

---

*Supplementary Materials*

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

Jin Zhang<sup>1</sup>, Bo Dong<sup>1,2,3,\*</sup>, and Likun Yang<sup>1,\*</sup>

- <sup>1</sup> Sars-Fang Centre, MoE Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China; zhangjincoolcool@163.com (J.Z.).  
<sup>2</sup> Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China.  
<sup>3</sup> Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China.  
\* Correspondence: bodong@ouc.edu.cn (B.D.); yanglikun@ouc.edu.cn (L. Y.)
- 

**Citation:** Zhang, J.; Dong, B.; Yang, L. Molecular Characterization and Expression Analysis of Putative Class C (Glutamate Family) G Protein-Coupled Receptors in Ascidian *Styela clava*. *Biology* **2022**, *11*, 782. <https://doi.org/10.3390/biology11050782>

Academic Editor: De-Li Shi

Received: 17 April 2022

Accepted: 18 May 2022

Published: 20 May 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



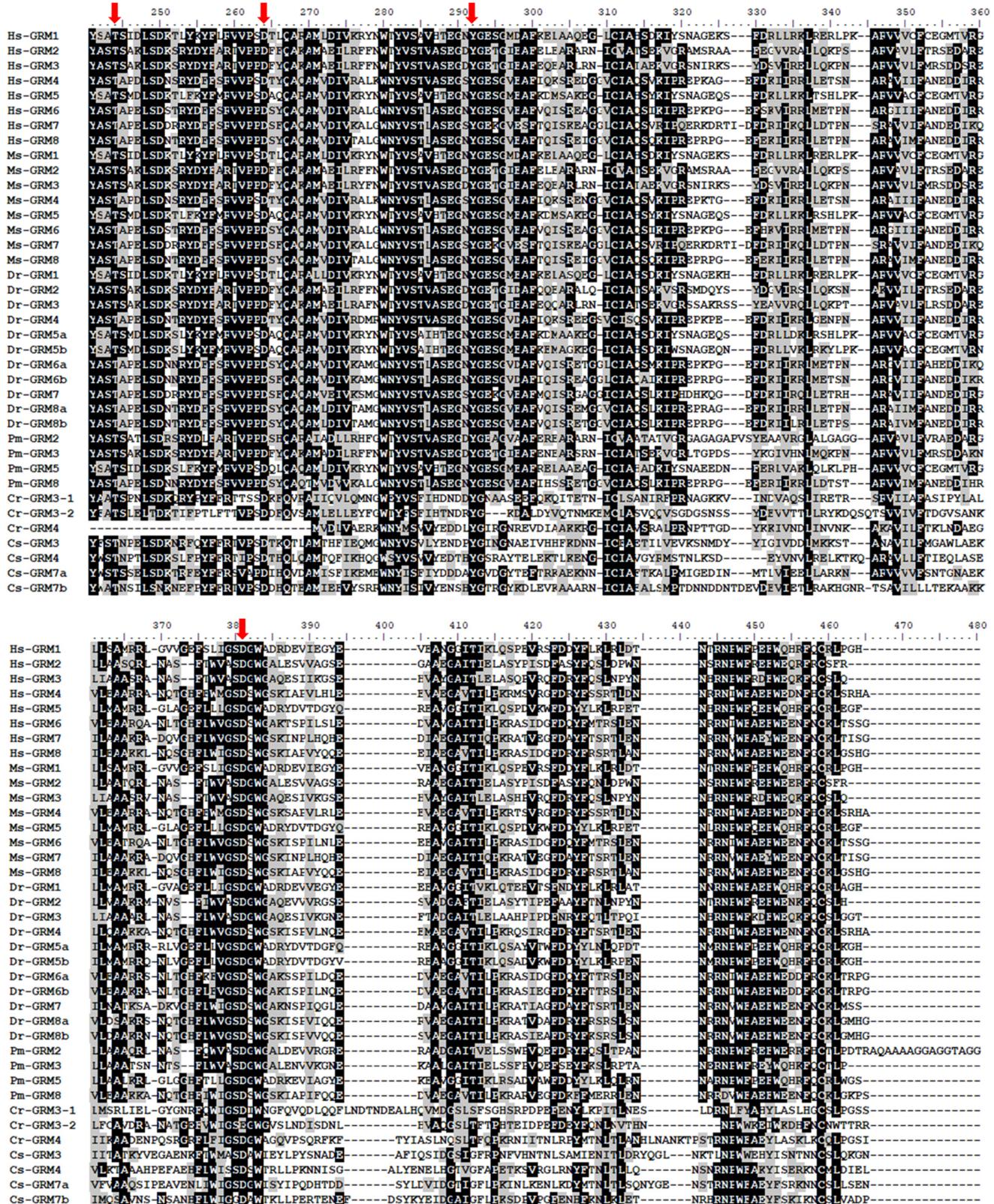
**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

