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Exploration of Chemosensory Ionotropic Receptors in Cephalopods: the IR25 gene is expressed in the Olfactory Organs, Suckers, and Fins of *Sepia officinalis*

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ABSTRACT

While they are mostly renowned for their visual capacities, cephalopods are also good at olfaction for prey, predator and conspecific detection. The olfactory organs and olfactory cells are well described but olfactory receptors -genes and proteins- are still undescribed in cephalopods. We conducted a broad phylogenetic analysis of the ionotropic glutamate receptor family in molluscs (iGluR), especially to identify IR members (Ionotropic Receptors), a variant subfamily whose involvement in chemosensory functions has been shown in most studied protostomes. A total of 312 iGluRs sequences (including 111 IRs) from gastropods, bivalves and cephalopods were identified and annotated. One orthologue of the gene coding for the chemosensory IR25 co-receptor has been found in *Sepia officinalis* (*Soff-IR25*). We searched for *Soff-IR25* expression at the cellular level by *in situ* hybridization in whole embryos at late stages before hatching. Expression was observed in the olfactory organs, which strongly validates the chemosensory function of this receptor in cephalopods. *Soff-IR25* was also detected in the developing suckers, which suggests that the unique « taste by touch » behavior that cephalopods execute with their arms and suckers share features with olfaction. Finally, *Soff-IR25* positive cells were unexpectedly found in fins, the two posterior appendages of cephalopods, mostly involved in locomotory functions. This result opens new avenues of investigation to confirm fins as additional chemosensory organs in cephalopods.

Key Words: cephalopod, ionotropic glutamate receptor, fin, olfactory organ, sucker

INTRODUCTION

As active marine predators, cephalopods (Mollusca) are mostly renowned for their visual capacities and their ability for camouflage. Their eyes and the associated optic lobes reach a size and complexity only comparable to that of vertebrates (Young 1988). They even possess light sensors all over their body (Ramirez and Oakley 2015; Kingston et al. 2015). But cephalopods are also good at chemoreception (Di Cosmo and Polese 2017). In *Sepia officinalis*, the common cuttlefish of Europe and North Africa, olfaction is associated with prey and predator detection (Boal and Golden 1999; Romagny et al. 2012; Maselli et al. 2020), intraspecific communication (Boal and Golden 1999; Boal and Marsh 1998; Zatylny et al. 2000) including mating choice (Boal 1997) and short-distance navigation (Alves et al. 2008). At the neuroendocrine level, olfaction is one of the key senses controlling reproductive behaviors in cephalopods (Polese et al. 2015). Coleoid cephalopods (cuttlefish, octopuses, and squids) also possess two unique and fascinating features, namely their arms and suckers, which are not only motor organs able to touch, seize and catch objects but also able to discriminate different surfaces and tastes (Wells 1963, 1964).

Similar to terrestrial animals, olfaction in cephalopods is mediated by sensory cells able to detect molecules arriving from a distance source at their specialized olfactory organs. Rhinophores of nautiluses are quite similar to rhinophores and tentacles of gastropods, but olfactory organs of coleoids broadly resemble two nostrils located behind each eye (von Kölliker 1844). They contain well-described types of ciliated sensory cells (Graziadei 1964; Graziadei and Gagne 1976; Wildenburg and Fioroni 1989; Polese et al. 2016), whose role in chemoreception have been confirmed by physiological assays (Mobley et al. 2008). But we still have little information on how the diversity of odorant cues is perceived and integrated (Mobley et al. 2007). Behind the diversity of cell types, a large diversity of neurotransmitters

has been described (Scaros et al. 2018, Scaros et al. 2020) and in the central nervous system, there is nothing resembling the vertebrate glomeruli that could potentialize odor discrimination (Scaros et al. 2018). Finally, chemoreceptors for olfaction are still undescribed in cephalopods. Only recently, new receptors have been identified in the sucker rims, where cephalopods are able to "smell" or "taste" by touch. The sucker rims host several types of sensory neurons (Graziadei 1964; Graziadei and Gagne 1976; Sakaue et al. 2014; Bellier et al. 2017), including chemo-tactile neurons expressing contact-dependent chemoreceptors belonging to the Acetylcholine Receptor family (van Geisan et al. 2020).

The objective of this paper is to identify olfactory chemoreceptors in *Sepia officinalis* and to validate their expression in chemosensory organs. In molluscs, olfactory receptors may belong to diverse protein families (Derby et al. 2016). Among them, the ionotropic glutamate receptors (iGluRs) constitute a large family of ligand-gated ion channels (Figure 1), which are best characterized by their roles in synaptic communication in vertebrate nervous systems (Mayer and Armstrong 2004). The family is divided into diverse subtypes based on their ligand binding properties and sequence similarity: NMDA receptors, Epsilon receptors (lost in Protostomes) and receptors of the AKDF family (AKDF for AMPA, Kainate, Delta and Phi receptors). AMPA receptors mediate the majority of fast excitatory synaptic transmission in the vertebrate brain. Kainate receptors appear to have a modulatory role in this process and NMDA receptors are involved in synaptic and neuronal plasticity (Croset et al. 2010). Recently, a broad analysis of the iGluR family was conducted among metazoa, in which mollusc sequences from *Crassostrea gigas* (bivalve), *Lottia gigantea* (gastropod) and *Octopus bimaculoides* (cephalopod) were included (Ramos-Vicente et al. 2018).

In protostomes, a variant subfamily of iGluRs, the Ionotropic Receptors (IRs), was first identified as a new class of chemosensory receptors in *Drosophila melanogaster* (Benton et al. 2009) and then in several other species. They are supposed to have evolved from an AKDF ancestor (Figure 1A), while acquiring a chemosensory function and the capacity to detect chemical signals from the external environment (Croset et al. 2010). In insects, for instance, a high diversification has led to a large family of antennal specific IRs (Croset et al. 2010). The IR25 receptor is supposed to be the oldest member of this family, since *IR25a* gene orthologs are found in molluscs, nematodes, crustaceans and insects, suggesting that this receptor may have fulfilled a chemosensory function in the protostome ancestor (Figure 1A). It displays a broad expression in many olfactory and gustatory neurons in arthropods (Corey et al. 2013; Groh-Lunow et al. 2015; Guo et al. 2013; Zbinden et al. 2017; Rytz et al. 2013) and gastropods (Croset et al. 2010; Liang et al. 2016).

According to Ramos-Vicente et al. (2018), molluscs are likely to possess members of NMDA, AMPA, Kainate, and Delta subfamilies, as well as IR25 and specific IRs for chemoreception (Figure 1A). In order to explore the diversity of iGluRs in cephalopods and to identify IRs in *Sepia officinalis*, we realized a large survey of the iGluR family in molluscs, using all available sequences, from public databases, former studies in gastropods (Croset et al. 2010; Liang et al. 2016; Ramos-Vicente et al., 2018) and transcriptomes of *Sepia officinalis* (Bassaglia et al. 2012). Our phylogenetic analyses confirmed the existence of all expected iGluRs families in bivalves, gastropods and cephalopods. We identified one IR25 ortholog in cephalopods, belonging to a mollusc-specific IR25 subfamily. In *S. officinalis*, we synthesized a riboprobe of the IR25 gene (*Soff-IR25*) and performed *in situ* hybridizations (ISH). Our results strongly suggest the involvement of *Soff-IR25* in chemoreception as we found it expressed in olfactory organs and suckers. The unexpected

expression of *Soff-IR25* found in the fins is discussed, as it may provide evidence for a chemosensory role for these organs.

Material and Methods

Ethic statements

All animal procedures were performed in compliance with the European Union (Directive 86/609) and French law (Decree 87/848) regulating animal experimentation. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Animal rearing and tissue collection

Fertilized clutches of *Sepia officinalis* eggs were collected in the English Channel from the marine stations in Luc-sur-Mer (Université de Caen Basse-Normandie) and from Blainville-sur-Mer (SMEL) between April and July and kept at 18°C in oxygenated sea water in the laboratory. Specimens were sampled daily to obtain a complete collection of different stages over the approximate 30 days of development. The darkly pigmented egg capsule and chorion were removed in sea water. After viewing with a stereo dissecting microscope to determine stages of development according to Lemaire (1970), embryos were anesthetized by progressive temperature lowering on ice. For ISH, embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), for different durations depending on the stage. After several washes in PBS, embryos were dehydrated in baths of increasing methanol concentration, and then stored at -20°C in 100% methanol until use. For RNA extraction, whole embryos or dissected embryos were immersed in RNAlater (Ambion, Austin, TX, USA).

Molluscan iGluR collection

Available protein sequences of different mollusc species were screened in NCBI non-redundant (nr) databases. Already published sequences (nucleic or amino acid) from *Aplysia californica*, *Lottia gigantea* (Croset et al. 2010), *Crassostrea gigas*, *Octopus bimaculoides* (Ramos-Vicente et al., 2018) and *Biomphalaria glabrata* (Liang et al. 2016) were used as queries using either the BLASTx or tBLASTn algorithm, with standard parameters and with BLOSUM62 as a matrix of score. For each query, all significant hits (low primary cut-off with E-value=1.0) were collected until saturation of the collection. Our efforts were concentrated on the three main taxa of molluscs: bivalves with *Crassostrea gigas* (*Cgig*), *Crassostrea virginica* (*Cvir*), *Mizuhopecten yessoensis* (*Myes*), cephalopods with *Octopus vulgaris* (*Ovul*) and *Octopus bimaculoides* (*Obim*), gastropods with *Aplysia californica* (*Acal*), *Lottia gigantea* (*Lgig*), *Biomphalaria glabrata* (*Bgla*), *Elysia cholorotica* (*Echl*), *Pomacea caniculata* (*Pcan*). The same procedure was conducted with our own transcriptome database (Bassaglia et al. 2012) to extract a maximum of new sequences in *Sepia officinalis*.

