1 **Title**

2 Intrinsic apoptosis is evolutionary divergent among metazoans

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Summary

Apoptosis is characterised by an analogous set of morphological features[1] that depend on a proteolytic multigenic family, the caspases[2,3]. Each apoptotic signalling pathway involves a specific initiator caspase, upstream of the pathway regulation, which finally converges to common executioner caspases. Intrinsic apoptosis, previously known as the mitochondrial apoptotic pathway, is often considered as ancestral and evolutionary conserved among animals[2,4–8]. First identified in the nematode *Caenorhabditis elegans*, intrinsic apoptosis was next characterised in fruit fly *Drosophila melanogaster* and mammals. Intrinsic apoptosis depends on the key initiator caspase-9 (named Ced-3 and Dronc in *Caenorhabditis* and *Drosophila*, respectively), the activator Apaf-1 and the Bcl-2 multigenic family[2,6,9]. Many functional studies have led to a deep characterisation of intrinsic apoptosis based on those classical models. Nevertheless, the biochemical role of mitochondria, the pivotal function of cytochrome c and the modality of caspases activation remain highly heterogeneous and hide profound molecular divergences among apoptotic pathways in animals[8,10]. Independent of functional approaches, the phylogenetic history of the signal transduction actors, mostly the caspase family, is the Rosetta Stone to shed light on intrinsic apoptosis evolution. Here, after exhaustive research on CARD-caspases, we demonstrate by phylogenetic analysis that the caspase-9, the fundamental key of intrinsic apoptosis, is deuterostomes-specific, while it is the caspase-2 which is ancestral and common to bilaterians. Our analysis of Bcl-2 family and Apaf-1 confirm the high heterogeneity in apoptotic pathways elaboration in animals. Taken together, our results support convergent emergence of distinct intrinsic apoptotic pathways during metazoan evolution.

Keywords

Intrinsic apoptosis, initiator CARD-caspase, apoptotic network evolution, phylogeny

RESULTS AND DISCUSSION

Apoptosis, a regulated cell death, occurs during metazoan development, tissue homeostasis and regeneration[11–13]. Pioneering works in *Caenorhabditis* established a molecular network of apoptosis decision, execution, and engulfment-degradation[2,4,14]. Next, investigation of apoptotic cascade key components from *Drosophila* and mammals imposed the paradigm that the intrinsic apoptotic "molecular program" is conserved throughout animal evolution[2,4–7]. However, recent researches revealed evolutionary divergences between these models, with major functional implications[15,16]. Based on evolutionarily conserved or divergent features of the cell death machinery among metazoans, we investigated the evolutionary history of several major actors of intrinsic apoptosis. We conducted exhaustive research of Apaf-1 and extensive phylogenetic analyses of initiator CARD-caspases and Bcl-2 multigenic family proteins from major animal phyla and describe their evolutionary patterns. It is apparent that in spite of common functional similarities, each actor of these pathways has a distinct evolutionary history that led us to consider the structural and functional organisation of intrinsic apoptotic components as the result of evolutionary convergence among animals. Thus, the three animal models (nematode, fly, mouse) which were used to elaborate a unified concept of intrinsic apoptosis machinery, not only present major functional divergences, but are markedly distinct in their protein architecture, whose origin and evolutionary history followed very different molecular pathways.

Initiator caspases of the intrinsic apoptosis pathways are not homologous

Initiation and execution of apoptotic signalling pathways are fundamentally linked to the complex diversification of the caspases multigenic family, having a widely extended repertoire encompassing all metazoan phyla[17–20].

Caspases are a class of proteases composed of three protein domains; the pro-domain, the small P10, and the large P20[9,21]. Initiator caspases, specific to an apoptotic pathway, have a specific Caspase Recruiting Domain (CARD) pro-domain or two Death Effector Domains (DED) pro-domains. The two distinct intrinsic and extrinsic apoptosis involve specifically the caspase-9 (CARD pro-domain) and the caspases-8-10 (DED pro-domains), respectively. Both pathways converge on common executioner caspases activation, triggering apoptosis execution[2,3].

Due to the pivotal role of caspase-9 as initiator in intrinsic apoptosis, our main analyses focused on CARD-caspases. We confirmed their distribution in most animal phyla and reconstructed their phylogenetic relationships (Figure 1, Table S1). Regardless of the phylogenetic methodology used for the analysis, three strongly supported (PP> 0.99) monophyletic groups were identified: caspase-9, caspase-2 and a more heterogeneous group named here [Inflammatory Caspases + Caspase-Y], respectively. Although relationships among these three clades differ depending on the methodology employed, their monophyly remains robust and together they form a clade strictly corresponding to bilaterian animals (PP = 0.87; BS>90), while the sequences of [Cnidaria + Ctenophora + Placozoa] form a divergent paraphyletic group named Caspase-X (Figure 1, Supplementary Figure 1).

Caspase-2 appears to be widely distributed among bilaterians and topology of the gene is congruent with the three major groups of eumetazoan [Deuterostomia + Ecdysozoa + Lophotrochozoa/Spiralia], reflecting an ancestral origin in bilaterians. However caspase-9 is restricted to deuterostome animals. Conversely, the clade [Caspase-Y + Inflammatory

Caspase] appears to be restricted to [Vertebrata and Lophotrochozoa]. The robustness of the deutostomes-specific caspase-9 clade was explored (Supplementary Figure 2), and its strict diversification among [Vertebrata + Cephalochordata + Echinodermata + Hemichordata] was confirmed (the relative position of the cephalochordate *Branchiostoma belcheri* 9A paralogous gene remains unstable). However, consistent with previous studies on the ascidian *Ciona intestinalis*[22] , the five representatives of ascidian genomes studied are devoid of any caspase-9, probably confirming the loss of this gene in urochordates, the sister-group of vertebrates[23]. Within bilaterians, the clade-specific acquisition of caspase-9 is a major event, probably leading to the specific functionalities observed in mammals, such as the mitochondrial outer membrane permeabilisation (MOMP) allowing cytochrome c release.

Unexpectedly, *Drosophila* Dronc and *Caenorhabditis* Ced-3 are distinctly identified as orthologous to caspase-2 of vertebrates, and not to caspase-9, as was previously reported and largely accepted[2,6]. All Dronc/Ced3 proteins of insects, horseshoe crab (Xiphosura), and nematodes form a well-defined, strongly supported (PP = 1), monophyletic clade showing only one orthologous gene per species (except horseshoe crab). Thus, the caspase-2 clade of ecdysozoans is the sister group of caspase-2 of [Lophotrochozoa + Deuterostomia]. The absence of identifiable caspase-2 in echinoderms or hemichordates can probably be interpreted as a specific loss in the latter group (i.e. Ambulacraria).

Taken globally, our analysis shown that the usually considered conserved and ancestral initiator caspases of intrinsic apoptosis are not orthologs but divergent.

The caspase activation regulator apoptosome is highly divergent in metazoans

In intrinsic apoptosis, initiator caspase activation depends on their recruitment by a pivotal and considered shared component, the apoptosome platform[2,15]. Apoptosome

115 formation relies on critical protein interactions which are *Caenorhabditis* Ced-4, *Drosophila*
116 Dark, and human Apaf-1 with their respective initiator caspases, Ced-3, Dronk, and
117 procaspase-9[15]. CARD and other protein domains (NOD, arm) are highly conserved in Apaf-1,
118 Dark and Ced-4[24–26]. However, excepted the majority of nematode species[16], Apaf-1 possesses
119 WD40 repeats at its C-terminus to bind to the cytochrome c. This binding is required in
120 mammals for Apaf-1 oligomerisation and apoptosome formation[26,27], while Ced-4 and Dark do
121 not require cytochrome c for their assembly into an apoptosome[15,28]. Taken with major
122 structural and regulating assembly differences between the octameric Dark, tetrameric Ced-
123 4 and heptameric Apaf-1 apoptosomes, it reveal evolutionary divergences between animal
124 apoptosomes formation and procaspases activation mechanisms, probably differentially
125 modulating the cell death execution pathway[29].