10 20 30 40 50 60 70 80 90 100 110 120

Hs-GRM1 -----MVLGLLFFFFPAIFLEVSLLPRSPGRKVLLAGASSQRSAVAFMDGIVIIGALFSEVHOPPAEKVPERICCEIREQYGIQFVEAMF  
Hs-GRM2 -----MGS-----LLALLALLLWG---AVAEG---PAKVKLTLEGDLVLGGLFVEOK---GPAEEDCGEVNEHRGICRLEAML  
Hs-GRM3 -----MRMLTRLQVLTIALFSKGFL---SLGDHNPLRREIRIEGDILVLGGLFVEINEK---GTGTEECGRINEDRGICRLEAML  
Hs-GRM4 -----MPGKRGGLGWNNWAR-----LPLCLLSSLYGPWMPSSLGKPKGHPHMNSIRIDGDIITLGGLFVEHGR---GSEGKICGELKKEGICRLEAML  
Hs-GRM5 -----MARPRRAREPL-----LVALLPLAWLAQAGLARA-----GSVRLAGSLTLGGLFFVHAR---GAAGRACQQLRKKECCVERLEAML  
Hs-GRM6 -----MVQLRKLLRVLT-----LMKFPCCVLEVLLCALAAAARGQEMYAPHSIRIEGDVTLGGLFVEHAK---GPSGVICGDLKRENHGICRLEAML  
Hs-GRM7 -----MVCERGRSASC-----CFPLLTAKFWYLITMMORTHS---QEYAHISIFVDPDIIILGGLFVEHAK---GERGVICGDLKRENHGICRLEAML  
Ms-GRM1 -----MVRLLLTFPPMFLEMISILPRMPDRKVLLAGASSQRSAVAFMDGIVIIGALFSEVHOPPAEKVPERICCEIREQYGIQFVEAMF  
Ms-GRM2 -----MES-----LTLRFLALLLRRG---AVAEG---PAKVKLTLEGDLVLGGLFVEOK---GPAEEDCGEVNEHRGICRLEAML  
Ms-GRM3 -----MRMLTRLQVLMALFSKGFL---SLGDHNFMFRRERIEGDILVLGGLFVEINEK---GTGTEECGRINEDRGICRLEAML  
Ms-GRM4 -----MSGKGGWAWWWAR-----LPLCLLSSLYGPWMPSSLGKPKGHPHMNSIRIDGDIITLGGLFVEHGR---GSEGKICGELKKEGICRLEAML  
Ms-GRM5 -----MVLILLISVLLRKED-VRGSQASERRVVAEMGDIIGALFSEVHOPTVDKVERFCGAVREQYGIQFVEAMF  
Ms-GRM6 -----MGRLR-----VLLLWLAWLSQAGIAHGA-----GSVRLAGSLTLGGLFFVHAR---GAAGRACQQLRKKECCVERLEAML  
Ms-GRM7 -----MVQLGKLLRVLT-----LMKFPCCVLEVLLCVALAAAARGQEMYAPHSIRIEGDVTLGGLFVEHAK---GPSGVICGDLKRENHGICRLEAML  
Ms-GRM8 -----MVCERGRSTSCP-----CFPLLTAKFWYLITMMORTHS---QEYAHISIFVDPDIIILGGLFVEHAK---GERGVICGDLKRENHGICRLEAML  
Dr-GRM1 -----MRMLIIRMNFFIVFLPCTLRLHHLSTERAVSRAAS--RSVAFMDGDIIGALFSEVHOPPSAEKAVERCCEIREQYGIQFVEAMF  
Dr-GRM2 -----MHRSLRTMAQRSAHFRGGPSWPFLHLLLILLARTEQALAIPGYNTDTSKKEITVMDGIDLVIIGALFSEVHGR---GEGAQDCGIRNTORGICRLEAML  
