

*Supplementary Materials*

# Molecular Characterization and Expression Analysis of Class C (Glutamate Family) G Protein-Coupled Receptors in Ascidian *Styela clava*

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130 140 150 160 170 180 190 200 210 220 230 240

Hs-GRM1 HTLDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--RDERD--GINRCLPDG--QSLPPGRTKKHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Hs-GRM2 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--ADGSR--HICPGDG--YATHGDAPTAICGVIGSGSSSVIAICVNLLQLFNIPQIS

Hs-GRM3 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--VDEAE--YICPGDG--YAIQENPIPLIACGVIGSGSSSVIAICVNLLQLFNIPQIS

Hs-GRM4 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDGTE--VFCGGG--PPIITKP-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Hs-GRM5 HTJLRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--EEEE--GLVRCVD--GSSSFRRSKKHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Hs-GRM6 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--DDEVG--VRCGGV--PPLRAPPEFVVVAICASSSVIAICVNLLQLFNIPQIS

Hs-GRM7 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDTSV--VRCNGE--PEVVFVK-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Hs-GRM8 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDASD--VRCANGD--PPIITKP-DRISGVIGASSSSVIAICVNLLQLFNIPQIS

Ms-GRM1 HTLDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--RDERD--GINRCLPDG--QTLPPGRTKKHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Ms-GRM2 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--ADGSR--HICPGDG--YATLSADAPTAICGVIGSGSSSVIAICVNLLQLFNIPQIS

Ms-GRM3 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--VDEAE--YICPGDG--YAIQENPIPLIACGVIGSGSSSVIAICVNLLQLFNIPQIS

Ms-GRM4 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDGTE--VFCGGG--PPIITKP-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Ms-GRM5 HTJLRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--EEEE--GLVRCVD--GSSSFRRSKKHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Ms-GRM6 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--DDEAS--VRCGGV--PPLRAAPPEFVVVAICASSSVIAICVNLLQLFNIPQIS

Ms-GRM7 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDTSV--VRCNGE--PEVVFVK-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Ms-GRM8 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDASD--VRCANGD--PPIITKP-DRISGVIGASSSSVIAICVNLLQLFNIPQIS

Dr-GRM1 HTLDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--RDERE--GPKVCGEN--PSAQPPDKKHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Dr-GRM2 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--VDGSE--YICPGDG--YAIHDDPLTAICGVIGSGSSSVIAICVNLLQLFNIPQIS

Dr-GRM3 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--VDGTE--FICPGDG--YALQDPSPLTAICGVIGSGSSSVIAICVNLLQLFNIPQIS

Dr-GRM4 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--RDGTD--VFCGGG--PPIITKP-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Dr-GRM5a HTLDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--EEEE--GMARCTE--GGGTPMKGKHHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Dr-GRM5b HTLDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--DESEEEAGGARCG--PGATPMKGKHHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Dr-GRM6a FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDTSV--IRGSGE--QPIITKP-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Dr-GRM6b FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDNSD--VFCNGE--PPIITKP-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Dr-GRM7 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDTSV--VRCNGE--PEVVFVK-ERVVGVIGASSSSVIAICVNLLQLFNIPQIS

Dr-GRM8a FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDGSD--VRCANGD--QPIIFAKP-DRISGVIGASSSSVIAICVNLLQLFNIPQIS

Dr-GRM8b FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDSTD--VFCASGE--QPIIFAKP-DRISGVIGASSSSVIAICVNLLQLFNIPQIS

Fm-GRM2 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--VEETE--FVCPGDG--YAVQCKRPLTAICGVIGSGSSSVIAICVNLLQLFNIPQIS

Fm-GRM3 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--VDGTD--FVCPGDG--YAIHGNGAHHIACVIGSGSSSVIAICVNLLQLFNIPQIS

Fm-GRM5 HTLDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFRDLSLISI--GDPKDAAAAAANAANAAPGVGRCQAPVAKRHHIACVIGSGSSSVIAICVNLLQLFNIPQIA

Fm-GRM8 FALDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--RDTSV--VRCNGE--AEVFTKP-ERISGVIGASSSSVIAICVNLLQLFNIPQIS

