apoptotic BAK, BAX, and BOK, and a large number of antiapoptotic genes including MCL1 and several BCL2 like genes.

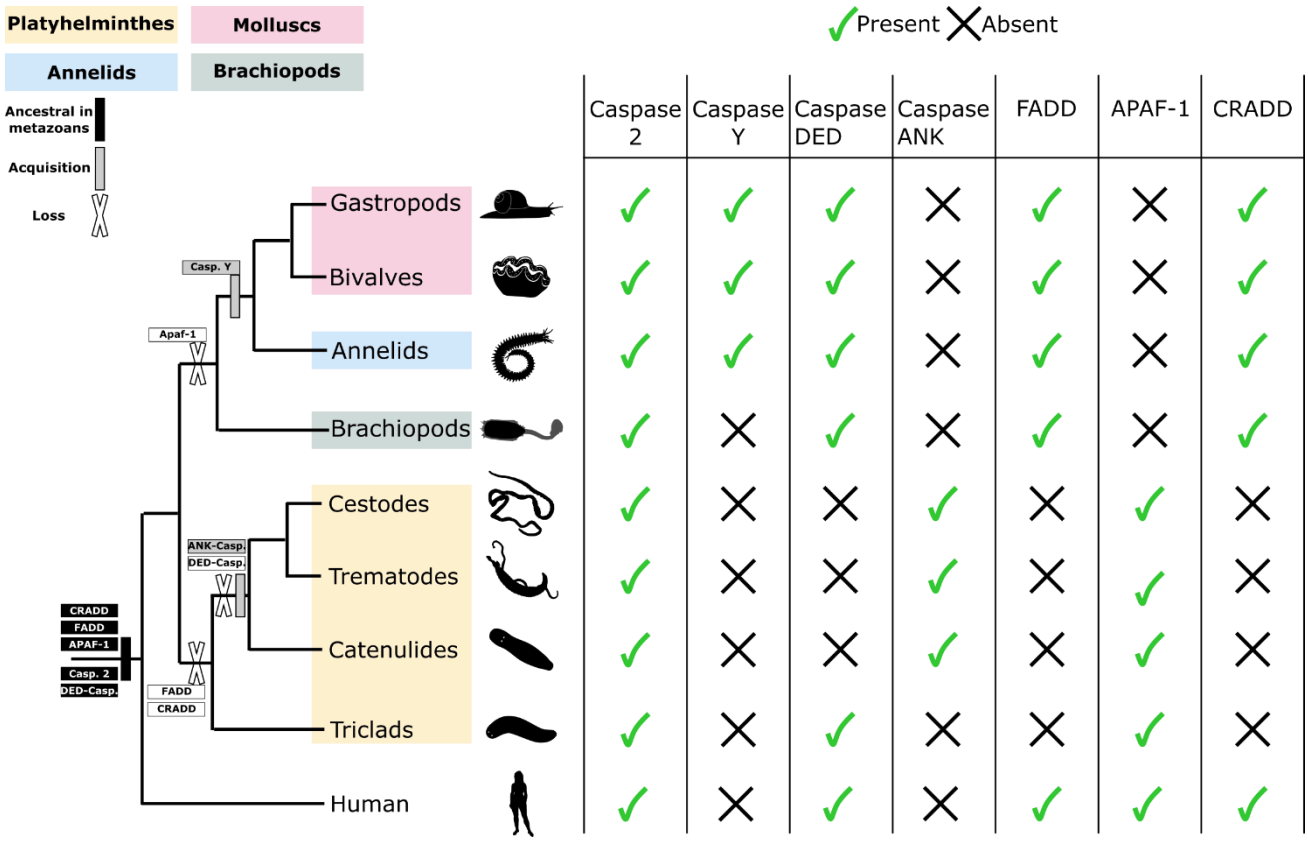


Figure 8: Summary of presence and absence of initiator Caspases and their potential activators in lophotrochozoans. Caspase-2 is the shared and ancestral common initiator in lophotrochozoans. Caspase-Y is a specific acquisition of molluscs and annelids. DED-Caspases are lost in parasitic platyhelminths, which also acquired the specific ANK-Caspases. CARD-Caspases are likely to be activated by APAF-1 in platyhelminths and by CRADD in molluscs, annelids, and brachiopods. FADD, activator of DED-Caspase, is lost in all platyhelminths. This apoptotic network suggests a loss of extrinsic apoptosis in flatworms, a divergence in CARD-Caspase activation modality within lophotrochozoans.

