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Molecular Cloning and Expression of Taste Receptor Gene T2R1 of Obscure Puffer, *Takifugu fasciatus*

Yijin He[1], Song Ma[2], Bo Liu[1,*], Ting Xue[1], Qunlan Zhou[1], Wu Jin[1] and Kui Chen[2,*]

[1]*Key Open Laboratory for Genetic Breeding of Aquatic Animals and Aquaculture Biology, Ministry of Agriculture, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, No. 9 Shanshui East Road, Wuxi 214081, China*
[2]*School of Life Sciences, Soochow University, Suzhou 215123, China*

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Authors' Contribution
YH, BL and KC conceived and designed the research. YH, SM and WJ conducted the experiment. YH, QZ, TX and BL analyzed the data. YH and BL wrote the paper. BL and KC revised the paper.

Key words
***Takifugu fasciatus*, Taste receptor gene, Differentially expressed.**

ABSTRACT

Obscure puffer, *Takifugu fasciatus* is a unique species of China which is distributed in the estuary of the Yangtze River, and belongs to the same genus with Red fin puffer *T. rubripes* which has been used as one of human genome model animal since mid-1990s. In this experiment, we cloned a pair of DNA fragment sequence including complete ORF (921bp) of taste receptor candidate gene *T2R1* of *T. fasciatus* and studied the differential expression spectrum of tissues in combination with bioinformatics analysis so as to understand the possible functions in response to chemical sensation behavior of taste receptors. The receptors when compared with *T. rubripes*, there was 99.2% identity in two kinds ORF sequences, with only 7 bases different, and there were 5 amino acids different between speculated fusion proteins. Under bioinformatics analysis, the *T2R1* gene without introns was consistent with the structural domains characteristics of this family genes. The translation protein of *T2R1* gene displayed with corresponding signal loci and functional domains of this gene family, and the result showed that the taste receptor translated by *T2R1* gene belongs to G protein-coupled receptor super-family. *T. fasciatus* taste receptor *T2R1* in various tissues appeared differentially expressed. The results showed *T2R1* were expressed in gill, spleen, heart, lips, skin and tongue tissue, suggesting that it is not only act as taste receptors, but also play other possible functions. Furthermore, the cloning and expression analysis of *T2R1* provide theoretical basis to further study function of *T2R1* for *T. fasciatus*.

INTRODUCTION

There are many research reports about the genus *Takifugu*. So far, the classification of this genus is commonly focused on the morphological, anatomical characteristics, cytogenetics and biochemical genetics research, but there are not many molecular genetic studies (Miyaki *et al*., 1995). Red fin puffer, *T. rubripes* has become one of model animal species for human genome DNA sequence research since mid-1990s (Elger, 1996). Therefore, the international studies afterward mainly focused on *T. rubripes* (Clark *et al*., 2001). There is one unique species of the genus *Takifugu* in China, it was named obscure puffer *T. fasciatus*, which is mainly distributed in the Yangtze River drainage and at the estuary of the Yangtze River (McClland, 1844). However, *T. fasciatus* is currently one of the species which is near extinction in the Yangtze River since there is a high demand for it as food for human consumption and as raw material for the pharmaceutical industry.

Taste receptor family No.2 mainly introduces a bitter taste (Conte *et al*., 2002; Scott, 2004; Go, 2006). Many members of the *T2R* family have a short amino terminal domain, the cytoplasmic inner loop and the adjacent transmembrane segments are very conservative, estimated to be a protein interaction site. However, the extracellular domain has great variability, estimated to be ligand binding site (Andres-Barquin and Conte, 2004).

As *T2R* was identified late, the relevant research data is less than *T1R*. No introns were found in the *T2R* of the fish that had been obtained; this is the same as mammals. In fish, only 1~2 genes of *T2R* are identified, far less than the mammalian species (22 in Human beings, 33 in Mouse) (Conte *et al*., 2003). Ishimaru *et al*. (2005) recently isolated a novel *T2R* gene *zf997-5* from several zebra fish (*Danio rerio*) like *T2R* gene. Similarly, in terms of vertebrate hormone receptors, there was only one objective gene found in zebra fish, far less than 137 of the mouse, but only two of the primate genome (Rodriguez *et al*., 2002; Pfister and Rodriguez, 2005). This indicates that

* Corresponding author: chenk@suda.edu.cn; liub@ffrc.cn
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the receptor expression in the chemical sensory system is very different from different species. Fish and mammals may only have a simplified *T2R* receptor system, it is also possible that other members of the *T2R* family have not yet been discovered, which needs further investigation.