Cr-GRM3-1 YTIKRVNDVIMLPVLTITIDEMADCTIDTFAIRKIVQNYLPQQLSQ--QSTRNNSYKCVIGSGSSSVIAICVNLLQLFNIPQIS

Cr-GRM3-2 YTVQKRVSYNEMNITLGRFIEDTQNSQIALSTLGVLPKQCNHTHP--VCFPEPCPSIAGVIGPESSVIALSLAHNALLTETIS

Cr-GRM4

Cs-GRM3 FALDRIADPELLPVLRLGARILDTCSRDTYALEQSLIEFVHASLTK--LNDP--EPRLICGVIGASSSSVIAICVNLLQLFNIPQIS

Cs-GRM4 MILDVNRDRKILPEMELGTYMIDTAMSHITIGQIVTEFIPLFSEAT--TSCSTS--KTLSKK-SMFAIGASSSSVIAICVNLLQLFNIPQIS

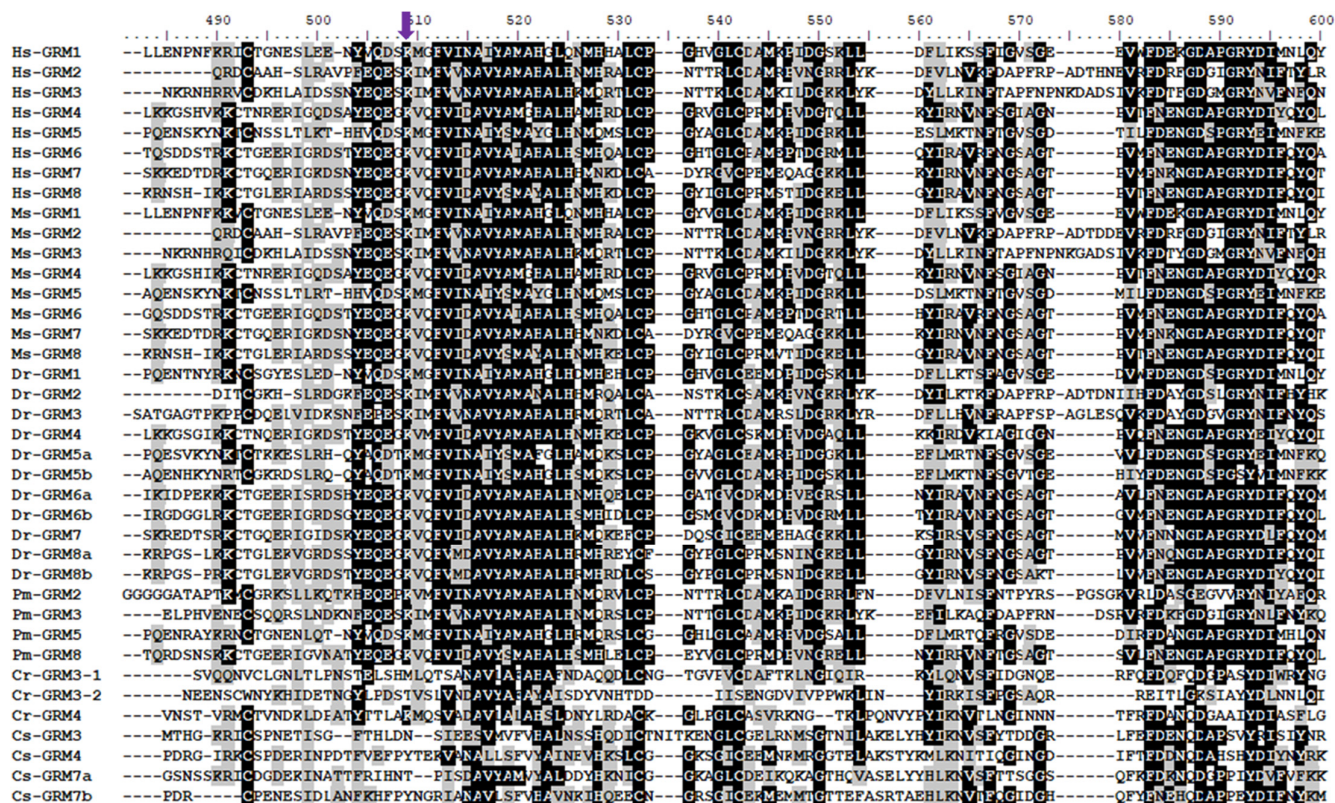
Cs-GRM7a MILDRIADPELLNITLGCEIIDSCHSSVALEQSLIEFVHASLTK--ICQGN--SDNDSKHHILTAIGASSSSVIAICVNLLQLFNIPQIS

