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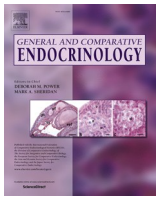

Global analysis of ligand-gated ion channel conservation across Platyhelminthes☆

Víctor Hugo Caña-Bozada [a,b,*], Ahmed A.Z. Dawoud [c], Ivana Ramos-de la Cruz [a], Lizeth C. Flores-Méndez [d], Josué Barrera-Redondo [e], Jesús Briones-Mendoza [f], Luis A. Yañez-Guerra [c,g,**]

[a] Centro de Investigación en Alimentación y Desarrollo, A.C. Unidad Mazatlán en Acuicultura y Manejo Ambiental, Mazatlán 82112 Sinaloa, Mexico
[b] Centro de Investigación para la Salud en América Latina (CISeAL), Pontificia Universidad Católica del Ecuador (PUCE), Quito, Ecuador
[c] School of biology. University of Southampton, University Road, SO17 1BJ Southampton, UK
[d] Universidad Autónoma de Occidente, Unidad Regional Mazatlán. Av. del Mar, Tellería, Mazatlán 82100 Sinaloa, Mexico
[e] Department of Algal Development and Evolution, Max Planck Institute for Biology Tübingen 72076 Tübingen, Germany
[f] Carrera de Biología, Facultad de Ciencias de la Vida y Tecnologías, Universidad Laica "Eloy Alfaro" de Manabí, Ciudadela Universitaria vía San Mateo, Manta, Ecuador
[g] Institute for Life Sciences. University of Southampton, University Road SO17 1BJ Southampton, UK

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ABSTRACT

Ligand-gated ion channels (LGICs) are critical for neurotransmission, mediating responses to neurotransmitters and hormones, and influencing diverse physiological processes. This study identifies and classifies LGICs across Platyhelminthes, with a particular focus on parasitic neodermatans, which impact human and animal health. Using bioinformatics tools, we analyzed LGICs from 41 neodermatan species and expanded our investigation to encompass vertebrates, other invertebrates, and non-bilaterians to trace LGIC evolutionary pathways across Metazoa. We identified 2,269 putative LGICs within neodermatan species, which we classified into the cys-loop, ASIC/Deg/ENaC, iGluR, and P2X families. Our phylogenetic and clustering analyses reveal lineage-specific patterns with distinct evolutionary trajectories for each LGIC family in neodermatans compared to free-living platyhelminths and other taxa. Notably, the ASIC/Deg/ENaC family displayed the greatest degree of neodermatan-specific divergence, while cys-loop and P2X families were more conserved across taxa. To provide insight into their potential physiological roles, we analyzed LGIC expression patterns in *Schistosoma mansoni*, revealing widespread expression across neuronal and muscle cell types. The distribution of acid-sensing ion channels (ASICs) in both neurons and muscles suggests a role in neuromuscular signalling, while the P2X receptor (Smp_333600) exhibited sex-specific expression, potentially indicating distinct functional roles in males and females. Additionally, several cys-loop acetylcholine and GABA receptors showed differential neuronal and muscle expression, highlighting their likely contributions to cholinergic and inhibitory neurotransmission. These findings underscore the relevance of LGICs in parasite physiology, particularly in neuromuscular and sensory processes, and suggest potential targets for antiparasitic interventions.

1. Introduction

Ion channels are transmembrane proteins that regulate ion transport across cell membranes, playing essential roles in the nervous and muscular systems. They are classified by ion selectivity, including potassium (K+), sodium (Na+), calcium (Ca2+), and anion channels (Hübner and Jentsch, 2002), and by activation mechanisms, such as voltage-gated and ligand-gated ion channels (LGICs) (Gao et al., 2020). LGICs operate through ligand binding, with primary families including the ionotropic glutamate receptors (iGluRs), the acid-sensing ion

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* Corresponding author at: Centro de Investigación en Alimentación y Desarrollo, A.C. Unidad Mazatlán en Acuicultura y Manejo Ambiental, Mazatlán 82112, Sinaloa, Mexico.
** Corresponding author at: School of biology. University of Southampton, University Road, SO17 1BJ Southampton, UK.
E-mail addresses: vhcana@puce.edu.ec (V.H. Caña-Bozada), l.a.yanez-guerra@soton.ac.uk (L.A. Yañez-Guerra).

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channels/degenerin channels/epithelial Na + channels (ASIC/Deg/ENaCs), the purinergic ion channels (P2X), and the cys-loop channels (Collingridge et al., 2009).

LGICs are critical in the endocrine system, facilitating rapid hormonal signaling and modulating essential endocrine functions. By mediating the effects of neurotransmitters and hormones (e.g., neuropeptides, acetylcholine [ACh], gamma-aminobutyric acid [GABA], and glycine) on target tissues, LGICs are key to processes like hormone release, metabolism, and physiological responses (Stojilkovic et al., 2010; Zemková and Stojilkovic, 2018; Liu et al., 2023). Their role in the neuroendocrine axis highlights the sophisticated interaction between the nervous and endocrine systems (Zemková and Stojilkovic, 2018). Given the importance of LGICs, these receptors have likely contributed to organismal adaptation. For example, DEG/ENaC channels may have facilitated the transition of tetrapods to low-salinity environments during land colonization (Wichmann and Althaus, 2020).

Neodermata, parasitic platyhelminths, are distinguished by a unique neodermis derived from the mesoderm, forming a syncytial structure without individual cellular boundaries (Tyler and Tyler, 1997). This distinctive feature is critical for their parasitic lifestyle, allowing them to efficiently absorb nutrients and evade host immune responses (Tyler and Hooge, 2004). This parasitic group includes three classes: cestodes (tapeworms), trematodes (flukes), and monogeneans, subclassified into Monopisthocotylea and Polyopisthocotylea. Many Neodermata members cause diseases impacting human and animal health. For instance, the cestode *Taenia solium* (pork tapeworm) poses a significant threat, particularly in developing nations. Its lifecycle involves both humans and pigs, with infection occurring via undercooked pork, causing taeniasis (Garcia and Del Brutto, 2005), which can progress to cysticercosis upon ingestion of eggs shed by adult worms. Neurocysticercosis, a severe form, causes neurological complications like seizures, headaches, and cognitive impairment (Garcia and Del Brutto, 2005; Ndimubanzi et al., 2010; Moyano et al., 2014). Schistosomiasis, caused by trematodes such as *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*, affects over 200 million people globally, with an estimated loss of 1.53 million disability-adjusted life years (Gryseels et al., 2006; Rinaldo et al., 2021). Intestinal and urogenital schistosomiasis result in severe health issues, including anemia, stunted growth in children, bladder cancer, and infertility.

These examples highlight the importance of understanding LGICs in Neodermata, as these channels are central to neurotransmission processes that influence physiology and behavior (Olsen and Sieghart, 2008; Albuquerque et al., 2009; Traynelis et al., 2010; Lemoine et al., 2012; Miller and Yeh, 2017). Despite their significance, the functionality of only a few LGICs has been explored in Neodermata, including two acetylcholine-gated chloride channels (ACCs), three glutamate-gated chloride receptors, and a P2X receptor from *S. mansoni* (Agboh et al., 2004; Dufour et al., 2013; MacDonald et al., 2014). Researching these channels could prove useful for parasite control and development of novel antiparasitic drugs. In helminths, ion channels are primary pharmacological targets (Rana and Misra-Bhattacharya, 2013), and in human and veterinary medicine, approximately 18 % of FDA-approved drugs target ion channels (Santos et al., 2017). For instance, in *S. mansoni*, the inhibition of the acetylcholine-gated chloride channel by arecoline and nicotine caused near-complete paralysis in schistosomula (MacDonald et al., 2014). Additionally, another channel, glutamate-gated chloride (GluCl), has been shown to be inhibited by thymol and propofol, demonstrating its susceptibility to various modulators (Lynagh et al., 2014), indicating its potential as a pharmacological target.

While some studies on LGICs in Neodermata exist, they largely focus on individual species (Johnson et al., 2023; Tolstenkov et al., 2023; Tsai et al., 2013). An exception is Preza et al. (2018), which analyzed several LGIC types across neodermatans, identifying the absence of GABA ionotropic receptors in cestodes and trematodes, and the widespread presence of inhibitory ACh-gated chloride channels and glutamate-gated chloride receptors. Aside from GABA receptors, these were detected in Cestoda, Monopisthocotylea, and Trematoda, providing insights into protein loss processes in Neodermata evolution. However, that study lacked a comprehensive representation of Monogenea (one Monopisthocotylea species and no Polyopisthocotylea species) and did not examine ASIC/Deg/ENaC channels, limiting the understanding of LGIC diversity in Neodermata.

Genomic data for the four major Neodermata groups allow analysis of the functional and evolutionary dynamics of LGICs in parasitic platyhelminths. Biological innovations in neodermatans and platyhelminths raise several evolutionary questions: Did novel LGICs evolve with the emergence of neodermatans or were significant innovations more pronounced during the emergence of Platyhelminthes? Which LGIC families were essential for evolutionary developments in both groups and did these innovations primarily result from gene duplication or sequence divergence? In this study, we identified and classified LGICs in 41 neodermatan species, examining their distribution across taxonomic levels and comparing their evolution within the species tree, including vertebrates, invertebrates, and non-bilaterians. Finally, we aim to identify taxon-specific sequences that may hold pharmacological potential for managing neodermatan parasitic species.

2. Methods

2.1. Molecular data

We identified LGICs based on predicted proteins from the transcriptomes, genomes, or ESTs of 41 Neodermata species. These species are distributed across four major neodermatan lineages: 16 cestodes, 18 trematodes, 5 monopisthocotyleans, and 2 polyopisthocotyleans. The data were retrieved from the NCBI database, WormBase ParaSite (WBPS17), and different studies (Hahn et al., 2014; Howe et al., 2017; Konczal et al., 2020; Vorel et al., 2021; Caña-Bozada et al., 2022a) (Supplementary Table S1). For the EST data of *Neobenedenia melleni*, we predicted ORFs and putative proteins using TransDecoder 5.5.0 (https://transdecoder.github.io) (Haas et al., 2013). To avoid overestimating sequences in the transcriptomes and EST, the longest isoforms per gene was extracted using the Trinity script "get_longest_isoform_seq_per_trinity_gene.pl" (Haas et al., 2013). To assess the completeness of the transcriptomes, genomes, or ESTs, we ran BUSCO v5.2.1 (Manni et al. 2021) in protein mode and with the lineage set to "eukaryote" with the database "metazoa_odb10" (Creation date of the database: September 2022, number of BUSCOs: 954). Missing genes varied across groups, ranging from 22.2 % to 74.8 % in cestodes, 32.8 % to 61.3 % in monopisthocotyleans, 22.7 % to 56.8 % in polyopisthocotyleans, 18.2 % to 45.8 % in trematodes, 10.3 % to 18.8 % in free-living platyhelminths, and 0 % to 19.7 % in reference species (Supplementary Table S2). Since many platyhelminth species in our study lack high-quality genomes, limiting comparative analyses, we exercised caution in identifying gene losses, particularly in Monopisthocotylea and Polyopisthocotylea, due to their lower dataset completeness and the limited number of species analyzed. In contrast, gene losses in Trematoda and Cestoda, where at least 16 species were examined and missing data were lower (with missing gene values of 18.8 % and 22.2 %, respectively), are more likely to be genuine.