Taste receptor gene is an important gene for chemical sensing behavior researching in animals. Many studies were conducted on the mechanism and distribution of taste receptors in mammals and other higher vertebrates (Adler *et al*., 2000; Chandrashekar *et al*., 2000; Shi and Zhang, 2007; Upadhyaya *et al*., 2010; Singh *et al*., 2011; Dai *et al*., 2011), but little is reported in fish and other lower vertebrates (Marui and Caprio, 1982; Ogawa and Caprio, 1999; Yasuoka *et al*., 2004; Oike *et al*., 2007; Aihara *et al*., 2008; Yasuoka and Abe, 2009). For example, Gao *et al*. (2017) reported that T1Rs was expressed at higher levels than T2Rs, and T1Rs showed the highest expression in barbell, followed by that in the gill, and then in the skin, while very low or no expression in the intestine and liver in channel catfish. In another study, T1R and T2R genes were expressed in taste bud cells in lips, gill rakers, and pharynx of zebra fish (Ishimaru *et al*., 2005). The current research investigates the existence of this gene and its expression distribution of expression profile in *T. fasciatus*, which will acquire the molecular information of this gene, and contribute to the basis for intensive study of test behavior in fish species.

First of all, according to the *T2R1* sequence of the taste receptor related genes on GenBank, we obtained the research results and the gene sequence of fish taste receptor gene of several *D. rerio*, *T. rubripes* and *Oryzias latipes*. Specific primers were designed by the sequence of these genes, which was used to clone the taste receptor candidate gene of *T. fasciatus*. Furthermore, we conducted the taste receptor gene expression analysis of related differences in tissues, and bioinformatics analysis to explore the evolution of taste receptor gene family and the relationship between the species evolution and possible functional role.

MATERIALS AND METHODS

Experimental animals

All experiments were approved by the Institutional Animal Care and Use Committee of the Ministry of Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences and were undertaken in accordance with the national legislation for fish welfare established by the Ministry of Science and Technology of the People's Republic of China. Experimental *T. fasciatus* were provided by the National Puffer Stock Farm Jiangsu Zhongyang Group Co., Nantong, Jiangsu. The fish (50 in total) were reared in a thermostat aquarium for domestication, being fed with commercial compound diet for *T. fasciatus*. The ambient conditions for rearing were 25°C and feeding one time every day. The sample tissues of gill, spleen, heart, lips, skin and tongue were collected from 6 fish.

RNA extraction

Total RNA was extracted from the lips and other tissues using RNAiso Plus (Takara, Dalian, China) according to the manufacturer's protocol (Rio *et al*., 2010). Briefly, after 100 µg tissue samples were ground and pulverized, 1000 µ l of RNAiso Plus was added with repetitive pipetting until the tissues were completely lysed. Next, 0.2 volumes of chloroform was added, and the mixture was left at room temperature for 5 min. and then centrifuged at 12,000 *g* for 5 min. The supernatant was removed, and an equal volume of anhydrous isopropanol was added to precipitate the RNA. The absorptions at 260 nm (*A*260) and at 280 nm (*A*280) were measured with a spectrophotometer, and the *A*260/*A*280 ratio was used to assess RNA quality before the samples were stored at –80°C. RNA samples were treated by RQ1 RNase-Free DNase prior to RT-PCR (Dalian Takara Co., Ltd.) to avoid genomic DNA amplification.

Table I.- Sequence of PCR primer used in this study.

PCR	Primer	Sequences (5' to 3')
Partial cDNA PCR	T2R1 F1	ATGCTTGAATCAGATGATTTG
	T2R1R1	CTACTCCCCTTCTGCATTAAT
5'RACE PCR	T2R1 F2	GAGTCCTCGCCAACCTTT
	T2R1R2	GACAAGCCCGAGAAGAGC
3'RACE PCR	T2R1 F3	AACTGTGGCTGTCATTATTT
	T2R1R3	CAAGTGTTTGTTGGAGCAG
Quantitative PCR	T2R1 F1	ATGCTTGAATCAGATGATTTG
	T2R1R2	CTACTCCCCTTCTGCATTAAT
β–Actin3	F1	CCCCATCGAACACGGAATCG
	R1	CGCTCGGCAGTGGTAGTGAA