126 Here we conducted exhaustive analysis by reciprocal BLAST and phylogenetic analyses
127 of Apaf-1 homologs (data not show) and confirmed its ubiquity in the majority of metazoan
128 phyla (Table S2). Remarkably, Apaf-1 orthologs were absent from all urochordates and
129 molluscs in accordance with previous work[22,25,30–33], and from *Capitella teleta* (Annelida),
130 suggesting independent losses in those animals or, a less parsimonious hypothesis, multiple
131 independent evolution of modular proteins from the common ancestor of metazoans[25]
132 (Figure 2). In non-bilaterians, Apaf-1 gene family has not been found in *Pleurobrachia pileus*
133 genome (Ctenophora), but seems present in other early diverging metazoan phyla [Porifera,
134 Cnidaria and Placozoa] ([25,34,35], our data). However, their CARD domains were highly divergent
135 from caspase-9 domains of vertebrates and comparative analysis revealed unrelated complex
136 apoptosis networks.

137 Importantly, analyses from several genomes (cnidarian, nematode, fly, amphioxus, sea
138 urchin, human) clearly identified three well-defined independent clades of paralogous genes

among metazoans, highlighting that Ced-4, Dark and Apaf-1 are not homologous between ecdysozoans and vertebrates[25]. The divergent evolution history of structural actors of the apoptosome platforms confirm functional analyses carried out so far suggesting that caspase structure and interaction differs among taxa and clades[16].

Despite its fundamental role in the apoptotic cell death pathways and also in non-apoptotic functions[36–38], diverse metazoan phyla have independently experienced adapter protein Apaf-1 ortholog losses or independent molecular evolution. Consequently, the modality of apoptosome formation and the subsequent caspases activation are convergent among metazoan, and the similar mechanisms observed are hypothetised to be related to relaxation of functional constrains on molecular Apaf-1-like molecules oligomerisation process.

Finally, in-depth analysis of apoptosome structure argues in favour of distinct evolutionary origins, most likely modulated by functional interactions involving distinct initiator caspases.

The regulation of apoptosis by the Bcl-2 family is convergent among metazoans

Intrinsic apoptosis is ultimately regulated by the Bcl-2 proteins, composed of several Bcl-2 homologous (BH) domains[39,40]. In mammals, the balance between pro-survival (four BH1-BH4 domains) and pro-apoptotic proteins (Bax/Bak/Bok and BH3-only) of the Bcl-2 controls initiation of intrinsic apoptosis. Conformational changes of the three-dimensional structures and interactions between Bcl-2 actors enables the assembly of pore-like structures controlling MOMP[41].

Multiple sequence alignments of metazoan Bcl-2 family proteins (Supplementary Figure 3, Table S3) (but with over-represented chordates reflective of greater availability of

163 vertebrate genomes) confirm the distribution of Bcl-2 in Metazoa[16,24,40,42]. Consistent with
164 functional Bcl-2 classification, proteins clustered into five monophyletic groups (i.e. three 'pro-
165 apoptotic' clades: Bok, Bak, Bax and two less supported pro-survival - 'anti-apoptotic' groups:
166 Bcl-2/W/XL and a more complex Bcl-B / Mcl-1 / Bfl-1 clade) (Supplementary Figure 3).
167 However, respective relationships among each of these well-supported groups were not
168 clearly resolved, and some divergent or less characterised sequences from molluscs such as
169 *Biomphalaria glabrata* (Bcl-like2, Bcl-like3), from the cnidarian *Hydra vulgaris* (Bcl-like1) or
170 from the urochordate *Ciona intestinalis* (Bcl-like1) were not strictly attributed to a particular
171 class.

172 *Caenorhabditis* is deprived of pro-apoptotic Bcl-2 and possesses only the pro-survival
173 Ced-9 (orthologous to vertebrates Bcl-2/w/xl), and two BH-3 only proteins (Egl-1 and Ced-13).
174 Conversely, only two pro-apoptotic Bok-like close paralogs (Debcl and Buffy) were present in
175 *Drosophila* (Figure 2, Supplementary Figure 3), but their functions remains unclear[43]. In both
176 animals, there is no MOMP and apoptosome assembly does not require cytochrome c
177 binding[44]. Presence of both anti- and pro-apoptotic Bcl-2 in molluscs underlines the divergent
178 particularities observed inside Protostomia. Contrarily, similarities of Bcl-2 family composition
179 are observed within some deuterostomes (mammals and echinoderms).

180 Finally, if multiple Bcl-2 genes were acquired early in metazoan evolution, and despite
181 conservation of almost homologous genes, key differences accumulate, making initiation
182 mechanisms in intrinsic apoptotic signalling pathways convergent among animals.

183

184 **Apoptotic mitochondrial pathways are convergent among metazoans**

185 Functional evidences emphasise that caspase-2 members play a critical role in various
186 cell deaths, but have independently involved in a range of non-apoptotic functions, including

cell cycle regulation, DNA repair and tumour suppression[45–48]. This implication of caspase-2 in a myriad of signalling pathways and interaction with a panel of adaptor molecules demonstrates its functional versatility[45,46,49–52]. As previously reported and unlike other initiator caspases studied, our phylogenetic analyses corroborate the wide distribution of caspase-2 in bilaterians and suggest its ancestral multifunctionality.

The major structural and functional similarities that led to an erroneous interpretation of the phylogenetic position of Ced-3 and Dronc underlines a probable common evolutionary origin of caspase-2 and -9 genes. We propose here that caspase-9 originates from deuterostome-specific duplication of a caspase-2-like gene, followed by functional specialisation of paralogs. In the case of vertebrates (and probably in Cephalochordates), the two families of paralogs have been preserved. Caspase-2 retains multifunctional activity, and in mammals can interact with PIDDosome platform containing P53, adapter molecules RAIDD, and signalling complex DISC, activating both extrinsic, intrinsic, and DNA damage pathways[51,53–55]. Conversely, the caspase-9 gene underwent a functional divergence in connection with its specialisation in allosteric interactions with the apoptosome[3].

Due to its pivotal role as a mediator of genomic stability through involvement in cell proliferation, oxidative stress, aging and cell death, the molecular divergence of the caspase-2 gene is highly constrained during evolution, probably because destabilisation of any signalling cascade is sufficient to initiate tumorigenesis. Therefore, purifying selection is likely important during caspase-2 evolution, except during the radiation of deuterostomes, when it could have been relaxed due to the duplication and the modification of the functionalisation of caspase duplicates (cf. Duplication-Degeneration-Complementation model)[56–58]. This model could explain the loss of caspase-9 concomitantly with a caspase-2 duplication in urochordates (Figure 1, Supplementary Figure 2). Similarly, orthologs of caspase-2 have been

lost, or strongly diverged, while caspase-9 orthologs present a *de novo* relative expansion in *Strongylocentrotups purpuratus* (Echinodermata) and *Saccoglossus kowalevskii* (Hemichordata).

Due to generalised gene losses in ecdysozoans and in comparison with other bilaterians, *Caenorhabditis* and *Drosophila* apoptotic pathways are generally considered simpler than those of vertebrates[9,10]. However, what distinguishes ecdysozoans from lophotrochozoans (molluscs, annelids, and their relatives) and deuterostomes is not only a smaller number of genes but also above all the shape of pathways organised around different paralogous genes. The absence of orthologous relationships among genes results in a very different structural organisation of platforms but also generates important functional divergences (i.e. mechanisms of regulating assembly, CARD-CARD interactions with procaspases)[15].