Cs-GRM7b MVVDVNRDENILPEITIGTIGTMINNRSGLIYIMQVINEFFPLSDLIQ--QCEQVN--FTNCKKPLFAGIAGASSSVIAICVNLLQLFNIPQIS



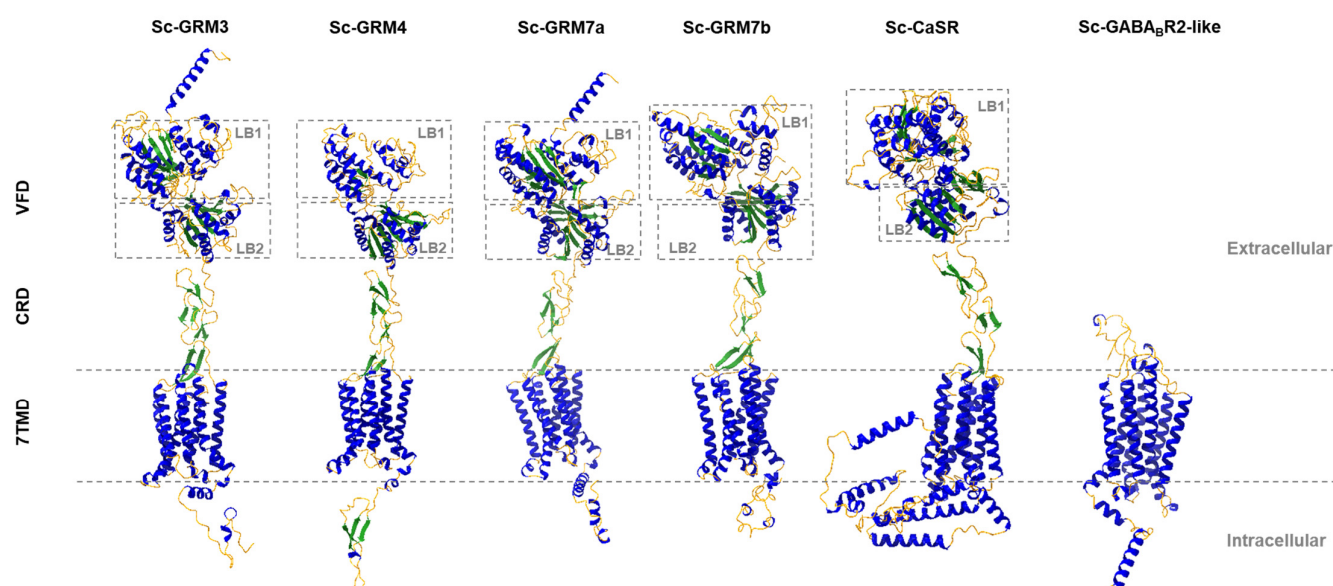
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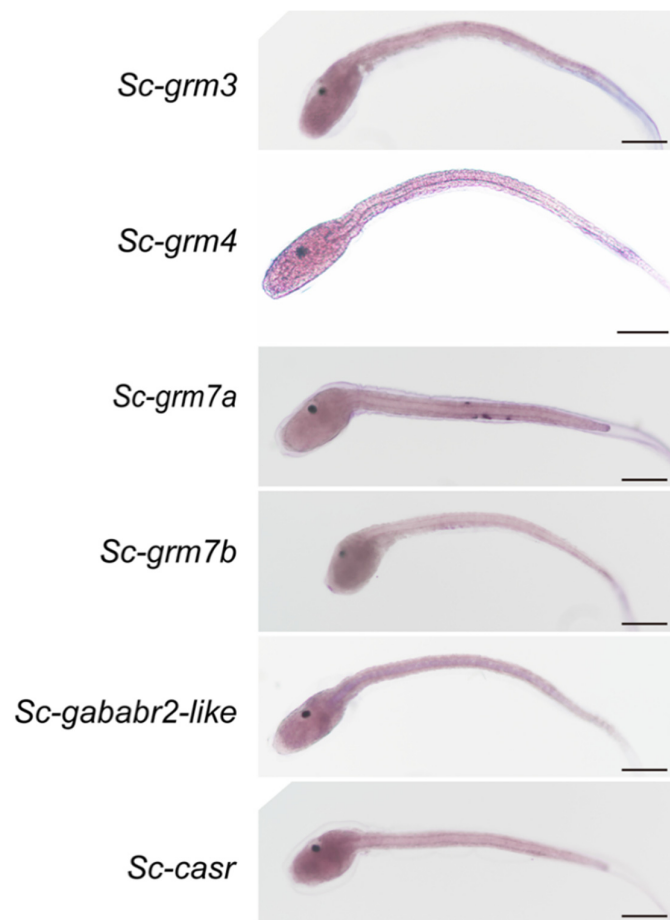
**Figure S1.** Sequence alignment of VFDs for representative GRMs in different species. The conserved residues are shaded. The critical amino residues for L-glutamate binding are indicated by arrows. These residues, from N-terminal to C-terminal, are arginine (R), serine (S), threonine (T), aspartate (D), tyrosine (Y), aspartate (D), and lysine (K). Red arrows indicate conserved residues in all the species, and purple arrows indicate residues not conserved in ascidians. The information for residues involved in L-glutamate binding can be found on Uniprot (<https://www.uniprot.org>) and previous study [59].