Cloning the complete cDNA sequence of T2R1

The *T2R1* primers were designed based on the sequences of the taste receptor gene *T2R1* of *D. rerio* (*Danio rerio, T2R1a,* AB200903; *T2R1b,* AB200904) (Lalitha, 2000). Samples (1µg) of total RNA from lips and other tissues were retrotranscribed using a cDNA synthesis kit (TakaraBio Inc., Dalian, China) with oligo (dT18) primer. PCR used 2 µ l of synthesized cDNA as a template.

All reactions contained 25 µ l of 200 nM *T2R1* F1 and R1 primers (Table I), 200 µ M of each dNTP, 2 mM MgCl2 and 1.2 U of rTaq DNA polymerase (TakaraBio Inc.). The amplification protocol was as follows: predenaturation at

95°C for 30 s, 30 cycles of denaturationat 95°C for 10 s, annealing at 57°C for 30 s and a final elongation step at 72°C for 45 s. All PCR products were electrophoresed in 1.5% (w/v) agarose gel stained with ethidium bromide to estimate the molecular mass of theamplicons. The target band of predicted size was gel-purified using a Gel Extraction kit (Takara Bio Inc.), cloned into the pMD-18-T vector (Takara Bio Inc.) and sequenced by Biosun Biotech (Shanghai, China). All experiments were performed in triplicate.

The 5'and 3'ends of the *T2R1* cDNA were obtained according to the manufacturer's protocol for the RACE kit (Takara). The other primers (Table I) were designed for the RACE reaction. The PCR products were subjected to electrophoresis in a 1.5% (w/v) agarose gel and were purified using a Gel Purification kit (Takara). The purified product was recovered, cloned into the pMD-18-T vector and then sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

T2R1 *expression patterns in different tissues*

Fluorescent quantitative real-time (RT) PCR (qRT-PCR) was used to analyze the *T2R1* expression level. Specific primers (Table I) were designed according to the conserved regions of the *T2R1* mRNA sequences from *T. fasciatus* in GenBank (EU272034.1) to detect its expression level. The β-actin gene was selected as the internal control gene, and the length of the fragment was about 200 bp. All primers were synthesized by Shanghai Biocolor, Bio Science and Technology Company (Shanghai, China). PCR products were 150- to 250-bp long.

RT-PCR used a 7500 RT-PCR system (Applied Biosystems, USA). RT-PCR reaction solution consisted of 10.0 µL of SYBRpremix Ex TaqTM (2×), 1.6 µL of primer (10 µM), 2.0 µL of RT reaction mix (cDNAsolution), 0.4µL ROX reference dye or dye II (50×) and 6.0 µL of dH_2O. The thermocycling conditions for the target genes were as follows: initiated with a denaturation step at 50 °C for 2 min, 95 °C for 3 min; followed by forty cycles at 95 °C for 15s, 60 °C for 60s, respectively. Melting curve analysis was performed at 95 °C for 15 s and 60 °C for 60 s over a range of 60-95 °C to verify that a single PCR product was generated. The florescent flux was then recorded and the reaction continued at 95 °C for 30 s, 60 °C for 15 s. We measured the dissolution rate between 65 and 92 °C. Each increase of 0.2 °C was maintained for 1 s and the fluorescent flux was recorded. The standard curve and amplification efficiency of T2R1 and β-actin for RT-PCR were below: T2R1: Y=-0.319x+9.89, R2=0.996; β-actin: Y=-0.324x+9.657, R2=0.990. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Bioinformatics analysis

By using the BLAST (Altschul *et al*., 1997) identity search tools, which NCBI provides and FASTA identity & similarity search tools, which EBI provides; and compare validation by using BLAST in the dbEST and dbWGS database; analyze the structure of nucleic acid and protein sequences by using DNAstar (Burland, 2000) and DNATools (Curran nad Tevedibrink, 2013); predict molecular weight and isoelectric point by using the tool kit in EXPASY site (http://prosite.expasy.org/prosite.html); predict the structure of gene exons and introns by using Sim4 procedure; predict structure domain by using SMART (http://smart.embl-heidelberg.de/) software; predict the secondary structure of protein by using SOPMA and nnpredict software, which EXPASY site offers; conduct signal peptide analysis of proteins by using the neural network analysis of SignalP (http://www.cbs.dtu.dk/services/SignalP/); analyze and forecast protein transmembrane region by using online tools TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/); perform their subcellular localization with online tools PSORTb (http://www.psort.org/psortb/); analyze the functional sites of protein sequence by using ScanProsite (Castro *et al*., 2006) and PROSCAN software (Chambers *et al*., 2008).