Consistent with mammalian caspase-2 functions, *Caenorhabditis* Ced-3 has both initiator as well as executioner activities but is activated by the Ced-4 platform in a specific manner[8,51,59,60]. Unlike the organisation of the mammal apoptosome, the *Caenorhabditis* Ced-4 platform presents neither MOMP nor the release of cytochrome c[4,10]. Due to its interaction with Dark (Apaf-1 paralog), the only CARD-caspase in *Drosophila* (Dronc) has been wrongly classified as caspase-9[6,61]. Likewise, involvement of Dronc in various processes such as compensatory cell proliferation, inhibition of cell migration or spermatid differentiation, brings this protein closer to the functionalities of caspase-2 clade[62,63].

Like other protostomes, molluscs are deprived of caspase-9 but caspase-2 orthologs have been identified in bivalves and were suspected to function in “a caspase-9-like manner”[64]. Hence, caspase-2 is responsible for apoptotic process during larval metamorphosis in the oyster *Crassostrea gigas*, but more surprisingly, despite the absence of caspase-9 and Apaf-1

(and thus, of a mammalian-like apoptosome), this peculiar pathway is amazingly associated with cytochrome c release[30,33,65–68]. The complexity of intrinsic apoptosis in molluscs seems to be important, but divergent from what was observed in ecdysozoans or vertebrates, and more specifically shows a putative expansion of initiator and executioner caspases that participate both in immunity, stress responses, and apoptosis (Figure 2)[33,64,69–72].

Unexpectedly, mammals present almost the unique case (with probably the cephalochordates) in which both caspase-2 and caspase-9 are conserved and involved in apoptosis. This putative functional redundancy (i.e. recruitment, autoactivation or transactivation, homodimerisation and subsequent interchain proteolytic cleavage) undoubtedly led to the functional specialisation observed for caspase-9. This appears to be fundamentally linked to the mammalian mitochondrial pathway and non-apoptotic activity most often indirectly via caspase-3 activation[73–76]. Finally, echinoderms seem to uniquely have an intrinsic apoptosis similar to mammals, with a caspase-9, Bcl-2, Apaf-1, and a MOMP with cytochrome c release (Figure 2)[5,77].

Although we can envisage a weak parsimonious scenario that showcases a common ancestral apoptotic pathway in deuterostomes (but implying independent secondarily losses in hemichordates, cephalocordates and urochordates), the similarities observed between echinoderms and mammals more probably reflect functional convergences based on independent recruitment of apoptotic actors.

CONCLUSION

The apoptotic networks of *Caenorhabditis* and *Drosophila* do not exemplify ancestral conditions from which mammalian-grade apoptotic complexity emerged but are on the

contrary, and as suggested recently, the result of a derived condition specific to ecdysozoans among animals [24,69,78,79].

The core components of intrinsic apoptotic pathways, especially initiator caspases and the apoptosome platform, are not ancestral in metazoans. Our phylogenetic analyses highlight an unexpected evolutionary history: while the bilaterian caspase-2-mediated apoptotic toolkit emerged ancestrally and remains multifunctional, the caspase-9 mediator of the mammalian apoptosome is specific to deuterostomes.

The major functional divergences in mitochondrial apoptotic pathways observed among animals[8,80–82] mainly originated in the recruitment of paralogous actors from the same multigenic families reflecting the adaptive processes specific to each taxon, which lead ultimately to convergent evolutionary histories. Interestingly, this richness of the apoptotic genetic repertoire was suggested to be links to the persistence of stem cells in adults from different phyla[83,84].

Finally, mitochondria-mediated apoptosis, like other programmed cell deaths, has likely evolved before and throughout metazoan diversification to shape developmental processes, immune response, or to adapt cellular environment to environmental constraint.

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be address to the Lead Contact, Gabriel Krasovec (gabriel.krasovec@nuigalway.ie).

Materials availability

Any request should be address to the Lead Contact.

Data and code availability

No code was generated during this study. Source data are available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

No experimental model was used during this study.

METHOD DETAILS

Sequences dataset construction

Putative metazoans CARD-caspases were identified by using tBLASTn and BLASTp searches with human caspases (caspase-1 to 10), Ced-3, and Dronc as query on NCBI, ANISEED (ascidians), EchinoBase (*Strongylocentrotus purpuratus*), and neurobase.rc.ufl.edu (*Pleurobrachia bachei*) databases, and followed by reciprocal BLAST. After identification of CARD-caspases in target species, sequences were added as query to conduct BLAST searches in close relative (i.e. identified CARD-caspases of *Crassostrea gigas* were used as query to look for in other molluscs). Sequences with an e-value inferior to 1e-10 was retained. All identified sequences were analysed with ScanProsite (ExPaSy)[85] and InterProScan (EMBL-EBI)[86] to double check the presence of specific caspases domains. Another sequence was added to verify its identification proposed as a caspase-2 in literature: *Crassostrea angulata* casp-2[68]. Caspases family are short proteins (containing the large common P20 and the small P10 domains) with a high number of genes per species that rapidly limits the relevance of the phylogenetic analyses. To reduce the artefact branching and unreadable topology, the dataset was built using CARD-caspase gene repertoires of selected species and in order to maximize

phylogenetic diversity across Metazoa. A full list of all caspase sequences is provided in Table S1.

Metazoan Bcl-2 were identified by using tBLASTn and BLASTp searches with human Bcl-2 as query on NCBI, are followed by reciprocal BLAST. All identified sequences were analysed with ScanProsite (ExPaSy) and InterProScan (EMBL-EBI) to double check the presence of BH domains. Because of their too short sequences, BH3-only were not taking in account. A full list of all Bcl-2 sequences is provided in Table S3.

Multiple alignments of protein sequences were generated using the MAFFT software version 7[87] with default parameters and also Clustal Omega[88] to verify the congruence of the different alignments. All sequences were then manually checked in BIoEdit 7.2 software[89] to verify the presence of the specific domains previously identified. Gblocks version 0.91b[90] was used to remove vacancies and blur sites. Final alignments are composed of 230, 235, and 147 amino acids for metazoan CARD-caspases alignment, deuterostomian CARD-caspases alignment, and metazoan Bcl-2 alignment, respectively.

APAF-1 detection

Identification of metazoan Apaf-1 was made by tBLASTn and BLASTp using human APAF-1, nematode Ced-4, and fly Dark as query on NCBI, ANISEED (ascidians), and neurobase.rc.ufl.edu (*Pleurobrachia bachei*) databases, and followed by reciprocal BLAST. E-value threshold was specified to be 0.1 to increase the chance of finding sequences that match. Potential resulting sequences were analyzed with ScanProsite and also InterProScan. A full list of all Apaf-1 sequences is provided in Table S2.

Phylogenetic analysis

Phylogenetic analyses were carried out from the amino-acid alignment by Maximum-Likelihood (ML) method using PhyML 3.1[91], combined ML tree search with 1000 bootstrap replicates, and tree were visualized using Seaview[92]. Best amino-acid evolution model to conduct analysis were determined using MEGA11[93] and are WAG and LG model for CARD-caspases alignments and Bcl-2 alignment, respectively.

