scientific reports

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Anatomy and transcriptomics of the common jingle shell (Bivalvia, Anomiidae) support a sensory function for bivalve tentacles

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Animals have evolved numerous mechanisms to perceive and interact with the environment that can be translated into different sensory modalities. However, the genomic and phenotypic features that support sensory functions remain enigmatic for many invertebrates, such as bivalves, an ecologically and economically important taxonomic group. No repertoire of sensory genes has been characterized in bivalves, representing a significant knowledge gap in molluscan sensory biology. Here, we gather multiple lines of evidence to explore the specialized sensory function of bivalve tentacles in the common jingle shell, *Anomia simplex*. In addition to applying microscopy techniques, we performed transcriptome sequencing of dissected tentacles using phylogenetically-informed annotation to identify candidate receptors. Our results demonstrate the expression of candidate GPCRs, including one opsin type, five small-molecule receptors, and 11 chemosensory-related receptors, supporting the involvement of sensory neurons in the organ, likely in association with the ciliated receptor cells observed along the tentacle surface. In addition, we identified seven ionotropic receptors as putative chemosensory receptors and one member of the Piezo mechanosensitive ion channel, which might be involved in touch sensation by ciliated sensory receptors. Our results provide the first evidence of putative sensory genes expressed in a bivalve sensory organ, representing an important starting point to investigate chemosensation in this class.

Keywords Chemosensation, Ciliated receptors, GPCR, Inotropic receptor, Mollusks, Piezo

Sensory biology explores how animals perceive and interact with their environment, providing critical insights into adaptive pathways and potential responses to a changing world. Photo-, mechano-, and chemo- sensation are examples of sensory modalities widespread across terrestrial and aquatic animals[1]. Chemosensation involves chemical communication with external cues from different distances and media[2]and is triggered by the binding of ligands to protein-based receptors[3]. This complex interaction covers an immense variety of receptor molecules and ligand types, such as organic compounds, hormones, amino acids, peptides, neurotransmitters and lipids[2]. The evolutionary history of chemical sensing is complex through massive diversification of gene families, changes to downstream signaling pathways, independent gene recruitment, and structural convergence that have expanded ligand-binding repertoires of taxonomic lineages[1,4–7]. Even though discoveries have been made over the past decade describing new invertebrate chemoreceptors and their functional properties[8–10], chemosensation is still largely uncharacterized for most taxa due to the enigmatic nature of their sensory organs and receptors.

Tentacles are flexible, soft-tissue extensions of the body used to sense olfactory and tactile stimuli and often assist in non-sensory functions such as feeding and protection in most animal phyla, including mollusks. In bivalves, tentacles are elongated processes that bear numerous ciliated cells[11], which may contain sensory cilia if they protrude from the surface of sensory neurons and contain protein receptors and key components of signal transduction. However, morphology alone cannot distinguish specialized mechano- and chemoreceptors in those animals[12]. Other approaches, including RNA-seq, can provide additional data to determine sensory modalities. In Pteriomorphia, a clade comprised of oysters, scallops, and relatives, tentacles are abundant in the mantle margin along the shell edge where they interact with the environment. Within this clade, the common jingle shell *Anomia simplex* (Anomiidae) is an appropriate species for exploring the functions of bivalve tentacles. Young and adult individuals of *Anomia* are attached to the substrate by a cemented byssus and, similar to most

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bivalves, are capable of very limited mobility[13]. While these animals can sense light, they lack photoreceptor organs[14] and rely on their numerous tentacles as the primary source of interaction with the surrounding environment. Those features represent a common condition among bivalves and possibly a similar sensory system. If tentacles are specialized organs that "taste" the water and the substrate using their long structure to capture environmental cues, then we expect several chemosensory protein receptors to be expressed along these organs. Also, if multimodal sensory capabilities are present, photo- and mechano- receptors will occur as well.

Chemosensory systems mainly rely on membrane chemoreceptors[15]. Unlike bivalves, cephalopods and gastropods have been studied considerably for chemosensation, with a focus on three major classes of chemoreceptors: chemotactile receptors (CR), which are only known from cephalopods[16], G-protein coupled receptors (GPCR), and ionotropic glutamate receptors (iGluR). The superfamily of GPCRs represents one of the largest groups of receptor proteins, including seven transmembrane proteins that bind to extracellular cues and mediate intracellular signaling via activation of specific G-proteins[17]. In particular, rhodopsin-like (Class A) GPCRs are the most diverse clade and extremely abundant among animals, binding to a myriad of ligands, including odorants, pheromones, neuropeptides, small molecules, and light-sensitive molecules[18]. Class A GPCRs comprise several chemosensory receptors in invertebrates, including those expressed in the olfactory organ of the gastropod *Aplysia*[19], which receptors are phylogenetically related to the remarkably diverse chemosensory receptors of nematodes[20]. Few studies have characterized GPCR repertoires in bivalves[21,22], and these studies did not investigate chemosensation. Consequently, candidate chemosensory GPCRs are still unknown for the second most species-rich molluscan class.

Most ionotropic glutamate receptors (iGluRs) are chemoreceptors associated with internal signaling in synaptic transmission and are frequently divided into groups based on ligand-binding properties and sequence similarity[4]. Ionotropic receptors (IRs) represent a diverse clade within the iGluRs, usually forming complexes containing up to four subunits to create ligand-gated ion channels. While recently investigated in invertebrates, including mollusks[5,23], IRs are absent in deuterostomes, and it has been hypothesized that IRs might have provided the protostome ancestor with additional ability to detect external chemical signals[4]. Despite extensive characterization of IRs in arthropods[8,24,25], studies focusing on molluscan IRs are still scarce[5,23,26], highlighting a gap of knowledge in the group.

Here, we explore the specialized sensory function of bivalve tentacles, gathering multiple lines of evidence to investigate different sensory modalities in the common jingle shell *Anomia simplex*. No chemosensory repertoires have been characterized in bivalves, representing a significant knowledge gap in molluscan sensory biology. Additionally, bivalve tentacles are regarded as sensitive to tactile stimuli, a function that has hitherto received nearly no attention. In addition to applying different microscopy techniques to examine tentacle anatomy, we performed transcriptome sequencing of *A. simplex* tentacles. Then, we combined data mining and phylogenetically informed annotation to identify putative receptors in the tentacles, mainly those used for chemosensory perception. Like cephalopods and gastropods, *A. simplex* expresses Class A GPCRs that closely resemble chemosensory-related genes. The diversity of tentacle GPCRs includes receptors for small-molecule neurotransmitters and neuropeptide receptors, supporting the involvement of sensory neurons in the organ, likely in association with the ciliated receptor cells observed along the tentacle surface. In addition, we identified IRs as putative chemosensory receptors among other sequences of iGluRs. Our results provide the first evidence of putative sensory genes expressed in a bivalve sensory organ, establishing a foundation for further comparative and functional studies on chemosensation.

Results

Tentacle anatomy

Approximately 10 tentacles per millimeter are distributed along the mantle margin of *A. simplex*, with more than 200 of them following the shell edge on each side of the animal (Fig. 1a, b). Tentacles are long, slender projections originating from the middle mantle fold (Fig. 1c). Longitudinal muscle fibers running from the base to the tip of each tentacle (Fig. 1d, e) likely control the substantial contractions observed in living animals (JAA, pers. obs.). A tentacle nerve is present at the center of the organ, surrounded by the longitudinal musculature (Fig. 1f). Tentacles are unpigmented and translucent, with a rugose external appearance (Fig. 2a). Both short tentacles (~0.5 mm, Fig. 2b, c) and long tentacles (~2 mm, Fig. 2d) are present. All tentacles have sparse and regularly distributed ciliated receptors (Fig. 2b-d). Each ciliated receptor cell has a ciliary tuft with several long cilia (~8 µm) at the center (Fig. 2e, f). Numerous neurite projections were observed leaving the tentacular nerve to the epithelium, reaching the ciliary rootlets of the ciliated receptor cells (Fig. 2g-j).

Transcriptome sequencing and annotation

Sequencing of *A. simplex* tentacle produced a total of 22,772,062 raw reads subjected to quality trimming and adaptor removal, yielding 21,855,942 clean reads that were *de novo* assembled into 105,804 transcripts with a 93.51% fragment mapping rate. The BUSCO output for the *A. simplex* transcriptome against the obd10 metazoan database was C:81.9% [S:66.2%, D:15.7%], F:11.6%, M:6.5%, n:954. In addition, the ExN50 statistics showed that the maximum N50 value was on E70% with 4,060 bp length. Transcripts were reduced to 23,709 clusters of peptides by CD-HIT. The summary of the sequencing statistics and transcriptome assembly is detailed in Table 1.