Statistical analyses

We used SPSS (version 11.5) software followed by Turkey's-b test to determine the differences. Diverse little letters above histogram bars show significant differences ($P<0.05$) among different tissues in Turkey's-b test All the results were expressed as means ±standard deviations (Ẍ±SD).

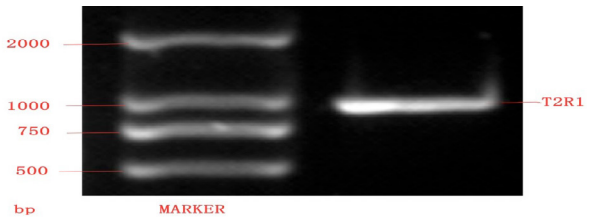

Fig. 1. Amplification of taste receptor T2R1 gene of *Takifugu fasciatus.*

RESULTS

The cloned taste receptor gene T2R1

By cloning and sequencing, we obtained the candidate taste receptor *T2R1* gene of *T. fasciatus* with the full length of 921 bp (Fig. 1) and an open reading frame, encoding 306 amino acids, to the end of the terminator for the gene.

The ratio of A+T was 46.91% and G+C, 53.09% (NCBI accession number: EU272034).

Differential expression of T2R1 *in different tissues*

T2R1 expression was determined in different tissues from *T. fasciatus*, including the gill, spleen, heart, lips, skin and tongue. The *T2R1* gene is expressed in all 6 tissues (Fig. 2) but at different levels. The expression levels were lower in the tongues of *T. fasciatus*, whereas the expression levels were higher in the skins and lips of *T. fasciatus* than those of gill, spleen, heart.

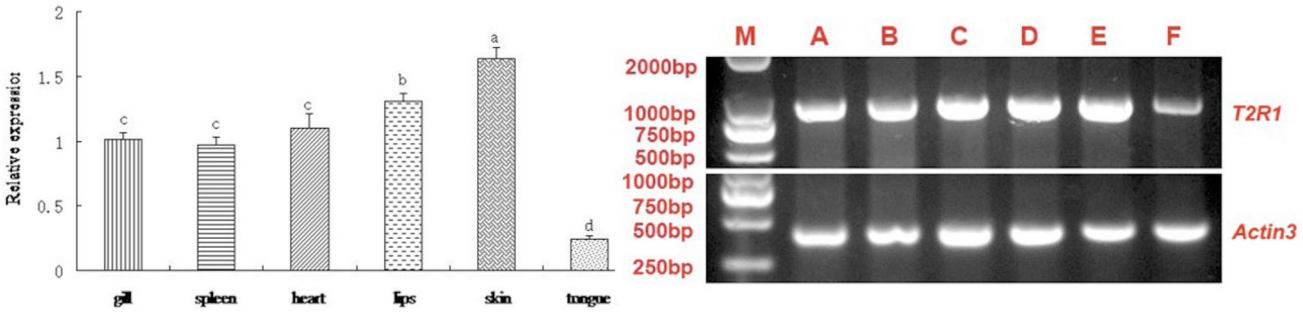

Fig. 2. Relative expression of T2R1 in different tissues of *Takifugu fasciatus*, A, gill; B, spleen; C, heart; D, lips; E, skin; F, tongue.