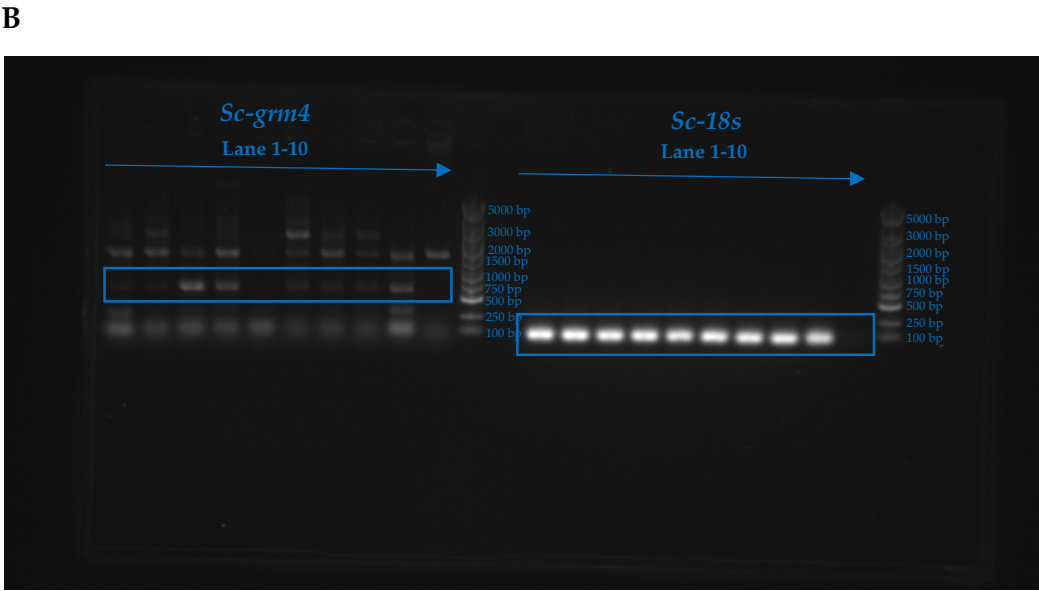
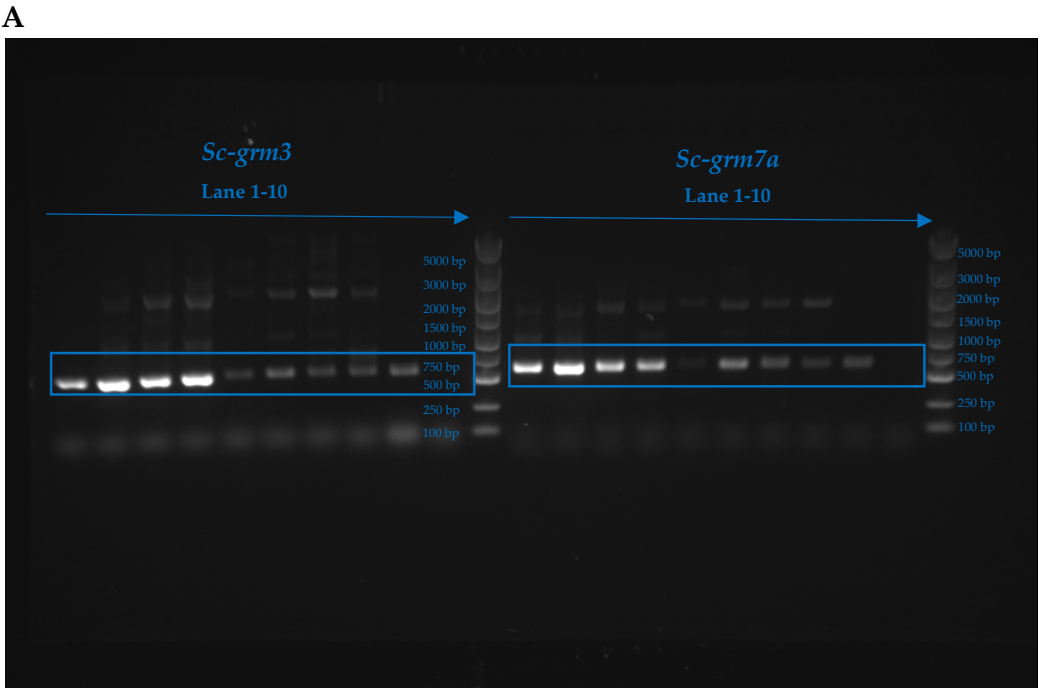
**Figure S2.** Tertiary structure prediction of *S. clava* Class C GPCRs by RoseTTAFold. Structures were predicted by deep-learning method using RoseTTAFold Public Server and visualized in the Ribbon diagram, in which the  $\alpha$ -helices,  $\beta$ -sheets, and random coils are shown in blue, green, and yellow, respectively. The VFD of each receptor contains lobe 1 (LB1) and lobe 2 (LB2).