In total, 17,553 unique transcripts were annotated for 229,341 GO terms divided into three main categories: cellular components (67,459; 29.4%), molecular functions (52,451; 22.8%), and biological processes (109,431; 47.7%) (Supplementary Information, Fig. S1). Among the cellular components category, annotated transcripts were mostly involved in "cell" (15,027; 83.4%) and "cell part" (15,027; 83.4%). Molecular functions category was dominated by "binding" (12,935; 71.8%) and "catalytic activity" (7,852; 43.6%). Among biological processes,

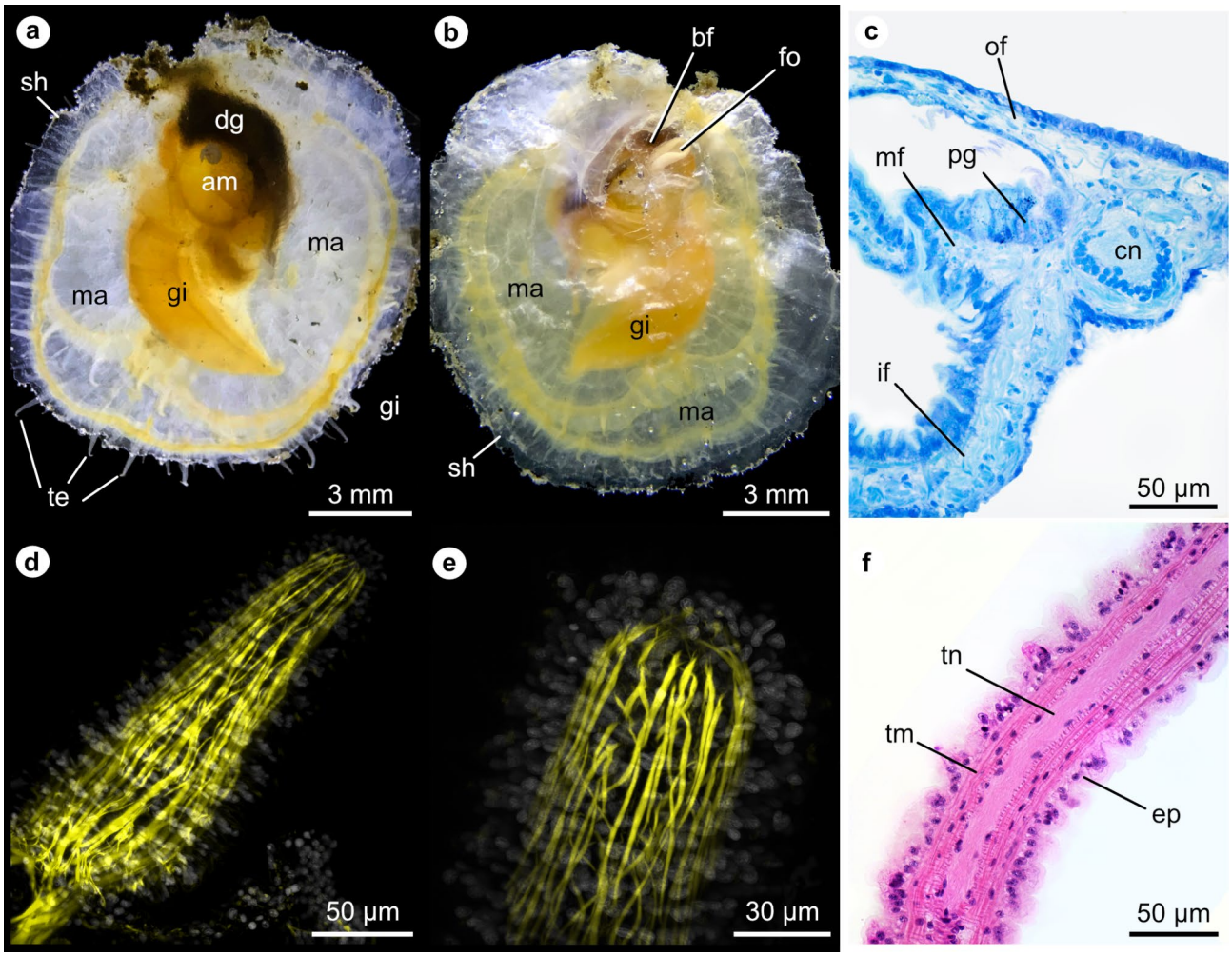

Fig. 1. Anatomy of the common jingle shell *Anomia simplex* and tentacles. **(a)** Right view of a live specimen, dorsal region at the top, and anterior region to the left. The body can be observed through the transparent shell. **(b)** Left view of a live specimen, dorsal region at the top, and anterior region to the right. **(c)** The histological section through the mantle margin shows the three mantle folds, including the middle fold where tentacles are located. Toluidine blue staining. **(d)** Longitudinal muscle fibers (yellow) in the tentacle; nuclei (grey). Confocal microscopy image. **(e)** Detail of tentacle musculature (yellow). Confocal microscopy image. **(f)** Longitudinal histological section of a tentacle showing the central tentacle nerve (tn), musculature, and epithelia. Hematoxylin and eosin staining. Abbreviations: *am*, adductor muscle; *bf*, byssal foramen; *cn*, circumpallial nerve; *dg*, digestive gland; *ep*, epidermis; *fo*, foot; *gi*, gill; *if*, inner mantle fold; *ma*, mantle; *mf*, middle mantle fold; *of*, outer mantle fold; *pg*, periostracal groove; *sh*, shell; *te*, tentacle; *tm*, tentacle musculature; *tn*, tentacle nerve.

the most common annotated transcripts are "cellular process" (14,214; 78.9%) and "metabolic process" (10,894; 60.5%). Interestingly, "response to stimulus" (6,987; 38.8%) and "signaling" (4,372; 24.3%) represent the fifth and eleventh most frequent annotated transcripts (Supplementary Information, Fig. S1). In addition, 18,185 unique transcripts were annotated against the Pfam database, generating 44,941 annotations, and 15,643 were matched to the KEGG database. Finally, 30,083 transcripts contain signal peptides, and 4,947 were predicted as transmembrane proteins. The functional annotation of the tentacle transcriptome is summarized in Table 1.

Putative chemosensory GPCRs

Among predicted transmembrane proteins, 145 transcripts were identified as GPCR according to Pfam domains (PF00001/7TM_1, PF00002/7TM_2, PF00003/7TM_3). However, we restricted our analysis to 47 sequences predicted to contain at least 6 or 7 transmembrane helices, as expected for a complete GPCR. Candidate sequences were analyzed in a phylogenetic framework with 30 GPCRs of different classes (A – C) from two bivalve species, the scallop *Mizuhopecten yessoensis* and the oyster *Crassostrea virginica*. Our results indicate that the tentacle transcriptome of *A. simplex* expresses 41 rhodopsin-like GPCRs (Class A), five adhesion GPCRs (Class B), and one glutamate GPCR (Class C) (Fig. 3). Among Class A GPCRs, only a single photoreceptor protein was identified including a conserved lysine residue necessary for retinal binding in the tentacle

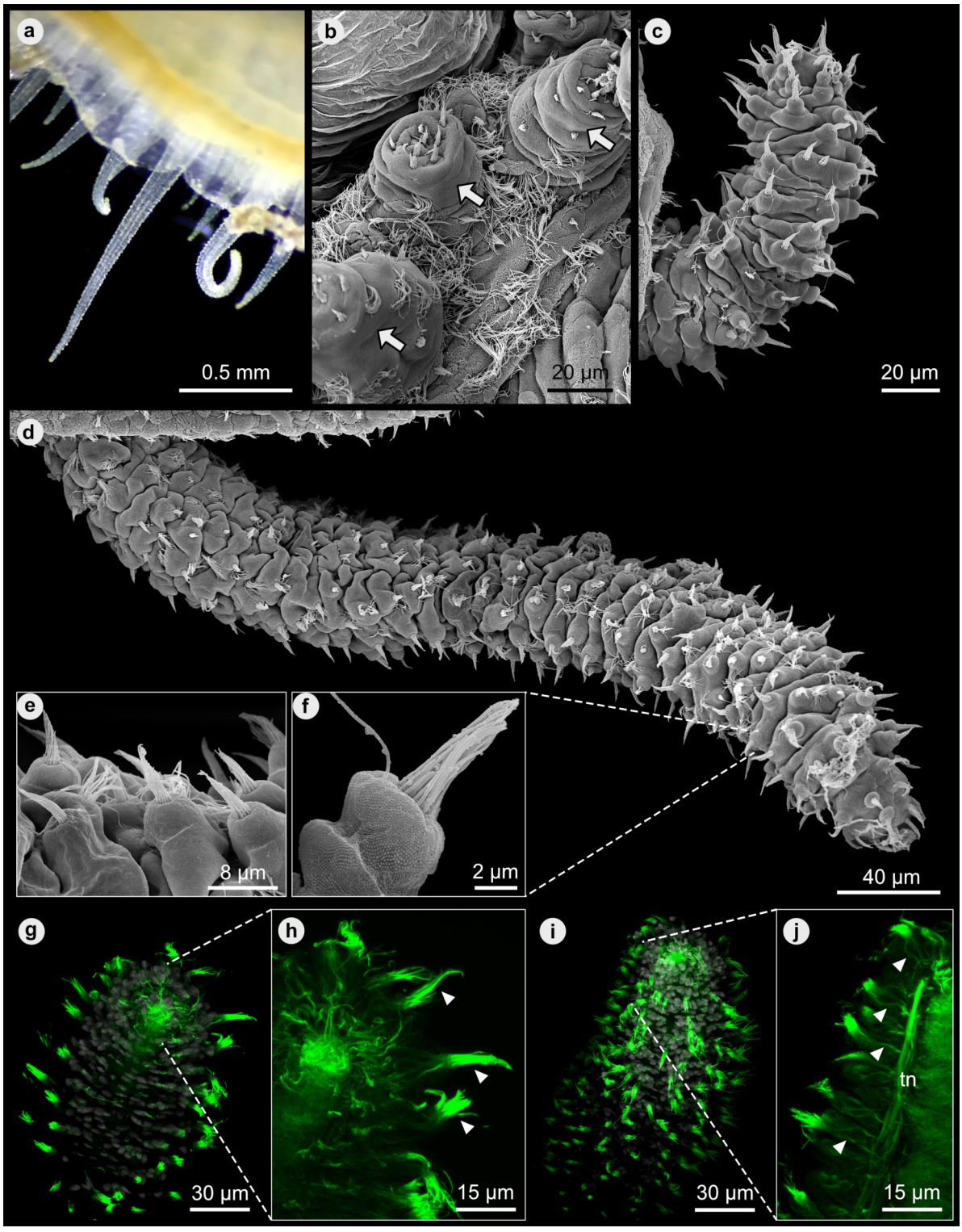

Fig. 2. Detailed anatomy of *A. simplex* sensory tentacles, including images obtained by scanning electron microscopy (b-f) and confocal microscopy (g-j; tubulin in green, nuclei in grey). **(a)** Detail of a long, extended tentacle in a live specimen. **(b)** Short tentacles (arrows) with ciliated receptor cells concentrated at the tip. **b.** Ciliated receptor cell distribution in a long tentacle. **d.** Ciliated receptor cells regularly spread along the long tentacle extension. **e.** Detail of the ciliated receptor cells. **f.** Detail of the ciliary tuft at the apical region of the receptor cell. **g.** Tip of the tentacle showing neurites from the tentacle nerve and ciliary tufts. **h.** Detailed of the ciliated receptor cells (arrows) and branching neurites in the center of the tentacle. **i.** Distribution of ciliary tufts. **j.** Detail of the connection between ciliary tufts and ciliary rootlets and between ciliary rootlets and neurites from the tentacle nerve (arrows). Abbreviations: tn, tentacle nerve.