Dr-GRM3 -----MSSAMGRMIGWSNHHHQ-----MWS--AVRALLNLSSAGLQQ---SRGDS--PRKEIRIDGIVLGGLFVEHEK---GMGMDCDEGRINEDRGICRLEAML  
Dr-GRM4 -----MSSAMGRMIGWSNHHHQ-----GSALVFLYLFMTALAARTKGP--GHTHLSNRIDGIDSLGGLFVEHAR---GHEKACGELKKEGICRLEAML  
Dr-GRM5a -----MGGFHLLVFLVLIQNGRLGPTRILAAEAQSERRVLAHETGDIIGALFSEVHOPPADKVERFCGAVREQYGIQFVEAMF  
Dr-GRM5b -----MVIICSLALLWWRMTSLVSTAQSNERRVVAHTGDIIGALFSEVHOPPADKVERFCGAVREQYGIQFVEAMF  
Dr-GRM6a MTSHPFLPSLDHQVRMFRVLSCTTC-----ASTWPLRLLWVWLPSCS-SVHQHSHPSHSIRIEGDITLGGLFVEHAR---GPAGVFCGEIKKEGICRLEAML  
Dr-GRM6b MTSQLYAPS--QSRHHRIIRAPKPH-----VGSILHIMIILTMQFTPESQAASSDQPHSIRIEGDITLGGLFVEHAR---GPAGIECGEVKREKGICRLEAML  
Dr-GRM7 -----MACFPWRFVWIIQALVDTVTS-QEIYAPHSIRIEGDITLGGLFVEHAR---GVSGEFGCDIKRENHGICRLEAML  
Dr-GRM8a -----MDLVCLSLRT-----QYOLLVFLCYWFVVFSLQKLETPTPIEYAHSLRDGIDLVIIGALFSEVHAR---GERGVICGELKKEGICRLEAML  
Dr-GRM8b -----MLSAFVWYG-----CLLLTGTVTRSISQRTDPLTPPAPEYAHSLRDGIDLVIIGALFSEVHAR---GERGAECGELKKEGICRLEAML  
Pm-GRM2 -----KEAM-----  
Pm-GRM3 -----MPPSPPPPRHRHRRRHQHHNHRRAFPPLLALHAAAACVCSASRPEPSGRKEAERKEQELIVGGLFVEHGR---GGSKDCGPPINEARGIQFVEAMF  
Pm-GRM5 -----MRWSLAPRFAVAIILPRLLAGAQGTPGRRIVARKRGGIDLIGALFSEVHOPPADKVSERCCCEIREQYGIQFVEAMF  
Pm-GRM8 -----MHVAPSRCSRTSPWLSSHCARSPLLGARLAATPMTPPPMPPLLILLLPEPVSAQDRATHSIRIEGDITLGGLFVEHGR---GPAGVACGEIKKEGICRLEAML  
Cr-GRM3-1 -----MILLEVILISIQVACVCYVKLANQATAQPVSSPEEFVIRLGCPFSTYPR-----RNDEHCDETKVLRSNOFELMAI  
Cr-GRM3-2 -----MGLFHRYHEVYSMEGLIMAGLFPHQH-----ECGLDLEDMGSTOREAMF  
Cr-GRM4 -----  
Cs-GRM3 -----MDLFDAKSFCTIVMNLICIKTLTNASEEYVDPAEVIYGDIVQFRDHTIIAVLLPITTSDENENRNEFRNEGIOMVEIN  
Cs-GRM4 -----  
Cs-GRM7a -----MSIMSVLRSRTLFCCEVIVASQSAHBLIGS-QHMK--QSRSGAPMQFRDGDIIILGAAMQVTNEANGCVEELT--QMYLVEIN  
Cs-GRM7b -----MVKKLGNRQCFLLLCLOAALATSW-----PKSKKNIVQHRUGDIIIGGFVYVPQEDQ---GIQIENEVIN