Table 1: dN/dS ratio calculated on the ORF using SNAP v2.1.1. Apoptotic actors with CARD-domains have a *ratio* indicating a high selection pressure (dN/dS<1), in opposite to apoptotic actors with DED domains (FADD and DED-Caspases) characterised by a relaxation of selection pressure (dN/dS>1). dN/dS<1 are red, dN/dS>1 are blue.

	dN	dS	dN/dS
APAF1	2.1121	2.4243	0.87122056
CRADD	2.0132	2.2225	0.90582677
FADD	2.4174	2.2275	1.08525253
ANK-Caspase	2.3185	2.459	0.94286295
CARD-Caspase (Lophotrochozoa)	2.5445	2.7253	0.93365868
CARD-Caspase (Molluscs, annelids)	2.5052	2.7432	0.91324001
CARD-Caspase (Platyhelminths)	1.7109	2.2011	0.77729317
DED-Caspase	2.9928	2.8498	1.05017896

Supplementary Table 1: List of sequences used to run phylogenetic analysis on all types of Caspases.

Species	Accession number	prodomain	Data Base
Aplysia californica	XP_005113266.1	CARD	NCBI
Aplysia californica	XP_012945247.1	DED	NCBI
Aplysia californica	XP_012945422.1	CARD	NCBI
Aplysia californica	XP_005099863		NCBI
Aplysia californica	XP_005092532		NCBI
Aplysia californica	XP_012944029		NCBI
Aplysia californica	XP_012940622		NCBI
Biomphalaria glabrata	XP_013082356.1	CARD	NCBI
Biomphalaria glabrata	XP_013094741.1	DED	NCBI
Biomphalaria glabrata	XP_013088924		NCBI
Biomphalaria glabrata	XP_013073999		NCBI
Biomphalaria glabrata	XP_013074008		NCBI
Biomphalaria glabrata	XP_013065656		NCBI
Biomphalaria glabrata	XP_013067788		NCBI
Biomphalaria glabrata	XP_013088924		NCBI
Echinococcus granulosus	KAH9285259.1	CARD	NCBI
Echinococcus granulosus	KAH9278879.1	ANKYRIN	NCBI
Echinococcus granulosus	KAH9283631.1		NCBI
Echinococcus granulosus	KAH9280007.1		NCBI
Haliotis diversicolor	QHY92978.1	DED	NCBI
Haliotis diversicolor	AYK02334.1	CARD	NCBI

Haliotis diversicolor	AYK02335		NCBI
Haliotis diversicolor	ABY87390		NCBI
Octopus bimaculoides	XP_014783442.1	CARD	NCBI
Octopus bimaculoides	XP_014790087.1	CARD	NCBI
Octopus bimaculoides	XP_014784582.1	CARD	NCBI
Octopus bimaculoides	XP_014780506.1		NCBI
Octopus bimaculoides	XP_014783598.1		NCBI
Octopus bimaculoides	XP_014775611.1		NCBI
Octopus bimaculoides	XP_014775614.1		NCBI
Octopus bimaculoides	XP_014789170.1		NCBI
Octopus bimaculoides	XP_014775609.1		NCBI
Octopus bimaculoides	XP_014768177.1		NCBI
Platynereis dumerilii	comp214042_c1_seq4	CARD	PdumBase
Platynereis dumerilii	comp218890_c0_seq4	DED	PdumBase
Platynereis dumerilii	comp223063_c0_seq9	CARD	PdumBase
Platynereis dumerilii	comp217549_c0_seq1	Z-BINDING DOMAIN	PdumBase
Platynereis dumerilii	comp219364_c1_seq2		PdumBase
Platynereis dumerilii	comp216671_c1_seq8		PdumBase
Platynereis dumerilii	comp219121_c0_seq2		PdumBase
Platynereis dumerilii	comp214042_c1_seq4		PdumBase
Platynereis dumerilii	PladumEVm029732		Paré et al, 2023
Platynereis dumerilii	PladumEVm031996		Paré et al, 2023
Platynereis dumerilii	PladumEVm010476		Paré et al, 2023
Platynereis dumerilii	PladumEVm027269		Paré et al, 2023
Schistocephalus solidus	VDL94269.1		NCBI
Schistocephalus solidus	VDM00376.1	CARD	NCBI
Schistocephalus solidus	VDL97252.1	ANKYRIN	NCBI
Schistosoma mansoni	ACU88130.1		NCBI
Schistosoma mansoni	ACU88129.1		NCBI
Schistosoma mansoni	XP_018646351.1	CARD	NCBI
Schistosoma mansoni	XP_018647858.1	ANKYRIN	NCBI
Spirometra erinaceieuropaei	VZI50365.1		NCBI
Spirometra erinaceieuropaei	VZI03839.1		NCBI
Spirometra erinaceieuropaei	VZI43280.1		NCBI
Spirometra erinaceieuropaei	VZI32601.1	ANKYRIN	NCBI
Taenia asiatica	VDK35885.1		NCBI
Taenia asiatica	VDK22955.1		NCBI
Taenia asiatica	VDK23513.1	CARD	NCBI
Taenia asiatica	VDK23326.1	ANKYRIN	NCBI

Supplementary Table 2: List of sequences used to run phylogenetic analysis on initiator Caspases.