Bayesian analyses were performed using MrBayes (v3.2.6)[94] 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 the posterior probability. The run for metazoan CARD-caspases alignment was carried out for 2 000 000 generations with 15 randomly started simultaneous Markov chains (1 cold chain, 14 heated chains) and sampled every 100 generations. The run for deuterostomian CARD-caspases alignment was carried out for 500 000 generations with 5 randomly started simultaneous Markov chains (1 cold chain, 4 heated chains) and sampled every 100 generations. The run for metazoan Bcl-2 alignment was carried out for 5 000 000 generations with 20 randomly started simultaneous Markov chains (1 cold chain, 19 heated chains) and sampled every 100 generations.

ML boostrap values higher than 50% and Bayesian posterior probabilities are indicated on the Bayesian tree (Figure 1; Supplementary Figures 2, 3)

For the metazoans caspase-CARD phylogeny, outgroup used is the only one caspase with a pro-domain Card of the Porifera *Amphimedon queenslandica* (XP_003383519) (Figure 1). Analyses of caspase-Card were made independently at the deuterostomes scale with four different outgroup to test their effect on the stability of the topology: *i*) caspase-Card-Y of the annelid *Capitella teleta* (ELT97848.1), *ii*) caspase-Card 2 of the mollusk *Aplysia californica* (XP_005113266), *iii*) caspase-Card-X2 of cnidarian *Hydra vulgaris* (NP_001274285.1) *iv*) caspase-Card Ced-3 of the ecdysozoan *Caenohabditis elegans* (AAG42045.1) (Supplementary

Figure 2). For the metazoans Bcl-2 phylogenies, outgroup used to test their effect on the stability of the topology are: *i*) Bcl-2 like1 (XP_003383425.1) and Bcl-2 like2 (XP_003387574.1) of Porifera *Amphimedon queenslandica* (Supplementary Figure 3).

ACKNOWLEDGEMENTS

Authors acknowledge Sébastien Darras (Sorbonne Université, Banyuls-sur-mer), Christine Vesque (Sorbonne Université, Paris), Jérôme Gros (Institut Pasteur, Paris), Sabine Hennequin (Sorbonne Université, Paris), Uri Frank (NUIG, Galway), and Helen Horkan (NUIG, Galway) for helpful comments. GK was supported by a Ph.D. fellowship from the French Ministry of Education, Research and Innovation.

AUTHOR CONTRIBUTIONS

JP and EQ managed the project. GK made BLAST, phylogenetic analysis, and figures. GK and EQ wrote the manuscript.

DECLARATION OF INTERESTS

Authors declare no competing interests.

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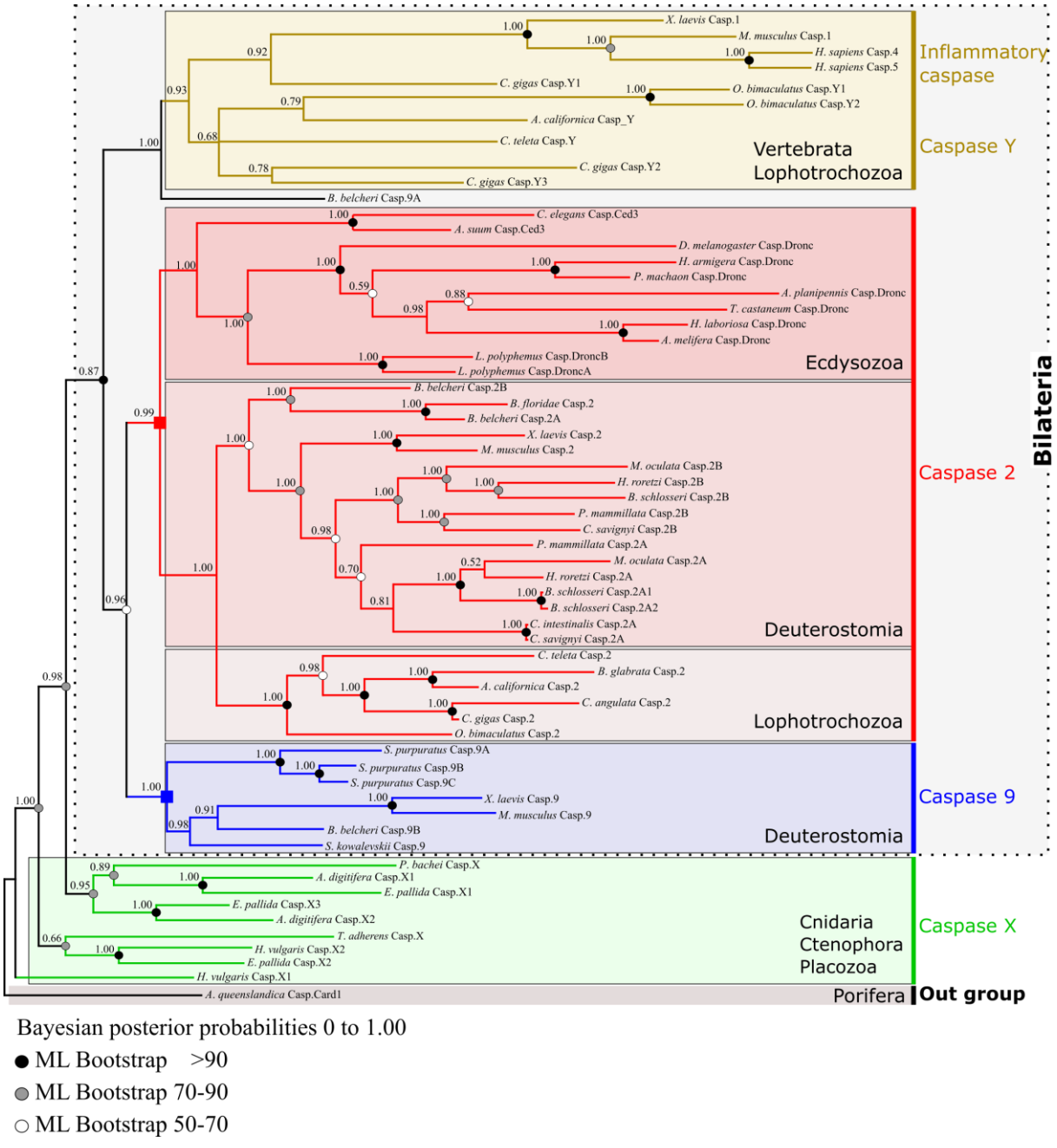

Figure 1:

Topology of metazoan CARD-caspases phylogeny obtained by Bayesian inference. Maximum likelihood method produces same topology. Three strongly supported monophyletic groups are identified: caspase-9, caspase-2 and a more heterogeneous group named here [Inflammatory Caspases + Caspase-Y]. Together they form a clade strictly corresponding to