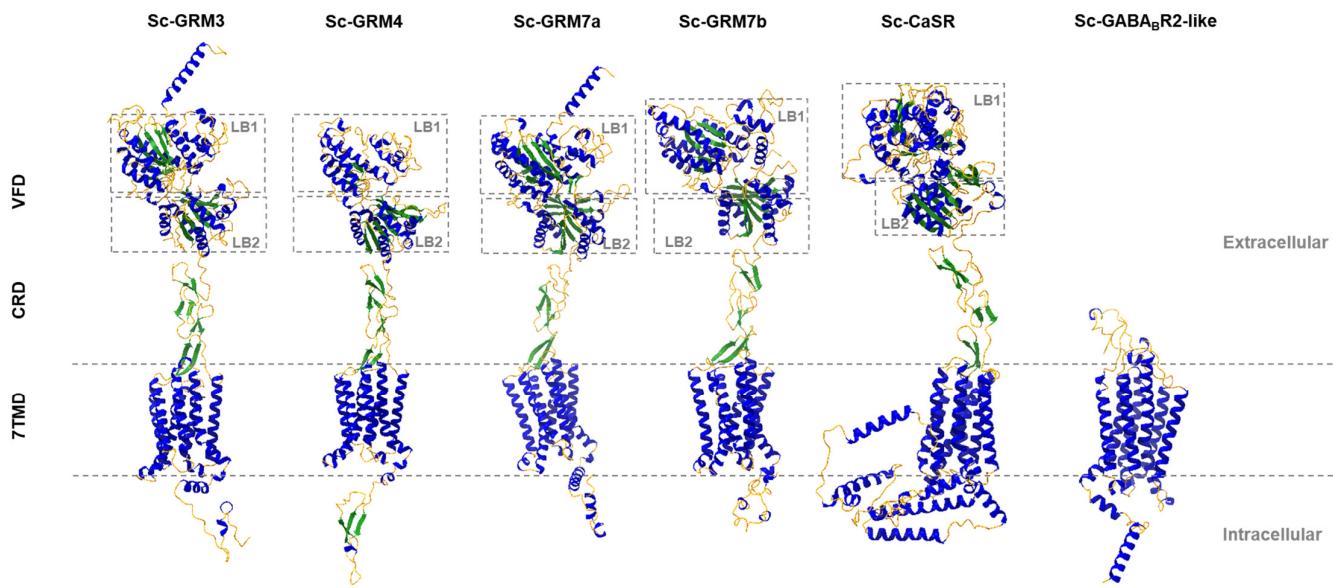
130 140 150 160 170 180 190 200 210 220 230 240