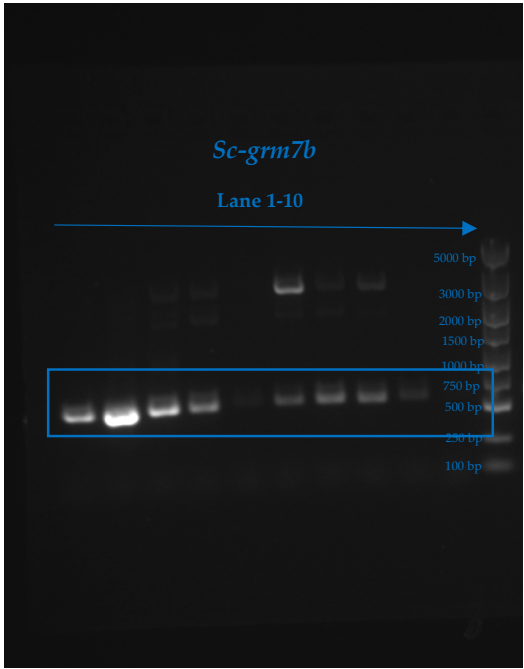
**Figure S3.** Whole-mount in situ hybridization of genes encoding *S. clava* Class C GPCRs in swimming larvae (negative controls with sense probe). Scale bar: 100  $\mu$ M.