608 bilaterian animals. Caspase-2 is widely distributed among bilaterians [Deutostomia +
609 Ecdysozoa + Lophotrochozoa/Spiralia] reflecting an ancestral origin. Conversely, caspase-9 is
610 strictly restricted to deuterostomian animals. Sequences of non-bilaterians (Cnidaria +
611 Ctenophora + Placozoa) form divergent paraphyletic groups. We used as outgroup the unique
612 CARD-caspase of the Porifera *Amphimedon queenslandica* (XP_003383519).
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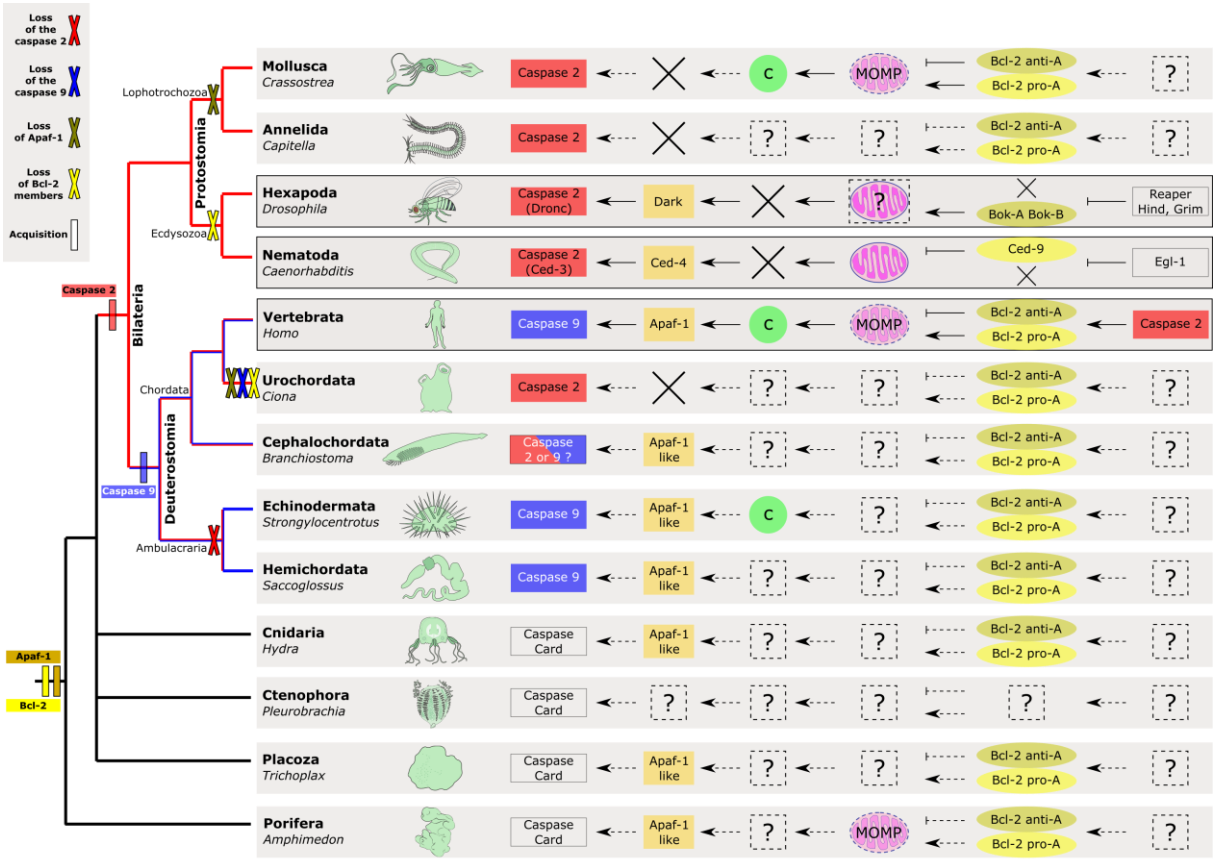

Figure 2:

Reconstruction of convergent hypothetical intrinsic apoptotic pathways among metazoans according to molecular actors detected and identified in their genomes. Variability of intrinsic apoptotic pathways among animals emerged from convergences and recruitment of distinct actors having independent evolutionary history. Caspase-2 is bilaterian-specific and the initiator of ecdysozoans intrinsic apoptosis. Caspase-9 is restricted to deuterostomes and the specific initiator of mammalian intrinsic apoptosis. Deuterostomes exhibit several losses (i.e. caspase 2 in cephalochordates, caspase 9 in urochordates) or duplication (i.e. caspase 9 in echinoderms), highlighting a putative evolutionary flexibility in apoptotic pathways establishment. Mitochondrial functions diverge among phyla and cytochrome c release thanks to the mitochondrial outer membrane permeabilisation (MOMP) is only specific to mammals

649 and possibly echinoderms. The convergent evolutionary histories certainly reflect a probable
650 phylum specific adaptive processes leading to parallel evolution of mitochondrial apoptotic
651 pathways observed among animals.
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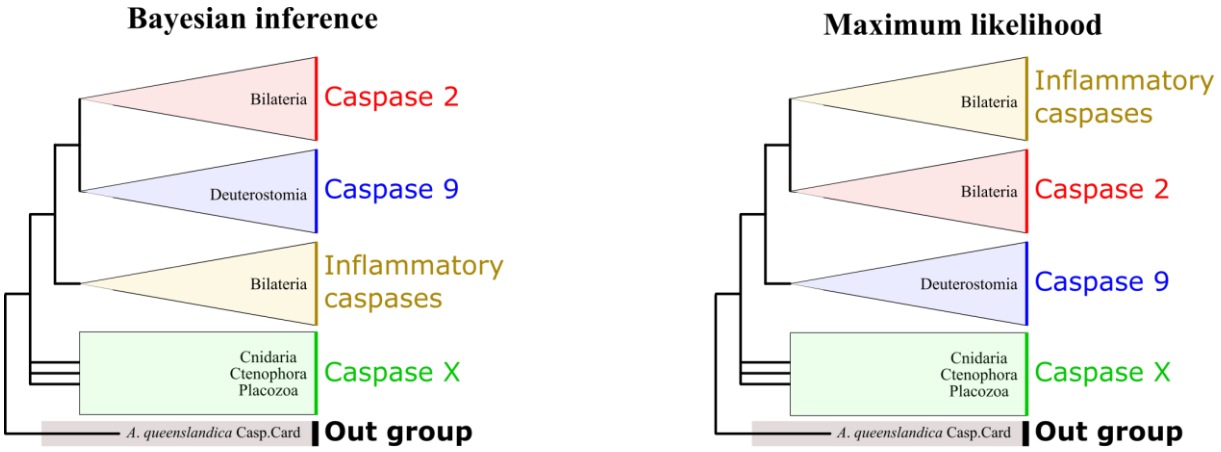
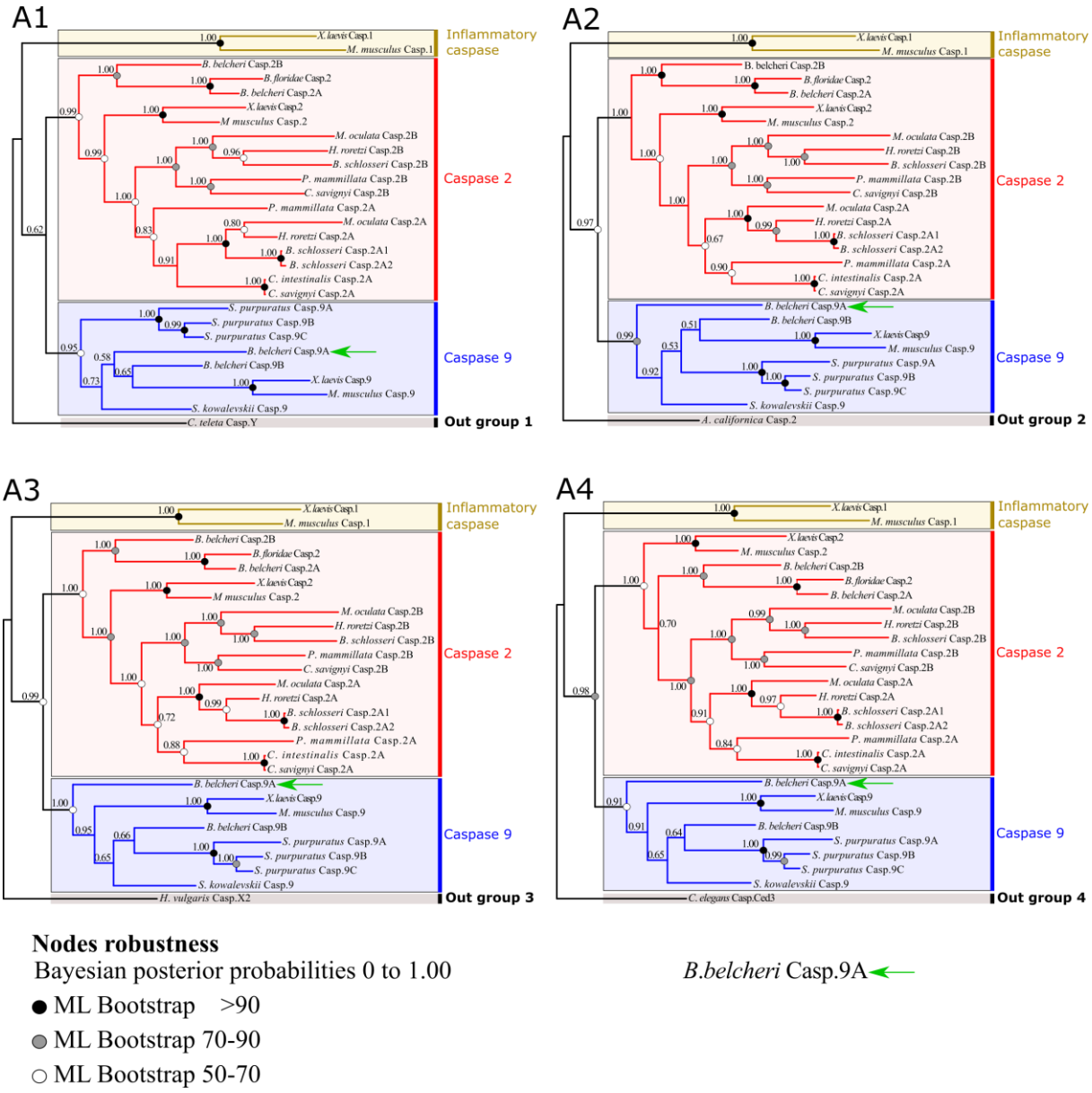