Hs-GRM1 HTLDRINADPILLPNITLGSEIRDSCWHSSVALEQSLEFIRDLSI-----RD2KD-GINELCPDG-----QSLPPGRTKHEIAVGIVPGSSVAIVVNLLQLEDFIPQIA  
Hs-GRM2 HADLRINADPILLP2VR1CAHILDSCKSDTDEALBQDLEFVAVSLRG---ADGSR-HICPDGS-----YATHGDAPTAINGIVCGSSISVSIIVANLLRLEFIPQIS  
Hs-GRM3 HADLRINADPILLDYLLEVR1GLVHILDTCSRDTYALEQSLEFVAVSLTK---VDEAE-YMCEDGS-----YATQENIPPLIIACVIVGSSSSSVIIVANLLRLEFIPQIS  
Hs-GRM4 HADLRINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3TE-VRCGSGG-----PP1ITKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Hs-GRM5 HTERINADPILLPNITLGCEIRDSCWHSSVALEQSLEFIRDLSI-----EEEE--GLVRCVD-----GSSSSFRSRKHIVCIVPGSSSVIAVVNLLQLEDFIPQIA  
Hs-GRM6 YALDQINSDPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD4SD-VRCGNG-----PPLRPAPPFVVAVVAGASSSSVIIVANLLRLEFIPQIS  
Hs-GRM7 YALDQINSDPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD4AS-VRCANG-----PEVFEVKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Ms-GRM1 HADLRINADPILLPNITLGSEIRDSCWHSSVALEQSLEFIRDLSI-----RD2KD-GLNCLCPDG-----PPIFTKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Ms-GRM2 HADLRINADPILLP2VR1CAHILDSCKSDTDEALBQDLEFVAVSLRG---ADGSR-HICPDGS-----YATLSDAPTAINGIVCGSSISVSIIVANLLRLEFIPQIS  
Ms-GRM3 HADLRINADPILLDYLLEVR1GLVHILDTCSRDTYALEQSLEFVAVSLTK---VDEAE-YMCEDGS-----YATQENIPPLIIACVIVGSSSSSVIIVANLLRLEFIPQIS  
Ms-GRM4 HADLRINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3TE-VRCGSGG-----PPIITKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Ms-GRM5 HTERINADPILLPNITLGCEIRDSCWHSSVALEQSLEFIRDLSI-----EEEE--GLVRCVD-----GSSS-FRSKKHIVCIVPGSSSVIAVVNLLQLEDFIPQIA  
Ms-GRM6 YALDQINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD4AS-VRCGNG-----PPLRPAPPFVVAVVAGASSSSVIIVANLLRLEFIPQIS  
Ms-GRM7 YALDQINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD4AS-VRCANG-----PEVFEVKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Dr-GRM1 YTLLDRINADPILLPNITLGCEIRDSCWHSSVALEQSLEFIRDLSI-----RD2RE-GPKWCVEGN-----PSAQAPPTRKEIACVIVPGSSSVIAVVNLLQLEDFIPQIA  
Dr-GRM2 IALDRINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVSLTK-----VDDSE-YTCSDGS-----YAIHDDVPLAISCVIGSSISVSIIVANLLRLEFIPQIS  
Dr-GRM3 HADLRINADPILLVVLGEVILDTCSRDTYALEQSLEFVAVSLTK-----VDDTE-FICPDGS-----YALQEDSPLAICVIVGSSSSSVIIVANLLRLEFIPQIS  
Dr-GRM4 HADLRINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3TD-VRCQGG-----SPLITKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Dr-GRM5a HADLRINADPILLPNITLGCEIRDSCWHSSVALEQSLEFIRDLSI-----EEEE--GMARCKSTE-----GGGTPMGRKHIVCIVPGSSSVIAVVNLLQLEDFIPQIA  
Dr-GRM5b HADLRINADPILLPNITLGCEIRDSCWHSSVALEQSLEFIRDLSVAA-----DESEEAGGGAKCAD-----PGATPMGRKHIVCIVPGSSSVIAVVNLLQLEDFIPQIA  
Dr-GRM6a YALDQINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3TD-IRCSNG-----QPIIRKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Dr-GRM6b YALDQINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3SD-VRCNSN-----PPIIPKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Dr-GRM7 YALDQINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3SD-VRCGNG-----PEVFEVKP-EVVKCVIGASSSSSVIIVANLLRLEFIPQIS  
Dr-GRM8a HADLRINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3SD-VRCANG-----QPIIFAKP-DRIICVIVGAAASSSVIIVANLLRLEFIPQIS  
Dr-GRM8b HADLRINADPILLPNITLGCAHILDTCSRDTYALEQSLEFVAVLAE-----RD3SD-VRCASG-----QPIIFAKP-DRIICVIVGAAASSSVIIVANLLRLEFIPQIS  
Pm-GRM2 HADDINSAFLP2VR1CAVFLVLDTCRDTYALEQSLEFVAVRSSLQR-----VEETE-FVCPDGS-----YAVQQKRPLFIACVIVGSSSSSVIIVANLLRLEFIPQIS  
Pm-GRM3 HADLRINADPILLP2VR1GLVHILDTCSRDTYALEQSLEFVAVRSSLQR-----VDTSD-FMCDDGT-----YAHGGNAQFIAACVIVGSSSSSVIIVANLLRLEFIPQIS  
Pm-GRM5 HADLRINADPILLP2ITLGCEIRDSCWHSSVALEQSLEFIRDLSA-----GDPDKDAAAAAAAVAVAPGGVGRQCBAPVRAKRHEIVLIGPGSSSVIAVVNLLQLEDFIPQIA  
Pm-GRM8 HADLRINADPILLP2ITLGCEIRDSCWHSSVALEQSLEFIRDLSA-----RD7SD-VRCNG-----AIFVFTKP-ERIICVIVGASSSSSVIIVANLLRLEFIPQIS  
Cr-GRM3-1 YTIEBVNNDTMLEVTLTIDEADCTIDTFALEIIVONYLPQQLSQ-----QSTRNNNSYCVIPESSSVAVSIPTRARLLEDFOSIS  
Cr-GRM3-2 YAVQRVNSVNPENNFTLGFRIEDTONQSIALSRTLJVLPHCNNCNTHYP-----VCPFPCPSLVACTVPEESSSVALLSAHACNLALTEIS  
Cr-GRM4 -----  
Cs-GRM3 -----MAIEPVVADKEKLP2ITLGTVIINTCLSVPTNLTKVIFDV-RKYILDN-QCHNSC-----LNPD-EPRILACV1GAMSSSVSMQ1JARLQVEMIPQIS  
Cs-GRM4 -----MIDQVNVDDKXILQMLGTMIDTFAMDSHTIQIVTEFIPFLSEAT-TSCSTS-----KTLSSKK-SMAFGICASSSVS1VSIETARLQVEMIPQIS  
Cs-GRM7a -----MIDDEIINANDEILTG14LGTVMIDTCSSDVEALRVRIMDILPLALDKR-ICGONC-----SEDESKKHILACLGICASSSSVSMQ1JARLQVEMIPQIS  
Cs-GRM7b -----MVDQVWNDENILP2ITLGTMINTNRSGD1MFOIVNEFPPLFSDLQ-OFCEVN-----FTNCKKKPLFAFICASSSTVSIHTSP1LQGYSIPOIS