2.2. Identification of LGICs

To identify LGICs, we searched for known LGIC family domains from the Pfam database (version 35) (Finn et al., 2016) in the predicted proteins of Neodermata using PfamScan v1.6.4 (Mistry et al., 2007) with the parameter "-e_seq 1". We specifically used the Pfam domains PF02931 and PF02932 for the cys-loop family, PF10613 and PF00060 for the iGluR family, PF00858 for the ASIC/Deg/ENaCs family, and PF00864 for the P2X family (Supplementary Table S3). We retained the proteins with a bit score $\geq$ 11. To detect potential contaminant sequences, retained proteins were aligned against the UniRef90 database

(147,407,377 proteins; accessed June 19, 2022) (UniProt Consortium, 2021) using Diamond v2.0.15 (Buchfink et al., 2021) with the option "–sensitive" (e-value < $1e^{-3}$) and default parameters. Following the approach of Caña-Bozada et al. (2022a), we retrieved sequences with the best hits for Protostomia (taxid: 33317), treating any remaining sequences as contaminants.

We also aligned our protein sequences against the UniProtKB/TrEMBL database (accessed October 17, 2022) (Apweiler et al., 2004) to detect and remove any non-LGIC false positive matches from the PfamScan results. We limited our sequence search to Metazoan proteins (taxonomy_id: 33,208 (33,434,238 proteins), using Diamond with the option "–sensitive" (e-value < $1e^{-3}$) and default parameters. We removed proteins whose best hits were neither LGICs nor proteins with LGIC-associated Pfam domains. To reduce redundant sequences, the predicted proteins of each of the 41 species were clustered with a 95 % identity threshold using the CD-HIT v4.6 software (Fu et al., 2012). A schematic representation of the pipeline is presented in Fig. 1A.

For comparative purposes in the phylogenetic analyses, LGIC sequences were identified in the free-living platyhelminths *Macrostomum lignano*, *Schmidtea mediterranea*, and *Bothrioplana semperi* (Laumer et al., 2015; Howe et al., 2017) (Supplementary Table S4) along with 12 other species representing Cnidaria, Ecdysozoa, Lophotrochozoa, Ambulacraria, and Chordata using the same methodology as the Neodermata sequences (Supplementary Tables S5 and S6). Within Ecdysozoa and Chordata, the species *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans* were included for having well-annotated LGIC sequences.

2.3. Identification of taxonomically-restricted LGICs in Neodermata and LGICs shared across other taxonomic levels

To examine the distribution of LGICs specific to Neodermata or shared across broader taxonomic levels (Metazoa, Protostomia, Spiralia, Lophotrochozoa, and Platyhelminthes), we analyzed the results obtained from alignments using Diamond. For that, the LGIC protein sequences of the 41 neodermatan species were aligned against five protein sequence datasets downloaded from the UniProtKB/TrEMBL database (accessed October 17, 2022) (Apweiler et al., 2004), following a similar methodology to Caña-Bozada et al. (2022a,b). The datasets were downloaded using the filter "taxonomy_id": 1) Metazoa excluding Protostomia [(taxonomy_id:33208) NOT (taxonomy_id:33317)], 2) Protostomia excluding Spiralia [(taxonomy_id:33317) NOT (taxonomy_id:2697495)], 3) Spiralia excluding Lophotrochozoa [(taxonomy_id:2697495) NOT (taxonomy_id:1206795)], 4) Lophotrochozoa excluding Platyhelminthes [(taxonomy_id:1206795) NOT (taxonomy_id:6157)]. A fifth dataset was constructed with sequences of 5) Platyhelminthes excluding Neodermata [(taxonomy_id:6157) NOT (taxonomy_id:37945) NOT (taxonomy_id:6178) NOT (taxonomy_id:6199)]. In addition, this fifth dataset included sequences obtained from WormBase ParaSite (Howe et al., 2017) and the study of Laumer et al. (2015) (Supplementary Table S7). To obtain predicted proteins from Laumer et al. (2015), the sequences were submitted to TransDecoder. These datasets enabled us to identify lineage-specific LGICs and assess their similarity across four major neodermatan lineages (Trematoda, Cestoda, Monopisthocotylea, and Polyopisthocotylea) and non-neodermatan taxa (Metazoa, Protostomia, Spiralia, Lophotrochozoa, and Platyhelminthes).

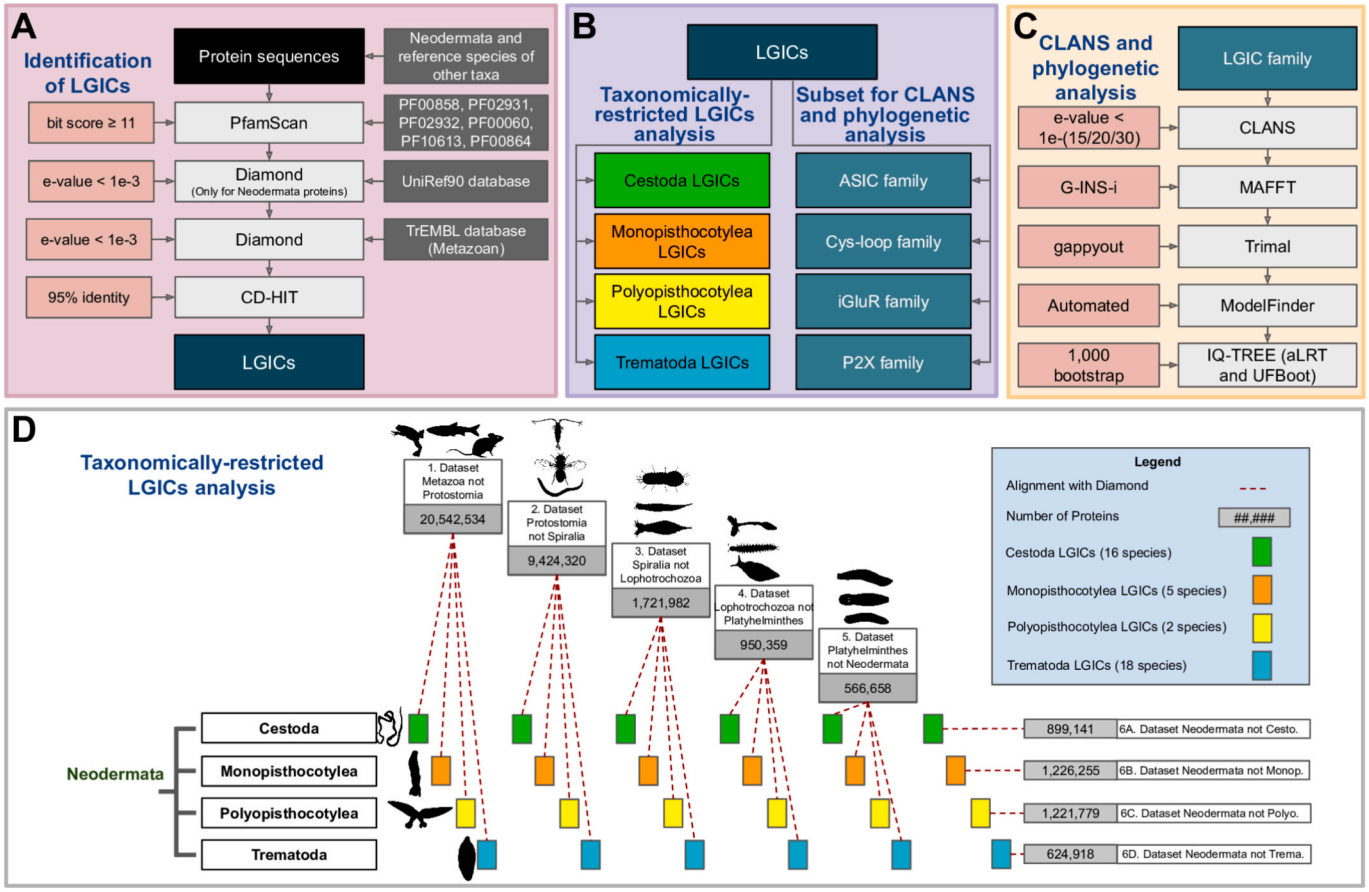

Fig. 1. Bioinformatic pipeline for the identification, classification, and exploration of diversification patterns of LGIC sequences of Neodermata. A) Identification of LGIC sequences of Neodermata and other metazoans. B) Subset of LGIC sequences used for CLANS, phylogenetic, and taxonomically-restricted LGICs analyses. C) Programs and parameters used for CLANS and phylogenetic analyses. D) Alignment of Neodermata LGICs with several datasets using the software Diamond.

We constructed four protein sequence datasets to pinpoint lineage-specific proteins and evaluate their similarity within neodermatans. These datasets included the sequences of 6A) Neodermata excluding Cestoda; 6B) Neodermata excluding Monopisthocotylea; 6C) Neodermata excluding Polyopisthocotylea; and 6D) Neodermata excluding Trematoda. The proteins of each platyhelminth species were aligned against each dataset using Diamond with the previously mentioned parameters. The neodermatan proteins were not aligned against proteins belonging to their same major neodermatan lineage. A schematic representation of the aligned databases is presented in Fig. 1D.

Neodermata LGICs with e-values greater than $1e^{-3}$ were classified as taxonomically restricted at these taxonomic levels. To evaluate differences in the proportion of taxonomically restricted LGICs across various taxonomic levels, pairwise statistical comparisons were performed using the non-parametric Dunn test, with p-values adjusted via the Holm method. This analysis was carried out using the grouped_ggbetweenstats function from the R package ggstatsplot v0.11.1 (Patil, 2021).

2.4. Clustering and phylogenetic analyses of LGICs

Clustering and phylogenetic analyses of LGICs were conducted to explore the relationship among the obtained LGICs from Neodermata, free-living platyhelminths, and other metazoans (Fig. 1B, C). The clustering analysis was performed with the CLANS software (Frickey and Lupas, 2004). For each LGIC family, a CLANS analysis was performed, and the clustering level was assessed using P cutoff values of $1e^{-15}$, $1e^{-20}$, $1e^{-30}$, $1e^{-40}$, and $1e^{-50}$. Based on their connection to the main cluster, a P cutoff value of $1e^{-30}$ was selected for the cys-loop and P2X families, $1e^{-20}$ for the IGluR family, and $1e^{-15}$ for the ASIC/Deg/ENaC family. Sequences with at least one connection to the main cluster were pre-filtered and used for the phylogenetic analysis, thus removing divergent or truncated sequences that could interfere with the analysis. The LGIC sequences of species *H. sapiens*, *D. melanogaster*, and *C. elegans* served as reference proteins to annotate the cluster maps.

Each cluster, representing different LGIC families (cys-loop acetylcholine, cys-loop GABA, iGluR, ASIC/Deg/ENaC, and P2X), underwent sequence alignment with MAFFT 7.31 (Katoh and Standley, 2013) using the iterative refinement method G-INS-i and gap removal with Trimal (Capella-Gutiérrez et al., 2009) using the gappyout mode. Maximum-likelihood phylogenetic trees were constructed using IQ-TREE2, with the Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT) branch support (1,000 replicates) and ultrafast bootstrap (1,000 replicates). IQ-TREE was run allowing ModelFinder (Kalyaanamoorthy et al., 2017) to determine the best model (−mset) from WAG, LG, Blosum62, Dayhoff, JTT, and Poisson, based on the Bayesian information criterion, for building each tree. The best model determined for the ASIC/Deg/ENaC family was WAG + F + R9, for the cys-loop family were LG + R10 (GABA channels) and WAG + F + R10 (Acetylcholine channels), for the iGluR family was LG + R9, and for the P2X family was WAG + F + R7. Visualization and annotation of the trees were performed using FigTree v1.4.2 (available from https://tree.bio.ed.ac.uk/software/figtree/).