Species	Accession number	Domain	Name	Data Base
Aplysia californica	XP_005113266.1	CARD	Casp.2	NCBI
Aplysia californica	XP_012945247.1	DED	Casp.DED	NCBI
Aplysia californica	XP_012945422.1	CARD	Casp.Y	NCBI
Biomphalaria glabrata	XP_013082356.1	CARD	Casp.2	NCBI
Biomphalaria glabrata	XP_013094741.1	DED	Casp.DED	NCBI
Bulinus truncatus	KAH9513478.1	CARD	Casp.Y1	NCBI
Bulinus truncatus	KAH9499437.1	CARD	Casp.Y2	NCBI
Capitella teleta	ELU00968.1	CARD	Casp.2	NCBI
Capitella teleta	ELU00616.1	CARD	Casp.2	NCBI
Capitella teleta	ELT90563.1	CARD	Casp.2	NCBI
Capitella teleta	ELT91797.1	DED	Casp.DED	NCBI
Capitella teleta	ELT97848.1	CARD	Casp.Y	NCBI
Crassostrea gigas	XP_011419292.1	CARD	Casp.2	NCBI
Crassostrea gigas	AKP95634.1	DED	Casp.DED	NCBI
Crassostrea gigas	XP_034301971.1	DED	Casp.DED	NCBI
Crassostrea gigas	XP_011414267.1	CARD	Casp.Y1	NCBI
Crassostrea gigas	XP_011432762.1	CARD	Casp.Y2	NCBI
Crassostrea gigas	XP_011449817.1	CARD	Casp.Y3	NCBI
Dimorphilus gyrociliatus	CAD5125939.1	CARD	Casp.2	NCBI
Dimorphilus gyrociliatus	CAD5115032.1	DED	Casp.DED	NCBI
Dugesia japonica	BAP74679.1	DED	Casp.DED	NCBI
Echinococcus granulosus	XP_024352296.1	CARD	Casp.2	NCBI
Echinococcus granulosus	KAH9278879.1	ANK	Casp.Ank	NCBI
Echinococcus multilocularis	CDS36952.1	ANK	Casp.Ank	NCBI
Gigantopelta aegis	XP_041374485.1	DED	Casp.DED	NCBI
Haliotis diversicolor	QHY92978.1	DED	Casp.DED	NCBI
Hydatigera taeniaeformis	VDM32914.1	CARD	Casp.2	NCBI
Hymenolepis diminuta	VDL11723.1	ANK	Casp.Ank	NCBI
Hymenolepis microstoma	CDS29941.1	ANK	Casp.Ank	NCBI
Lamellibrachia satsuma	KAI0215549.1	DED	Casp.DED	NCBI
Macrostomum lignano	PAA59014.1	CARD	Casp.2	NCBI
Macrostomum lignano	PAA54824.1	PROTEIN KINASE DOMAIN	Casp.PK	NCBI
Mesocestoides corti	VDD79553.1	CARD	Casp.2	NCBI
Mesocestoides corti	VDD77147.1	ANKYRIN	Casp.Ank	NCBI
Mytilus californianus	ADB80146.1	DED	Casp.DED	NCBI
Mytilus californianus	ADB80147.1	DED	Casp.DED	NCBI
Mytilus galloprovincialis	AIS73193.1	DED	Casp.DED	NCBI
Mytilus galloprovincialis	ADZ24778.1	CARD	Casp.Y	NCBI
Octopus bimaculoides	XP_014783442.1	CARD	Casp.2	NCBI
Octopus bimaculoides	XP_014790087.1	CARD	Casp.Y1	NCBI
Octopus bimaculoides	XP_014784582.1	CARD	Casp.Y2	NCBI