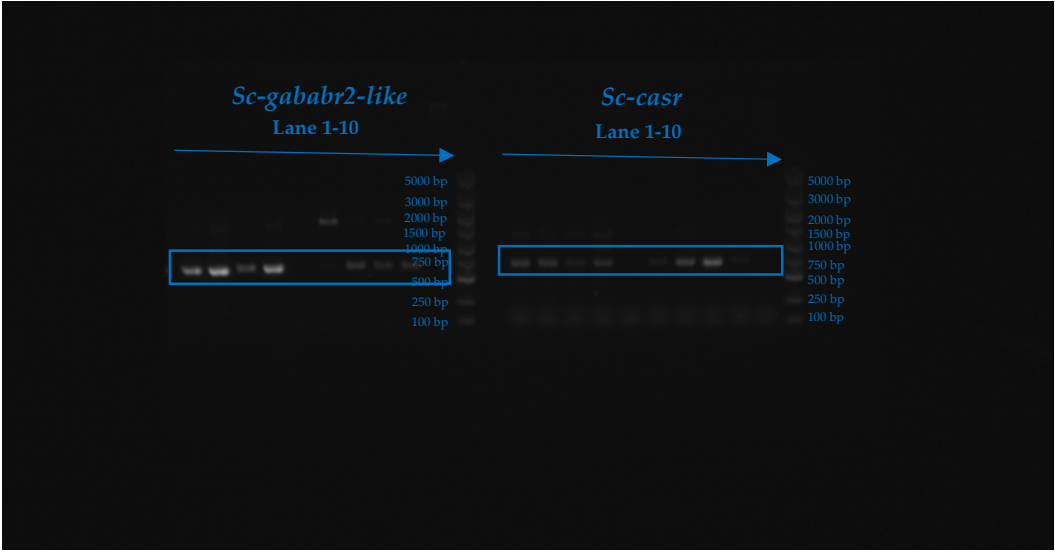




C



D

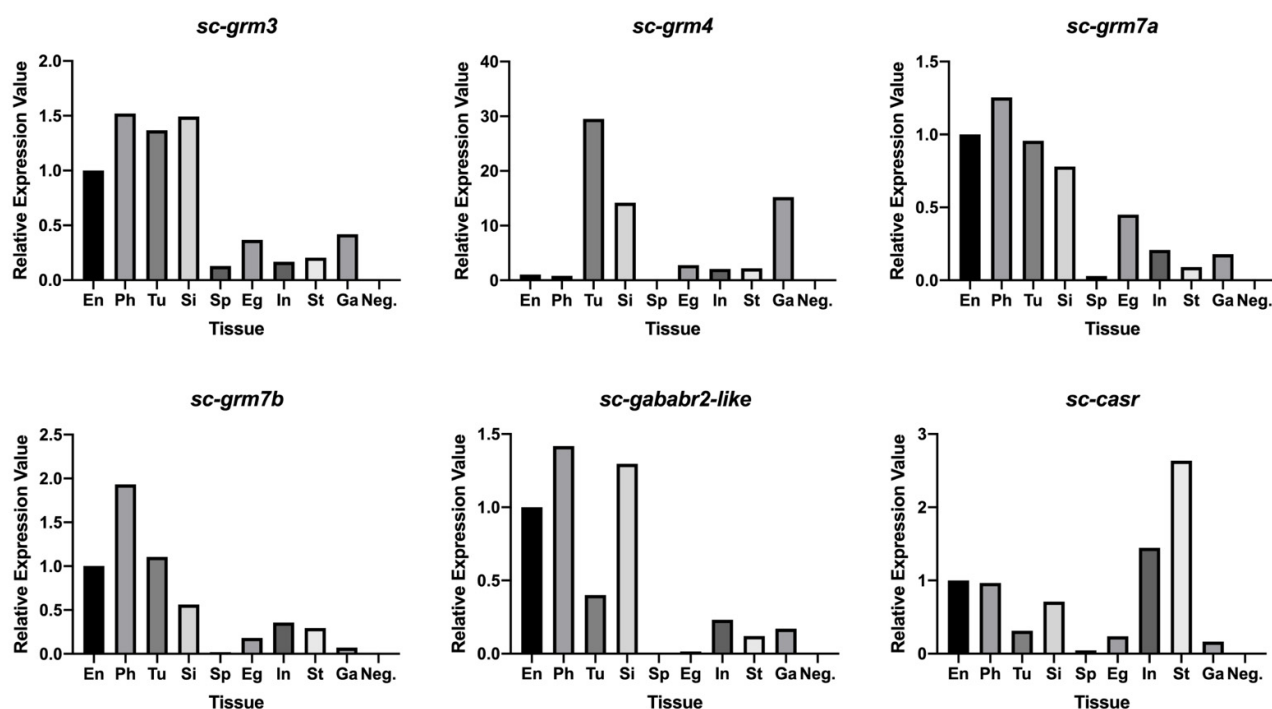


E

Gene Name	Intensity									
	En	Ph	Tu	Si	Sp	Eg	In	St	Ga	Neg.
sc-grm3	26115.054	40905.246	35248.125	38506.196	3083.276	9303.468	4197.276	5058.690	9490.054	0.000
sc-grm4	1364.104	1149.154	39735.974	19146.560	142.556	3683.024	2713.660	2814.832	18002.167	0.000
sc-grm7a	31291.347	40424.660	29564.589	24164.711	841.991	13654.004	6215.225	2654.376	4866.033	0.000
sc-grm7b	22531.468	44875.075	24595.125	12569.054	422.991	3965.861	7686.761	6234.518	1402.719	0.000
sc-gababr2-like	26430.882	38625.539	10470.711	33878.368	152.021	378.092	5871.418	3001.276	3931.640	0.000
sc-casr	14842.761	14780.489	4600.054	10416.075	645.790	3423.761	20544.660	36763.610	2144.468	0.000
sc-18s	51269.903	52838.903	50597.439	50680.853	47086.782	49594.146	49039.560	48201.439	44493.459	0.000

Gene Name	Ratio									
	En	Ph	Tu	Si	Sp	Eg	In	St	Ga	Neg.
sc-grm3	0.509	0.774	0.697	0.760	0.065	0.188	0.086	0.105	0.213	N/A
sc-grm4	0.027	0.022	0.785	0.378	0.003	0.074	0.055	0.058	0.405	N/A
sc-grm7a	0.610	0.765	0.584	0.477	0.018	0.275	0.127	0.055	0.109	N/A
sc-grm7b	0.439	0.849	0.486	0.248	0.009	0.080	0.157	0.129	0.032	N/A