2.5. Characterizing the expression of LGICs in Schistosoma

To investigate the expression profiles of LGICs in *S. mansoni*, we utilised the single-cell sequencing data generated by Wendt et al. (2020) (GSE146737). Briefly, single-cell RNA sequencing (scRNA-seq) data was obtained from sorted cells of adult male (n = 16,042), adult female (n = 13,515), and age-matched virgin female (n = 14,085). The dataset includes 20,312 features and 43,642 cells, clustered into 86 groups based on gene expression patterns. These 86 groups were then categorised into 68 distinct cell types, following the methodology described by Wendt et al. (2020). In their analysis, clusters were merged based on shared gene expression profiles. For example, the clusters for neoblasts (clusters 0, 1, 2, 6, 7, and 37) and neoblast progeny (clusters 4 and 8) were combined, as were the clusters for neuron 1 (clusters 10, 60, and 68) and neuron 6 (clusters 24 and 26). Other merged categories include parenchyma (clusters 11, 12, and 51), flame cells (clusters 14 and 41), S1 cells (clusters 3, 9, 32, and 42), and tegument (clusters 36 and 63). This resulted in the final classification of 68 distinct cell types, which include neurons (n = 31) and muscles (n = 8) (Supplementary Table S8).

We used R v4.2 and the Seurat package v4.3 (Satija et al., 2015) to analyse the entire dataset as well as each sex group individually. We examined the expression of 35 LGIC genes across the cys-loop, ASIC/Deg/ENaC, iGluR, and P2X families. First, we subset the data to focus on relevant cells and normalised the expression counts using the "LogNormalize" method with a scale factor of 10,000. We then scaled the data using the 2,000 most variable features to standardise gene expression counts before analysis. Expression levels were compared using the Wilcoxon rank-sum test, restricting the analysis to genes expressed in at least 1 % of the cells within each cell-type group as defined by (Wendt et al., 2020) and requiring a minimum log fold-change (LogFC) of 0.1 between clusters. Multiple comparisons were adjusted using the Bonferroni correction. A scatter plot was generated using Python v3.11 and the Seaborn package v0.13 (Waskom, 2021) to visualise the average LogFC (dot colour) and the percentage of cells expressing each LGIC gene (dot size) across pooled muscle and neuron clusters, as well as across sex groups. The full dataset, including gene IDs, p-values, log fold-change values, expression percentages, cell-type clusters, adjusted p-values, and LGIC family classifications, is provided in Supplementary Table S8. The analysis code is publicly available at (https://github.com/ad2n15/LOGIC_Platyhelminthes).

3. Results

3.1. Identification of LGICs

We identified 2,269 putative LGICs in the 41 neodermatan species (Supplementary Table S9). Information about the identified proteins is shown in Supplementary Table S10, and the amino acid sequences of all LGICs of Neodermata and other metazoans are available in Supplementary File S1. Domain analysis led to the classification of the proteins into four LGIC groups (Supplementary Table S9). The cys-loop family was the most abundant with 987 proteins, followed by the ASIC/Deg/ENaC family with 650 proteins, the iGluR family with 459 proteins, and the P2X family with 173 proteins.

3.2. Evolution of ligand-gated ion channels

3.2.1. Neodermata LGICs that are shared across diverse taxonomic groups or restricted to specific taxonomic hierarchies

Our analysis revealed that Neodermata LGICs can be either shared across a broad range of taxa or restricted to specific taxonomic groups. In the case of the ASIC/Deg/ENaC family, a large proportion of neodermatan LGICs (35 %) were found to be specific to Platyhelminthes (Fig. 2), suggesting that these proteins likely originated during the expansion of flatworms. Analysis of the cys-loop and iGluR families revealed that taxonomically restricted proteins likely originated with the appearance of lophotrochozoans, as these sequences were found to be shared between flatworms and other lophotrochozoan taxa. In contrast, the P2X family showed no evidence of restricted sequences.

3.2.2. Clustering and phylogenetic analyses of ligand-gated ion channels

Through clustering and phylogenetic analyses, the relationships between LGICs from Neodermata, free-living platyhelminths, and other metazoans were investigated. Clustering analysis (Supplementary Figs. S1-S3) facilitated the grouping of LGICs with reference proteins and the filtering of sequences that were subsequently used in the phylogenetic analyses. This process led to the exclusion of 160 cys-loop sequences of Neodermata, 50 iGluR sequences, 2 P2X sequences, and 129 ASIC/Deg/ENaC sequences (Supplementary Table S9). Following

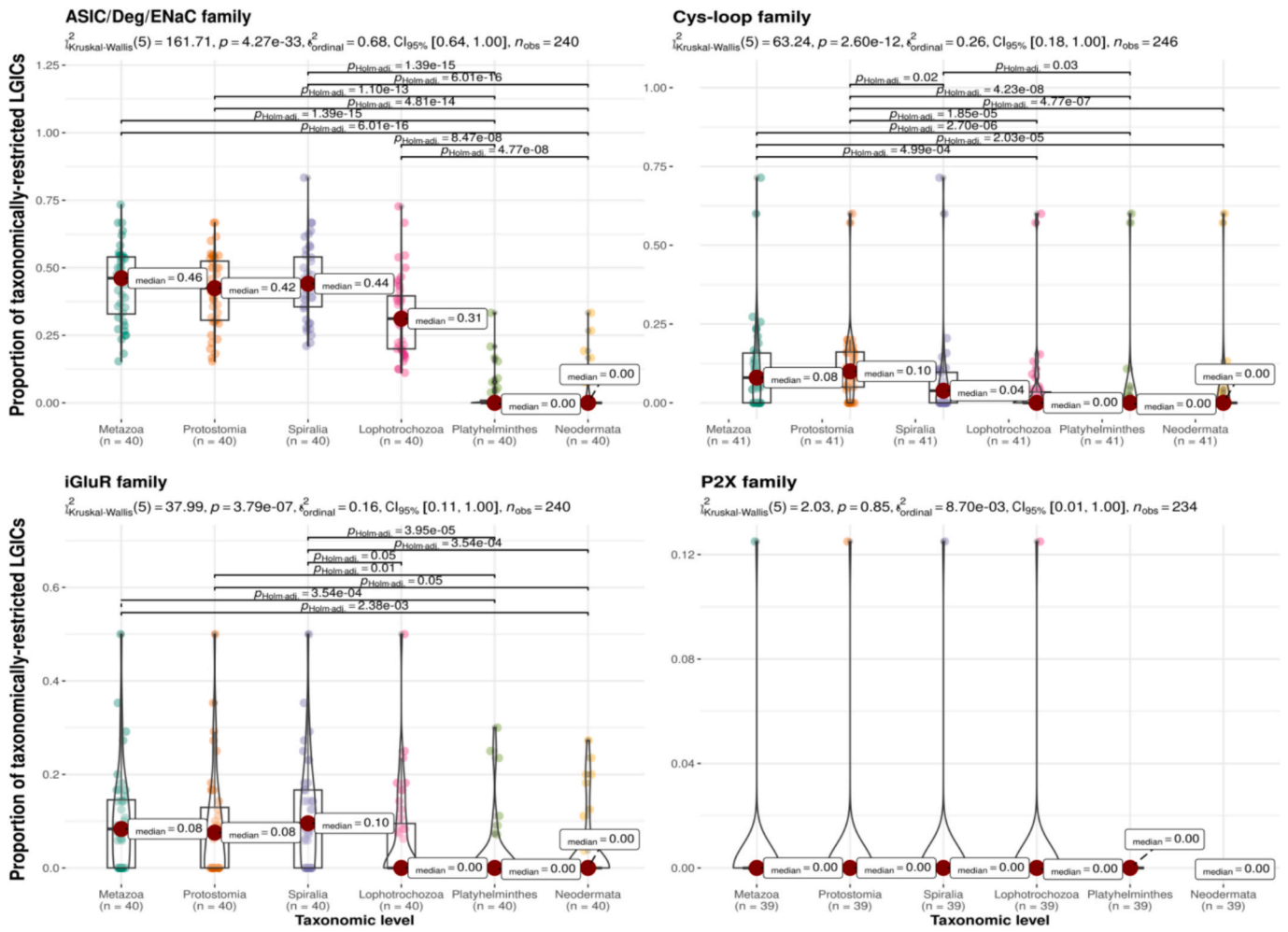

Fig. 2. Box-and-violin plots showing the proportion of taxonomically-restricted LGICs for each Neodermata species at each taxonomic level. Box elements show the median and the upper and lower quartiles. Red dots display the median. The Kruskal-Wallis statistic and the corresponding p-values are shown. P-values obtained from the post-hoc Dunn test were corrected using the Holm method for pairwise comparisons. Bars show significant comparisons (p-values < 0.05). The highest proportions indicate expansion processes with respect to a certain taxon, while the lowest values indicate fewer new proteins.

data filtering, phylogenetic analyses were performed for each cluster containing reference proteins. The CLANS analysis of cys-loop channels revealed 2 well-defined clusters: one containing acetylcholine (ACh) and serotoninergic (5-HT) channels and the other containing GABA channels (Fig. 3). Consequently, phylogenetic analyses were conducted for each cys-loop cluster independently. Sequences used in phylogenetic analyses are available in Supplementary File S2. A summary of the counts of all clusters obtained from annotation using phylogenetic trees for each taxon are presented in Supplementary Tables S11 and S12. Protein identifiers of each cluster obtained from the phylogenetic trees are presented in Supplementary Table S13 and Supplementary Figs. S4-S8.

Phylogenetic analyses of acetylcholine channels of the cys-loop family revealed the presence of six clusters containing proteins of Neodermata, all of which also contained proteins from free-living platyhelminths (Fig. 4 and Supplementary Fig. S4). Additionally, free-living platyhelminths contained three unique clusters not shared with Neodermata. A cluster containing proteins from Neodermata, free-living platyhelminths, and Lophotrochozoa (Inhibitory_AChR_Lop_Pla_Neo) included two ACCs from *S. mansoni* (Sm-ACC-1 and Sm-ACC-2), suggesting that this is an inhibitory ACh receptor cluster. Inhibitory_AChR_Lop_Pla_Neo represented the second-largest cluster of Neodermata LGIC members (Fig. 4). Our results did not identify a relationship between ACCs from *S. mansoni* and those of *C. elegans*, with this latter clustering more closely with GABA channels.

Two clusters containing proteins from Neodermata and free-living platyhelminths (ACh_Ecd_Lop_Pla_Neo and ACh_Ecd_Lop_Pla_Neo_2) showed a close phylogenetic relationship with human acetylcholine receptor subunits alpha 1–6, beta 2–4, gamma, delta, and epsilon. However, no proteins from Trematoda were observed in these clusters. Another cluster containing proteins from Neodermata and free-living platyhelminths (Ach_alpha7_Ecd_Lop_Pla_Neo) showed phylogenetic similarity to human acetylcholine receptor alpha 7. Proteins related to acetylcholine receptor alpha 7 were absent in Polyopisthocotylea, possibly due to the limited number of species from this parasitic group.

The 5-hydroxytryptamine 3 (5HT3) receptor subfamily was mostly found in Chordata species. However, two sequences from the free-living platyhelminth *M. lignano* were found to be homologous (5HTR-like_Pla_2). Also, a cluster containing proteins from Neodermata and free-living platyhelminths (5HTR-like_Lop_Pla_Neo), along with a cluster from free-living platyhelminths (5HTR-like_Pla) and some clusters from other metazoans, showed a close phylogenetic relationship with 5HT3 receptors, although they are not true orthologues. Among Neodermata proteins, no Cestoda proteins displayed close phylogenetic relationship to 5HT3 receptors. Experimental characterization would be necessary to confirm if these receptors respond to serotonin or other related compounds.