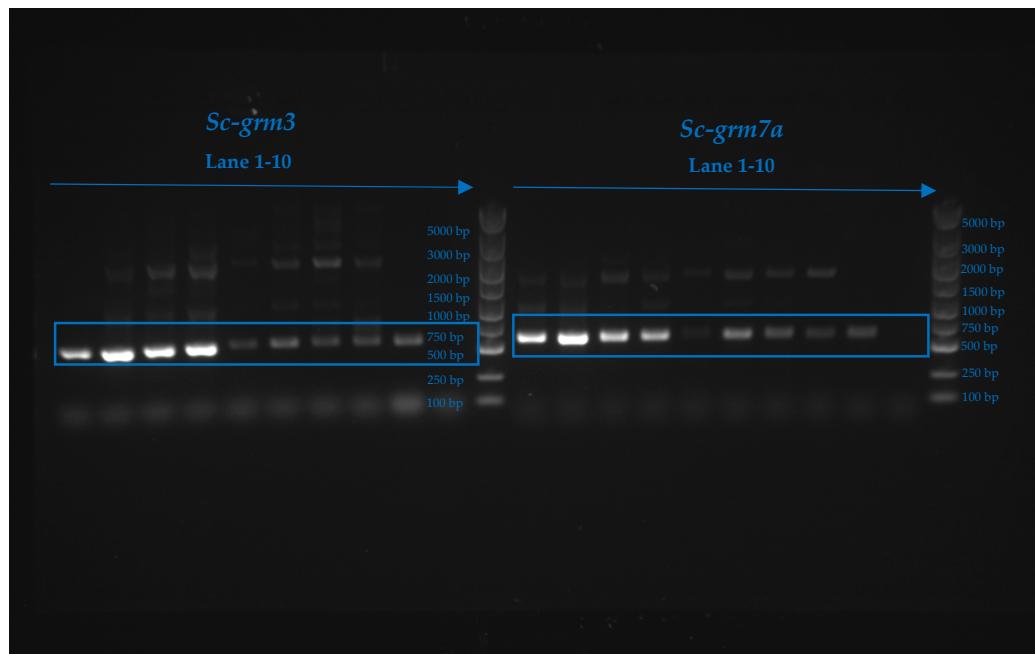
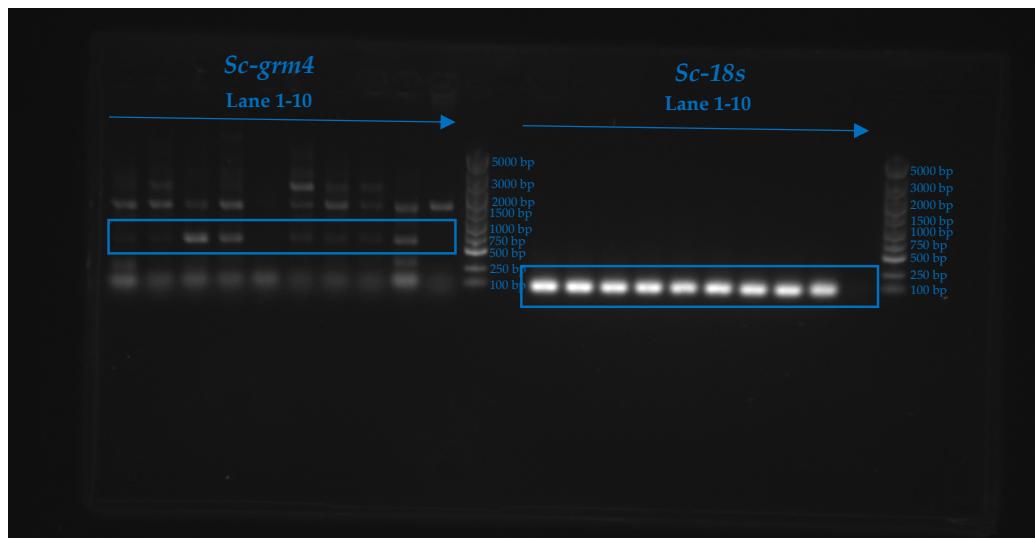
**Figure S1.** Sequence alignment of VFDs for representative GRMs in different species. The conserved residues are shaded. The critical amino residues for L-glutamate binding are indicated by arrows. These residues, from N-terminal to C-terminal, are arginine (R), serine (S), threonine (T), aspartate (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].