sc-gababr2-like	0.516	0.731	0.207	0.668	0.003	0.008	0.120	0.062	0.088	N/A
sc-casr	0.290	0.280	0.091	0.206	0.014	0.069	0.419	0.763	0.048	N/A
sc-18s	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	N/A

Gene Name	Relative Expression									
	En	Ph	Tu	Si	Sp	Eg	In	St	Ga	Neg.
sc-grm3	1.000	1.520	1.368	1.492	0.129	0.368	0.168	0.206	0.419	N/A
sc-grm4	1.000	0.817	29.517	14.199	0.114	2.791	2.080	2.195	15.207	N/A
sc-grm7a	1.000	1.254	0.957	0.781	0.029	0.451	0.208	0.090	0.179	N/A
sc-grm7b	1.000	1.933	1.106	0.564	0.020	0.182	0.357	0.294	0.072	N/A
sc-gababr2-like	1.000	1.418	0.401	1.297	0.006	0.015	0.232	0.121	0.171	N/A
sc-casr	1.000	0.966	0.314	0.710	0.047	0.238	1.447	2.635	0.166	N/A
sc-18s	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	N/A



**Figure S4.** The original DNA gel images with densitometry readings related to Figure 6. (A) Whole DNA gel for RT-PCR of *Sc-grm3* and *Sc-grm7a*; (B) Whole DNA gel for RT-PCR of *Sc-grm4* and *Sc-18s*; (C) Whole DNA gel for RT-PCR of *Sc-grm7b*; (D) Whole DNA gel for RT-PCR of *Sc-gababr2-like* and *Sc-casr*; (E) The intensity values and calculated ratios of bands in DNA gels, and relative expression levels of *S. clava* Class C GPCR genes in different tissues.