Phylogenetic analyses of CLANs containing GABA channels revealed the presence of four clusters containing proteins of Neodermata, three of them containing proteins of free-living platyhelminths (Fig. 5 and Supplementary Fig. S5). In addition, free-living platyhelminths presented

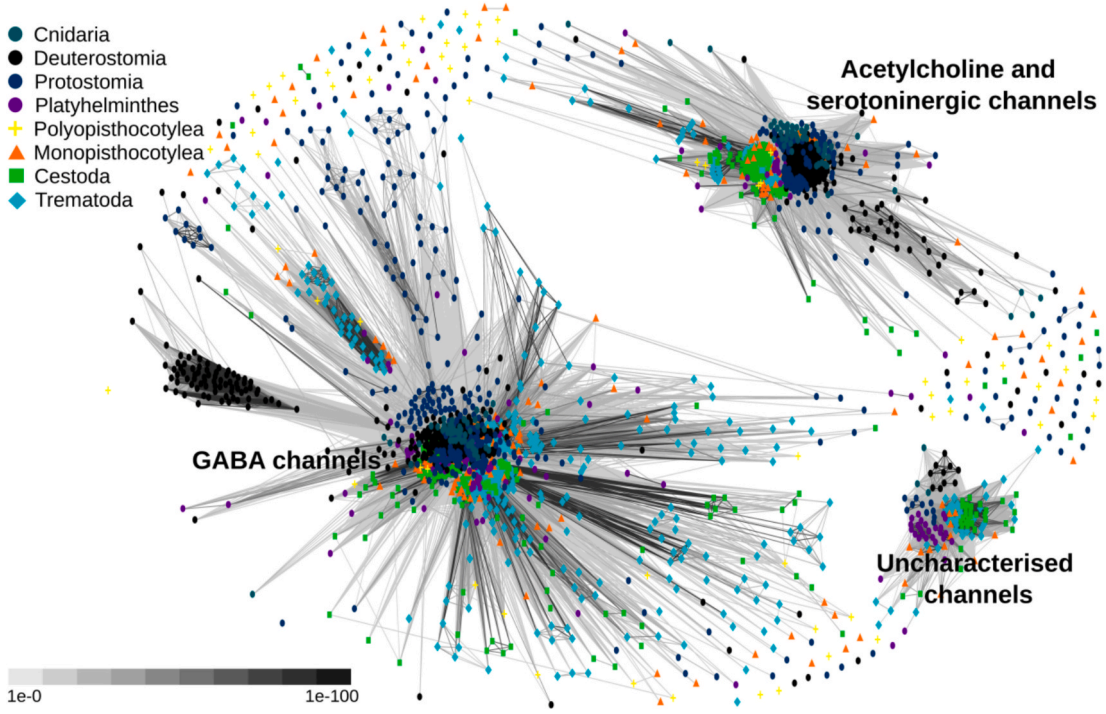

Fig. 3. Cluster map of cys-loop family receptors across Metazoa. Nodes correspond to individual channel sequences belonging to the cys-loop family and are colored by taxon as indicated by the legend. Edges correspond to BLAST connections with p-values $< 1e^{-30}$. The general locations of the acetylcholine and GABA channels from humans are indicated in the figure.

another cluster, not shared with Neodermata. A cluster containing proteins of Neodermata (only proteins of Monopisthocotylea), free-living platyhelminths, and other metazoans (Ecdysozoa and Lophotrochozoa) (GABA_alpha_gamma_Ecd_Lop_Pla_Neo) are closely-related with receptors alpha, gamma from humans (GABA_alpha_gamma_Amb_Cho_Cep). Some proteins of Monopisthocotylea grouped in a cluster (GABA_EXP-1_RDL_Ecd_Neo) with the protein EXP-1, a excitatory GABA receptor, from *C. elegans* and RDL from *D. melanogaster*. A cluster containing proteins of Neodermata (only proteins of Monopisthocotylea), free-living platyhelminths, and other metazoans (Ambulacraria, Ecdysozoa, and Lophotrochozoa) (GABA_beta_theta_Amb_Ecd_Lop_Pla_Neo) showed a close phylogenetic relationship with receptors beta, and theta from humans (GABA_beta_theta_Cho). Only the cluster GluCl_Pla_Neo, which also included proteins from free-living platyhelminths, contained proteins from the four major neodermatan lineages. This cluster included the SmGluClreceptors (SmGluCl-1, SmGluCl-2 and SmGluCl-3) from *S. mansoni* that have been experimentally characterized and demonstrated to be activated by glutamate. Notably, the results indicate the absence of GABA ionotropic receptors in most Neodermata, suggesting that this cluster likely consists of GluCl channels. Glycine receptors were only identified in Chordata species. Although a group of anionic glutamate receptors was present in Ecdysozoa, it was phylogenetically distinct from the anionic glutamate receptors found in platyhelminths.

The proteins of the ASIC/Deg/ENaC family formed two clear clusters in the CLANS analysis, which are consistent with the two clades in the phylogenetic analysis, ASIC and Deg/ENaC clades (Fig. 6; Supplementary Fig. S6). Within the ASIC clade, a cluster containing proteins from Neodermata (ASIC_Lop_Pla_Neo), as well as from free-living platyhelminths and Lophotrochozoa, was identified. Similarly, within the Deg/ENaC clade, a cluster was identified that included proteins from Neodermata and free-living platyhelminths (ENaC_Pla_Neo). ENaC_Pla_Neo stood out for containing the largest number of LGICs from Neodermata and free-living platyhelminths (448 proteins and 166 proteins, respectively) among all the LGIC clusters identified in this study. Additionally, it is the most phylogenetically distant cluster within the Deg/ENaC clade. Two other ENaC clusters were identified in free-living platyhelminths: FaNaC_FaNaC-like_Ecd_Lop_Pla and ENaC_Pla, where FaNaC_FaNaC-like_Ecd_Lop_Pla includes FMRFamide-gated sodium channels (FaNaCs). The FaNaC_FaNaC-like_Ecd_Lop_Pla cluster was annotated as such because it grouped genes previously identified as members of the FaNaC family, including *Octopus bimaculoides* (tr_A0A0L8FW45) and *Lottia gigantea* (tr_V4CL22), which have shown electrophysiological responses to FMRFamide and related peptides (Dandamudi et al., 2022).

Phylogenetic analyses of iGluR revealed the presence of nine clusters containing proteins of Neodermata, six of them also containing proteins from free-living platyhelminths (Fig. 7A and Supplementary Fig. S7). In addition, free-living platyhelminths presented other four clusters, not shared with Neodermata. Within the AKDF subfamily, a cluster containing proteins of Neodermata, free-living platyhelminths, and other metazoans (Ecdysozoa and Lophotrochozoa) (AMPA_Ecd_Lop_Pla_Neo) presented phylogenetic closeness to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors from humans (AMPA_Cep_Cho). In addition, a specific cluster of Neodermata proteins was identified (AMPA_Neo), which was also close to AMPA receptors. Notably, in both AMPA receptor clusters of Neodermata were absent proteins of Trematoda. A cluster containing proteins of Neodermata, free-living platyhelminths (KAINATE_Pla_Neo) presented phylogenetic closeness to Kainate receptors. Proteins from other metazoans (Ambulacraria, Cephalochordata, Ecdysozoa, and Lophotrochozoa) are also closely-related to KAINATE receptors. Phi receptors only were identified in Cephalochordata (phi_Cep) and delta receptors in Ambulacraria and Chordata, including Cephalochordata (Delta_Amb_Cep_Cho). In addition, within the AKDF subfamily, it was identified two clusters consisted of proteins from free-living platyhelminths and other Lophotrochozoa, but not from Neodermata (AKDF_Amb_Cep_Lop_Pla and AKDF_Lop_Pla). The epsilon subfamily was identified in Cnidaria, Cephalochordata, and a species of Monopisthocotylea Epsilon_Cni_Cep_Neo.

Within the N-methyl-D-aspartate (NMDA) subfamily, a cluster containing proteins of Neodermata, free-living platyhelminths, and other

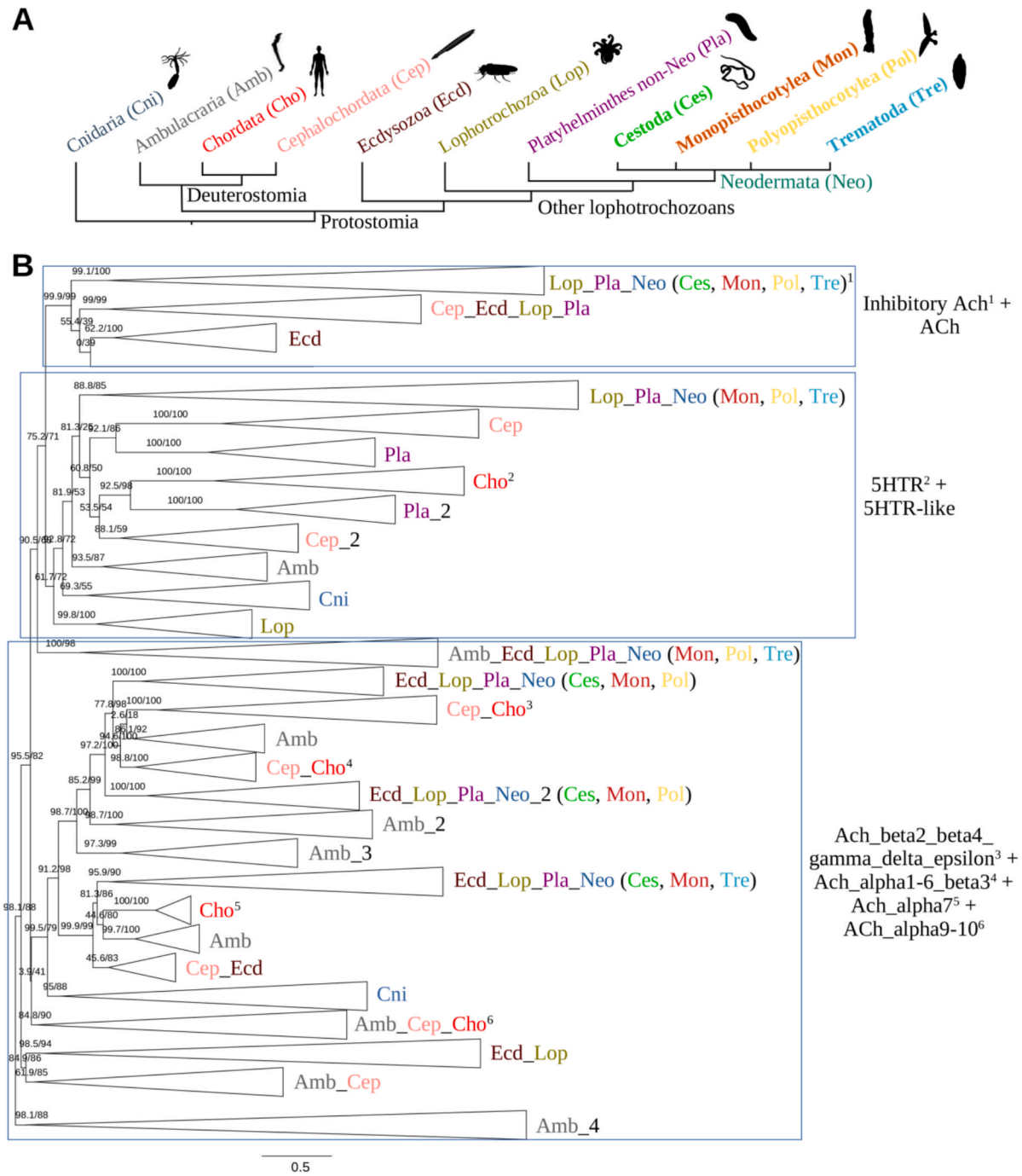

Fig. 4. Phylogenetic analysis of acetylcholine channels. A) Phylogeny of species used in the phylogenetic analyses. B) Phylogeny of acetylcholine channels from neodermatan species and proteins from other taxa including free-living platyhelminths. The branches of some clusters are collapsed. The midpoint-rooted phylogenetic tree was constructed using the SH-aLRT and ultrafast bootstrap with 1000 replicates. The WAG + F + R7 model was implemented. Each taxon is presented by a color.