Protein tree building and validation of molluscan iGluR

All collected amino acid sequences of putative mollusc iGluR-like proteins were aligned together with non-molluscan iGluR sequences from Ramos-Vicente et al. (2018). Alignment was performed using ClustalW Multiple Alignment (Thompson et al. 1994) and examined in BioEdit Sequence Alignment Editor (Hall 1999). Maximum Likelihood (ML) trees were built under the Jones-Taylor-Thornton (JTT) model of substitution with 500 bootstrap replicates for node support (MEGA5, Tamura et al. 2011), using a portion of 500 aligned residues covering the Ligand Binding Domain (LBD) and the Transmembrane Domain (TMD) (from domain S1 to domain M3, see Figure 1B, 1C). Sequences that were too short and of too low

quality were removed. Trees were viewed and graphically edited with MEGA5. Sequences of plant iGluR (GLR) and of sponge iGluR (GluL) were used to root trees (Ramos-Vicente et al. 2018). Collected molluscan sequences that did not well cluster with other Metazoan iGluRs were considered as non-iGluR and were removed. The final collection of molluscan sequences was listed in Supplementary Data S1.

Gene and protein nomenclature

Among all the molluscan iGluR sequences we collected, some had annotations and names from previous studies, some had no annotation or not confirmed annotations (e.g. automated annotations on NCBI). According to the phylogeny we obtained and to the available annotations, we attributed a name to all molluscan sequences. This name was used, together with the accession number, to label sequences in our trees. All data about the sequences, their family assignment and the name we attributed, were summed up in Supplementary Data S1. Names were given as follows. Receptor names are preceded by a four-letter species abbreviation consisting of an uppercase initial letter of the genus name and three lower case initial letters of the species name (see above). We respected the NC-IUPHAR nomenclature for iGluRs (Collingridge et al. 2009): each species name is followed by ‘‘Glu’’ and a letter representing the subtype of the receptor (GluK for Kainate, GluA for AMPA, GluN for NMDA and GluD for delta) or IR for Ionotropic Receptors. Distinct subclades were annotated with additional numbers or letters: directly concatenated when orthology or paralogy was similar to that found outside molluscs (e.g. GluN1, GluN2 and GluN3, IR25a) and with a “-m” for mollusc-specific subclades (e.g. GluN3-mA, GluN3-mB, GluA-mA, GluK-m6). Finally, if multiple copies of an ortholog existed for a species they are given the same name followed by a point and a number (e.g. *Cvir*-GluK-m7.1 and *Cvir*-GluK-m7.2).

Reverse Transcription PCR of *Soff-IR25*

Total RNA was extracted from whole *S. officinalis* embryos or from hand-dissected tissues (arms, brain, optic lobe, olfactory organs, funnel, mantle skin, gills, muscle, and fin) using NucleoSpin RNA set for NucleoZol (Machery-Nagel, Germany) according to manufacturer's instructions. cDNA was synthesized using 500ng of these total RNA and a Omniscript® Reverse Transcription First-strand cDNA synthesis Kit (Qiagen,Valencia, CA, USA) providing a final 20µL volume of cDNA. A couple of primers were designed to amplify a 590 pb fragment of *Soff-IR25* coding for most of the S2 and M3 conserved domains, *Soff-IR25*-F (5'-GGCAGTTTGGCACTACCCTA-3') and *Soff-IR25*-R (5'-TGGATTCACTGAAGGCAGGA-3'). PCR amplifications were performed with 1µL of cDNA template using REDTaq DNA polymerase (Eurogentec) in a C1000 Touch Thermocycler (BioRad) under the following conditions: 95°C for 5 min. + (95°C for 1 min., 55°C for 1 min., 72°C for 1 min.) for 30 cycles + 72°C for 5 min. The amplification of a 250 pb fragment of *Sepia officinalis* beta-actin (accession number HM157277.1) was used as a control for all cDNA templates: *Soff-Act*-F (5'-TTCCAGCCTTCCTTCTTGGG-3') and *Soff-Act*-R (5'-GGAGTATTTACGCTCGGGGG-3').

***Soff-IR25* RNA *in situ* hybridization**

The amplified fragment of *Soff-IR25* was purified with a NucleoSpin PCR Clean-Up kit (Machery-Nagel, Germany), cloned into pDrive Cloning Vector (Qiagen,Valencia, CA, USA) and sequenced by Eurofin Biotech (Germany). RNA probes were then generated by *in vitro* transcription using digoxigenin-11-UTP (Dig RNA labelling mix kit, Roche, Meylan, France). Antisense and sense probes were obtained with T3 or T7 polymerase (Roche). RNA probes were purified by cold precipitation with lithium chloride and anhydrous alcohol. Sense RNA probes were used as negative controls. *In situ* hybridizations were performed on

whole embryos of *S. officinalis* at stage 28 (Lemaire 1970). The protocol was adapted from Scaros et al. (2018) and from Sinigalia et al. (2018). The embryos were rinsed in PTW (PBS, 0.1% Tween-20) at room temperature and permeabilized by 10 mg/mL proteinase K for 30 min. After two rinses in 2mg/mL glycine and two acetylation steps (in 0.1M Triethanolamine / 0.25% acetic anhydride and second with 0.90% acetic anhydride), the embryos were post-fixed 1 hr in 4% PFA. After rinse in a saline sodium citrate buffer (2X SSC: 0.3 M sodium citrate, 3 M NaCl, pH 8.0), the embryos were incubated at 65°C for 5 hr in the pre-hybridization buffer (50% deionized formamide, 2X SSC, 0.1% Tween 20, 50 µg/ml heparin, 50 µg/mL tRNA, and 10% dextran sulfate). The hybridization buffer included the addition of 45 ng of probe/mL of pre-hybridization buffer and was left overnight at 65°C. After progressively stringent rinses in SSC (2X) and SSC (0.2X), at 65°C, embryos were then transferred to MABT (100 mM maleic acid, 150 mM NaCl, 100 mM NaOH, 0.1% Tween-20, pH 7.5) and incubated for 1 hour in a blocking solution (1% Blocking Reagent [Roche], 5% fetal bovine serum, in MABT) at room temperature. Embryos were then incubated in polyclonal sheep anti-DIG antibody (Roche) conjugated to alkaline phosphatase (AP) and diluted 1:1000 in blocking solution overnight at 4°C. Embryos were thoroughly rinsed with MABT and left at 4°C in an AP buffer (100 mM Tris–HCl pH 9.5, 100 mM NaCl, 0.1% Tween-20) overnight. Finally, AP buffer, with 50 mM $MgCl_2$, was added to the embryos twice for 30 min each before development of staining with the addition of 100 µg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 80 µg/mL nitroblue tetrazolium chloride (NBT) until the desired contrast was reached. After several PBS rinses, embryos were post-fixed with 3.7% formaldehyde for 24h, and finally rinsed with PBS. The total number of embryos used for ISH, including controls, was 6.

For histological visualization of staining, hybridized embryos were impregnated in 0.12M phosphate buffer pH 7.2 with 15% saccharose at 4°C for twice 24 h. Then, they were

included in Neg-50™ embedding medium (Thermo Scientific) and blocks were frozen in 2 min at −60°C with PrestoCHILL (Milestone, USA). Sections of 20 µm were performed using cryostat HM 560 MV (Microm Microtech, France).

Observation and Imaging

Whole embryos were viewed under a Leica M16 2F microscope and photographed using BK Plus digital imaging system (Dunn Inc, USA) with a Canon EOS 5DSR. Sections were viewed under a Leica DMLB microscope and imaged using a Leica MD 190 HD camera for color pictures. Final figures were constructed using Photoshop CC 2015 (Adobe Systems, Inc., San Jose, CA) with adjustments for size, contrast, and brightness only. Schematic diagrams were drawn using Adobe Photoshop CC 2015.

RESULTS

Phylogenetic survey of iGluRs in Molluscs

For an unambiguous identification of IRs sequences in *S. officinalis*, we first decided to construct a complete and informative molecular phylogeny of iGluR proteins from molluscs. Previously described sequences of iGluR and IR proteins in molluscs (Croset et al. 2010; Liang et al. 2016; Ramos-Vicente et al., 2018) were used as initial queries to blast and mine available mollusc databases (NCBI) and our own *S. officinalis* transcriptome. A total of 312 sequences was collected in molluscs (full list in Supplementary Data S1), all possessing the conserved Pfam domains (PF10613 and PF0060) coding for the iGluR-specific ligand-gated ion channel (see Figure 1B-C; Finn et al. 2008). To confirm their affiliation to the iGluR family, a large phylogenetic analyze (ML algorithm) has been performed in addition with non-molluscan iGluR sequences (from Ramos-Vicente et al. 2018). The alignment file and

the general tree we obtained are provided in Supplementary Data S2 and S3. Inside this tree, molluscan sequences clustered into monophyletic subtrees with very good bootstrap values (often 100, never under 80), all containing clear clusters of sequences for each taxon we investigated (gastropods, bivalves and cephalopods). The following paragraphs detail how each of these subtrees have been annotated, mostly on the basis of their branching to other non-molluscan sequences but also according to annotations of already published sequences.

Four molluscan subtrees gathered with GluN sequences (NMDA receptors) (Figure 2). Since the Bilateria radiation, the NMDA receptor family is subdivided into three subfamilies GluN1, GluN2 and GluN3 (Ramos-Vicente et al. 2018). This subdivision is conserved in molluscs: they possess a single GluN1 family, a single GluN2 family, but obviously two versions of GluN3 (we called mA and mB) according to the two molluscan subtrees we found inside the whole GluN3 branch.

Inside the AKDF family, GluA (AMPA receptors) and GluK (Kainate receptors) are two sister groups (Figure 1A). Three molluscan subtrees gathered with other non-molluscan GluA (Figure 3). These trees contained sequences that were already annotated as GluA in *Aplysia*, *Biomphalaria* and *Lottia* (Croset et al. 2010, Liang et al. 2016, Ramos-Vicente et al. 2018). Since these sequences were diversely named in previous studies (R1 to R5, alpha to zeta), we decided to simply call these groups mA, mB and mC (m for mollusc). In the Kainate family, two molluscan subtrees were delineated and called m7 and m10 (in reference to two already annotated sequences GluR7 and GluR10 in *Biomphalaria* and *Aplysia*).