Sequencing statistics	Count
Raw reads (paired-end)	22,772,062
Clean reads (paired-end)	21,855,942
Q30 (%)	88.77%
GC content (%)	39%
Assembly statistics	
Total assembled bases	82,704,955
Total number of transcripts	105,804
Total number of unigenes	90,554
Median contig length (bp)	403
Average length of unigenes (bp)	781.68
GC content (%)	35.63%
Raw reads mapped to contigs (%)	93.51%
N50 (bp)	1,353
Highest ExN50 value (% Ex)	70
E70N50 value (bp)	4,060
BUSCO completeness (%)	81.9%
Coding DNA sequences	
Number of longest ORF	40,529
Number of peptide sequences	30,083
Number of CD-HIT clusters (nt)	26,554
Number of CD-HIT clusters (pep)	23,709
Functional annotation	
Pfam annotated	18,185
GO annotated	17,553
KEGG annotated	15,643
Predicted proteins with signal peptides	30,083
Predicted transmembrane proteins	4,947

Table 1. Summary of *de novo* assembly and functional annotation of *Anomia simplex* tentacle transcriptome.

transcriptome. Our phylogenetic results indicate that the opsin sequence belongs to the retinochrome group, sister to peropsins (Supplementary Information, Fig. S2). Sequences with predicted Pfam domains for the B class (PF00002/7TM_2) are traditionally regarded as members of the secretin family. However, our analysis supports these sequences in a clade formed by adhesion GPCRs, which share the same 7TM_2 domain, reinforcing the importance of phylogenetic analysis for proper sequence classification. One of these adhesion GPCR sequences was annotated as a cadherin EGF LAG seven-pass G-type receptor 1 (CELSR1) in the KEGG database.

As Class A GPCRs are remarkably diverse, we further investigated the presence of neurotransmitters and chemosensory-related genes within the expressed repertoire. Blast searches against a curated selection of small-molecule receptors for different metazoan groups revealed five putative small-molecule receptors in the bivalve tentacles. Phylogenetic analysis of these candidates supports that two are biogenic amine receptors (Fig. 4), including one putative serotonin receptor (5HT1) and one putative octopamine receptor (octβ). The other small-molecule receptors include one putative adenosine receptor (AdoR) and two eicosanoid (prostaglandin) receptors (PGE). Functional annotation against the KEGG database suggests that some of the incomplete Class A GPCRs expressed in the bivalve tentacles are peptide and neuropeptide receptors. However, we opted to keep a stringent analysis focused on the phylogenetic classification of sequences with at least 6 of the 7 transmembrane helices.

Putative chemosensory genes of Class A GPCR were investigated based on blast searches against a curated selection of sequences from different invertebrate groups, including gastropods. We identified 19 possible candidates containing serpentine domains (srw/srsx/srx) expressed in the tentacles of *A. simplex*, with 11 blastp hits against an invertebrate chemosensory GPCR collection[10]. Our analysis supports a clade of gastropod-specific, planarian-specific, and nematode-specific chemosensory genes (Fig. 5). One transcript of *A. simplex* is nested among chemosensory receptors that contain the srw domain and FMRFamide receptor-like annotation (Fig. 5). Nine transcripts were grouped in a clade formed mostly by sequences including the srsx domain and likely associated with neuropeptide receptors (Fig. 5). Interestingly, one transcript is grouped with putative chemosensory orphan receptors without the srw/srsx/srx domains (Fig. 5). In total, from 47 GPCRs found in the *A. simplex* tentacles, we were able to annotate 17 receptors potentially involved in chemoreception functions, i.e., 16 Class A GPCRs, including five small-molecule neurotransmitter receptors and 11 chemosensory receptors. Annotated candidates are summarized in Table 2.

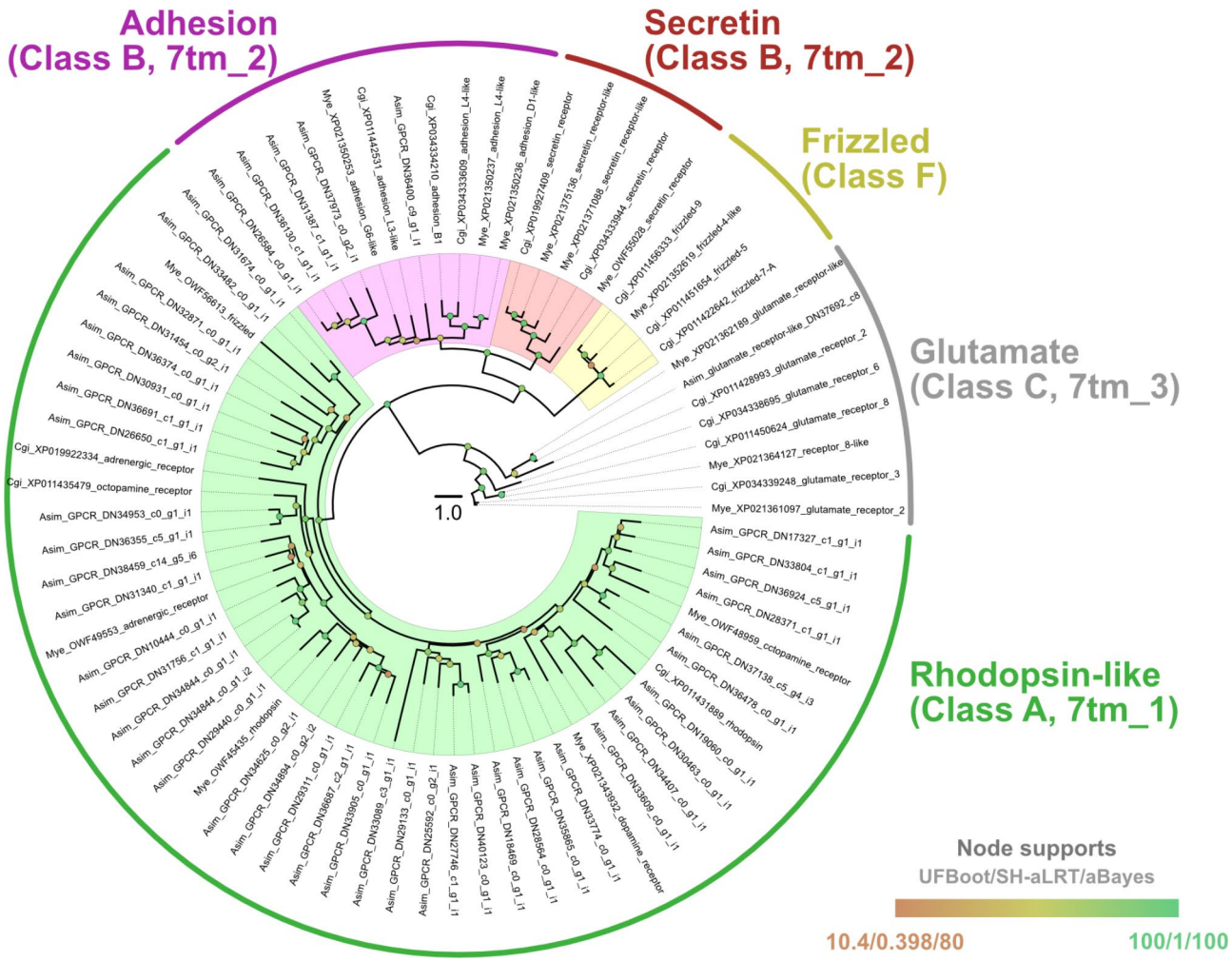

Fig. 3. Maximum likelihood phylogenetic tree of most complete GPCRs found in the tentacle transcriptome of *A. simplex*. We also included 30 representative protein sequences from all major GPCR classes of two bivalve species, the scallop *Mizuhopecten yessoensis* and the oyster *Crassostrea virginica*, retrieved from the NCBI database. Colors represent the five main classes of GPCRs. The model of substitution is LG + F + R5 according to BIC as selected by ModelFinder. Support values (ultrafast bootstrap, SH-like approximate likelihood ratio test, and aBayes test) are indicated by a colored gradient on the nodes.

Putative iGluRs and IRs

We identified 13 putative sequences of iGluR based on the presence of two Pfam conserved domains, PF00060 and PF10613. Our phylogenetic analysis, including 412 molluscan iGluR sequences from previous studies, revealed that *A. simplex* tentacles express six iGluRs, including two GLuN receptors (GLuN1 and GLuN2) from the clade of NMDA receptors, one GluA-mC receptor from the clade of AMPA receptors, two GluK-m7 receptors from the clade of Kainate receptors, and one GluR-m9 receptor, a mollusk-specific clade of iGluR (Fig. 6). In addition, we identified seven IRs, a clade of ligand-gated ion channels with likely chemosensory roles in protostomes. These are phylogenetically classified with high node supports to the clades IR-D, IR-A, and IR-25 (Fig. 7). Annotated candidates are summarized in Table 2.

Mechanosensitive ion channels

Even though no transient receptor potential (TRP) channel has been found, we identified one Piezo-type mechanosensitive ion channel in *A. simplex* tentacle transcriptome based on the presence of two Pfam conserved domains, PF15917 and PF12166. The sequence has 35 helices according to TMHMM prediction, which is within the expected range for this family of transmembrane receptors. Phylogenetic analysis supports all molluscan sequences in a single clade, and the sequence of *A. simplex* is nested in the bivalve clade, close to the scallop *Ylistrum balloti*, which is also a member of the bivalve clade Pteriomorphia (Fig. 8).