metazoans (Ecdysozoa, and Lophotrochozoa) (NMDA1_Ecd_Lop_Pla_Neo) showed to be orthologues of the NMDA1 receptors from humans (NMDA1_Cep_Cho). Proteins from other metazoans (Ambulacraria and Cnidaria) also showed orthology to NMDA1 receptors. A cluster containing proteins of Neodermata, free-living platyhelminths, and other metazoans (Ecdysozoa, and Lophotrochozoa) (NMDA2_Ecd_Lop_Pla_Neo) is orthologous to NMDA2 receptors from humans (NMDA1_Cep_Cho). Other metazoan organisms (Ambulacraria and Cnidaria) also present orthologous sequences to the NMDA2 receptors. Proteins with phylogenetic relationships to NMDA3 receptors from humans (NMDA3_Cho) are not found in Neodermata but are observed in free-living platyhelminths (NMDA3_Pla and NMDA3_Ecd_Lop_Pla) and other metazoans such as Ambulacraria, Ecdysozoa, and Lophotrochozoa.

Additionally, the analysis identified a cluster containing proteins of Neodermata, free-living platyhelminths, and other Lophotrochozoa (Ion_rec_8a_25a-like_Lop_Pla_Neo) that showed homology to a cluster containing the ionotropic receptor 8a and 25a of *D. melanogaster* (Ion_rec_8a_25a_Ecd); and two clusters containing proteins of Neodermata and free-living platyhelminths (Ion_rec_21a_93a-like_Lop_Pla_Neo and Ion_rec_21a_93a-like_Pla_Neo) that presented phylogenetic relationship to a cluster containing the ionotropic receptors 21a and 93a of

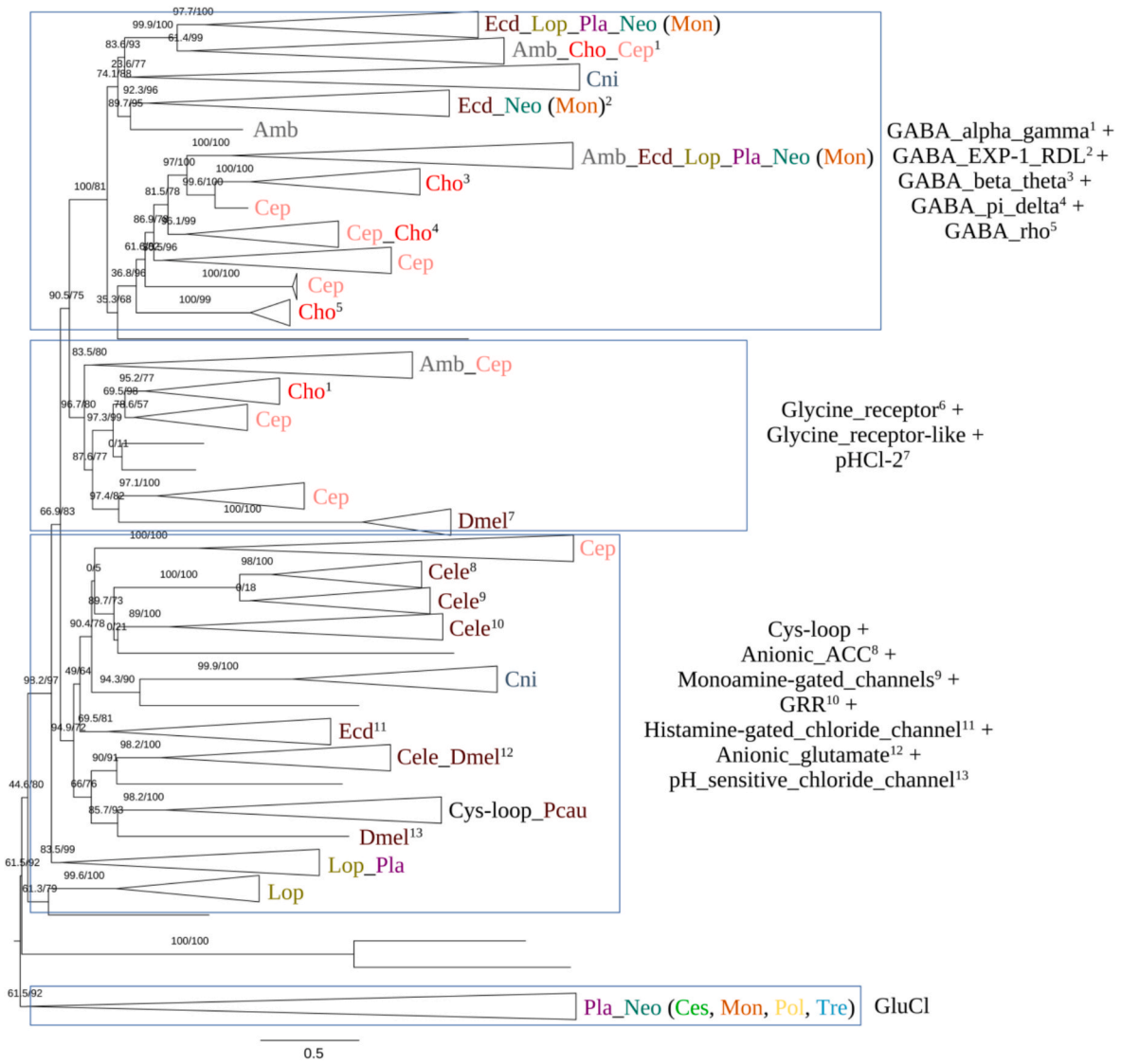

Fig. 5. Phylogeny of GABA channel sequences from neodermatan species and proteins from other taxa including free-living platyhelminths. The branches of some clusters are collapsed. The midpoint-rooted phylogenetic tree was constructed using the SH-aLRT and ultrafast bootstrap with 1000 replicates. The LG + R10 model was implemented. For details on the abbreviation of each taxon and its corresponding color, see Fig. 4A.

D. melanogaster (Ion_rec_21a_93a_Ecd_Lop).

Phylogenetic analysis of the P2X family produced shorter branch lengths than those found in the other LGIC families, and the proteins grouped mainly according to the phylogenetic proximity of each taxon, demonstrating a high level of conservation of these channels when compared to any other channel families. Platyhelminths proteins were the most distant among all taxa (P2X_Pla_Neo and P2X_Pla) and did not cluster with proteins from other Lophotrochozoa species as expected (Fig. 7B and Supplementary Fig. S8). Despite this, all proteins of the P2X family can be grouped into the same clade. Within the P2X_Pla_Neo cluster, the *S. mansoni* schP2X protein was identified (Agboh et al., 2004). A summary of the number of proteins identified in each LGIC family is presented in Fig. 8.

3.2.2.1. Cell-type-specific expression of LGIC's in Schistosoma mansoni. Following the identification of homologous sequences for LGIC genes in platyhelminths, we performed an expression analysis in the representative parasitic species *S. mansoni* to assess the transcriptional activity of these genes in specific cell types. For this analysis, we used the single-cell transcriptomic dataset from Wendt et al. (2020), which comprises 43,642 cells, categorized into 68 distinct cell types. We analyzed the dataset to examine the expression profiles of 35 LGIC genes across five receptor families: ASIC/Deg/ENaC, cys-loop ACh, cys-loop GABA, P2X, and iGluR. This approach enabled us to generate an analysis (Supplementary Table S8) detailing the expression levels of each LGIC across all cell types and for different sexes (males, females, and immature-females [virgin-females]). For this study, we focused on a distilled dataset representing gene expression in muscle (n = 8) and neuronal (n = 31) cell types, which were clustered together for analysis..

Our findings revealed distinct expression patterns in muscle and neuronal cell types (Fig. 9). Notably, ASICs, including Smp_346800, Smp_334250, Smp_144110, Smp_093210, Smp_083980, Smp_052630, and Smp_342000 (highlighted in blue), exhibited variable expression patterns across both muscle and neuronal populations, with Smp_093210 being the most upregulated in neurons. The P2X receptor (Smp_333600) was the only purinergic receptor with detectable expression at these thresholds, being strongly expressed in neuronal cells in males, females, and virgin females. Interestingly, this gene was also expressed in muscle cells, but only in male organisms. Among cys-loop acetylcholine receptors (highlighted in red), Smp_305740, Smp_139330, Smp_142700, Smp_037960, Smp_176310, and Smp_337330 were highly expressed in both neurons and muscle cells.