Inside the AKDF family, we also found monophyletic molluscan subtrees, with strong bootstraps (higher than 99) (Figure 4). Four of them were called “mollusc-specific GluR” since these sequences did not cluster with any other non-molluscan sequences. We called them GluR-m6 and GluR-m9 (according to previously annotated sequences *Acal*-GluR6,

Acal-GluR9) and GluR-m11 and GluR-m12 (not previously described sequences). Another group contained several sequences that were already annotated as GluD, the Delta receptors that have been lost in most Protostomes (Ramos-Vicente et al. 2018). One of its remarkable features is the high number of paralogs that most species possess (e.g. up to 4 in *O. bimaculoides*).

Description of IRs in Molluscs

The IR25 subfamily is shared by all protostomes and has been consistently found in molluscs in a monophyletic subtree with good bootstrap values (Figure 5A). However, two unexpected features were observed. First, the typical IR25a subfamily shared by all protostomes is lacking cephalopod sequences. Second, the IR25-like sequences we found in cephalopods belong to a well delineated group (bootstrap value of 100), close to the IR25a group and that we called IR25b in respect with the IR25b sequences previously described in *Lottia* (Croset et al. 2010). The arthropod-specific IR8 group is close but distinct from both IR25a and IR25b groups (high bootstrap value of 100). Interestingly, the IR25b group appears to be specific to molluscs. Consequently, gastropod and bivalve species possess two IR25 forms (paralogs IR25a and IR25b), while cephalopods only have genes from the IR25b-like group. A comparative multiple amino acid sequence alignment of all collected mollusc IR25 (together with arthropod IR8) is provided in Figure 5B. We point out some of the amino-acid positions that unambiguously discriminate sequences from the IR25a and IR25b groups, thereby eliminating any hypothesis of artifactual subdivision of the IR25 family.

Finally, inside the AKDF group, 93 sequences gathered into four large subtrees containing most of the already published IRs found in *Lottia gigantea*, *Aplysia californica* (Croset et al. 2010) and *Biomphalaria glabrata* (Liang et al. 2016). We arbitrarily named them them IR-A, IR-B, IR-C and IR-D (Figure 6). IR-B and IR-C groups were respectively

specific to *L. gigantea* and *A. californica*. IR-D group contained sequences from gastropods and bivalves only. The IR-A group contained sequences from all mollusc groups, and then is the only group that contains IRs from cephalopods. Two sequences from *A. californica* were found in the IRs group while previously annotated as GluK (*Acal-IRA.5* and *Acal-IRD.1*, Figure 6). Conversely, two sequences annotated as IRs in *L. gigantea* turned out to be GluN (IR274, Figure 2) and mollusc-specific GluR (IR288, Figure 4).

Spatiotemporal expression patterns of *Soff-IR25*

We performed all our investigations on late embryos (stage 28, a few days before hatching, Lemaire 1970). They possess all the organs of future juveniles and already react as such in their egg capsule. They also have the advantage of being small enough for whole-mount hybridization technique. RT-PCR of *Soff-IR25* were conducted on cDNA from different dissected tissues of late embryos. Investigated tissues included potential chemosensory regions (arms, olfactory organs, skin), as well as non-sensory tissues (gills), central nervous structures (brain, optic lobe) and motor organs (mantle muscle, fin, funnel). Significant amplifications were obtained with cDNA of olfactory organs, arms and fins (Figure 7).

To further evaluate the expression of *Soff-IR25* in chemosensory cells, we analyzed its cellular spatial distribution by whole-mount ISH in late embryos of *S. officinalis* (see Figure 8 for dorsal and ventral views). No staining was observed using a sense riboprobe for *Soff-IR25* (Supplementary Figure S2A, B), while discrete staining was clearly visible in different parts of the animal bodies using an antisense riboprobe (Figure 8E). Expression at the cellular level was confirmed and determined following cryostat sectioning (Figure 9). Positive *Soff-IR25* cells were observed in both olfactory organs of the embryos (Figure 8E, 9A, A', A"). At these stages of development, each olfactory organ is a roundish organ made of a sensory

pseudostratified epithelium hosting OSNs (Scaros et al. 2018). These placodes later invaginate to form a pit, like a nostril, behind the eyes. Thin sections have shown that staining is restricted to the outer layer of cells, which corresponds to the layer of olfactory sensory neurons (OSNs; Figure 9A', A"). Higher magnification confirmed the nature of these positive cells, as some of them showed the typical shape of olfactory organs OSNs in *S. officinalis* (Scaros et al. 2018). We also detected expression of *Soff-IR25* in the developing suckers of all ten arms (Figure 8E, 9B, B', B"). At these stages of development, suckers are small bulges that later develop an inner rim (Kimbara et al. 2020). Positive cells were detected at the edge of these developing rings, and on the lateral sides of the suckers (Figure 9B"), which corresponds to the location of the well-described sensory neurons of adult suckers (Bellier et al. 2017). We also observed that *Soff-IR25* was expressed in a discrete band all along the border of the fins (Figure 8E, 9C, C', C"). Thin sections showed that positive cells were located on the ventral face of the fins only (Figure 9C").

We found no evidence of staining in other parts of the embryos, neither from the external observation nor from thin sections of the entire embryos. Other colorations were confirmed as background noises (see Supplementary Data S4). They mostly correspond to cavities or sinuses (like eyes, shell or funnel) that typically retain compounds and provoke unspecific non-cellular staining.

Discussion

iGluRs in Mollusc and Cephalopods

A good annotation of the iGluRs and IRs in molluscs was a prerequisite for the investigation of potential chemosensory IRs in cephalopods. Using non-molluscan sequences in addition to molluscan sequences collected from databases, the *in silico* phylogenetic analyses we

performed provided 20 unambiguous mollusc-specific subtrees, with high bootstrap values: 4 subtrees of molluscan GluN (Figure 2), 3 subtrees of GluA and 2 subtrees of GluK sequences (Figure 3), one GluD subtree (Figure 4), 2 subtrees of IR25 sequences (IR25a, IR25b, Figure 5), 4 subtrees of unknown AKDF families (GluR-m6, m9, m11, m12, Figure 4) and 4 subfamilies we associated to IRs (Figure 6). Through this work, 68 "hypothetical" or "uncharacterized" sequences (22%) now have annotations and family assignments have been corrected for 44 sequences (14%) and refined for 61 "iGluR sequences" (19%) (See Supplementary Data S1).

Deep phylogenetic analyses have now clearly established that bivalves and gastropods are sister clades and that cephalopods are sister to them (Kocot et al. 2011; Tanner et al. 2017). Here, with our phylogenetic analysis of molluscs iGluRs, we had too few and too short sequences to have high expectations of obtaining such a topology in our molluscan subtrees. Nonetheless, cephalopod sequences were at the basal node of 8 out 15 molluscan subtrees (GluN1, GluN2, GluN3-mA, GluN3-mB, GluA-mB, GluK-m7, GluR-m6, m11, m12) (Figure 2, 3, 4). In other subfamilies (7 out of 15), the basal node was sometimes that of the gastropod sequences (GluA-mC, GluD) or that of bivalves (GluK-m10, GluA-mA, GluA-mB, GluR-m9, IR25b) (Figure 3, 4).

Regarding cephalopods, these are 57 sequences that have been analyzed (see Table 1), including 17 new sequences from *Sepia officinalis*, and 36 already published sequences whose annotations have been corrected or refined (Table 1). Some of these sequences are likely to code for the glutamate receptors that have been detected by immunochemistry and located in the central and peripheral nervous system of cephalopods: GluN in *S. officinalis* and *O. vulgaris* (Di Cosmo et al. 2004), GluA in *Loligo vulgaris* (Di Cosmo et al. 1999; Di Cosmo et al. 2006).

IRs and *IR25* in Molluscs and Cephalopods

Alongside NMDA and AKDF receptors, we identified sequences of iGluRs variants, or IRs (including the IR25 subfamily), a group of iGluRs that is expected to have undergone high diversification as they gain a chemosensory function (Croset et al. 2010). Our analyses discriminated 64 IR sequences in gastropods, including 32 new sequences for *L. gigantea* and *A. californica* (Figure 6, Supplementary Data S1), as compared to those listed in Croset et al. 2010. In bivalves, these are 24 sequences previously annotated as "GluR" that are now listed as IRs (Supplementary Data S1). In cephalopods, we found five IRs (Figure 6, Table 1) including two forms in *S. officinalis* (*Soff-IRA1, Soff-IRA2*). Thus, duplication of IRs seems to have been more frequent in gastropods than in bivalves and cephalopods, unless these numbers only reflect a lower effort of transcriptome sequencing of chemosensory organs in the two last groups.

Concerning the IR25 subfamily (Figure 6), the evolutionary story seems more complex than in other iGluRs. Actually, two groups of IR25-like sequences were delineated in Molluscs (Figure 5). First, we described an IR25a group that is close to the IR25a arthropods sequences (bootstrap value of 100 at the node) but that is surprisingly empty of cephalopod sequences (probably a specific loss early before cephalopod radiation). Second, we described an IR25b-like group that contains sequences from all mollusc groups (including cephalopods) but with no equivalent in arthropods (probably a specific duplication of IR25a in molluscs or in lophotrochozoans). The arthropod-specific IR8 group is close to both IR25 groups (bootstrap value of 100 at the node) but is clearly distinct from them. We did not find any significant evidence for a closer relationship between the molluscan group IR25b and the arthropod group IR8 (bootstrap value of 41, Figure 5) but, with such a low resolution, the possibility that mollusc genes in IR25b are orthologs of arthropod IR8 genes can neither be rejected. For

all these reasons, we retained the IR25b denomination for the molluscan sequences and reserved the "IR8" denomination to the arthropod subtree (consistently with Croset et al. 2010).