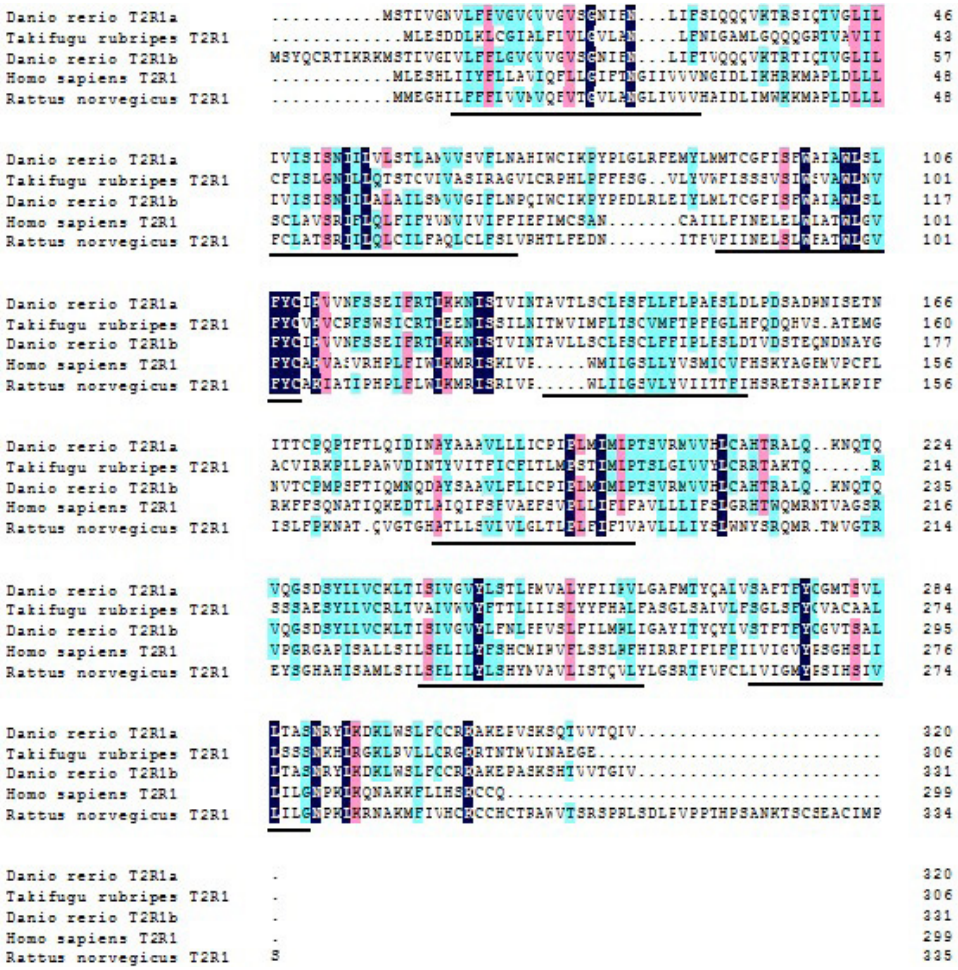

Fig. 3. The similarities of T2R1 amino acid sequences between *T. fasciatus* with other animals. Note: the amino acids are numbered along the left and right margin. The transmembrane domains are underlined.