Supplementary Figure 1:

Monophyly of each CARD-caspase group is conserved using Bayesian inference and maximum likelihood analyses. Caspase-9, caspase-2 and [Inflammatory Caspases + Caspase-Y] groups remain conserved, confirming the bilaterian-specificity of caspase-2 and deuterostomian-specificity of caspase-9. Relationships among these three clades differ depending on the methodology employed. Divergent sequences of non-bilaterians animals (Cnidaria + Ctenophora + Placozoa) always form a paraphyletic group of caspases.



Supplementary Figure 2:

Phylogeny of CARD-caspases at the deuterostomian scale. Maximum likelihood and Bayesian inference methods produce similar topologies. Despite unstable position of the cephalochordate *Branchiostoma belcheri* caspase-9A (green arrow), it unequivocally appears to belong to caspase-9 group. Numbers given correspond to posterior probabilities. We used respectively as outgroup the *Capitella teleta* caspase-Y (ELT97848.1) (**A1**), the *Aplysia*

californica caspase-2 (XP_005113266) (**A2**), the *Hydra vulgaris* caspase-X2 (NP_001274285.1) (**A3**), and the *Caenorhabditis elegans* Ced3 (AAG42045.1) (**A4**).

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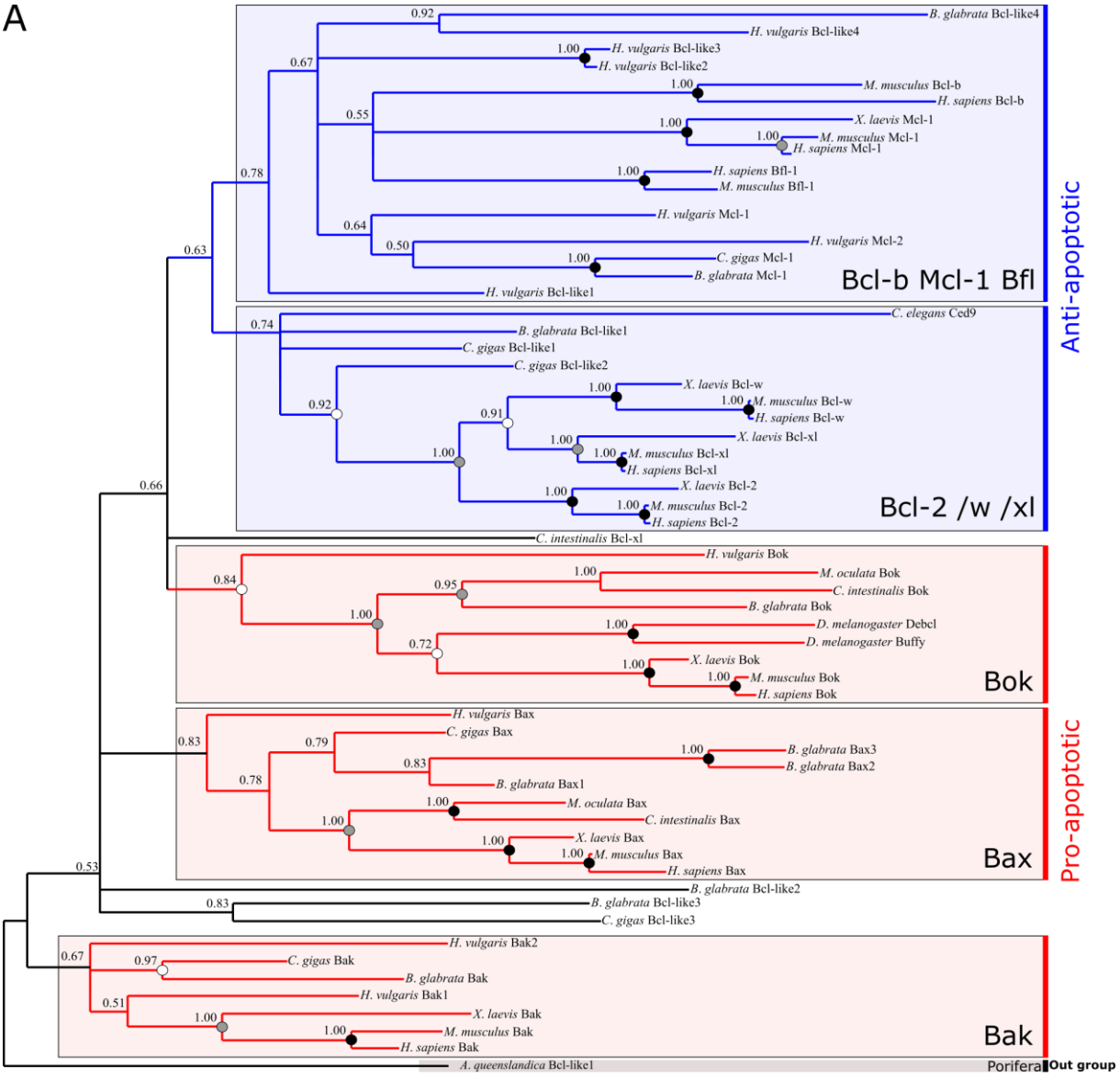
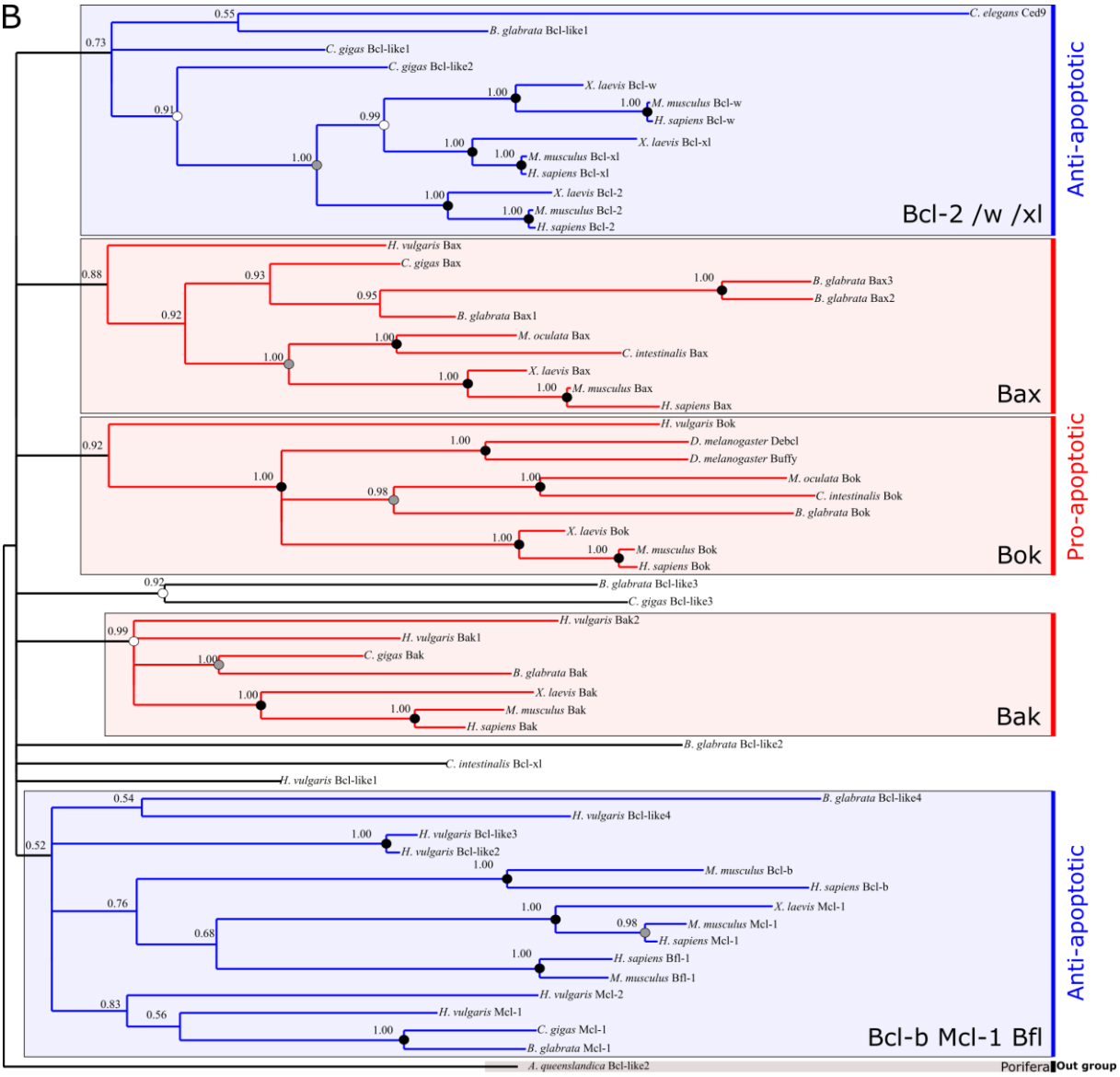