If IR25a is known to be involved in chemosensory functions (Croset et al. 2010), what about the IR25b-like form specifically found in molluscs? We cloned the *Soff-IR25* gene and performed RT-PCR and ISH on late embryos of *S. officinalis*. Both techniques demonstrated that *Soff-IR25* is significantly expressed in three different organs: the olfactory organs, the suckers and the fins (Figure 7, 8, 9). RT-PCR amplifications were also obtained at very low level in other tissues with no perceptible hybridization staining (Figure 7). Similar results in two gastropods have shown that IR25a can also be detected in non-sensory structures such as CNS and reproductive tracts (Croset al. 2010; Liang et al. 2016) suggesting a possible role in endocrine-mediated signaling.

***SoffIR25a* is expressed in the olfactory organs**

Our RT-PCR and ISH experiments have shown that *Soff-IR25* is expressed in the olfactory organs of *S. officinalis* (Figure 7, 8, 9), supporting a conserved olfactory role of the IR25 receptor in cephalopods, even though it belongs to the IR25b-like group. In other protostomes, IR25a is detected in a variety of olfactory organs, such as antennae of insects (in the locust *Schistocerca*: Guo et al. 2013; in *Drosophila*: Rytz et al. 2013), antennules and antennae of crustacea (Corey et al. 2013; Groh-Lunow et al. 2014; Zbinden et al. 2017), rhinophores and tentacles of gastropods (in *Aplysia*: Croset et al. 2010; in *Biomphalaria*: Liang et al. 2016). The olfactory organ of cephalopods therefore represents a third type of organ in which IR25 may mediate the odorant detection.

Soff-IR25 expression is located at the surface of the olfactory organ (Figure 9A"), i.e. in the epithelium hosting chemoreceptive neurons (Wildenburg 1990; Scaros et al. 2018). Up

to five types of OSNs were described in the olfactory epithelium of cephalopods (Emery 1976; Wildenburg 1997; Polese et al. 2016), with at least three different neurotransmitters (Nitric Oxide, FMRFamides, histamine, Scaros et al. 2018) and specific odorant responsiveness and transduction pathways (Mobley et al. 2007, 2008). Since *Soff-IR25* is not expressed in the whole epithelium, we hypothesize that IR25 is likely not the sole chemosensory receptor in the olfactory organs. Further studies will be required to determine whether *Soff-IR25* positive cells are associated with a specific population of OSNs and/or with a specific neurotransmitter.

Soff-IR25 **is expressed in suckers**

Soff-IR25 positive cells were also found in some cells of the developing suckers (Figure 9B, B', B"). Suckers of cephalopods' arms are fascinating organs able to seize objects or prey but also to discriminate objects with different surfaces (Wells 1964) or different tastes (Wells 1963; Wells et al. 1965; Anraku et al. 2005). In the late embryos we studied, suckers are small bulges in the center of which an invagination will form the future inner rim (Kimbara et al. 2020). *Soff-IR25* positive cells were observed at the periphery of the sucker bulge but also around the future rim (Figure 9B"), at the very same location and at the same stages that early sensory cells described by immunostaining (Buresi et al. 2014). This result suggests that these cells are one of the first chemosensory cells of the suckers (Bellier et al. 2017), ready to operate after hatching.

In 1963, Wells named "taste by touch" the capacity of *Octopus vulgaris* to discriminate objects with different chemical characteristics (Wells 1963). Recently, chemo-tactile receptors have been recently described in the sucker rim of *Octopus* species (van Geisan et al. 2020). They belong to a cephalopod-subfamily of Acetylcholine Receptors and seem able to detect poorly soluble natural products (van Geisan et al. 2020). Our results show

that these receptors are obviously note the sole to mediate odor detection. The IR25 receptor is also a possible actor of this capacity and it is interesting to note that, if so, the IR25 receptor would be both involved in "smell" (olfactory receptors) and "taste" (suckers). Arms and suckers are synapomorphies of cephalopods. Several molecular arguments now confirm that the arm crown surrounding the mouth opening in coleoid cephalopods is derived from the ancestral pedal area of molluscs (Boletzky 1988; Shigeno et al. 2008; Buresi et al. 2016) and has evolved as efficient tools to catch prey but also to taste them. As such, they are probably not homologous to oral tentacles in gastropods. But similar selective pressures have operated in both types of organs, since in gastropods the IR25a receptor is also found both in olfactory rhinophores and in oral tentacles located in the mouth vicinity (Croset et al. 2010; Liang et al. 2016).

Soff-IR25 **is expressed in the fins**

The discovery of a *Soff-IR25* expression in the fins of *Sepia officinalis* was unexpected. Fins are motor appendages involved in the control of slow locomotion and fine motion. They contain fibres of motoneurons controlling the fin muscles, and fibres controlling the chromatophore structures, responsible for the versatile skin color patterns (Gaston and Tublitz 2004). A single study has suggested a sensory function in the cephalopod fins, with the description of mechanoreceptors in the fins of *S. officinalis* (Kier et al. 1985). Here, our *in situ* hybridizations detected expression of *Soff-IR25* in epithelial cells of fins, which suggests that these positive cells may be primary sensory cells and probably chemosensory cells, maybe similar to that of the olfactory organs. Together with the suckers at the anterior side of the animal and the two lateral olfactory organs close to the mantle opening, fins would add a posterior dimension to the detection of odors in *S. officinalis*. New fields of exploration are now open and future investigations must be conducted to describe the chemosensory cells, to

validate any fin olfactory capacities by detecting behavioral or physiological reactions to odors and to unravel the sensory pathways to more central nervous structures, ganglia or brain lobes.

Summary and evolutionary perspectives

Soff-IR25 is the first chemosensory receptor whose expression has been investigated in a cephalopod. Its olfactory function is not formally demonstrated here but strongly suggested by the nature of the organs in which its expression has been detected, especially the olfactory organs (Figure 8, 9). Our results on late embryos suggest that juveniles hatch with a full array of odor detectors, anteriorly with their arms, laterally with their olfactory organs and posteriorly with their fins. Numerous features of *Soff*-IR25 yet remain to be unraveled: the molecules it can bind with or the other receptors it may operate with, the type of sensory cells expressing it, or the evidence of its involvement in olfaction in later juveniles and adults.

Soff-IR25 belongs to a large family of orthologous genes, whose relationship with chemosensation and olfaction is now deeply established from insects, and crustaceans, to gastropods (Croset et al. 2010) and now cephalopods. Regarding molluscs, bivalves remain the forgotten group to explore, and an effort must be undergone to understand the potential specific functions of the two groups of paralogous genes *IR25a* and *IR25b* we delineated in gastropods and bivalves (Figure 6). We also described several members of variant iGluR (IRs) in molluscs, including two forms in *S. officinalis*, *Soff-IRA1* and *Soff-IRA2*. IR subunits have been found in the olfactory tissues of two divergent gastropod subclasses, pulmonates

(*Biomphalaria*) and opisthobranchs (*Aplysia*) (Croset al. 2010; Liang et al. 2016). Exploration of IRs in bivalves and cephalopods will probably demonstrate a similar general role for the IR family in chemosensory signaling in molluscs. More generally other potential chemosensory receptor families, like GPCRs, must be explored since they have been established as key receptors in olfaction for other mollusc species (Cummins et al. 2009) and may act in concert with IRs to contribute to the output of sensory neurons.

Data deposition

The new sequences of *S. officinalis* we reported and analyzed in this paper have been deposited in Genbank (18 sequences, from MW657339 to MW657356). See Dataset S1 for details.

Author Contributions

Conceived and designed the experiments: AA SB. Performed the experiments: AA, SV. Analyzed the data: AA SB. Wrote the paper: AA SB.

Competing interests

The authors have declared that no competing interest exists.

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Table 1 – iGluRs and IRs sequences collected and analyzed in Cephalopods.

Species	Accession Number	NCBI annotation	Name in previous publications	Family assignment (present study)	Attributed name	Effect of this study on family assignment
Doryteuthis opalescens	CAB65182.1	GluR		GluA-mC	Dopa_GluA-mC	more precise
Octopus bimaculoides	XP_014790759.1	NMDA 1		GluN1	Obim_GluN1	confirmed
	XP_014786064.1	NMDA 2A		GluN2	Obim_GluN2	confirmed
	XP_014786105.1	NMDA 3A		GluN3-mA	Obim_GluN3-mA	confirmed
	XP_014767729.1	NMDA 3A		GluN3-mB	Obim_GluN3-mB	confirmed
	XP_014780672.1	GluR2		GluA-mA	Obim_GluA-mA	more precise
	XP_014770574.1	GluR	GluA alpha	GluA-mB	Obim_GluA-mB	confirmed
	XP_014776221.1	GluR	GluA beta	GluA-mC	Obim_GluA-mC	confirmed
	XP_014777584.1	kainate 2	GluK beta	GluK-m7	Obim_GluK-m7.1	confirmed
	KOF77616.1	-	GluK gamma	GluK-m7	Obim_GluK-m7.2	confirmed
	XP_014788118.1	kainate 2	GluK alpha	GluK-m10	Obim_GluK-m10	confirmed
	XP_014790397.1	kainate 2		GluR-m6	Obim_GluR-m6	corrected
	XP_014768777.1	kainate 2		GluR-m9	Obim_GluR-m9	corrected
	XP_014776235.1	kainate 2		GluR-m11	Obim_GluR-m11	corrected
	XP_014776282.1	kainate 2		GluR-m12	Obim_GluR-m12	confirmed
	XP_014773477.1	delta 1		GluD	Obim_GluD.1	confirmed
	XP_014773476.1	GluR2		GluD	Obim_GluD.2	more precise
	XP_014771816.1	GluR2		GluD	Obim_GluD.3	more precise
	XP_014773475.1	GluR2		GluD	Obim_GluD.4	more precise
	XP_014768464.1	delta 2		IR25	Obim_IR25	corrected
	XP_014781666.1	GluR2		IR	Obim_IRA.1	more precise
	XP_014783750.1	GluR1		IR	Obim_IRA.1	more precise
Octopus vulgaris	XP_029648108.1	NMDA 1		GluN1	Ovul_GluN1	confirmed
	XP_029651610.1	NMDA 2B		GluN2	Ovul_GluN2	confirmed
	XP_029643902.1	NMDA 3A		GluN3-mA	Ovul_GluN3-mA	confirmed
	XP_029638855.1	NMDA 3A		GluN3-mB	Ovul_GluN3-mB	confirmed
	XP_029635632.1	2-like		GluA-mA	Ovul_GluA-mA	more precise
	XP_029641811.1	GluR		GluA-mB	Ovul_GluA-mB	more precise
	XP_029652493.1	kainate2		GluK-m7	Ovul_GluK-m7.1	confirmed
	XP_029656687.1	kainate 2		GluK-m7	Ovul_GluK-m7.2	confirmed