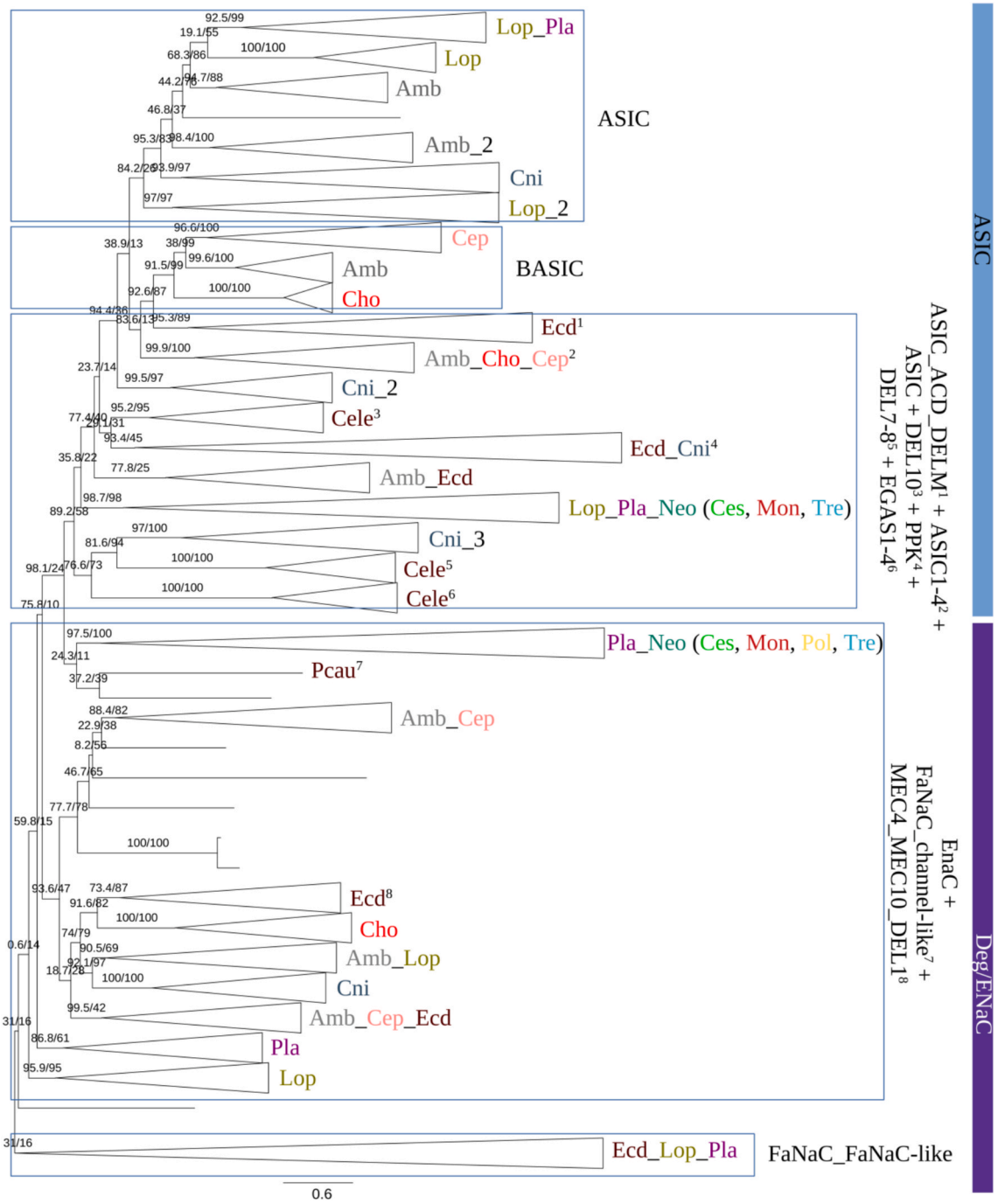

Fig. 6. Phylogeny of ASIC/Deg/ENaC channel sequences from neodermatan species and proteins from other taxa including free-living platyhelminths. The branches of some clusters are collapsed. The midpoint-rooted phylogenetic tree was constructed using the SH-aLRT and ultrafast bootstrap with 1000 replicates. The WAG + F + R9 model was implemented. For details on the abbreviation of each taxon and its corresponding color, see Fig. 4A.

Notably, Smp_337330 was downregulated in both neuron and muscle clusters in comparison to other cell types. Additionally, the ACh receptors Smp_306240 and Smp_157790 were exclusively expressed in neuronal cell types. Cys-loop GABA receptors (Smp_096480 (SmGluCl-1), Smp_015630 (SmGluCl-2), and Smp_334730, highlighted in green) also displayed neuronal expression patterns. Finally, only one ionotropic glutamate receptor (iGluR), Smp_140920, was expressed in neuronal populations of virgin females.

4. Discussion

The emergence of platyhelminths and neodermatans marks an important stage in evolutionary history, introducing distinct biological adaptations. To explore the role of LGICs (Ligand-Gated Ion Channels) in these transitions, we analysed their evolutionary dynamics in Neodermata through cluster-based and phylogenetic approaches. Our study focused on taxonomically restricted LGICs and the evolutionary patterns of cys-loop, ASIC/Deg/ENaC, iGluR, and P2X families.

We identified different evolutionary patterns in the neodermatan LGIC families, giving us clues on the molecular mechanisms behind their diversification. First, we observed an acquisition of a large number of taxonomically-restricted LGICs of the ASIC/Deg/ENaC family in platyhelminths (above 30 %). Secondly, a limited presence of taxonomically restricted LGICs was observed in the cys-loop, iGluR, and P2X families

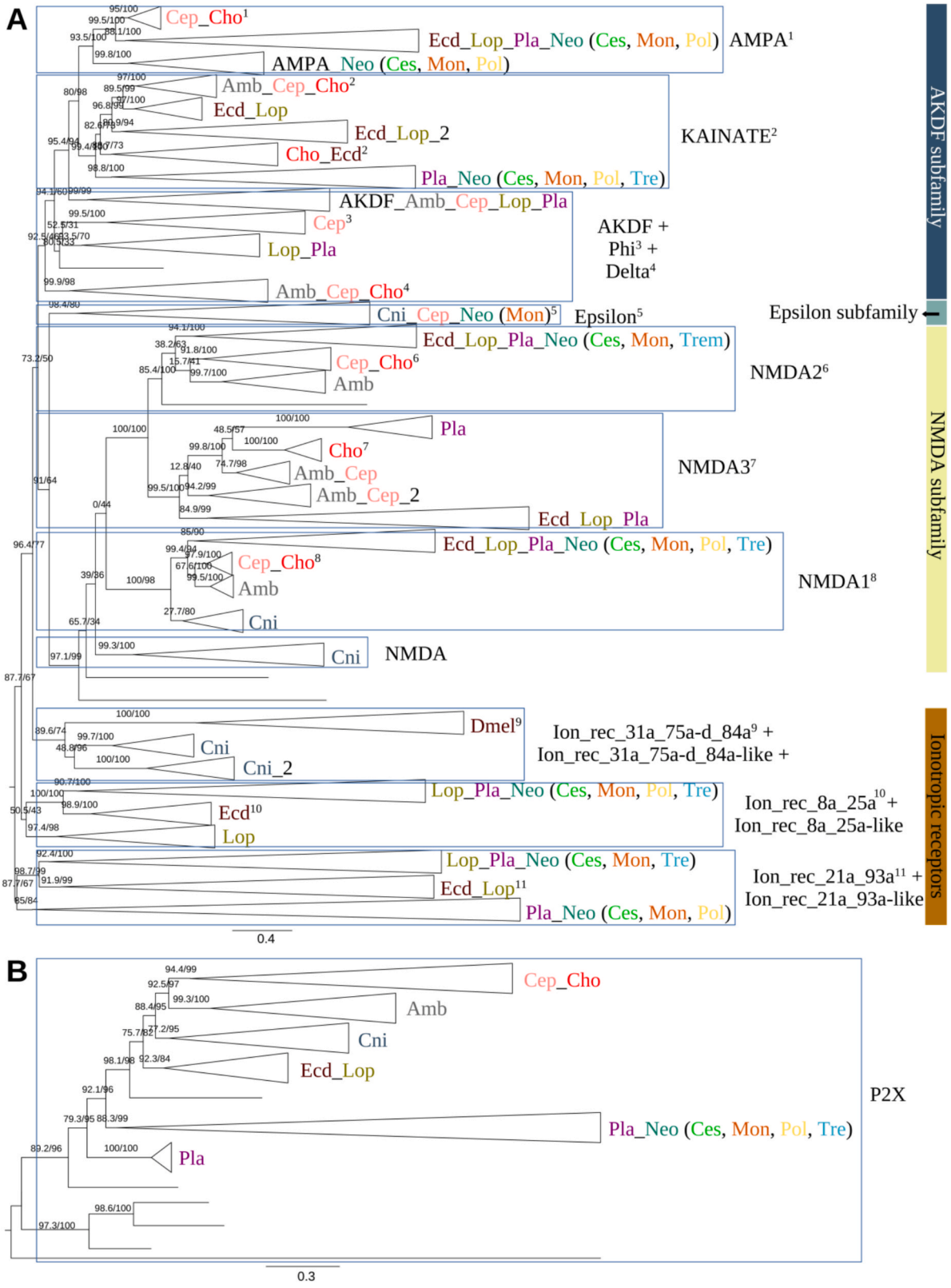

Fig. 7. A) Phylogeny of iGluR from neodermatan species and proteins from other taxa including free-living platyhelminths. The LG + R9 model was implemented. B) Phylogeny of P2X protein sequences from neodermatan species and proteins from other taxa including free-living platyhelminths. The WAG + F + R7 model was implemented. In both trees, the branches of some clusters are collapsed. The midpoint-rooted phylogenetic tree was constructed using the SH-aLRT and ultrafast bootstrap with 1000 replicates. For details on the abbreviation of each taxon and its corresponding color, see Fig. 4A.

Family	Cluster in trees	Ces	Mon	Pol	Tre	FLP	Lop	Ecd	Cep	Cho	Amb	Cni	Total Neo
ASIC family	ASIC*	32	10	-	31	24	25	2	-	-	11	29	73
	ASIC1-4	-	-	-	-	-	-	-	12	10	1	-	-
	ASIC_ACD_DELM	-	-	-	-	-	-	9	-	-	-	-	-
	BASIC	-	-	-	-	-	-	-	16	2	2	-	-
	DEL10	-	-	-	-	-	-	2	-	-	-	-	-
	DEL7 -8	-	-	-	-	-	-	2	-	-	-	-	-
	EGAS1-4	-	-	-	-	-	-	4	-	-	-	-	-
	ENaC*	133	60	26	229	169	8	1	14	8	36	7	448
	FaNaC_FaNaC-like	-	-	-	-	6	25	7	-	-	-	-	-
	FaNaC_channel -like	-	-	-	-	-	-	1	-	-	-	-	-
	MEC4_MEC10_DEL1	-	-	-	-	-	-	10	-	-	-	-	-
	PPK	-	-	-	-	-	-	34	-	-	-	1	-
Cys-loop family: Acetylcholine and 5HT receptor	5HTR	-	-	-	-	-	-	-	-	7	-	-	-
	5HTR-like	-	8	3	35	19	111	-	78	-	3	5	46
	ACh*	79	33	6	69	31	55	70	8	-	49	44	187
	ACh_alpha1 -6_beta3	-	-	-	-	-	-	-	7	15	-	-	-
	ACh_alpha7	5	18	-	41	8	3	16	1	4	2	-	64
	ACh_alpha9 -10	-	-	-	-	-	-	-	43	4	24	-	-
	ACh_beta2_beta4_gamma_delta_epsilon	-	-	-	-	-	-	-	11	13	-	-	-
	Inhibitory_AChR	67	29	9	169	23	19	-	-	-	-	-	274
Cys-loop family: GABA and Glycine channel	Anionic_ACC_channel	-	-	-	-	-	-	8	-	-	-	-	-
	Anionic_glutamate_Cys -loop	-	-	-	-	-	-	15	-	-	-	-	-
	Cys-loop*	-	-	-	-	3	23	-	28	-	-	6	-
	GABA*	-	-	-	-	-	-	-	7	-	-	-	-
	GABA_alpha_gamma	-	7	-	-	4	11	6	1	20	1	43	7
	GABA_beta_theta	-	4	-	-	3	5	4	1	7	1	-	4
	GABA_EXP-1_RDL	-	3	-	-	-	-	7	-	-	1	-	3
	GABA_pi_delta	-	-	-	-	-	-	-	19	5	-	-	-
	GABA_rho	-	-	-	-	-	-	-	-	6	-	-	-
	GluCl	118	30	8	82	19	-	-	-	-	-	-	238
	Glycine_receptor	-	-	-	-	-	-	-	-	10	-	-	-
	Glycine_receptor -like	-	-	-	-	-	-	-	16	-	44	-	-
	GRR	-	-	-	-	-	-	8	-	-	-	-	-
	Histamine-gated_chloride_channel_alpha1	-	-	-	-	-	-	5	-	-	-	-	-
	Monoamine-gated_channels pHCl-2	-	-	-	-	-	-	8	-	-	-	-	-
	pH_sensitive_chloride_channel	-	-	-	-	-	-	3	-	-	-	-	-
		-	-	-	-	-	-	1	-	-	-	-	-
iGluR Family	AKDF*	-	-	-	-	6	15	-	6	-	2	-	-
	AMPA	23	10	4	-	2	15	7	2	8	-	-	37
	Delta	-	-	-	-	-	-	-	29	4	3	-	-
	Epsilon	-	1	-	-	-	-	-	7	-	-	3	1
	Ion_rec_21a_93a	-	-	-	-	-	28	24	-	-	-	-	-
	Ion_rec_21a_93a -like	65	33	8	66	43	7	-	-	-	-	-	172
	Ion_rec_31a_75a -d_84a	-	-	-	-	-	-	7	-	-	-	-	-
	Ion_rec_31a_75a -d_84a -like	-	-	-	-	-	-	-	-	-	-	6	-
	Ion_rec_8a_25a	-	-	-	-	-	2	4	-	-	-	-	-
	Ion_rec_8a_25a-like	4	5	1	30	6	10	-	-	-	-	-	40
	KAINATE	25	6	6	39	9	7	18	2	10	1	-	76
	NMDA	-	-	-	-	-	-	-	-	-	-	4	-
	NMDA1	27	4	2	12	1	3	3	2	2	2	2	45
	NMDA2	17	3	-	16	3	7	4	5	7	3	-	36
	NMDA3	-	-	-	-	7	7	1	2	4	2	-	-
	phi	-	-	-	-	-	-	-	12	-	-	-	-
P2X family	P2X	59	25	3	83	18	2	1	1	13	5	3	170

Fig. 8. Summary of the number of proteins found in each LGIC family. For details on the abbreviation of each taxon and its corresponding color, see Fig. 4A.