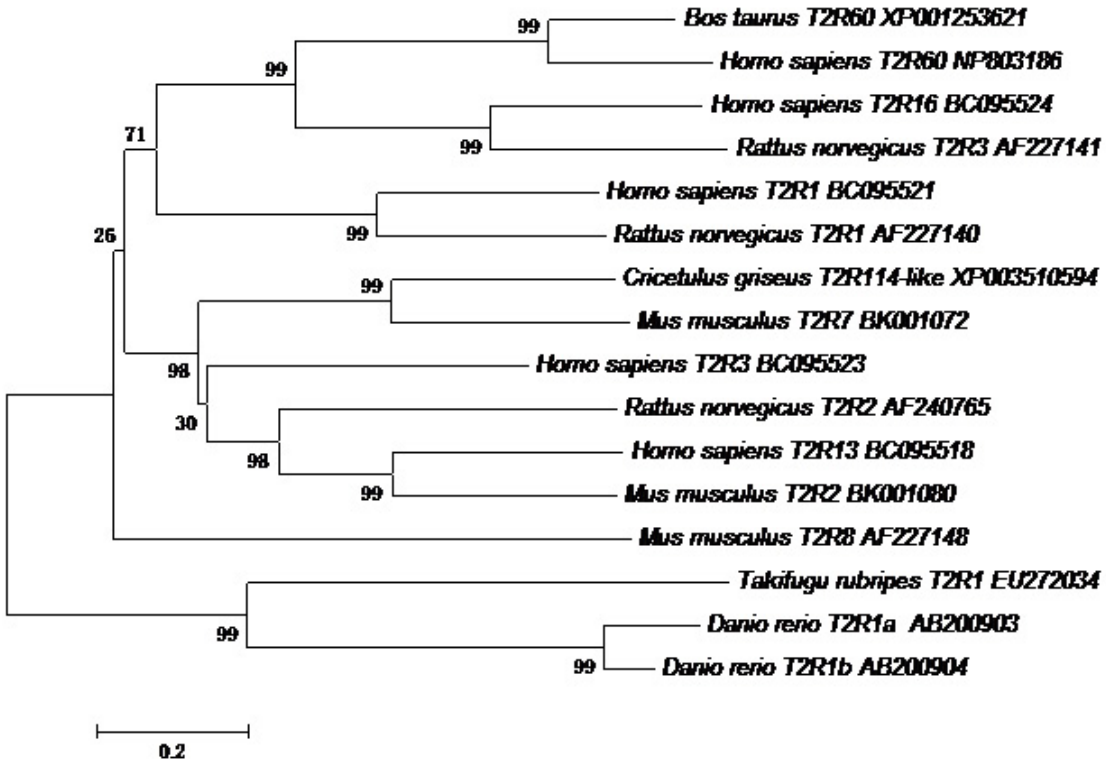

Fig. 4. Phylogenetic tree revealing the relationship of T2R1 in *T. fasciatus* relative to other T2Rs family members of other species. Note: the horizontal branch length is proportional to amino acid substitution rate per site. The numbers represent the frequencies (%) with the tree topology presented was replicated after 1000 iterations.

Bioinformatics analysis of T2R1

The identity of sequences of *T2R1* between *T. fasciatus* and *T. rubripes* reached 99.2%, with only 7 bases different, while the speculated fusion protein had 5 amino acids differentwhen compared with corresponding sequence of *D. rerio,* their identity with *T. rubripes* was consistent. However, when they were compared with the identity to corresponding families of other mammals, it was 80% to 90% homologous with *T2R39*, *T2R56* and *T2R60* fragments of cow, *Bos Taurus* and 91% homologous with *T2R43* fragment of dog, *Canis familiaris*. It was 88% homologous with *T2R124* fragment of horse, *Equus caballus* and 80% homologous with *T2R4* fragments of primates such as human being. The homologous part of the gene was found in the area near 270-320 bp of *T2R1* (Figs. 3, 4).

T2R1 by the cloned expansion of pre-mRNA transcripts obtained a complete body, the length of which was 921 bp, encoding 306 amino acids, the isoelectric point 8.533, and molecular size of 34.025 KD. By using online prediction tools SMART, which the Expasy site provides, the analysis showed that the amino acid sequence includes 7 transmembrane domains, which are consistent with the characteristics of G protein-coupled receptor family. Based on this analysis, in addition to 7 transmembrane domains, extra-territorial, from the 4-266 amino acid residues may be a HTTM (Horizontally Transferred Transmembrane Domain) region; the 9-168 amino acid residues be acid phosphatase protein family function domain; the 85-285 amino acid residues be a GTPase activated TBC domain; the 105-290 amino acid residues be structural domain similar to TRAM receptor (Fig. 5).

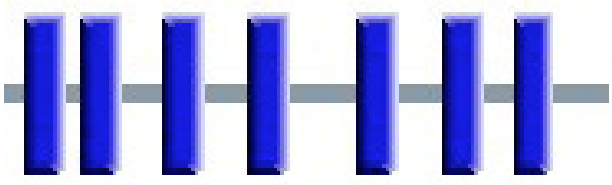

Fig. 5. The structural domain of *T. fasciatus* T2R1 protein Note: transmembrane region 1. 10-32bp; 2. 39- 61bp; 3. 81-103bp; 4. 124- 146bp; 5. 179- 201bp; 6. 222- 244bp; 7. 259- 278bp.