	XP_029644204.1	kainate 2	GluR-m6	Ovul_GluR-m6	corrected
	XP_029644262.1	kainate 2	GluR-m9	Ovul_GluR-m9	corrected
	XP_029638129.1	kainate 2	GluR-m11	Ovul_GluR-m12	corrected
	XP_029638127.1	kainate 3	GluR-m12	Ovul_GluR-m11	confirmed
	XP_029650697.1	GluR3	GluD	Ovul_GluD.1	more precise
	XP_029657881.1	delta 2	GluD	Ovul_GluD.2	confirmed
	XP_029650698.1	delta 2	GluD	Ovul_GluD.3	confirmed
	XP_029639446.1	delta2	IR25	Ovul_IR25	corrected
	XP_029637805.1	GluR2	IR	Ovul_IRA.2	more precise
Sepia officinalis	MW657340	-	GluN1	Soff_GluN1	first annotation
	MW657341	-	GluN2	Soff_GluN2	first annotation
	MW657343	-	GluN3-mA	Soff_GluN3-mA	first annotation
	MW657342	-	GluN3-mB	Soff_GluN3-mB	first annotation
	MW657354	-	GluA-mA	Soff_GluA-mA	first annotation
	MW657355	-	GluA-mB	Soff_GluA-mB	first annotation
	MW657356	-	GluA-mC	Soff_GluA-mC	first annotation
	MW657349	-	GluK-m7	Soff_GluK-m7.1	first annotation
	MW657350	-	GluK-m7	Soff_GluK-m7.2	first annotation
	MW657348	-	GluK-m10	Soff_GluK-m10	first annotation
	MW657352	-	GluR-m6	Soff_GluR-m6	first annotation
	MW657353	-	GluR-m9	Soff_GluR-m9	first annotation
	MW657347	-	GluR-m12	Soff_GluR-m12	first annotation
	MW657344	-	GluD	Soff_GluD	first annotation
	MW657339	-	IR25	Soff_IR25	first annotation
	MW657345	-	IR	Soff_IRA.1	first annotation
	MW657346	-	IR	Soff_IRA.2	first annotation
Sepiella japonica (Sjap)	QHI00147.1	NMDA 1	GluN1	Sjap_GluN1	confirmed

Figure Legends

Figure 1. The iGluRs and IRs families. A- Model for the evolution of iGluRs and IRs in animals (derived from Croset et al. 2010 and Ramos-Vicente et al. 2018). Animal ancestral iGluRs have evolved and diversified into various subfamilies: Lambda receptors, NMDA receptors and non-NMDA receptors subdivided into AMPA, Kainate, Phi and Delta receptors (the AKDF subfamily). According to Ramos-Vicente et al. (2018), molluscs (belonging to protostomes) are expected to possess NMDA, AMPA, Kainate and Delta receptors. In addition, specific families of iGluRs have evolved for chemosensory functions in protostomes: these are Ionotropic Receptors (IRs). IR25 is widely shared among protostomes, while specific IRs (including IR8) have evolved in specific taxa (e.g. antennal IRs in insects, Croset et al. 2010). B- Protein domain structure of IR25 in schematic form, showing an Amino-Terminal Domain (ATD), a Ligand-Binding Domain (LBD) constituted by two conserved regions S1 and S2, and a Transmembrane Domain (TMD) made of 4 helix domains (M1, M2, P, M3). C- 3D-model of *Soff*-IR25 obtained from Swiss-Model server (Biasini et al. 2014).

Figure 2. Characterization of GluN sequences (NMDA receptors) in Molluscs. Phylogenetic construct obtained with molluscan GluN and non-molluscan sequences (from Ramos-Vicente et al. 2018), rooted on plant GLRs and sponge GluL. GluN1 and GluN2 in molluscs are clearly delineated by two monophyletic subtrees, with internal subtrees for each taxon (gastropod, bivalve and cephalopod). In GluN3, mollusc sequences delineate two monophyletic subtrees (called GluN3-mA and GluN3-mB). Sequences from molluscs are labeled as follows: attributed name / accession number (if any) / asterisk(s) for sequences

analyzed in previous studies (two asterisks when annotation is confirmed) / their name in previous studies (if any). Non-molluscan sequences directly refer to that from Ramos-Vicente et al. (2018). Bootstraps of molluscan subtrees are encircled in yellow. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 3. Characterization of GluA (AMPA receptors) and GluK (Kainate receptors) in Molluscs. Phylogenetic construct obtained with molluscan GluA-GluK and non-molluscan sequences (from Ramos-Vicente et al. 2018), rooted on plant GLRs and sponge GluL. Three molluscan subtrees were found within the GluA family (neutrally called GluA-mA, GluA-mB and GluA-mC) and two molluscan subtrees were found in the GluK family (called GluK-m7 and GluK-m10, according to the names GluR7 and GluR10 given to some of these sequences in previous studies). Sequences from molluscs are labeled as follows: attributed name / accession number (if any) / asterisk(s) for sequences analyzed in previous studies (two asterisks when annotation is confirmed) / their name in previous studies (if any). Non-molluscan sequences directly refer to that from Ramos-Vicente et al. (2018). Bootstraps of molluscan subtrees are encircled in yellow. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 4. Characterization of other AKDF subfamilies in Molluscs. Phylogenetic construct obtained with molluscan AKDF sequences (except IRs) and non-molluscan sequences (from Ramos-Vicente et al. 2018), rooted on plant GLRs and sponge GluL. The AKDF family is rooted by AKDF sequences from sponges (*Oscarella carmela*, from Ramos-Vicente et al. (2018), and whose specific branch is compressed and delineated by the gray triangle GluAkdf. Close to the GluA and GluK subfamilies (compressed as orange and red

triangles), we found 4 mollusc-specific GluR (called m6, m9, m11, m12), the GluD-m molluscan family (Delta receptors) and the IR25 family (compressed here in blue, detailed in Figure 5). Sequences from molluscs are labeled as follows: attributed name / accession number (if any) / asterisk(s) for sequences analyzed in previous studies (two asterisks when annotation is confirmed) / their name in previous studies (in brackets, if any). Non-molluscan sequences directly refer to that from Ramos-Vicente et al. (2018). Bootstraps of molluscan subtrees are encircled in yellow. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 5. The IR25 family in Molluscs. A- Molecular phylogeny of IR25 receptors in molluscs. IR25 and IR8 sequences from arthropods have been added (Maximum Likelihood algorithm, 500 bootstrap replicates). Molluscs do not possess any IR8-like sequences. Cephalopod sequences of IR25-like receptors do not belong to the IR25a subgroup but to a sister group (IR25b-like) which is specific to molluscs. Sequences from molluscs are labeled as follows: attributed name / accession number (if any) / asterisk(s) for sequences analyzed in previous studies (two asterisks when annotation is confirmed) / their name in previous studies (in brackets, if any). Non-molluscan sequences directly refer to that from Ramos-Vicente et al. (2018). Bootstraps of molluscan subtrees are encircled in yellow. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. **B-** Partial alignment of amino acid sequences of the IR25 family in molluscs (plus four IR8 sequences from arthropods). A focus is made on two domains (final portion of S1 and M2): some discriminant amino-acid positions between IR25a and IR25b-like subgroups are highlighted by boxes.

Figure 6. Survey of the IRs in Molluscs. Phylogenetic construct obtained with AKDF molluscan including IRs and non-molluscan sequences (from Ramos-Vicente et al. 2018), rooted on plant GLRs and sponge GluL. Sister of the GluN group (violet triangle), the AKDF group is here mostly compressed (see GluA, GluK, GluD, IR25 and mollusc-specific GluR as colored triangles), with the exception of 4 molluscan subfamilies (arbitrarily labelled A, B, C and D), containing numerous molluscan sequences annotated as IRs in previous studies (Croset et al. 2010; Liang et al. 2016). Cephalopod sequences were only found in the A subfamily. Sequences from molluscs are labeled as follows: attributed name / accession number (if any) / asterisk(s) for sequences analyzed in previous studies (two asterisks when annotation is confirmed) / their name in previous studies (in brackets, if any). Non-molluscan sequences directly refer to that from Ramos-Vicente et al. (2018). Bootstraps of molluscan subtrees are encircled in yellow. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 7. Tissue expression of *Soff-IR25*. *Top*: schematic representation of a late embryo of *S. officinalis* (stage 28); *Bottom*: RT-PCR analysis of *Soff-IR25* gene expression in different tissues of late embryos of *S. officinalis*. Significant amplifications were obtained in arms, olfactory organs and fins. Control RT-PCR products for comparative analysis of gene expression correspond to beta-actin. All RT-PCR products were sequenced to confirm their identity.