(less than 10 % in each family). These evolutionary patterns shed light on the complex processes that have shaped the LGIC families in Platyhelminthes, laying the groundwork for deeper studies on their functional implications in these organisms. Finally, we mined available single-cell sequencing data to examine the expression of these genes in a representative platyhelminth species, the parasitic *S. mansoni*. This analysis provides insights into the cell-type specificity and functional significance of LGICs in neodermatan parasites.

4.1. *ASIC/Deg/ENaC family evolution*

The ASIC/Deg/ENaC family exhibited remarkable sequence divergence between platyhelminths and other metazoan taxa evident in the high proportion of taxonomically restricted LGICs, as well as in the phylogenetic analysis. Our phylogenetic analysis revealed two main clades, ASIC and Deg/ENaC channels, as observed in previous studies (Aguilar-Camacho et al., 2023; Elkhatib et al., 2023). Neodermata presented clusters of proteins in both main clades (ASIC_Lop_Pla_Neo and ENaC_Pla_Neo, respectively), with ENaC_Pla_Neo standing out as the best-represented cluster in platyhelminths (614 proteins) and being specific to this taxon with a clear phylogenetic separation from other metazoan clusters. The acquisition of taxonomically-restricted LGICs may reflect the evolutionary trajectory of platyhelminths, which have colonized diverse environments, from marine, freshwater, and terrestrial ecosystems (Noreña et al., 2015) to parasitic niches (Collins et al., 2017). Lineage-specific expansions of protein families are key to such adaptations (Lespinet et al., 2002; Aguilera et al., 2013). ENaC channels, which are present in metazoans and different single-celled eukaryotes (Studer et al., 2011; Elkhatib et al., 2023), are crucial for osmoregulatory adaptations in vertebrates and invertebrates (Chen et al., 2010; Wichmann and Althaus, 2020). For example, ENaCs enabled tetrapods to thrive in low-salinity environments (Wichmann and Althaus, 2020), while invertebrates like *Drosophila* use ASIC receptors for salt and food detection and water-related behaviors (Liu et al., 2003; Chen et al., 2010). In *C. elegans* these channels help to detect tactile stimuli (O'Hagan et al., 2005).

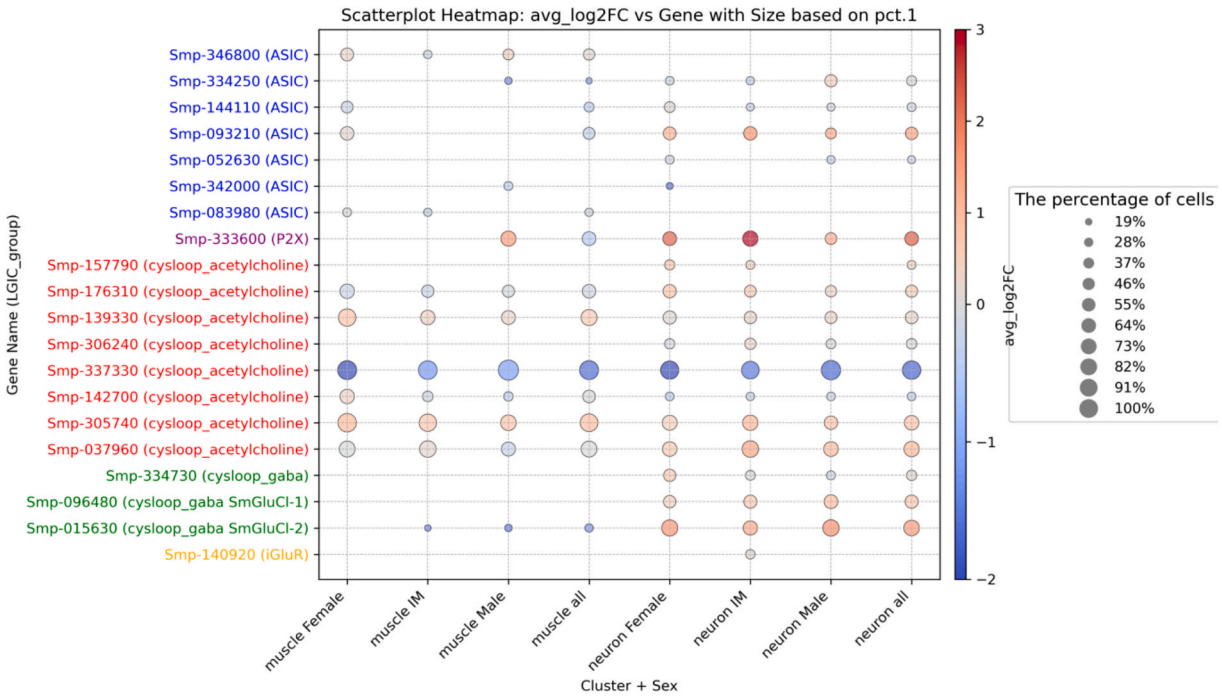

Fig. 9. Muscle and neuron cell-type expression of LGICs in *S. mansoni.* Heatmap depicting cell-specific expression of LGICs in *S. mansoni*. Genes are grouped by receptor type: ASICs (blue), P2X (purple), cys-loop acetylcholine receptors (red), cys-loop GABA receptors (green), and iGluRs (orange). Dot size represents the percentage of cells expressing each gene, while color indicates the log2 fold-change of the average expression between the assessed cell type (muscle or neuronal) and other cell types. Muscle and neuronal cells were further stratified by sex into males, females, and immature females (virgin females). Expression levels were compared using the Wilcoxon rank-sum test, with analysis restricted to genes expressed in at least 1% of cells within each cluster and a minimum log fold-change (LogFC) of 0.1 between clusters. For this figure, muscle and neuronal cell clusters were pooled; detailed expression data for specific neuron and muscle clusters are provided in Supplementary Table S8.

The absence of Neodermata proteins in several clusters (ASIC_Lop_Pla and FaNaC_FaNaC-like_Ecd_Lop_Pla) consisting of free-living platyhelminth and other metazoan proteins (Lophotrochozoa and/or Ecdysozoa) suggests that the parasitic lifestyle may have also led to gene loss events. However, our findings highlight that, despite these gene losses, certain protein groups have undergone significant expansions in Neodermata. While parasites commonly undergo genome reduction events, shedding genes that are no longer essential in their new environment, they still retain a substantial proportion of genes shared with free-living species (Jackson, 2015).

4.2. *P2X family evolution*

Our results show strong conservation of neodermatan LGICs within the P2X family, supported by traceable homologs across metazoans and short branch lengths in phylogenetic trees. This reflects the ancient origin of P2X receptors, dating back to early eukaryotes and found in fungi, plants, and protists (Verkhratsky and Burnstock, 2014). The strong conservation of P2X receptors is likely due to their unique activation by ATP (Hattori and Gouaux, 2012), a molecule critical for basic cellular functions, placing these receptors under strong purifying selection.

A notable observation is that P2X proteins predominantly cluster according to the phylogenetic proximity of each taxon, which may be linked to the degree of conservation within this family (Fig. 8B). However, platyhelminth P2X receptors did not group with other Lophotrochozoa, instead appearing as the most distant metazoan group, likely due to long branch attraction from higher substitution rates. Within the P2X_Pla_Neo cluster, we identified schP2X from *S. mansoni* (Agboh et al., 2004), which grouped with Neodermata and free-living platyhelminths, suggesting functional conservation in Platyhelminthes.

4.3. *Cys-loop family evolution*

Although phylogenetic analyses suggest an expansion of cys-loop receptors in Neodermata, they still show a close phylogenetic relationship with proteins from other metazoans, reflected in the limited acquisition of proteins restricted taxonomically to Neodermata. This pattern could be related to the ancient origins of the cys-loop family, similar to the proposed for the P2X family. It has been postulated that channels of this family arose in eukaryotes through horizontal gene transfer from prokaryotes before the emergence of the metazoan lineage (Tasneem et al., 2005). CLANS analysis split the cys-loop family into two groups: nAChRs and 5HT3 receptors, and GABA/glycine receptors, consistent with vertebrate classifications of cation-selective excitatory (ACh, 5HT3) and anion-selective inhibitory (GABA, glycine) receptors (Ortells and Lunt, 2005; Dent, 2006). Invertebrates, however, exhibit a wider diversity, including excitatory GABA receptors and inhibitory nAChRs and glutamate receptors (Dent, 2006), all of which were also found in Neodermata.

4.3.1. *ACH and 5HT3 receptors*

Although previous studies have evidenced the presence of 5HT3 receptors only in vertebrates (Preza et al., 2018), our study found that some clusters containing proteins from Neodermata, free-living platyhelminths, Lophotrochozoa, Ecdysozoa, and Cnidaria showed clustering to these receptors (5HT3-like receptors). Rao et al. (2023) similarly identified 5HT3-like receptors in invertebrates. However, it's worth noting that there were differences in protein annotations between the two studies. For example, the proteins we identified as 5HT3-like were not reported by Rao et al. (2023). In contrast, the proteins they annotated as 5HT3—such as Mlig023547g1, Mlig010174g1, and Mlig020283g2—grouped with other cys-loop proteins in our analysis, specifically with Inhibitory_AChR_Lop_Pla_Neo, ACh_Ecd_Lop_Pla_Neo,

and ACh_alpha7_Ecd_Lop_Pla_Neo, respectively. These differences likely stem from variations in the methodologies employed to identify these receptors. The presence of proteins with phylogenetic relationship to 5HT3 receptors suggests that evolutionary traces of their function could persist. An experimental functional characterization is the only way to know if these receptors will be activated by serotonin (5-hydroxytryptamine: 5HT). Serotonin (5HT) is a neurotransmitter that regulates diverse physiological and behavioral processes, including cell division, neuronal migration, emotion, cognition, and pain perception (Berger et al., 2009). In vertebrates, 5HT receptors comprise seven types (5HT1-5HT7), with six being GPCRs and only 5HT3 being ionotropic (Hannon and Hoyer, 2008). Serotonin primarily signals through GPCRs, as evidenced in *S. mansoni,* where Sm5HTR mediates serotonin's motor effects (Patocka et al., 2014). Notably, cestodes lack 5HT3-like receptors, suggesting adaptations to their parasitic lifestyle and potential divergence in serotonin signaling. This absence raises questions about whether invertebrates rely exclusively on GPCRs for 5HT signaling, highlighting the need for further investigation into ligand specificity and functionality.