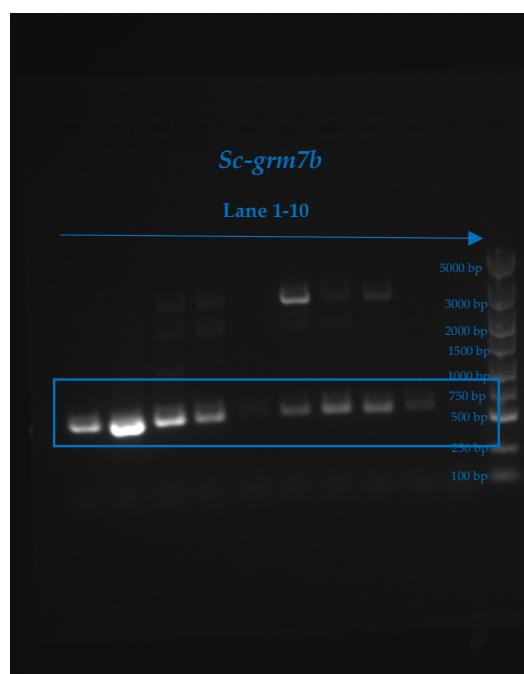
**Figure S2.** Tertiary structure prediction of *S. clava* Class C GPCRs by RoseTTAFold. Structures were predicted by deep-learning method using RoseTTAFold Public Server and visualized in the Ribbon diagram, in which the  $\alpha$ -helices,  $\beta$ -sheets, and random coils are shown in blue, green, 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.