Onchidium reevesii	QMS54626.1	CARD	Casp.Y	NCBI
Pecten maximus	XP_033728057.1	DED	Casp.DED	NCBI
Platynereis dumerilii	comp214042_c1_seq4	CARD	Casp.2	PdumBase
Platynereis dumerilii	comp218890_c0_seq4	DED	Casp.DED	PdumBase
Platynereis dumerilii	comp223063_c0_seq9	CARD	Casp.Y	PdumBase
Platynereis dumerilii	comp217549	Z-BINDING DOMAIN	Casp.Z_bin.	PdumBase
Rodentolepis nana	VDO03831.1	CARD	Casp.2	NCBI
Schistocephalus solidus	VDM00376.1	CARD	Casp.2	NCBI
Schistosoma bovis	RTG88383.1	ANK	Casp.Ank	NCBI
Schistosoma haematobium	XP_012801329.1	ANK	Casp.Ank	NCBI
Schistosoma japonicum	TNN06772.1	CARD	Casp.2	NCBI
Schistosoma mansoni	XP_018647858.1	ANK	Casp.Ank	NCBI
Schmidtea mediterranea	AEX93483.1	CARD	Casp.2	NCBI
Schmidtea mediterranea	AGY56134.1	DED	Casp.DED	NCBI
Spirometra erinaceieuropaei	VZI32601.1	ANK	Casp.Ank	NCBI
Taenia asiatica	VDK23513.1	CARD	Casp.2	NCBI
Taenia asiatica	VDK23326.1	ANK	Casp.Ank	NCBI
Tubifex tubifex	ACP41139.1	DED	Casp.DED	NCBI

Supplementary Table 3: List of sequences used to run phylogenetic analysis on FADD.

Species	Accession number	Name	Data base
Aplysia californica	XP_005094212.1	FADD	NCBI
Arenicola marina	UCK81522.1	FADD	NCBI
Biomphalaria glabrata	XP_013076075.1	FADD-a	NCBI
Biomphalaria glabrata	XP_013085925.1	FADD-b	NCBI
Candidula unifasciata	CAG5121212.1	FADD	NCBI
Capitella teleta	ELU17899.1	FADD	NCBI
Crassostrea gigas	NP_001295786.1	FADD	NCBI
Crassostrea virginica	XP_022313282.1	FADD	NCBI
Dreissena polymorpha	KAH3872045.1	FADD	NCBI
Elysia chlorotica	RUS72671.1	FADD	NCBI
Elysia marginata	GFS16290.1	FADD	NCBI
Gigantopelta aegis	XP_041362799.1	FADD	NCBI
Haliotis diversicolor	AOW44266.1	FADD	NCBI
Lingula anatina	XP_013392457.1	FADD	NCBI
Littorina littorea	QNL15271.1	FADD	NCBI
Mercenaria mercenaria	XP_045212635.1	FADD	NCBI
Mercenaria mercenaria	XP_045214861.1	FADD	NCBI
Mytilus edulis	CAG2221198.1	FADD	NCBI
Mytilus galloprovincialis	VDI34998.1	FADD-a	NCBI
Mytilus galloprovincialis	VDI37367.1	FADD-a	NCBI
Plakobranchus ocellatus	GFO38852.1	FADD	NCBI

Platynereis dumerilii	comp214744_c0_seq2	FADD-a	PdumBase
Platynereis dumerilii	comp218707_c0_seq2	FADD-b	PdumBase
Pomacea canaliculata	PVD39589.1	FADD	NCBI
Ruditapes philippinarum	QBB20029.1	FADD	NCBI

Supplementary Table 4: List of sequences used to run phylogenetic analysis on APAF-1.

Species	Accession number	Name	Data base
Macrostomum lignano	PAA76335.1	APAF-1-a	NCBI
Macrostomum lignano	PAA53960.1	APAF-1-b	NCBI
Sparganum proliferum	VZI22297.1	APAF-1	NCBI
Spirometra erinaceieuropaei	VZI41625.1	APAF-1	NCBI
Dibothriocephalus latus	VDN16554.1	APAF-1	NCBI
Taenia asiatica	VDK37537.1	APAF-1	NCBI
Hydatigera taeniaeformis	VDM18691.1	APAF-1	NCBI
Echinococcus granulosus	XP_024354545.1	APAF-1	NCBI
Echinococcus multilocularis	CDI98465.1	APAF-1	NCBI
Mesocestoides corti	VDD80977.1	APAF-1	NCBI
Schistosoma japonicum	KAH8853391.1	APAF-1	NCBI
Schistosoma haematobium	XP_035588882.1	APAF-1	NCBI
Schmidtea mediterranea	AEX93473.1	APAF-1	NCBI

Supplementary Table 5: List of sequences used to run phylogenetic analysis on CRADD.