Figure 8. Whole views of *S. officinalis* late embryos. A, B: dorsal views (drawing and picture); **-C, D**: ventral views (drawing and picture); **-E**: whole mount *in situ* hybridization of *Soff-IR25* showing specific staining in the olfactory organs, arms and fins (asterisks). Pink

coloration in funnel, ink sac and viscera are background noise (see Fig. S1). a1, a2, a3, a4, a4, a5: arms 1 to 5; ey: eye; fu: funnel; fi: fin; is: ink sac; ma: mantle; oo: olfactory organs; sh: shell. Scale bars: 1mm.

Figure 9. Details of *Soff-IR25* ISH staining in *S. officinalis* late embryos. **A, A' and A"**: details of *Soff-IR25* positive cells at the outer surface of the epidermal olfactory organs (arrows). Pictures A' and A" are obtained after cryostat sections of the whole mount ISH; **B, B', B"**: details of *Soff-IR25* positive cells in suckers, close to the inner rim (ir) (arrow) and to the lateral sides (arrowhead). B' and B" are obtained after cryostat sections of the whole mount ISH. **-C, C', C"**: details of *Soff-IR25* positive cells at the ventral side of the fins (arrows). Pictures C' and C" are obtained after cryostat sections of the whole mount ISH. Scale bars: A: 300µm; B: 200µm; C: 1mm; central column: 100µm; right column: 50 µm.

Figure 1

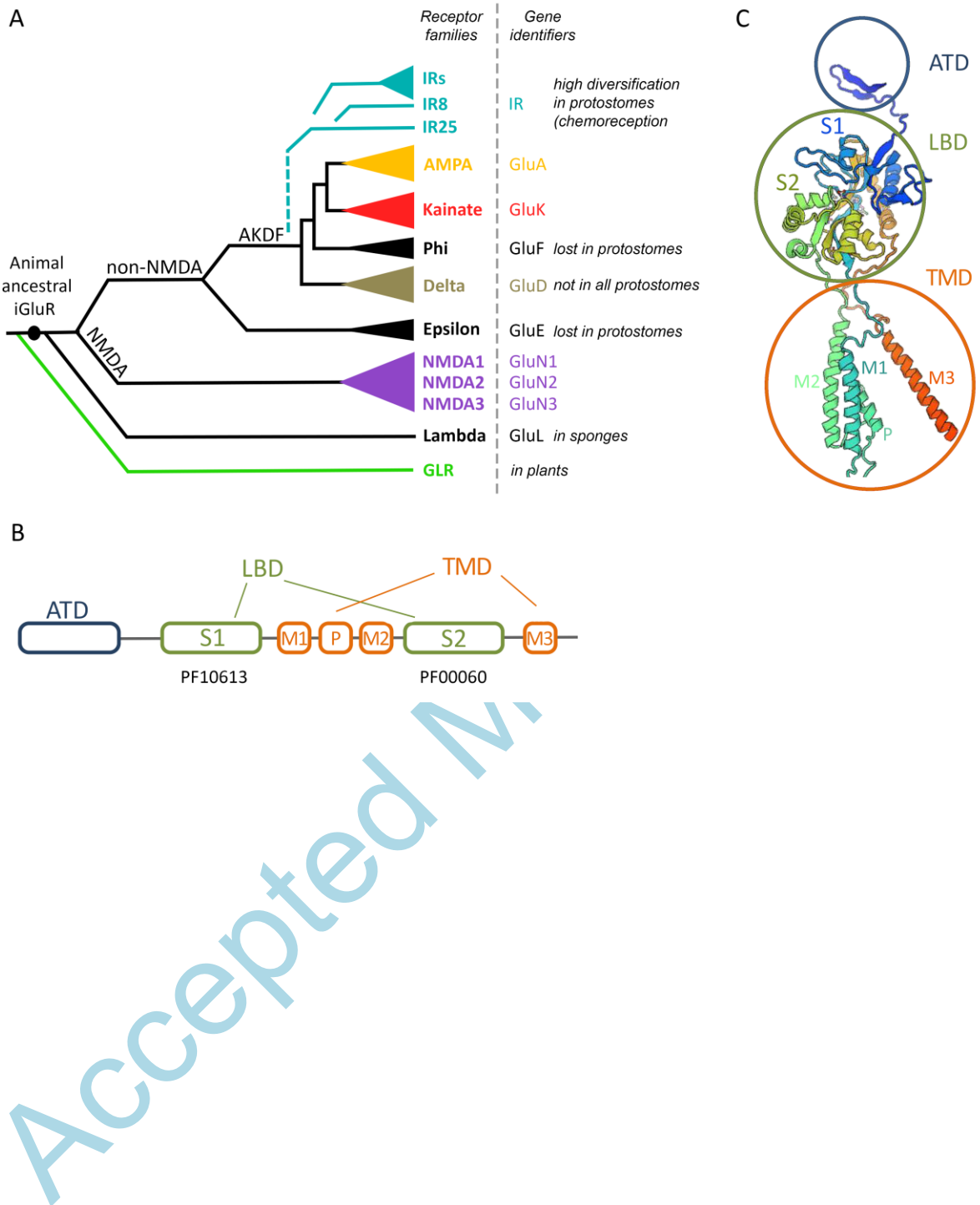
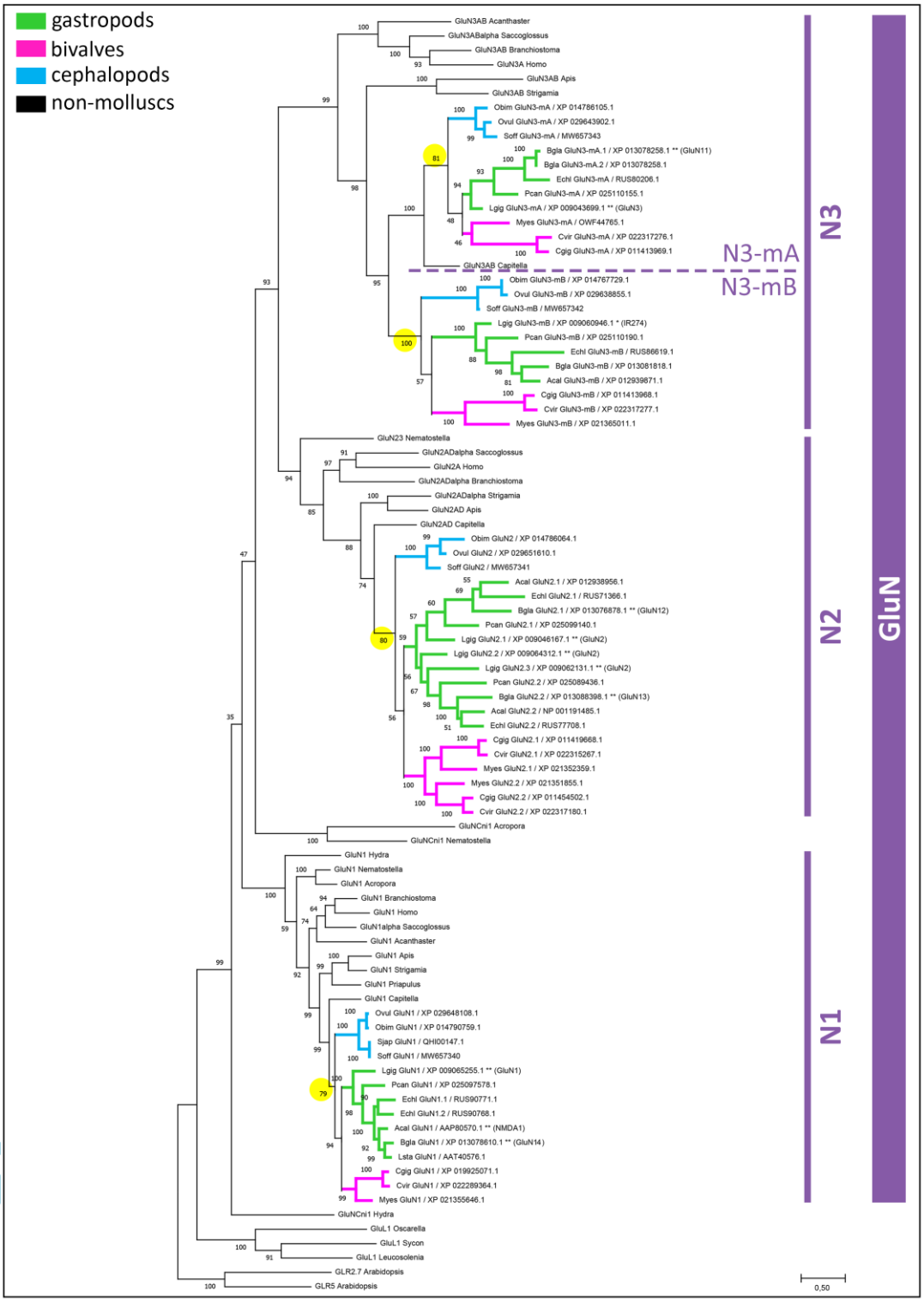