Discussion

For the first time, we provide anatomical evidence combined with molecular data to support tentacles as sensory organs in bivalves, using the common jingle shell *A. simplex*. Besides extensive distribution of highly innervated

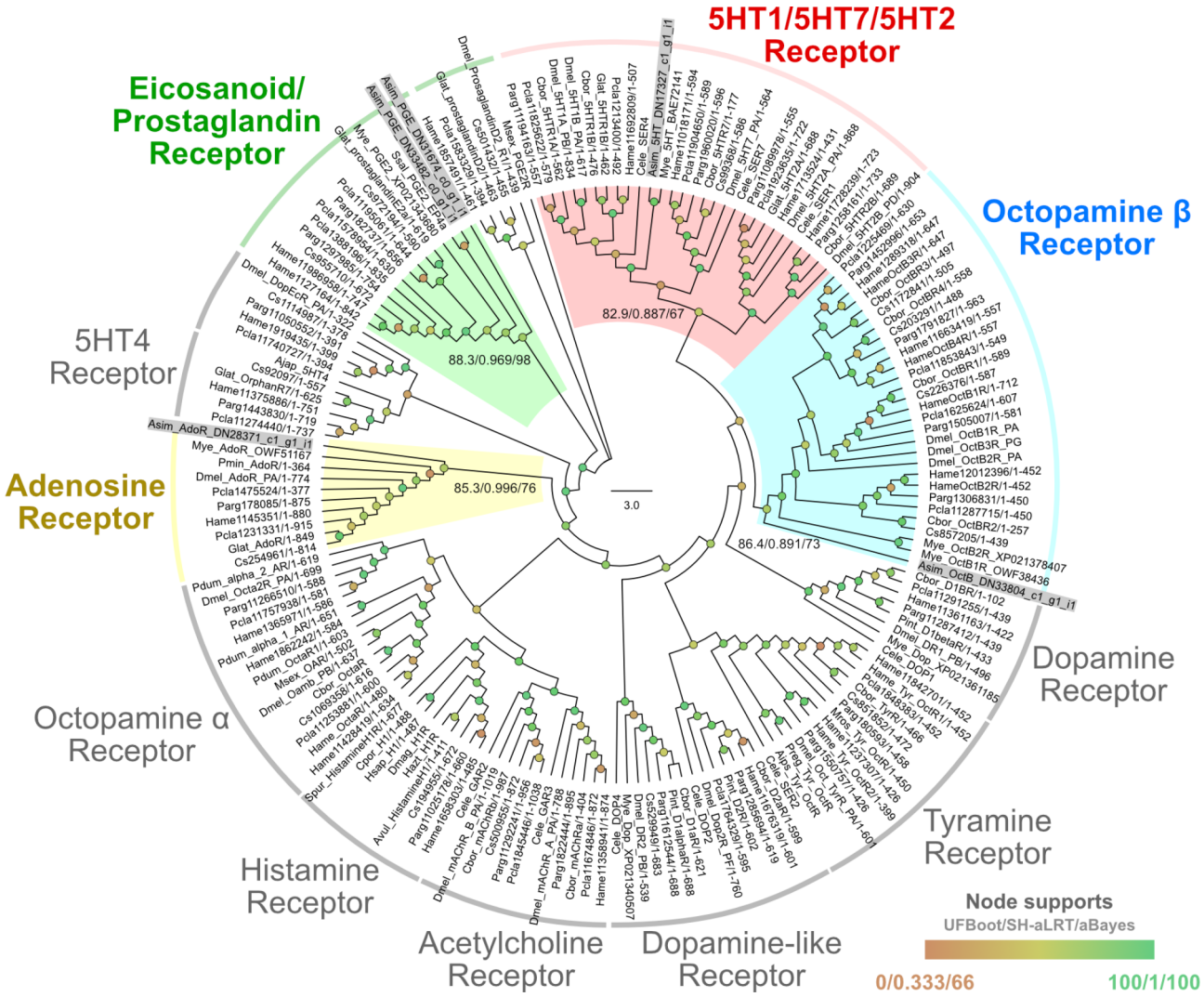

Fig. 4. Maximum likelihood phylogenetic tree of small-molecule receptors (Class A GPCRs) of *A. simplex* tentacles and other species. Clades containing protein sequences annotated in this study (terminal labels with gray background) are colored. Sequences for other species include nematodes, annelids, crustaceans, insects, echinoderms, and chordates retrieved from the small-molecule receptor collection in Rump et al. (2021). The model of substitution is Q.yeast + F + R6 according to BIC as selected by ModelFinder. Support values (ultrafast bootstrap, SH-like approximate likelihood ratio test, and aBayes test) are indicated on key nodes and by a colored gradient on all nodes.

ciliated receptors, the tentacles express putative transmembrane receptors from three major protein families: (1) chemosensory Class A GPCR, (2) chemosensory ionotropic receptor (IR), and (3) Piezo mechanosensitive ion channel.

Tentacles are a common structure shared by many bivalve groups, particularly the pteriomorphians[27]. In many mollusks, dense patches of ciliary coverage are frequently associated with mucociliary transportation required for cleansing and lubrication of soft tissues[28]. In the case of *A. simplex*, the scattered distribution of ciliary tufts on the tentacles does not support mucous transportation and suggests the presence of sensory ciliated receptors instead. However, as previously noted, specialized mechano- and chemoreceptors cannot be distinguished based exclusively on morphology[12]. The innervation of these ciliated receptors, including highly branching neurites and connection to the tentacle nerve, is also evidence for sensory roles, as observed in other bivalve tentacles[11]. Similarly, sparsely distributed ciliated receptors have been described for the tentacles of some scallop (Pectinidae)[29,30], and file clam (Limidae)[12]species, which exhibit even longer and more numerous tentacles. Anomiids are phylogenetically close to the scallops and relatives[31], and tentacles with uniformly distributed ciliated receptors seem to be a shared trait among these bivalves. In contrast, oysters (Ostreidae) and pearl oysters (Margaritidae) have putative ciliated receptor cells clustered at the tip of relatively shorter tentacles[11]. Despite differences in distribution, ciliated receptors are a common feature of bivalve tentacles, reinforcing anatomical evidence of sensory roles.

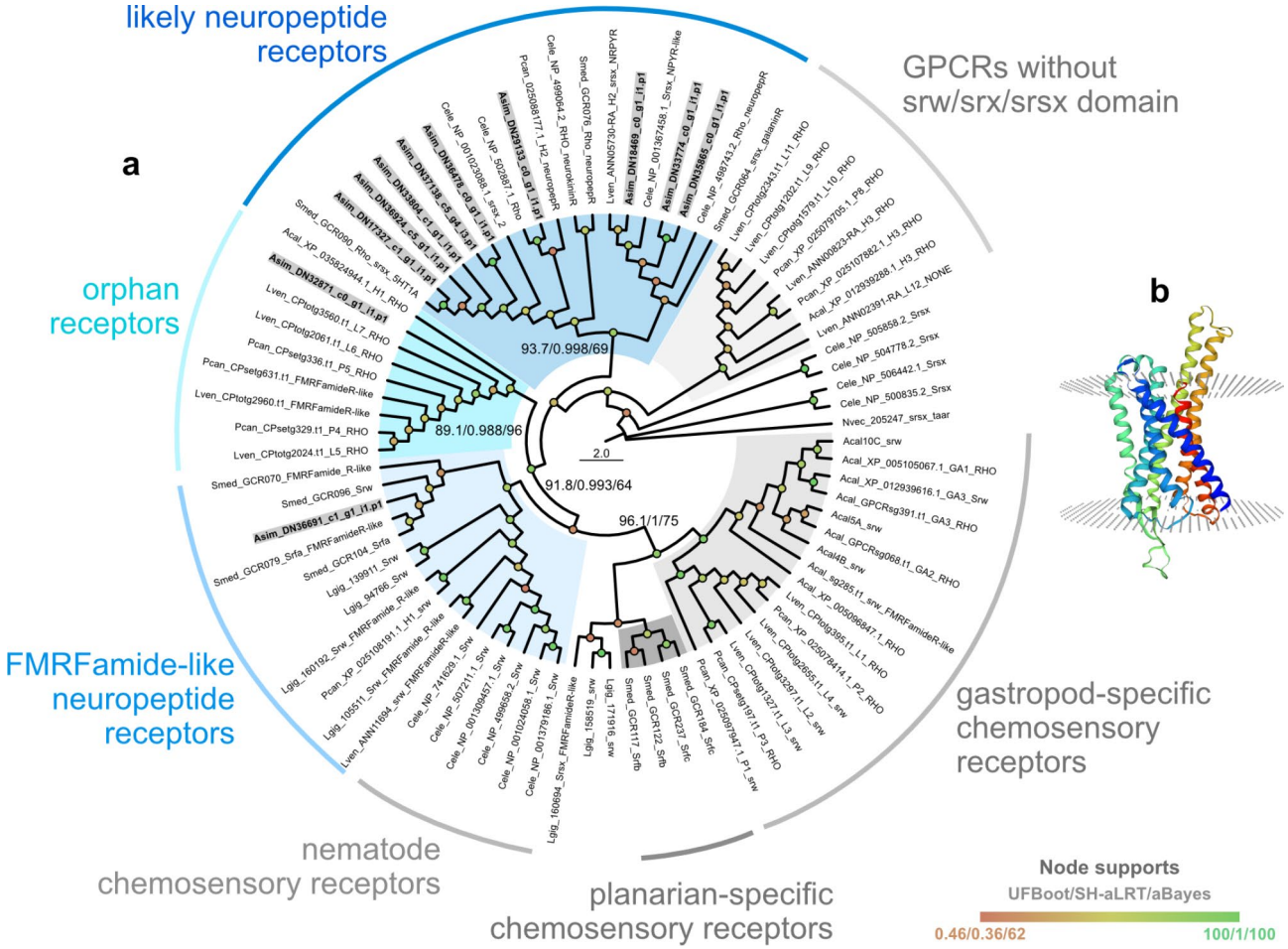

Fig. 5. **(a)** Maximum likelihood phylogenetic tree of putative chemosensory receptors (Class A GPCRs) of *A. simplex* tentacles and other invertebrate species. Protein sequences annotated in this study are highlighted with a gray background. Sequences for other species include nematodes, planarians, and gastropods retrieved from the chemosensory receptor collection in Rondon et al. (2024). The model of substitution is LG + F + R4 according to BIC as selected by ModelFinder. Support values (ultrafast bootstrap, SH-like approximate likelihood ratio test, and aBayes test) are indicated on key nodes and by a colored gradient on all nodes. **(b)** 3D model of the putative chemosensory receptor Asim_DN35865 (showing the seven transmembrane helices in different colors) predicted by the Swiss-Model server based on the neuromedin U receptor 2 template.