Species	Accession number	Name	Data base
Aplysia californica	XP_035826003.1	CRADD	NCBI
Biomphalaria glabrata	XP_013063451.1	CRADD	NCBI
Candidula unifasciata	CAG5119245.1	CRADD	NCBI
Crassostrea gigas	XP_034308208.1	CRADD	NCBI
Crassostrea virginica	XP_022301225.1	CRADD-a	NCBI
Crassostrea virginica	XP_022301224.1	CRADD-b	NCBI
Elysia chlorotica	RUS76462.1	CRADD	NCBI
Elysia marginata	GFS20672.1	CRADD	NCBI
Lingula anatina	XP_013399254.1	CRADD	NCBI
Lottia gigantea	XP_009058069.1	CRADD	NCBI
Ostrea edulis	XP_048741236.1	CRADD	NCBI
Plakobranchus ocellatus	GFN84464.1	CRADD	NCBI
Platynereis dumerilii	comp218707_c0_seq2	CRADD-a	PdumBase
Platynereis dumerilii	comp214744_c0_seq2	CRADD-b	PdumBase

Pomacea canaliculata	XP_025107107.1	CRADD	NCBI

Supplementary Table 6: List of sequences used to run phylogenetic analysis on BCL-2.

Species	Accession number	Name	Data base
Biomphalaria_glabrata	XP_013085524.1	Bax 1	NCBI
Biomphalaria_glabrata	XP_013096338_1	Bax 2	NCBI
Biomphalaria_glabrata	XP_013086802.1	Bax 3	NCBI
Biomphalaria_glabrata	XP_013070177.1	Bak	NCBI
Biomphalaria_glabrata	XP_013081872.1	Bcl-2 like 1	NCBI
Biomphalaria_glabrata	XP_013093137.1	Bcl-2 like 2	NCBI
Biomphalaria_glabrata	XP_013068612.1	Bcl-2 like 3	NCBI
Biomphalaria_glabrata	XP_013083436.1	Bcl-2 like 4	NCBI
Biomphalaria_glabrata	XP_013069706.1	Bok	NCBI
Biomphalaria_glabrata	XP_013065969.1	Mcl-1	NCBI
Crassostrea_gigas	XP_011424481.1	Bax	NCBI
Crassostrea_gigas	XP_011439700.1	Bak	NCBI
Crassostrea_gigas	XP_011449013.1	Bcl-2 like 1	NCBI
Crassostrea_gigas	ACH42081.1	Bcl-2 like 2	NCBI
Crassostrea_gigas	EKC18663.1	Bcl-2 like 3	NCBI
Crassostrea_gigas	XP_011436990.1	Mcl-1 1	NCBI
Crassostrea_gigas	EKC40007.1	Mcl-1 2	NCBI
Platynereis dumerilii	PladumEVm039898t3_1	Bak	Paré et al, 2023
Platynereis dumerilii	PladumEVm043631t1_1	Bax	Paré et al, 2023
Platynereis dumerilii	comp208044_c1_seq1_2	BCL2_like_A	PdumBase
Platynereis dumerilii	PladumEVm024408t5_1	Mcl-1 1	Paré et al, 2023
Platynereis dumerilii	comp214954_c0_seq5_1	Mcl-1 2	PdumBase
Platynereis dumerilii	comp223694_c0_seq4_1	Mcl-1 3	PdumBase
Platynereis dumerilii	PladumEVm036386t4_1	Bok	Paré et al, 2023
Lingula anatina	XP_013387174.1	Bak 1	NCBI
Lingula anatina	XP_013403489.1	Bax	NCBI
Lingula anatina	XP_023933050.1	Bcl-2 like 1	NCBI
Lingula anatina	XP_013381285.1	Bcl-2 like 3	NCBI
Lingula anatina	XP_013414238.1	Bok	NCBI
Lingula anatina	XP_013407221.1	Bak 2	NCBI
Lingula anatina	XP_013384166.1	Bcl-2 like 2	NCBI
Lingula anatina	XP_013403667.1	Mcl1	NCBI
Schistosoma mansoni	XP_018653371.1	Bak 2	NCBI
Schistosoma mansoni	XP_018649162.1	Bcl-2 like 2	NCBI
Schistosoma mansoni	XP_018654288.1	Bak 1	NCBI
Schistosoma mansoni	XP_018653633.1	Bcl-2 like 1	NCBI
Schistosoma mansoni	XP_018651996.1	Bcl-2 like 3	NCBI

Taenia asiatica	VDK40520.1	Bak	NCBI
Taenia asiatica	VDK34448.1	Bcl-2 like 2	NCBI
Taenia asiatica	VDK38296.1	Bcl-2 like 1	NCBI
Echinococcus granulosus	XP_024350043.1	Bax	NCBI
Echinococcus granulosus	CDS22448.1	Bcl-2 like 1	NCBI