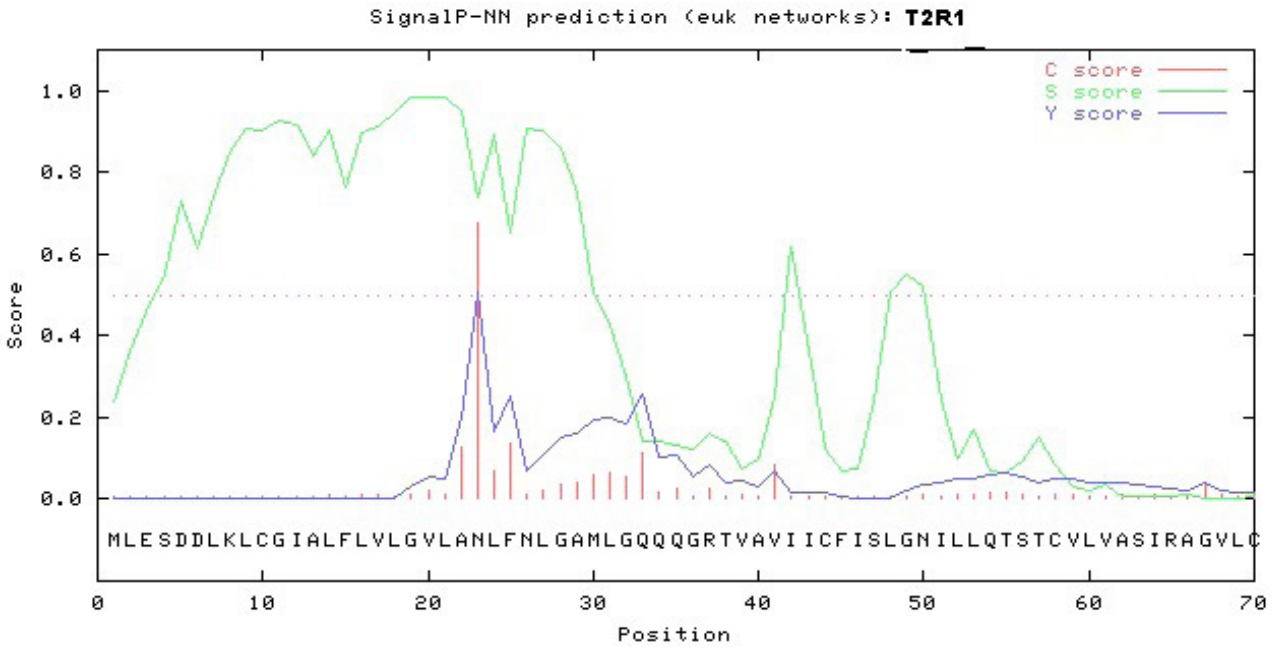

Fig. 6. Signal peptide analysis of T2R1 protein of *T. fasciatus*.

By using SignalP neural network analysis method, we conducted signal peptide analysis of the T2R1 protein. The results showed that the signal peptide was in 1-20 amino acids of the protein, with the scores of 0.98, and the split site was between the 22 and 23 amino acid residues (Fig. 6).

It could be predicted by TMpred online tool that there were 7 transmembrane helixes, which were from inside to outside and 7 transmembrane helixes, which were also from outside to inside. This strongly proved that the protein belonged to the 7 transmembrane receptor's family (Fig. 7). In the meantime, we used TMHMM to verify it, and the result showed that peptides from the 7-26 amino acid region were the transmembrane helixes, which were from inside to outside. This was consistent with the former predicted result.

PSORTb online tools were used to localize the gene in the subcellular level. The results showed that the protein was localized on the membrane, with the score as high as 10. Functional site analysis by software PROSCAN showed that the protein contained 2 N-glycosylation sites. This means the protein was a kind of glycoprotein. The protein sequence contained 4 protein kinase C phosphorylation sites, 3 casein kinase, phosphorylation sites, 3 N-myristoyl sites and 1 amidation site too. It showed the protein was probably related to the regulation of cell signal transduction.

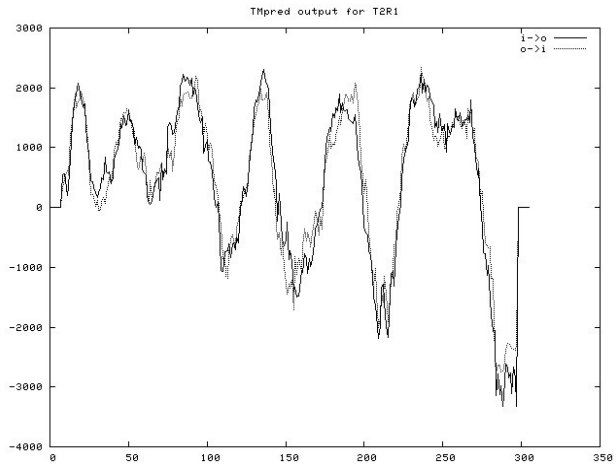

Fig. 7. Transmembrane helices prediction of T2R1 by TMpred online tool.