Similar to other invertebrates, acetylcholine receptors were identified in Neodermata, including both cation-selective and anion-selective nAChRs (Preza et al., 2018). Particularly, the identified anionic nAChR cluster included proteins from Neodermata, free-living platyhelminths, and other lophotrochozoans (Inhibitory_AChR_Lop_Pla_Neo), including the inhibitory nAChRs Sm-ACC-1 and Sm-ACC-2 from *S. mansoni* and LnAChR B, F, I, and K from *Lymnaea stagnalis* (van Nierop et al., 2005; MacDonald et al., 2014). While in vertebrates, nAChRs are typically cation-selective and mediate excitatory responses, in lophotrochozoans, such as mollusks, experimental studies have demonstrated the coexistence of two types of nAChR: excitatory cation-selective nAChRs and inhibitory anion-selective nAChRs (van Nierop et al., 2005). Similarly, some anion-selective nAChRs also have been characterized in the neodermatan *S. mansoni* (MacDonald et al., 2014). The cluster Inhibitory_AChR_Lop_Pla_Neo not only appears to be conserved among lophotrochozoans but is also one of the most well-represented groups. This highlights the significance of these receptors in lophotrochozoan species, underscoring their evolutionary and functional importance in the regulation of inhibitory cholinergic signaling. Additionally, our study did not identify a relationship between ACCs of *S. mansoni* (Sm-ACC) and those of *C. elegans*, showing that they likely descended from different ancestral lineages, consistent with the findings of van Nierop et al. (2005).

Analysis of cation-selective nicotinic acetylcholine receptor (nAChR) clusters revealed a pattern of receptor loss across major neodermatan lineages. Notably, the ACh_Cep_Ecd_Lop_Pla cluster lacked Neodermata proteins, despite containing representatives from Lophotrochozoa (e.g., free-living platyhelminths), Ecdysozoa, and Cephalochordata. This cluster includes the LnAChR H protein from Lymnaea stagnalis, a cation-selective nAChR (van Nierop et al., 2005). Its proximity to anion-selective nAChRs suggests a potential evolutionary shift toward anion selectivity within Lophotrochozoa, highlighting functional diversification in this receptor family.

Further analysis identified additional clusters with lineage-specific protein losses. The ACh_Amb_Ecd_Lop_Pla_Neo cluster lacked Cestoda proteins, while the ACh_Ecd_Lop_Pla_Neo and ACh_Ecd_Lop_Pla_Neo_2 clusters, phylogenetically related to human acetylcholine receptors (α1-6, β2-4, γ, δ, ε), showed no trematode proteins. Similarly, the Ach_alpha7_Ecd_Lop_Pla_Neo cluster, related to human α7 receptors, lacked Polyopisthocotylea proteins. All clusters comprised cation-selective nAChRs, including LnAChR A, C, D, E, and G from *L. stagnalis* (van Nierop et al., 2005). These losses reflect evolutionary divergence in platyhelminths, suggesting functional or evolutionary adaptations in cholinergic signaling across lineages.

4.3.2. GABA and glycine receptors

Phylogenetic analysis revealed that GABA receptors from various metazoans, including platyhelminths, cluster closely with human α, γ, β, and θ GABA receptors. Within Neodermata, only Monopisthocotylea proteins exhibited this phylogenetic proximity (GABA_alpha_-gamma_Ecd_Lop_Pla_Neo), consistent with findings by Preza et al. (2018) in *Gyrodactylus salaris*. The absence of these receptors in Cestoda, Polyopisthocotylea, and Trematoda suggests distinct neurotransmission mechanisms within Neodermata, highlighting key evolutionary divergences.

Monopisthocotylea proteins also clustered with the *C. elegans* EXP-1 receptor (GABA_EXP-1_RDL_Ecd_Neo), a GABA-gated cation channel involved in excitatory neuromuscular signaling (Beg and Jorgensen, 2003). This suggests that Monopisthocotylea may have retained or modified specific functional features of GABA receptors. Further studies are needed to determine whether these receptors mediate both inhibitory and excitatory GABAergic functions, as observed in *C. elegans* (Bamber et al., 1999; Beg and Jorgensen, 2003). Additionally, a cluster containing proteins from all major neodermatan lineages and free-living platyhelminths, along with the *S. mansoni* SmGluCl receptors, underscores the significance of GluCl receptors in flatworm neurotransmission. These receptors, absent in vertebrates but widespread in invertebrates (Putrenko et al., 2005; Dufour et al., 2013; Preza et al., 2018), possess a conserved glutamate-binding arginine residue essential for function (Dufour et al., 2013). The widespread conservation of this residue across flatworms suggests a fundamental role for glutamate in both excitatory and inhibitory neurotransmission.

4.4. iGluR family evolution

Neodermatan iGluR proteins are widely conserved across distant taxa, serving as crucial postsynaptic receptors for synaptic plasticity (Grant, 2016). This aligns with Ovsepian and Vesselkin (2014), who reported stable receptor numbers co-opted for various functions throughout evolution. These channels are present not only in metazoans but also in plants and prokaryotes, highlighting their biological significance (Tikhonov and Magazanik, 2009; Croset, 2010; van Giesen and Garrity, 2017).

Phylogenetic analysis revealed Neodermatan proteins clustering with AMPA, epsilon, KAINATE, NMDA1, NMDA2, and ionotropic receptors of *D. melanogaster*. While Ramos-Vicente et al. (2018) identified DELTA LGICs in Lophotrochozoa, these were absent from this analysis, as were Phi receptors in both Lophotrochozoa and Ecdysozoa. AMPA receptors in Neodermata formed two distinct clusters, one lacking free-living platyhelminths and both showing an absence of trematode proteins, suggesting evolutionary expansion, specialization, and lineage-specific losses.

The epsilon subfamily was found in Cnidaria, Cephalochordata, and a single Neodermatan species (Monopisthocotylea: *S. longicornis*), though its presence in Neodermata remains uncertain due to limited evidence (Ramos-Vicente et al., 2018). Additionally, Neodermatan proteins showed phylogenetic proximity to *D. melanogaster* ionotropic receptors 8a, 21a, 25a, and 93a, which are involved in sensory perception and thermoregulation (Croset et al., 2010; Knecht et al., 2016, 2017; Ni et al., 2016; Greppi et al., 2020). Given their conservation in Lophotrochozoa and Ecdysozoa, these receptors may play similar roles in non-arthropods, making them valuable for studying parasite control strategies.

Despite their broad conservation, only receptor 25a has been previously identified in Lophotrochozoa (Croset et al., 2010; Abuin et al., 2011). Its ancestral nature, resembling both NMDA and non-NMDA iGluRs, likely explains its persistence across Protostomia (Croset et al., 2010). In contrast, ionotropic receptors 31a, 75a, and 84a appear exclusive to arthropods (Croset et al., 2010). The absence of Ion_rec_21a_93a-like proteins in trematodes and polyopisthocotyleans suggests functional divergence, warranting further research into how these parasites have adapted their signaling systems for survival.

4.5. Cell-type expression analysis of LGICs

Our expression analysis of LGICs in *S. mansoni* provides insights into their potential physiological roles within the parasite. Expression data is essential in confirming the presence and functional relevance of these genes, as mere genomic identification does not guarantee their biological activity. By assessing gene expression patterns, we aimed to identify potential physiological roles and cellular contexts in which these LGICs might function. The widespread expression of ASICs across both muscle and neuronal populations suggests a role in neuromuscular signaling. The notable upregulation of Smp_093210 in neurons indicates potential involvement in neuronal excitability or sensory transduction.

The P2X receptor (Smp_333600) was the only purinergic receptor with detectable expression. Its strong expression in neuronal cells across males, females, and virgin females implies a conserved role in neuronal communication. Interestingly, its exclusive expression in male muscle cells suggests a sex-specific function, possibly related to male-specific behaviors or physiological processes. Sex differences in P2X receptor expression have been observed in other species. For instance, in rats, P2X4 receptor expression in spinal microglia is upregulated following nerve injury in males but not in females, indicating a sexually dimorphic role in pain signaling (Mapplebeck et al., 2018). Additionally, studies have reported sexual dimorphisms in the expression of various P2X receptors in microglia, with certain receptors exhibiting higher expression in males and others in females (Crain et al., 2009). These findings suggest that sex-specific expression of P2X receptors may be a conserved phenomenon across species, warranting further investigation in *S. mansoni.* Among cys-loop acetylcholine receptors, the high expression of receptors such as Smp_305740, Smp_139330, Smp_142700, Smp_037960, and Smp_176310 in both neurons and muscle cells indicates their involvement in cholinergic signaling pathways, essential for muscle contraction and neural communication (MacDonald et al., 2014). The downregulation of Smp_337330 compared to other receptors may suggest a more specialized or regulated role. The exclusive neuronal expression of Smp_306240 and Smp_157790 points to specific functions in neural transmission. Cys-loop GABA receptors (Smp_096480, Smp_015630, and Smp_334730) displayed neuronal expression patterns. Notably, while Smp_096480 and Smp_015630 cluster within the cys-loop GABA family of receptors, and are phylogenetically related to this channels. Experimental studies have demonstrated that they function as glutamate-gated chloride channels (GluCl-1 and GluCl-2, respectively), rather than GABA receptors. This indicates that, despite their classification, they are activated by glutamate and mediate inhibitory neurotransmission, contributing to neuronal excitability modulation and neural circuit balance, similar to cys-loop GABA receptors in other species (Beg and Jorgensen, 2003; Dufour et al., 2013). The expression of the iGluR Smp_140920 in neuronal populations of virgin females may indicate a role in excitatory neurotransmission, potentially linked to reproductive or developmental processes unique to this group.

These findings highlight potential avenues for further research into the functional roles of LGICs in neodermatans such as *S. mansoni*. Our data demonstrate that these receptors are not only present in the genome but are also actively expressed in specific cell types, providing a crucial first step towards understanding their roles in parasite physiology. This work serves as a foundation for future studies exploring their potential as drug targets, ultimately contributing to the development of novel therapeutic strategies against neodermatan parasites.

5. Conclusion

This research, through clustering, phylogenetic, and conservation analyses, provides valuable information for a deeper understanding of the evolutionary relationships and functions of LGICs in Neodermata. We observed different distribution patterns among LGICs in neodermatan species at various taxonomic levels. The distinct distribution pattern suggests potential variations in the evolutionary forces that have shaped LGICs across these taxonomic divisions, underscoring the divergence in their functional roles or regulatory mechanisms. Our phylogenetic and taxonomically-restricted LGICs analyses unveiled the proportion of LGICs shared across different taxonomic levels and those restricted to specific taxa, suggesting the presence of evolutionary innovations unique to neodermatan and non-neodermatan platyhelminth species. Unveiling the evolutionary dynamics of these receptors represents the initial phase in characterizing ion channels in neodermatans and exploring LGICs' potential as targets for novel antiparasitic drugs.

CRediT authorship contribution statement

Víctor Hugo Caña-Bozada: Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ahmed A.Z. Dawoud:** Writing – review & editing, Visualization, Software, Methodology. **Ivana Ramos de la Cruz:** Writing – review & editing, Visualization, Software, Methodology. **Lizeth C. Flores-Méndez:** Writing – review & editing, Visualization. **Josué Barrera-Redondo:** Writing – review & editing, Methodology. **Jesús Briones-Mendoza:** Writing – review & editing. **Luis A. Yañez-Guerra:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2025.114718.

Data availability

Supplementary Tables, the Supplementary Figures, and Supplementary Files are available in Supplementary Materials and at https://github.com/victorcana/Supplementary_LGIC_Platyhelminthes

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