Transcriptomic data of *A. simplex* provides the first insights into gene expression in the tentacles of a bivalve species. Function annotation in GO terms revealed transcripts associated with "binding" (71.8%), "response to stimulus" (38.8%), and "signaling" (24.3%), which are expected in sensory organs involved in the transduction of external and internal signals. As discussed below, we identified several candidates of chemosensory receptors, including GPCRs and IRs, in addition to a mechanosensitive ion channel. Therefore, our data significantly advances in identifying candidate receptors in bivalves. Based on current limitations of this study, future work would benefit from determining differentially expressed genes in the tentacles, including possible intraspecific and interspecific variation between organs from different body regions and under controlled experimental conditions.

GPCRs can be grouped into five main clades: rhodopsin-like (Class A), adhesion (Class B2), secretin (Class B1), glutamate (Class C), and frizzled (Class F)[32]. Our results show that most GPCRs expressed in the tentacles of *A. simplex* belong to the Class A group, including 41 out of 47 most complete transcripts. The overwhelming diversity and abundance of Class A GPCRs compared to the other classes is widespread among animals, as exemplified by the genomes of some gastropod species, in which more than 95% of the GPCR repertoire comprises Class A receptors[10]. Similarly, the first characterization of a bivalve GPCR content revealed a total of 586 GPCRs in the genome of the Pacific oyster *Magallana gigas*, with 454 genes assigned to Class A[21]. Not surprisingly, target GPCRs involved in oyster response to environmental stresses were mainly found in Classes A and B[21]. Recent evidence of human cells and *Drosophila* sensory neurons suggests that some adhesion GPCRs (Class B2) are involved in the perception and regulation of mechanosensing[33,34]. For example, adhesive junctions in mechanotransduction might be based on cadherin receptors[33], a GPCR family present in the tentacle transcriptome of *A. simplex*. Unfortunately, the lack of molecular and experimental information for

Protein family	Receptor Class	Domain description	Receptor subgroups	Total number of transcripts
G-protein coupled receptor (GPCR)	Rhodopsin-like (Class A)	7 transmembrane receptor, rhodopsin family (PF00001)	Small-molecule receptor	5
			Putative chemosensory receptor (sr domain)	11
			Others	25
	Adhesion (Class B)	7 transmembrane receptor, secretin family (PF00002)		5
	Metabotropic glutamate (Class C)	7 transmembrane sweet-taste receptor of 3 GCPR (PF00003)		2
Ionotropic Glutamate Receptor (iGluR)		Ligand-gated ion channel (PF00060) Ligated ion channel L-glutamate- and glycine-binding site (PF10613)	GluN, GluA, GluK, GluR-m9	6
			Ionotropic Receptor (IR)	7
Piezo mechanosensitive ion channel		Piezo family (PF15917) Piezo non-specific cation channel, R-Ras-binding domain (PF12166)		1

Table 2. Summary of putative sensory receptors annotated in the tentacle transcriptome of the bivalve *Anomia simplex*. Even though we identified 145 transcripts as GPCRs, we restrained our analysis to 47 sequences predicted to contain at least 6 or 7 transmembrane helices. Final numbers reflect totals after functional annotation and phylogenetic analysis.

invertebrate taxa, such as mollusks, severely hampers further speculations on the mechanoreception roles of adhesion GPCRs in non-model organisms.

Bivalve mollusks have light-sensitive tissues along the edge of shells that provide nondirectional photoreception[35]. Even though no eyespot is present in *A. simplex*, the expression of a photoreceptor protein in the tentacles is not surprising considering that most pteriomorphian bivalves live above the sediment (i.e., they are not burrowing species) and respond to light changes perceived by the mantle and nerves[35]. In addition, opsins are expressed in the mantle eyes and mantle of many pteriomorphian species, including mussels, oysters and scallops[36]. In fact, other eyeless bivalves, such as the European oyster *Ostrea edulis*, are known to respond to light changes with valve opening and closure[37]. The single opsin recovered in this study is a retinochrome, a type of photoisomerase opsin associated with the visual cycle, converting all-*trans* retinal back into 11-*cis* retinal but not necessarily with the perception of light[38]. The pervasive presence of retinochrome in the genome and transcriptome of mollusks suggests that other opsins might be present, particularly rhabdomeric opsins (G_q) that initiate phototransduction[39,40]. The absence of other opsins, such as the G_q-opsins, the most associated with vision in invertebrates, suggests that light detection does not play a significant role in the tentacles of *A. simplex*.

Among Class A GPCRs, neurotransmitter receptors have a critical role in sensory systems, enabling communication in central and peripheral sensory neurons. Our results based on functional annotation and phylogenetic analysis suggest that five Class A GPCRs identified in the tentacles of *A. simplex* are small-molecule receptors for serotonin, octopamine, adenosine, and prostaglandin. Even though data on neuroreceptor diversity and evolution in mollusks is scarce, GPCR repertoire in other invertebrates has proven to help understand chemosensation in organisms other than vertebrates. For example, octopamine and prostaglandin receptors are also highly expressed in the chemosensory organs of decapod crustaceans[7]and serotonin receptor expression, particularly 5HT1R and 5HT7R, suggests a mechanism of serotonergic modulation of olfactory sensory neurons in those species[7]. Interestingly, serotonin was identified in various body tissues of the scallop *Placopecten magellanicus*, including mantle nerves[41], and intense serotonergic immunoreactivity was observed in the tentacular nerve of the scallop *Nodipecten nodosus*[30], which adds to our data, supporting an extensive recruitment of serotonin receptors in bivalve tentacles. Nevertheless, functional characterization of neuroreceptors in *A. simplex* tentacles is still required to elucidate the precise ligands and the physiological mechanisms underlying signaling.

Even though many invertebrates, mainly arthropods, rely on chemosensory proteins other than GPCRs[42], many studies have demonstrated the diversity and expression of putative chemosensory GPCRs in invertebrate sensory systems. In echinoderms, such as the crown-of-thorns starfish, putative olfactory receptors are highly diverse GPCRs, many expressed in sensory tentacles and tube feet[43]. Among mollusks, gastropods seem to have a vast number of chemosensory-related gene copies in their genomes, mostly class A GPCRS, with experimental evidence that some of them are expressed in the rhinophores of the sea hare *Aplysia californica*[19]. Our study provides the first evidence of at least 11 putative chemosensory-related genes expressed in the tentacles of a bivalve species. As a genome assembly is not available for *A. simplex*, the number of GPCRs and chemosensory receptors for the species is likely substantially higher.

The second most represented domain in the gastropod GPCR repertoire is the serpentine receptor (sr; PF10324)[10]. Initially identified in *C. elegans*, the sr domain is present in a massive diversity of chemoreceptor genes, including large species-specific expansions[44]. Currently, knowledge of nematode chemosensory repertoire includes more than 1,000 chemosensory genes in different major sr families, likely including peptide receptors[6]. Here, we identified 19 transcripts with sr domains (srw/srx/srsx) expressed in *A. simplex*, with 11 of them sharing significant similarities with sequences from gastropods, planarians, and nematodes. Our results support these transcripts as putative chemosensory candidates and domain annotation suggests a neuropeptide receptor-like