Figure 2



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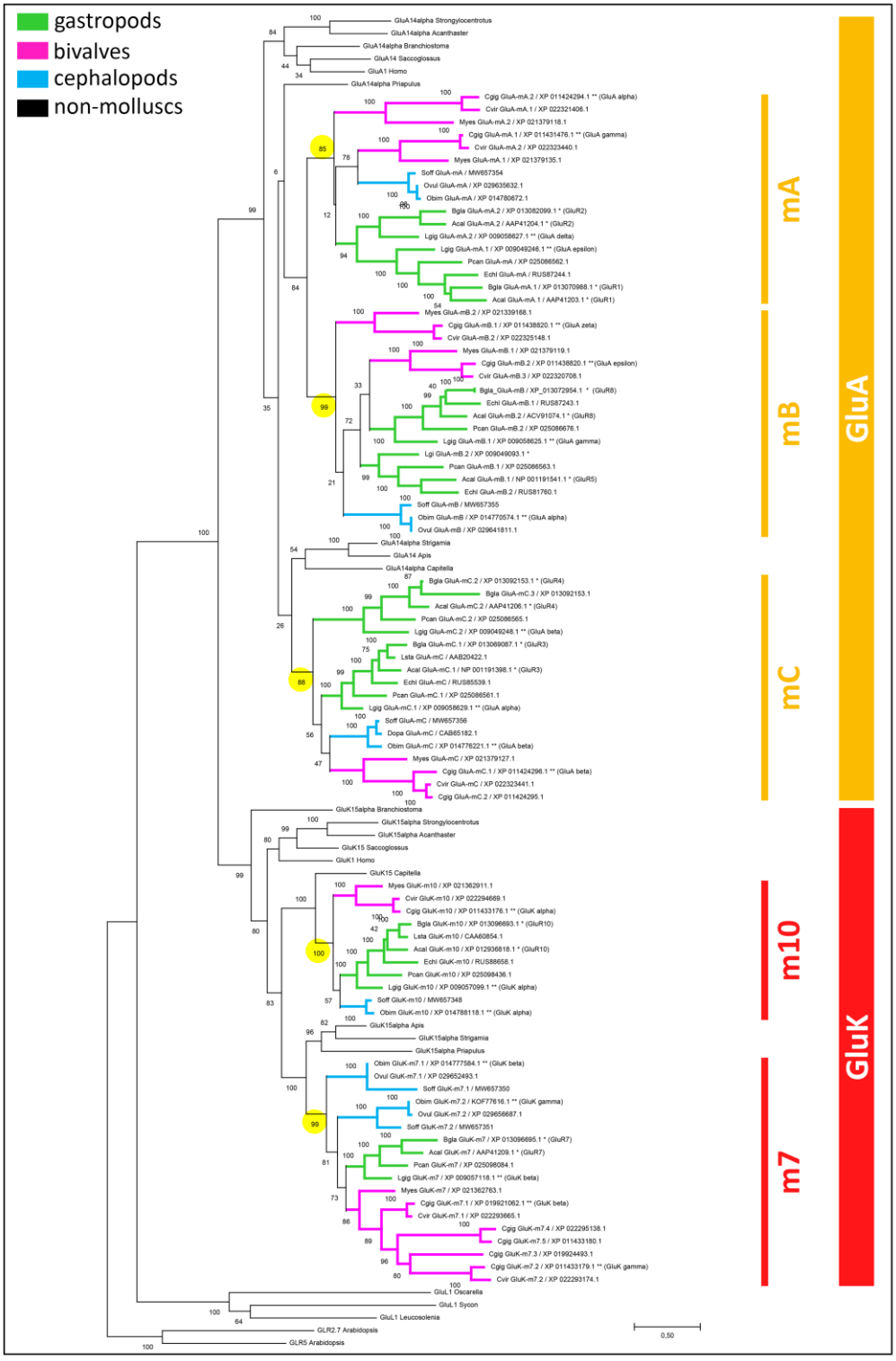

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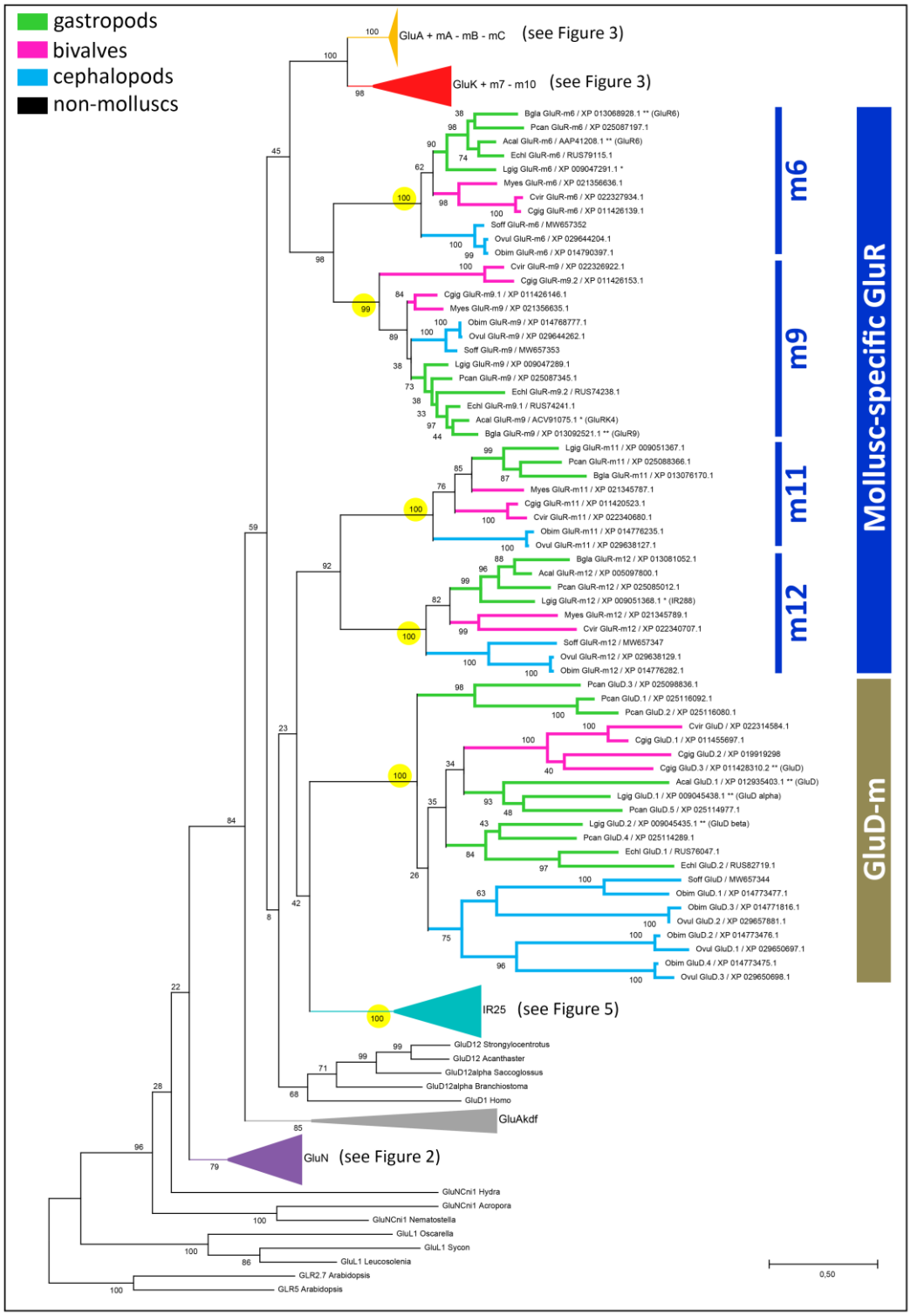
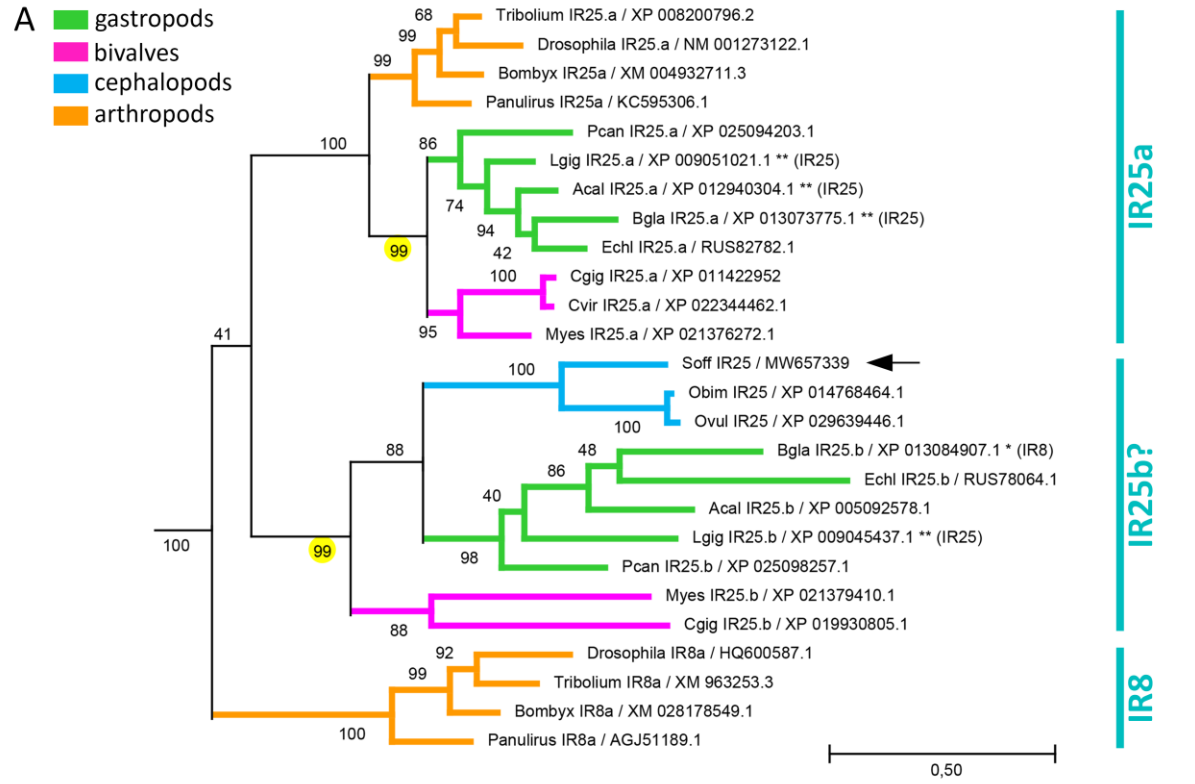