Bayesian posterior probabilities 0 to 1.00

● ML Bootstrap >90

● ML Bootstrap 70-90

○ ML Bootstrap 50-70



Bayesian posterior probabilities 0 to 1.00

● ML Bootstrap >90

● ML Bootstrap 70-90

○ ML Bootstrap 50-70

Supplementary Figure 3:

Topology of Bcl-2 family phylogeny from Bayesian inference and maximum likelihood at the metazoan scale. Bcl-2 proteins clustered into five monophyletic groups: three 'pro-apoptotic' clades (Bok, Bak, Bax) and two less supported 'anti-apoptotic' groups (Bcl-2/W/XL) and a more complex (Bcl-B/Mcl-1/Bfl-1) clade. Each group includes bilaterians as well as non- bilaterians sequences. Clustering into these five groups is consistent between analyses, while relationship

among them is not well resolved. Numbers given on branches correspond to posterior probabilities. We used respectively as outgroup the *Amphimedon queenslandica* Bcl-like 1 (XP_003383425.1) (**A**), and *Amphimedon queenslandica* Bcl-like 2 (XP_003387574.1) (**B**).

772 Supplemental table 1: List of CARD-caspases used for phylogenetic analysis.
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Species		Accession number	Name
Porifera	*Amphimedon queenslandica*	XP_003383519	Casp. Card
Cnidaria	*Acropora digitifera*	XP_015766400	Casp. X2
		XP_015768208	Casp. X1
	Exaiptasia pallida	KXJ12672.1	Casp. X1
		KXJ20965.1	Casp. X2
		KXJ12683.1	Casp. X3
	Hydra vulgaris	ACY95435.1	Casp. X1
		ACY95436.1	Casp. X2
Ctenophora	*Pleurobrachia bachei*	Sb 2658116	Casp. X
Placozoa	*Trochoplax adherens*	RDD44781.1	Casp. X
Lophotrocozoa	*Aplysia californica*	XP_005113266	Casp. 2
		XP_012945422.1	Casp. Y
	Biomphalaria glabrata	XP_013082356.1	Casp.
	Capitella teleta	ELU00616.1	Casp. 2
		ELT97848.1	Casp. Y
	Crassostrea angulata	AGN75137.1	Casp. 2
	Crassostrea gigas	XP_011419292	Casp. 2
		XP_011414267	Casp. Y1
		XP_011449817	Casp. Y3
		XP_011432762	Casp. Y2
	Octopus bimaculatus	XP_014783442.1	Casp. 2
		XP_014790087.1	Casp. Y1
		XP_014784582.1	Casp. Y2
Chelicerata	*Limulus polyphemus*	XP_013788138.1	Casp. 2
		XP_013776997.1	Casp. 2
Insecta	*Agrilus planipennis*	XP_018324425.1	Casp. 2
	Apis melifera	XP_016771440.1	Casp. 2
	Drosophila melanogaster	CAB53565.1	Casp. 2
	Habropoda laboriosa	XP_017788772.1	Casp. 2
	Helicoverpa armigera	AEK20835.1	Casp. 2
	Papilio machaon	XP_014362236.1	Casp. 2
	Tribolium castaneum	XP_001813274	Casp. 2
Nematoda	*Ascaris suum*	ERG86894.1	Casp. 2
	Caenorhabditis elegans	AAG42045.1	Casp. 2
Vertebrata	*Homo sapiens*	AAH17839.1	Casp. 4
		AAI13407.1	Casp. 5
	Mus musculus	NP_033937.2	Casp. 1
		EDL13489.1	Casp. 2
		AAH56447.1	Casp. 9
	Xenopus laevis	NP_001081223.1	Casp. 1
		NP_001081404.1	Casp. 2
		NP_001079035.1	Casp. 9
Echinodermata	*Strongylocentrotus purpuratus*	XP_789183.3	Casp. 9A
		XP_011661242.1 1	Casp. 9B