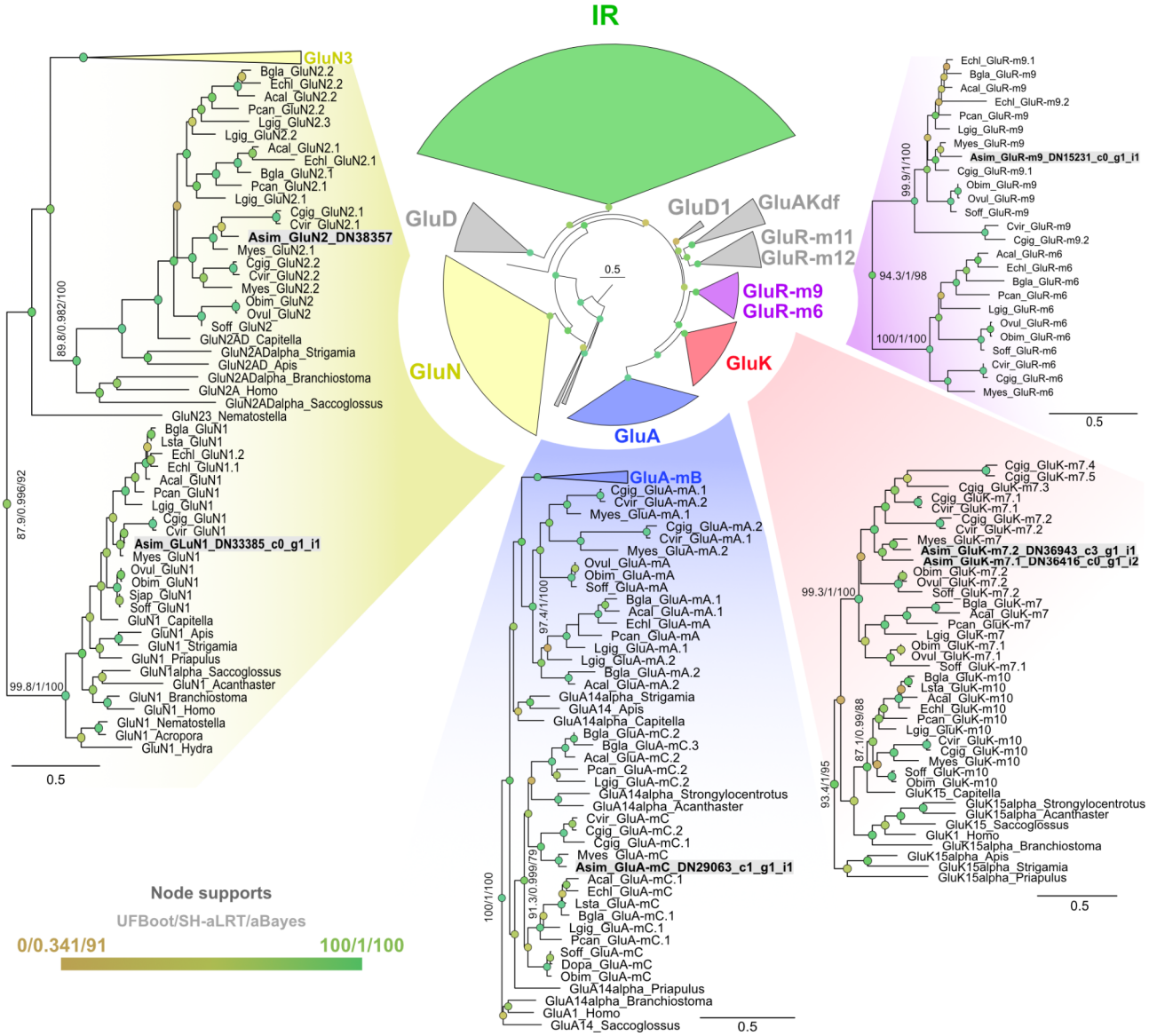

Fig. 6. Maximum likelihood phylogenetic tree of iGluRs of *A. simplex* tentacles and other species. The expanded clades indicated by colors, i.e., GluN (NMDA receptors), GluA (AMPA receptors), GluK (Kainate receptors), and GluR-m6/9 (mollusk-specific iGluR), represent the different iGluR subgroups that contain protein sequences annotated in this study (gray background). Sequences for other species include mostly mollusks and are based on the iGluR collection in Andouche et al. (2021). The model of substitution is LG + R8 according to BIC as selected by ModelFinder. Support values (ultrafast bootstrap, SH-like approximate likelihood ratio test, and aBayes test) are indicated on key nodes and by a colored gradient on all nodes. For the characterization of IRs, please see Fig. 7.

nature, including receptors of FMRFamide-like ligands. Remarkably, FMRFamide-like immunoreactivity was observed in the sensory afferents from the central nervous system of decapod crustaceans[45], and FMRFamide-like receptors are highly expressed in chemosensory neurons of decapod sensory organs[7]. Interestingly, FMRFamide-like immunoreactivity was detected in nerves and all three ganglia of the scallop *P. magellanicus*, suggesting neuropeptide regulation of central and peripheral functions[46]. Even though we provided the first step to understanding the identity of chemosensory receptors in bivalve tentacles, validating the sensory functions through functional and experimental studies remains necessary. As previously pointed out[6], the similarity of GPCRs containing the sr domain to peptide receptors in many animals may provide hints about the nature of the ligands in future experimental work.

An extensive survey of iGluRs in mollusks was conducted using available sequences from genomes and transcriptomes to identify candidate olfactory chemoreceptors[23]. Here, we identify six putative sequences of iGluRs in the tentacles of *A. simplex*, which are distributed in four receptor type clades. One of the expressed iGluRs is part of the AMPA receptors (Asim_GluA-mC), a clade that seems to have expanded in mollusks and

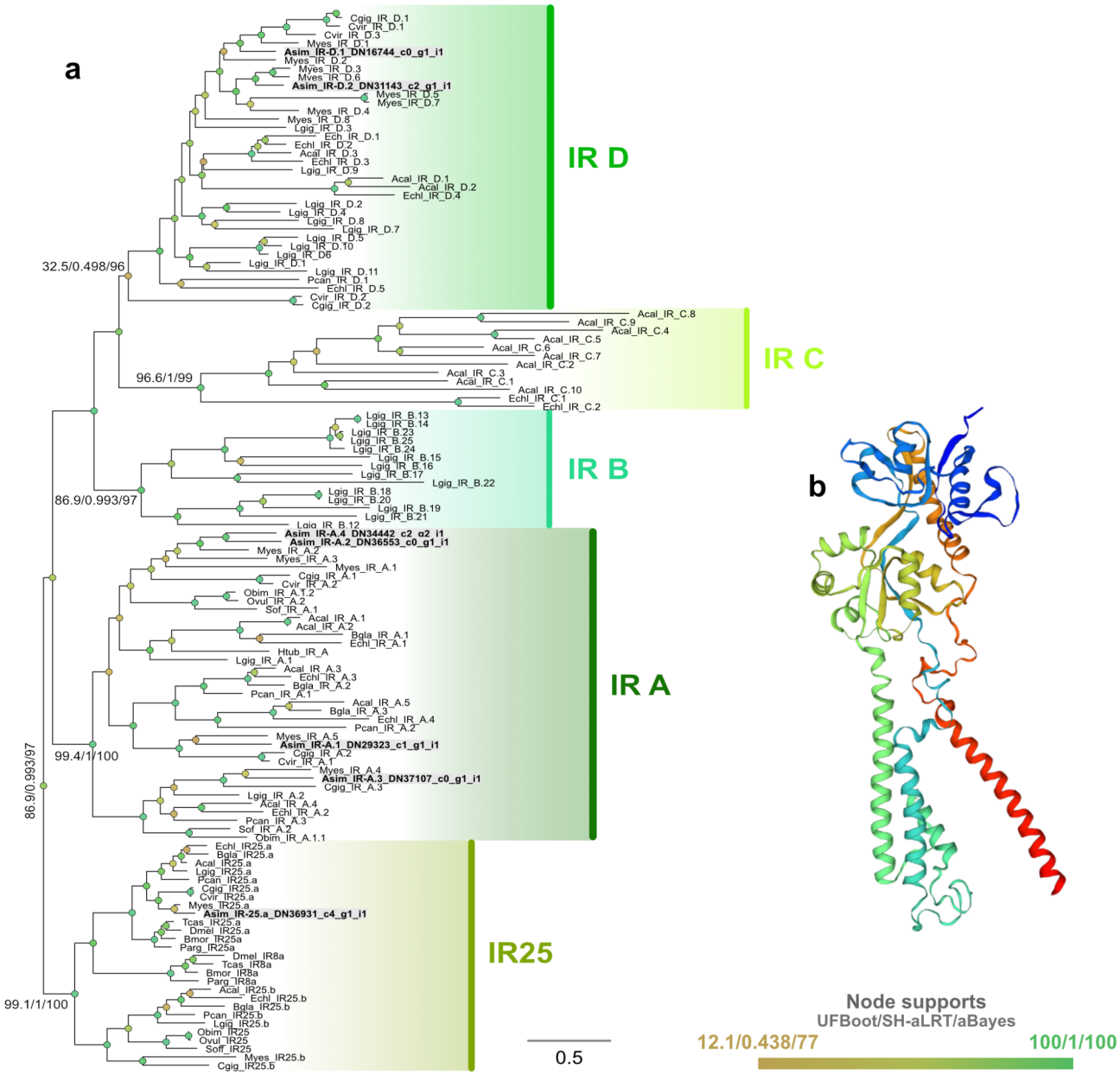

Fig. 7. **(a)** Maximum likelihood phylogenetic tree of IRs of *A. simplex* tentacles and other species. As a diverse group of iGluR variants (see Fig. 6), molluscan IRs are divided into five main clades, three of them (IR-A, IR-D, and IR25) containing protein sequences annotated in this study (terminal labels with gray background). Sequences for other species include mollusks and arthropods from the IR collection in Andouche et al. (2021). The model of substitution is LG + R8 according to BIC as selected by ModelFinder. Support values (ultrafast bootstrap, SH-like approximate likelihood ratio test, and aBayes test) are indicated on key nodes and by a colored gradient on all nodes. **(b)** 3D-model of Asim_IR-A.3_DN37107 (monomer) predicted by Swiss-Model server based on the template of glutamate receptor (GluA).

annelids[47], and two sequences (Asim_GluK-m7.1 and Asim_GluK-m7.2) are identified as Kainate receptors. Even though AMPA receptors are known to mediate fast excitatory synaptic transmission in vertebrates and Kainate receptors appear to have a modulatory function, their role is largely unknown in invertebrates[4,23]. Also, two sequences (Asim_GluN1 and Asim_GluN2) belong to the clade of NMDA receptors, which are potentially involved in synaptic and neuronal plasticity[4]. Finally, Asim_GluR-m9 is nested within a mollusk-specific clade of iGluR. Our results not only increase the number of known molluscan iGluRs, but also highlight putative receptors expressed in target tissues.