Figure 5



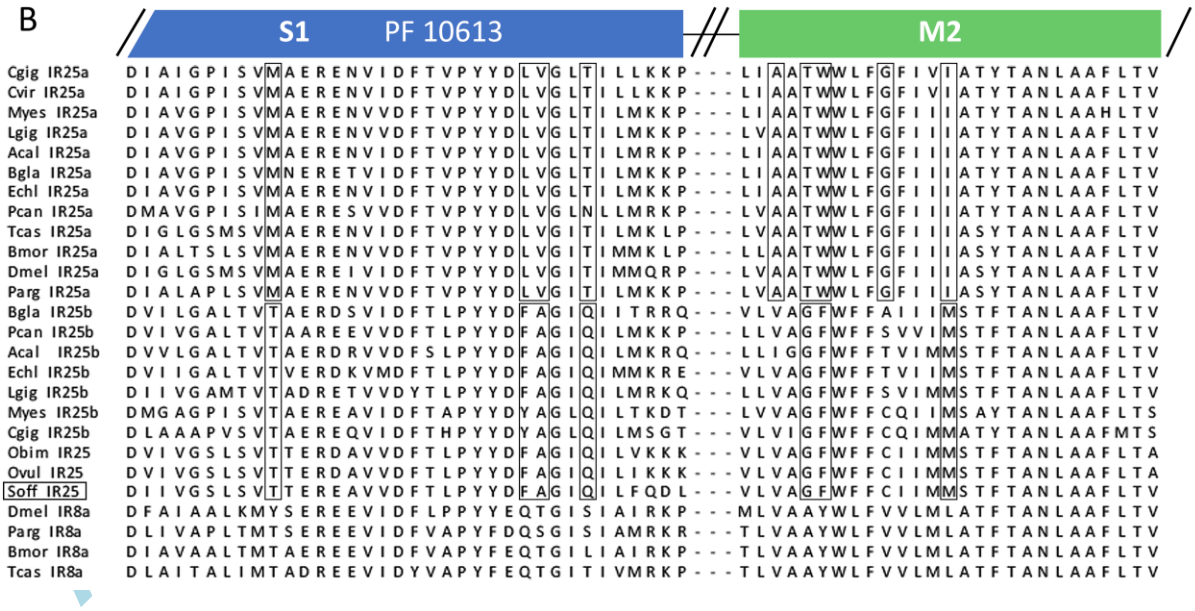
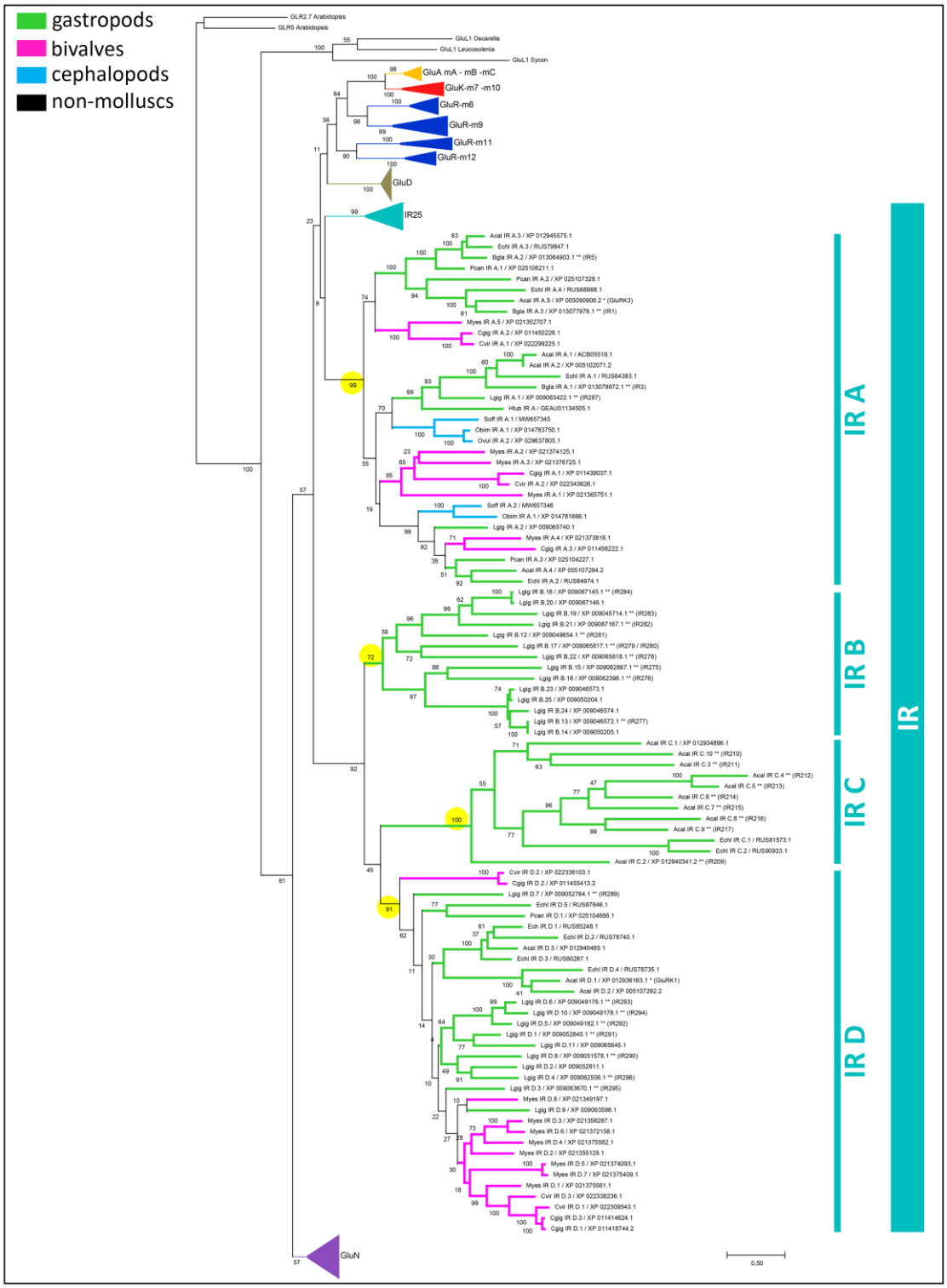


Figure 6



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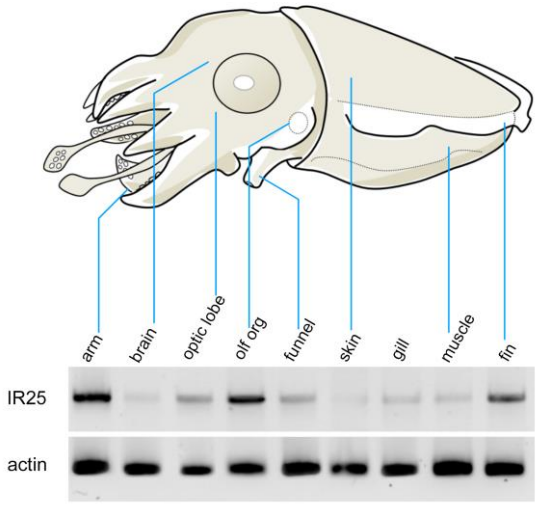

Figure 8

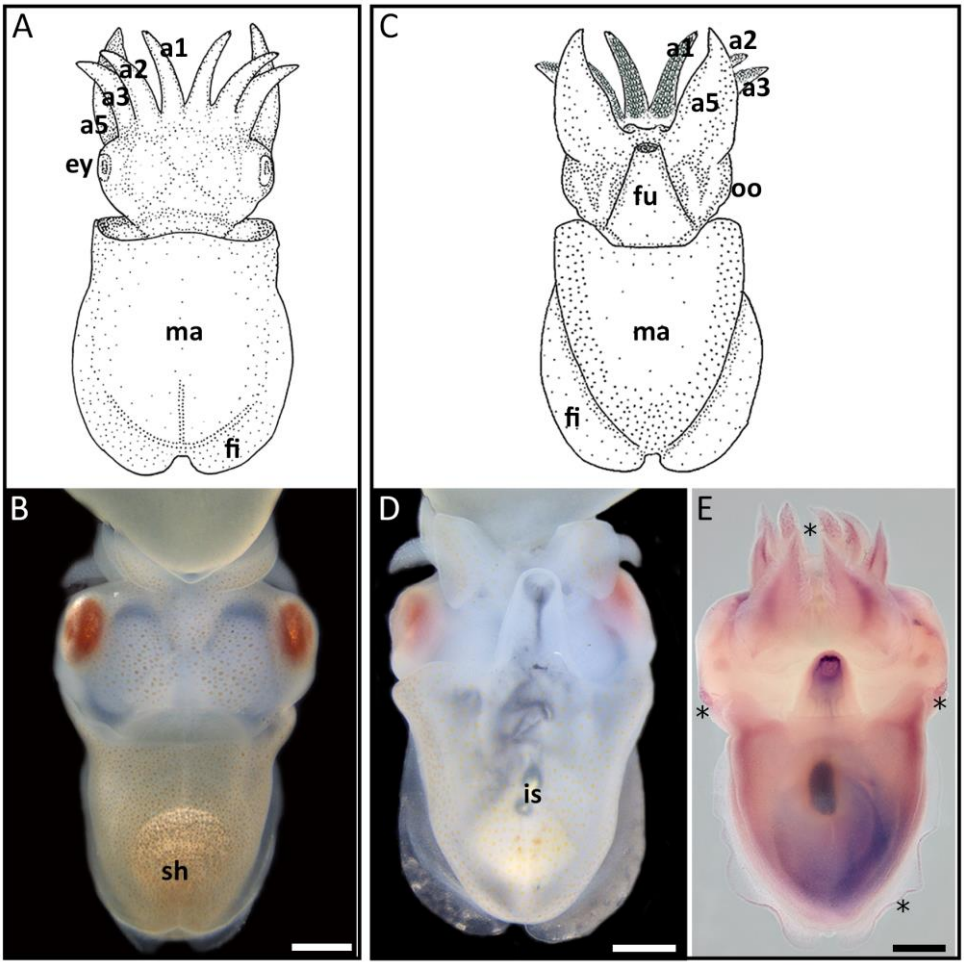

Figure 9