		XP_011661359.1	Casp. 9C
Hemichordata	*Saccoglossus kowalevskii*	XP_006811879.1	Casp. 9
Cephalochardata	*Branchiostoma belcheri*	XP_019646757.1	Casp. 2A
		XP_019642903.1	Casp. 2B
		XP_019623612.1	Casp. 9A
		XP_019644208.1	Casp. 9B
	Branchiostoma floridae	EEN68002.1	Casp. 2
Urochordata	*Botryllus schlosseri*	Boschl.CG.Botznik2013.chrUn.g02816.01.p	Casp. 2A1
		Boschl.CG.Botznik2013.chrUn.g08767.01.p	Casp. 2A2
		Boschl.CG.Botznik2013.chrUn.g09831.01.p	Casp. 2B
	Ciona intestinalis	KH2012:KH.C4.463.v1.A.ND1-1	Casp. 2A
	Ciona savignyi	CISAVI-CG-ENS81-R15-461426-463207-09349-P	Casp. 2A
		CISAVI-CG-ENS81-R54-461275-467018-10343-P	Casp. 2B
	Molgula oculata	Moocul-CG-ELv1_2-S113854-g13164-01-p	Casp. 2A
		Moocul-CG-ELv1_2-S103067-g09526-01-p	Casp. 2B
	Phallusia mammillata	PHMAMM-CG-MTP2014-S310-G07060-01-P	Casp. 2A
		PHMAMM-CG-MTP2014-S92-G02989-01-P	Casp. 2B
	Halocynthia roretzi	HARORE-CG-MTP2014-S35-G03390-01-P	Casp. 2A
		HARORE-CG-MTP2014-S130-G07320-01-P	Casp. 2B

789 Supplemental Table 2: List of Apaf-1 among metazoans. N/A: No APAF-1 has been identified.
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Species		Accession number
Porifera	*Amphimedon queenslandica*	XP_019855714.1
Ctenophora	*Pleurobrachia bachei*	N/A
Placozoa	*Trichoplax sp.*	RDD40813.1
Cnidaria	*Acropora digitifera*	XP_015776356.1
	Exaiptasia pallida	KXJ17575.1
	Hydra vulgaris	CDG72123.1
Nematoda	*Caenorhabditis elegans*	CAA48781.1
Chelicerata	*Limulus polyphemus*	XP_022244405.1 XP_022249946.1
Insecta	*Agrilus planipennis*	XP_025834330.1
	Apis mellifera	*XP_026298102.1*
	Drosophila melanogaster	NP_725637.1
	Habropoda laboriosa	XP_017794472.1
	Helicoverpa armigera	XP_021181657.1
	Papilio machaon	XP_014359375.1
	Tribolium castaneum	XP_015840766.1
	Aplysia californica	N/A
	Biomphalaria glabrata	N/A
Lophotrocozoa	*Crassostrea angulata*	N/A
	Crassostrea gigas	N/A
	Octopus bimaculatus	N/A
	Capitella teleta	N/A
Vertebrata	*Homo sapiens*	NP_863658.1
	Mus musculus	NP_033814.2
	Xenopus laevis	NP_001085834.1
Echinodermata	*Strongylocentrotus purpuratus*	XP_011682983.1 XP_011680781.1 XP_011680779.1
Hemichordata	*Saccoglossus kowalevskii*	XP_006818297.1
Cephalochordata	*Branchiostoma belcheri*	XP_019621685.1
	Branchiostoma floridae	XP_035681983.1 XP_035678916.1
Urochordata	*Botryllus schlosseri*	N/A
	Ciona intestinalis	N/A
	Ciona savignyi	N/A
	Molgula oculata	N/A
	Phallusia mammillata	N/A
	Halocynthia roretzi	N/A

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796 Supplemental table 3: List of Bcl-2 used for phylogenetic analysis.
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Species		Accession number	Names
Porifera	*Amphimedon queenslandica*	XP_003383425.1	Bcl-2 like 1
		XP_003387574.1	Bcl-2 like 2
Cnidaria	*Hydra vulgaris*	EF104645	Bak 1
		EU035760	Bak 2
		XP_012562061.1	Bax
		EU035764	Bcl-2 like 1
		EF104646	Bcl-2 like 2
		EU035765	Bcl-2 like 3
		EU035763	Bcl-2 like 4
		EU035761	Bok
		EF104647	Mcl-1 1
		EU035762	Mcl-1 2
Nematoda	*Caenorhabditis elegans*	AAA20080.1	Ced-9
Insecta	*Drosophila melanogaster*	AAF44120.1	Buffy (Bok-1)
		AAF26289.1	Buffy (Bok-2)
Lophotrocozoa	*Biomphalaria glabrata*	XP_013085524.1	Bax 1
		XP_013096338_1	Bax 2
		XP_013086802.1	Bax 3
		XP_013070177.1	Bak
		XP_013081872.1	Bcl-2 like 1
		XP_013093137.1	Bcl-2 like 2
		XP_013068612.1	Bcl-2 like 3
		XP_013083436.1	Bcl-2 like 4
		XP_013069706.1	Bok
		XP_013065969.1	Mcl-1
	Crassostrea gigas	XP_011424481_1	Bax
		XP_011439700_1	Bak
		XP_011449013_1	Bcl-2 like 1
		ACH42081_1	Bcl-2 like 2
		EKC18663_1	Bcl-2 like 3
		XP_011436990_1	Mcl-1 1
		EKC40007_1	Mcl-1 2
Vertebrata	*Homo sapiens*	AAA74466.1	Bak
		NP_001278357.1	Bax
		API71171.1	Bcl-2
		AAK48715.1	Bcl-B
		AAB09055.1	Bcl-w
		CAA80661.1	Bcl-xL
		NP_004040.1	Bfl-1
		NP_115904.1	Bok
		AAF64255.1	Mcl-1
	Mus musculus	NP_031549.2	Bak
		NP_031553.1	Bax
		AAH95964.1	Bcl-2

		Q9Z0F3.1	Bcl-B
		AAA51039.1	Bcl-xL
		AAB09056.1	Bcl-w
		Q07440.1	Bfl-1
		NP_058058.1	Bok
		NP_032588.1	Mcl-1
	Xenopus laevis	NP_001089587.1	Bak
		AAR84081.1	Bax
		BAH28834.1	Bcl-2
		AAI10791.1	Bcl-xl
		XP_018089640.1	Bcl-w
		NP_001139563.1	Bok
		ACI47310.1	Mcl-1
Urochordata	*Ciona intestinalis*	KH2012:KH.C4.794.v1.A.SL1-1	Bax
		KH2012:KH.S653.2.v2.A.SL1-1	Bcl-xl
		KH2012:KH.L87.39.v1.A.ND1-1	Bok
	Molgula oculata	Moocul.CG.ELv1_2.S96550.g08147.01.p	Bax
		Moocul.CG.ELv1_2.S112899.g12639.01.p	Bok

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818 **KEY RESOURCES TABLE**

Software and Algorithms		
ScanProsite	Gattiker et al., 2002[85]	https://prosite.expasy.org/scanprosite/
InterProScan	Quevillon et al., 2005[86]	https://www.ebi.ac.uk/interpro/search/sequence/
MAFFT 7	Katoh et Standley, 2013[87]	https://mafft.cbrc.jp/alignment/server/
Clusta Omega	Sievers et al., 2011[88]	https://www.ebi.ac.uk/Tools/msa/clustalo/
BioEdit 7.04	Hall, 1999[89]	https://bioedit.software.informer.com/7.2/
Gblocks 0.91b	Castresana, 2000[90]	http://molevol.cmima.csic.es/castresana/Gblocks_server.html
PhyML 3.1	Guindon et al., 2005[91]	http://www.atgc-montpellier.fr/phyml/versions.php
Seaview	Gouy et al., 2010[92]	http://doua.prabi.fr/software/seaview
Mega11	Tamura et al., 2021[93]	https://www.megasoftware.net/
MrBayes 3.1.2	Ronquist et Huelsenbeck, 2003[94]	https://nbisweden.github.io/MrBayes/download.html

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