**A****B**

C



D



E

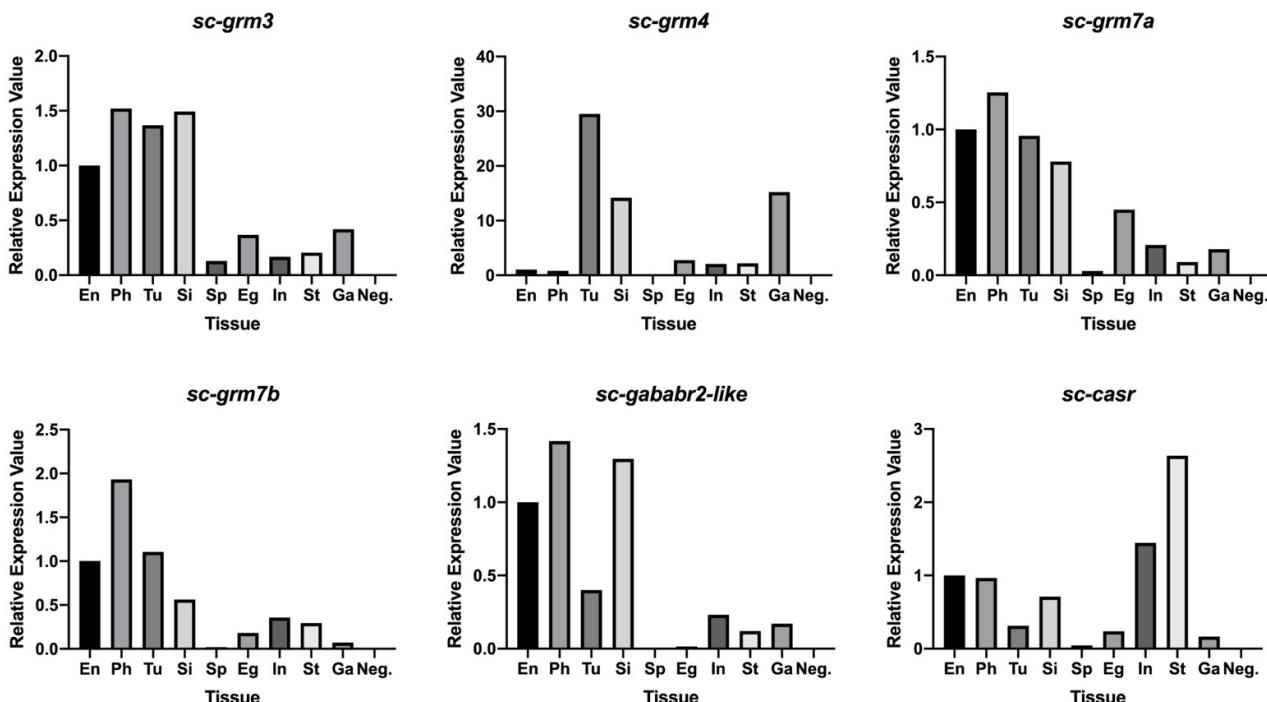
Gene Name	Intensity									
	En	Ph	Tu	Si	Sp	Eg	In	St	Ga	Neg.
sc-grm3	26115.054	40905.246	35248.125	38506.196	3083.276	9303.468	4197.276	5058.690	9490.054	0.000
sc-grm4	1364.104	1149.154	39735.974	19146.560	142.556	3683.024	2713.660	2814.832	18002.167	0.000
sc-grm7a	31291.347	40424.660	29564.589	24164.711	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.