Protostomes, especially arthropods, rely predominately on ionotropic receptors (IRs), a diverse group within iGluRs, rather than GPCRs for chemosensation[5]. For example, more than 200 IRs have been identified in the transcriptome of decapod crustaceans, many with high expression in chemosensory organs[25]. All molluscan IRs are clustered in a well-supported clade, including different subgroups, such as IR25 and others previously

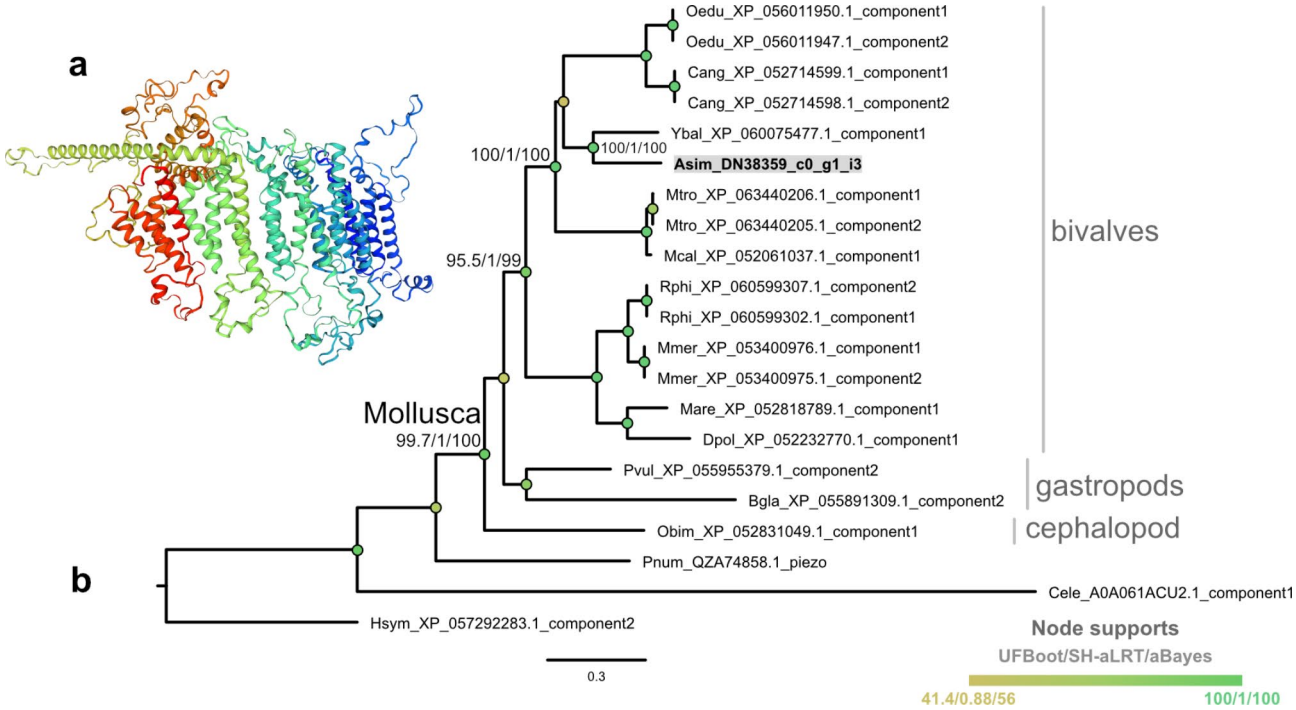

Fig. 8. **(a)** 3D-model of Asim_piezo_DN38359 (monomer) predicted by Swiss-Model server based on the template of piezo type mechanosensitive ion channel component 2. **(b)** Maximum likelihood phylogenetic tree of the Piezo mechanosensitive channel found in *A. simplex* tentacles and other species. Sequences for other molluscan and non-molluscan species (*Caenorhabditis elegans*, *Hydractinia symbiolongicarpus*, and *Platynereis dumerilii*) were gathered from NCBI based on predicted genes from available genomes. The model of substitution is Q.yeast + F + G4 according to BIC as selected by ModelFinder. Support values (ultrafast bootstrap, SH-like approximate likelihood ratio test, and aBayes test) are indicated on key nodes and by a colored gradient on all nodes.

named from IR-A to IR-D[23]. Our analysis supports such organization of molluscan IRs, showing that *A. simplex* tentacles express seven candidates from three main subclades (IR25, IR-A, and IR-D). Despite few studies, robust evidence supports the chemosensory functions of IRs in mollusks. In the gastropod *Aplysia californica*, 10 IRs are predominantly expressed in the rhinophore and oral tentacles, including IR25a, IR-A, and IR-C receptors, with some of them also being expressed in the skin and the central nervous system[4]. The pulmonate gastropod *Biomphalaria glabrata* has seven IRs, and the expression of IR25a in the cephalic tentacle supports its involvement in olfactory perception[26]. In the case of the cephalopod *Sepia officinalis*, three IRs were identified, and the specific expression of IR25 in the olfactory organs and suckers was validated[23]. Overall, we show that bivalve tentacles express a similar number of IRs compared to the gastropods investigated so far.

Our results provide the first evidence of IR expression in a bivalve sensory organ, supporting the likely chemosensory function of these receptors. IRs have hitherto been identified in several protostome clades, such as arthropods, nematodes, priapulids, brachiopods, annelids and mollusks (i.e., gastropods and cephalopods), probably being a synapomorphy of Protostomia[1,4]. The present finding of IRs in bivalve mollusks expands our knowledge of the prevalence of this family of receptors in protostome animals – particularly for Lophotrochozoa, which is currently understudied compared to Ecdysozoa[4]– opening doors for further characterization of the protostome IR repertoire and its functional diversification. In addition, sensory chemoreceptors appear to have a dynamic evolutionary history, and IRs illustrate one of several instances of neurotransmitters being co-opted into chemoreceptors[1]. Within this evolutionary labile gene family, the subclades of bivalve IRs found in this study suggest evolutionary novelty in bivalve chemosensation and highlight that the diversification of molluscan IRs is still likely underestimated. Future directions in understanding bivalve sensory systems include identifying organ-specific IRs and immunolocalization or in situ hybridization to validate the expression of candidate IRs in the ciliated receptor cells.

In striking contrast to photoreception and chemosensation, mechanosensation is largely unknown for mollusks. In mechanotransduction, mechanical stimuli, such as touch sensation, vibration, and pressure, are converted into electrochemical signals by specialized ion channels[48]. Here, we focus on the Piezo channels, a mechanoreceptor protein that has been identified in the tentacle transcriptome of *A. simplex*. One exceptional feature of the Piezo channels is the large number of predicted transmembrane regions, ranging from 10 to 40, including a propeller-shaped homotrimeric structure with a central cap[49]and a mechanogating mechanism[50]. Accordingly, the Asim_Piezo protein is predicted to contain at least 35 helices. In addition, Piezo channels show the ability to locally deform lipid membranes into a dome-like shape, which was experimentally proved to cause mechanotransduction activation[51].

To the best of our knowledge, the only record of expression of a Piezo receptor in a bivalve organ comes from the foot transcriptome of the chemosymbiotic clam *Loripes orbiculatus*(Lucinidae)[52]. In addition to providing the first phylogenetic analysis of Piezo ion channels in mollusks, our results indicate that one member is expressed in the sensory tentacles of *A. simplex*, supporting a likely role of mechanotransduction. In *Drosophila*, physiological evidence supports Piezo channel expression in sensory neurons responding to noxious mechanical stimuli[53]. A Piezo channel has also been identified in spider mechanosensory neurons and the nervous system, possibly contributing to mechanosensory transduction in the mechanosensilla[54]. Previous comparative analysis has suggested that most mechanosensory receptors, including Piezo channels, are highly conserved as part of the ancestral sensory toolkit of animals[1]. The roles of Piezo channels were not characterized in mollusks, highlighting a stimulating topic to explore in this megadiverse animal group. Based on the anatomy and life habits of sessile bivalves, we speculate that Piezo channels may be involved in touch sensation and seawater flow detection by ciliated sensory receptors in the tentacles. Ciliated cells have been proposed to act as sensory receptor cells to detect seawater flow in many marine taxa; however, physiological evidence is still lacking[55]. Overall, additional molecular and electrophysiological tools are required to validate and characterize the roles of Piezo channels in the mechanosensation of bivalve organs.

Sensory systems are required to intermediate environmental stimuli and organismal responses, impacting adaptive pathways and diversification. Here, we provide the first evidence combining anatomical features and molecular data to support tentacles as sensory organs in bivalves, a neglected group in chemosensation studies. Our work unravels the first repertoire of putative receptors in bivalve tentacles, identifying candidates from major protein families involved in different sensory modalities. The tentacles have their sensory role suggested by the expression of 17 GPCRs (Class A), including one opsin, five putative small-molecule receptors, and 11 putative chemosensory receptors, in addition to six iGluRs, seven IRs, and one Piezo mechanosensitive ion channel. Even though the total size of the sensory repertoire in *A. simplex* is currently difficult to estimate without a genome assembly as a reference, our results provide the first insight into photo-, mechano-, and chemo- sensation in the sensory organs of a bivalve species, expanding the knowledge of molluscan receptors. Overall, the results represent an exciting starting point for investigating chemosensation in mollusks other than cephalopods and gastropods, encouraging different perspectives, including molecular, physiological, and behavioral approaches.

Methods

Animal collection and tissue preservation

Four individuals of *Anomia simplex* were collected beneath stones from a rocky coast at Praia Grande (São Sebastião, SP, Brazil, 23°49'22.9 "S 45°24'60.0"W) during low tide in 2022. For RNA sequencing, multiple tentacles from two specimens were dissected under a stereomicroscope and immediately stored in RNAlater (Invitrogen) at −80°C until RNA extraction. For microscopy, the other specimens were anesthetized in a 7.5% solution of $MgCl_2$ for 2 h, and mantle margins bearing tentacles were dissected. For histology and scanning electron microscopy (SEM), tentacles were fixed in a Karnovsky solution for 3 h and stored in cacodylate buffer at 4°C[11]. For confocal laser scanning microscopy, tentacles were fixed in 4% paraformaldehyde (PFA) for 2 h and stored in phosphate buffer (PB) at 4°C[11]. Shells and body remains of dissected specimens were preserved in ethanol 95% and deposited in the Museum of Biological Diversity of the University of Campinas (MDBio, UNICAMP, Brazil) under the catalog numbers ZUEC-BIV 8565 and 8566.

Anatomic investigation of tentacles

For SEM, samples were post-fixed in 1% OsO_4 in cacodylate buffer for 1 h at 4°C[30]. After complete dehydration in a graded ethanol series, samples were critical point dried using CO_2 as intermediate in a Balzers CPD-030 (Electron Microscopy Sciences), mounted on stubs using conductive carbon adhesive tape, coated with gold in a Balzers SCD-050 sputter coater (Electron Microscopy Sciences), and analyzed in a Zeiss DSM 940. For confocal laser scanning microscopy, tentacles were permeabilized in phosphate buffer containing 2% Triton-X 100 (PBT) overnight. To visualize musculature, samples were incubated in a 1:40 dilution of Alexa Fluor 488 phalloidin (Molecular Probes) in PBT overnight at room temperature in the dark. For cilia and neurite investigation, samples were incubated in a 1:400 dilution of monoclonal alpha-tubulin antibody with Alexa Flour 488 conjugate (Molecular Probes) in PBT overnight at room temperature in the dark. After washes in PB, samples were mounted on microscope slides in Fluoromount-G with DAPI (Invitrogen) and analyzed in a Leica DM6 confocal microscope. Maximum projections were obtained from image stacks using ImageJ2[56]. For histology, samples were embedded in methyl-methacrylate resin (Leica Historesin Kit) after being completely dehydrated in ethanol. Serial sections of 3 µm were obtained with a Leica RM2255 microtome, stained with hematoxylin and eosin or toluidine blue, and analyzed in a Leica DMLB light microscope. All panels were produced in Affinity Designer v2.4.0.