DISCUSSION

Evolution of taste receptor gene T2R1

Studies related to taste receptor of fish are in its infancy stages. As a result, the data we collected was obtained from phylogenetic analysis in the database such as Genebank, the closest sequence we could search was other member's of the taste receptor family of mammals

(Go, 2006).

Compared with the known sequences of *T. rubripes* and *Brachydanio rerio,* the identity of homologous fragment of *T2R1* from homologous cloning could reach more than 98%. Therefore, it could be speculated that the identity of taste receptors in the same genus was higher, but there is instability of the sequences among different fish species.

From inter-specific comparisons, the identity of *T2R1* between the obscure puffer fish and other species were lower. There was an area near 270-320 bp, the identity of which was more than 80% compared with multiple taste receptor genes of No. 2 family of mammals, so it could be speculated that there was a conserved region of taste receptor gene of No. 2 family.

The success of taste receptor gene cloning of *T. fasciatus* was mainly based on the similarity and structure of the gene sequence. In regards to the research of function, regulations of the gene could be all round proved by RNA interference, gene knockout or transgenic method. The real characteristics of the expression of the gene could be known by immuno-histological chemical reaction, fluorescent quantitative PCR and in-situ hybridization. The direction of future research should be focused on the relationship between the regulation processes and the actual behavioral response.

Differential expression of T2R1 *in different tissues*

It could be known by analyzing the differential expression in several tissues of T. fasciatus that T2R1 could be expressed in gill, spleen, heart, lips, skin and tongue. Recent research shows that, T2Rs is expressed in the human lung and can relax bronchial smooth muscle by Ca^{2+} signaling pathway (An *et al*., 2012). At the same time, we found that the Gene Expression Omnibus (GEO) analysis was based on GenBank, GDS3834 / 5828 / TAS2R1 data show that T2R1 has more or less expression in a variety of normal tissues. Our results confirmed that the expression of T2R1 in multiple tissues of T. fasciatus, it is suggested that it is not only as a taste receptor, but also plays a role in other aspects.

The experiment is just a qualitative study of the expression of taste receptors, which does not express quantitative analysis. Further, detailed studies should be conducted with the interval design, with different gradient concentrations of chemical substances to stimulate, using Real Time-PCR or fluorescence in situ hybridization and other techniques so as to quantify the expression of the taste receptor gene in tissues. The use of the RNAi or genetically modified and other technologies is also essential in order to confirm its function. In addition to research focused on their functions, in the future, research work is needed in the regulation of gene networks.

Gene structure and the type of the protein of T2R1

It should be known by bioinformatics analysis of the speculated protein of *T2R1* that the protein had the same characteristics as the taste receptor gene family such as having 7 transmembrane helix structural domain; that the protein was localized on the membrane of the cells; that it had the obvious structure of signal peptide, horizontally transferred transmembrane domain (HTTM), TBC domain which could activate GTPase, and that it had the similar structure of TRAM receptor. All these from the structure proved that *T2R1* had the functions of membrane transferring, receptor and GTPase activation. From functional site analysis, the results showed that the protein contained several phosphorylation sites and glycosylation sites, which indicated that the protein may be related to the regulation of cell signal transduction, and which meant it was related to chemical sensory conduction too. The characteristics of the protein were matched with the receptor proteins of other kinds of eukaryotes (Upadhyaya *et al*., 2010, 2014; Singh *et al*., 2011, 2014). This discovery made the experiment more meaningful.

The structure and potential function of the gene were speculated by bioinformatics analysis, and it has confirmed the probability of the taste gene, which lay a good foundation for the further study about molecular expression and regulation of the taste gene. It could be speculated from the sites that taste receptor gene was highly conserved in the fish, so the results of family number 1 and 2 of *T. fasciatus* had a high reference value to the research of taste receptor gene of other kinds of fish. The paper revealed the function sites of taste receptor gene of *T. fasciatus*, which laid the foundation for further research related to the sensory signal transduction of chemical substances, and the reaction mechanism of taste cells to the special chemical substances. It is important for the development of the new attractant so as to provide target gene for the molecular controlling. It also demonstrates there is a need to promote the production of *T. fasciatus* for the application of science and technology.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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