Transcriptome sequencing and de novo assembly

Total RNA was extracted from pooled tentacles using TRIzol (Invitrogen, Thermo Fisher Scientific) following the manufacturer's instructions. The total RNA extracted was quantified using Qubit RNA Broad Range (Invitrogen), and integrity was checked by running agarose gel electrophoresis. Quality assessment was performed on Agilent 2100 Bioanalyzer, and mRNA was isolated using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, NEB). NEBNext Ultra RNA Library Prep Kit and NEBNext Multiplex Oligos for Illumina (NEB) were used to generate the cDNA library and provide adaptor ligation, respectively. Paired-end cDNA sequencing was performed on an Illumina NextSeq 500 platform (Mid Output KT v2.5, 150 cycles). Prior to assembly, raw reads were inspected using FastQC v0.12[57], trimmed, and quality filtered using Trimmomatic v.0.36[58] under default parameters. A *de novo* transcriptome of tentacles was generated using Trinity v2.1.1[59] and screened for predicted proteins with open reading frames (ORFs) using Transdecoder v5.5.0. Quality assessment

was performed by mapping reads back to the transcriptome assembly using Bowtie2 v2.5.1[60]. In addition, redundancy was removed using CD-Hit v4.8.1[61] using default parameters. Then, BUSCO v5.7.0[62] was run on the amino acid dataset using the obd10 metazoan database to assess the completeness of the assembly. Sequence data generated in this study have been deposited in the Sequence Read Archive with the primary accession code SRR29413220.

Functional annotation

Transcripts from the tentacle transcriptome were annotated using Trinotate v4.0.0 pipeline[63], including homology search via BlastX v2.12 and BlastP v2.13[64]to UniProt/SwissProt database. The outputs were used to generate GO terms[65]and KEGG annotations[66]. Protein conserved domain identification was performed by HMMER v3.4[67] using the *hmmscan* command against the Pfam 36.0 database for protein families and domains. Following Trinotate default parameters, the search uses Hidden Markov Models to annotate functional gene regions based on transcript sequence, using the domain noise cutoff (DNC) as a threshold to filter out sequences that are likely to belong to different families. Transmembrane domains (helices) were predicted by TMHMM v2.0[68] based on primary amino acid sequences and models for both alpha-helical and beta-barrel protein folds using default parameters. In addition, SignalP v6.0[69] was used to predict signal peptides. Annotations were loaded into an SQLite database, and the R package TrinotateR v1.0 was used to summarize the annotation reports.

Annotated sequences were screened for Pfam conserved domains (PF00001, PF00002, PF00003) related to the seven transmembrane regions of GPCRs. Protein sequences containing less than six predicted transmembrane helices were considered largely incomplete and removed from subsequent analyses. All retrieved GPCRs were then categorized into subclasses comprising rhodopsin-like (Class A, 7TM_1), secretin and adhesion (Class B, 7TM_2), and metabotropic glutamate (Class C, 7TM_3). For iGluRs, annotated sequences were screened for Pfam conserved domains PF10613 and PF0060 related to ligand-gated ion channels of the ionotropic glutamate receptor family[23,25]. Only sequences containing both domains were kept for subsequent analysis.

Classification and phylogenetic analysis of receptor candidates

We performed a phylogenetic analysis of *A. simplex* putative receptors and reference proteins to determine sequence homology and provide a phylogenetic classification of the targets. For GPCRs, we retrieved a reference set of 30 protein sequences from representatives of all major GPCR classes of two bivalve species, the scallop *M. yessoensis* and the oyster *C. virginica*, available in the NCBI database. We focused on identifying Class A GPCRs, including opsins, small-molecule neurotransmitter receptors, and other putative chemosensory receptors.

Photoreceptor proteins were investigated using PIA2 (https://github.com/xibalbanus/PIA2) to mine the dataset against a diverse panel of opsins[70]. The pipeline included BLAST searches and likelihood analysis to put unknown sequences into pre-calculated phylogenetic trees. Subsequently, candidates identified by PIA2 were visually screened for the conserved lysine residue (K296 in Bovine rhodopsin) necessary for retinal binding and further classified in a phylogenetic approach including an opsin collection for 23 pteriomorphian bivalves[40], along with outgroups[71]. Then, we investigated the diversity of Class A GPCRs focusing on low molecular weight neurotransmitters, such as biogenic amines, to identify possible receptors frequently expressed in sensory neurons associated with chemical synapsis. We used blastp with an e-value of 1e-30 to query receptors from our annotated Class A GPCRs against the 159 small-molecule receptor collection[7] for different metazoan groups. To include bivalve representatives in the subject dataset, we added seven receptors from the scallop *Mizuhopecten yessoensis*(i.e., serotonin, dopamine, octopamine, adenosine, and prostaglandin receptors), available in the NCBI database. To identify other putative chemosensory receptors within our transcripts, we searched for conserved serpentine domains srsx (PF10320), srw (PF10324), and srx (PF10328) associated with chemosensation and neuropeptide receptor-like domains. Then, we used blastp with an e-value of 1e-30 to query receptors from our results against an invertebrate chemosensory GPCR collection[10], including sequences for gastropods, nematodes, planarians, and cnidarians. Most chemosensory candidates in the dataset have not had their sensory function confirmed experimentally, neither in vivo nor in vitro. However, data for some gastropods and nematodes provide evidence of association with chemosensation[19,72]. All significant hits were used for subsequent phylogenetic analysis.

Classification of putative iGluRs was based on a large dataset[23], including 312 protein sequences with Pfam conserved domains PF10613 and PF0060 for ten molluscan species (three bivalves, two cephalopods, and five gastropods). We leverage this dataset, spanning all major iGluR groups and then focusing on IRs, to perform a phylogenetic analysis and classify our candidate transcripts. According to previous studies[23,73], nomenclature for iGluRs includes the species name followed by "IR" or "Glu", a letter representing the receptor group, and a letter or a number for subclades (e.g., Asim_GluK-m7.1).

As expected for a tactile sensory organ, we also investigated the expression of possible mechanoreceptors in the tentacles. Annotated transcripts were screened for the Pfam domains PF15917 and PF12166 related to the Piezo family of mechanosensitive ion channels and PF06011 for transient receptor potential (TRP) ion channels. To perform a phylogenetic assessment of possible candidates, we gathered 17 homolog sequences of Piezo channels predicted in the genomes of 12 molluscan species and three other invertebrates (*Caenorhabditis elegans*, *Platynereis dumerilii*, and *Hydractinia symbiolongicarpus*).

Each dataset, combining our transcripts with the reference sets, was aligned with MAFFT v7.453[74] using the "auto" parameter and quality trimmed using trimal v1.4[75] with the "-automated1" parameter, except for opsin sequences (with the "-gappyout" option). Maximum likelihood phylogenetic trees were generated in IQ-TREE2 v 2.1.3[76] after searching for the best-fitting model of protein sequence evolution using ModelFinder[77]. Branch support was determined with 10,000 replicates for ultrafast bootstrap, 10,000 replicates for SH-like approximate likelihood ratio test (SH-aLRT), and an approximate aBayes test[78,79]. Phylogenetic trees were visualized in FigTree v1.4.3. Bioinformatic analyses were conducted in Darwin Server (University of São Paulo) and Nova

Cluster (Iowa State University). A summary of all different datasets used for phylogenetic comparisons of sensory-related genes found in the tentacle transcriptome of *Anomia simplex*, including sources and taxonomic scope, is indicated in Supplementary Information, Table S1. All sequences, alignments, and tree files used during this study are available in a Figshare repository under DOI: https://doi.org/10.6084/m9.figshare.26058316.

Data availability

All sequences, alignments, and tree files used during this study are available in a Figshare repository under DOI: https://doi.org/10.6084/m9.figshare.26058316. Sequence data generated in this study have been deposited in the Sequence Read Archive with the primary accession code SRR29413220.

Received: 3 July 2024; Accepted: 13 December 2024
Published online: 28 December 2024

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Acknowledgements
The authors acknowledge funding provided by the grants 2019/18834-1 and 2022/02746-9 (São Paulo Research Foundation – FAPESP to JAA and JEARM), grant 307180/2020-4 (National Council for Scientific and Technological Development – CNPq to JEARM) and the United States National Science Foundation (NSF DEB 1754331 to JMS). The authors thank the Center for Marine Biology (CEBIMar–USP, Brazil) for the support in the analysis of live specimens and Centro de Facilidades de Apoio à Pesquisa (CEFAP–USP, Brazil) for providing technical assistance during sequencing. The research reported in this paper is partially supported by the HPC@ISU equipment at Iowa State University, some of which has been purchased through funding provided by the National Science Foundation under the Major Research Instrumentation Program (Grant 1726447 and 2018594). The authors also thank the invaluable assistance of Manuel A. Junior and Beatriz V. Freire during all RNA-seq steps, Federico D. Brown for providing access to the confocal microscope, and two reviewers for their thorough comments. This is a contribution of NP-BioMar (Research Center for Marine Biodiversity – USP).

Author contributions
J.A.A. and J.E.A.R.M. designed the study. J.A.A. collected the data and created the figures. J.A.A., K.E.M., and J.M.S. conducted the analyses. J.A.A. wrote the first draft of the manuscript. All authors edited and approved the final version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-83313-7.

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