Immune and gustatory roles of the channel catfish skin as revealed by comparative transcriptomic analyses

by

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Abstract

Fish scales are physical barriers protecting them from various external stresses, in particular, from pathogen infections. However, vast majority of catfishes are evolutionarily scaleless. How scaleless fish respond to external environment, particularly against pathogen infections, is of great interest to biologists. Besides, much effort has been put on the expression patterns of potential immune-related genes post bacterial challenges for the catfish skin. A number of genes have been identified as differentially expressed genes, which are expressed significantly higher at certain time points post bacterial challenges, suggesting their significant roles involved in the catfish skin immunity. However, the expression patterns of these genes in the catfish skin under normal status remains largely unknown. Recently, RNA-Sequencing of several channel catfish tissues and organs allows us to conduct intra-species transcriptomic comparison to learn about specific transcripts in the channel catfish skin involved in the immunity. Furthermore, the complete RNA-Sequencing of several armored fish species, along with the RNA-Seq datasets of the channel catfish skin, makes the skin transcriptomic comparison between scaleless fish and scaled fish feasible. Such comparisons should provide us insights into the special roles of the channel catfish skin against the pathogen invasion. In the present study, these objectives were achieved through extensively analysis of RNA-Seq datasets originated from Illumina sequencing platforms.

The catfish skin transcriptome was compared to that from four catfish tissues, barbel, gill, liver, intestine to determine skin-specific transcripts, especially those involved in immune responses. Also, the catfish skin transcriptome was compared to that of four other fish species, zebrafish, rhodeus, notothenia and stickleback, to determine the skin specific transcripts,

especially those involved in immune functions in the scaleless skin of catfish. My results indicated that the channel catfish skin is more involved in the host immunity than previously known. Inter-species transcriptomic comparison suggests that not only the percentage of catfish-specific genes involved in the immunity is the highest among all fish species used in our study, but also the percentage of catfish-specific GO term directly involved in the immunity is the highest, too. This might be related to the scaleless characteristic of the channel catfish skin.

Taste sensation plays pivotal roles for nutrient identification and acquisition. Channel catfish lives in turbid waters with limited vision, and taste sensation can be even more important for food seeking and survival. This biological process are mainly mediated by taste receptors expressed in taste buds distributed in several organs and tissues, including the barbel and skin. It has been known for a long time, that taste receptors as well as gustatory associated G proteins are involved in the gustation. Although many studies were previously conducted on mammalian taste receptor genes and gustatory associated G protein genes, studies of these genes in fish species, especially in the channel catfish, is lacking. Considering the importance of the gustation for fish feeding, along with significant roles of these genes involved in gustation, comprehensive studies for these genes in fish species are needed. Thus, upon the completion of the reference genome assembly and the availability of RNA-Sequencing datasets from various tissues and organs, the author identified these genes in the catfish genome first, and then characterized their expression patterns in various tissues and organs, providing a general understanding of these genes in both genomic and transcriptomic levels in the channel catfish.

In the present study, I identified a complete repertoire of taste receptor and gustatory associated G proteingenes in the catfish genome. A total of eight taste receptor genes were identified, including five type I taste receptor genes and three type II genes. Addition four

genes for gustatory associate G protein genes were also identified. Their genomic location, copy numbers, phylogenetic relations, orthologies, and expression were determined. Phylogenetic and syntenic analysis allowed determination of their evolution dynamics of these gene families. Furthermore, motif and dN/dS analyses allowed the inference of selection pressure imposed on these receptors. Expression patterns of catfish taste receptors and gustatory associated G proteins across organs are similar to the distribution of taste buds across organs. Expression comparison between catfish and zebrafish skin transcriptome provided evidence for potential roles of catfish skin in taste sensation.

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MALT	Mucosal Associated Lymphoid Tissue
TR	Taste Receptor

Chapter 1

Literature review

Fish Skin Immunity

Unlike armored fish species, channel catfish (*Ictalurus punctatus*) is scaleless, a characteristic makes its skin as the most external surface against pathogen infections from the outside environment. Actually, it's the mucosal surface of channel catfish skin functioned as a physical barrier to prevent the host from infections (Ellis, 2001; Magnadottir, 2006). The catfish skin mucosa, also named skin-associated lymphoid tissue (SALT), belongs to mucosa-associated lymphoid tissue (MALT), which is also composed of other MALTs, such as gut-associated lymphoid tissue (GALT) and gill-associated lymphoid tissue (GIALT) (Ángeles Esteban, 2012; Salinas et al., 2011). All of these tissues are important immune compartments, and play pivotal roles in the fish immune system. Comparing to mammals, teleost skin is completely different because it remains the active dividing ability in its outermost layer (Salinas et al., 2011). In turn, the epidermis of fish skin is non-keratinized (Rakers et al., 2010), which makes them is more vulnerable of pathogen invasions. Besides, several types of secretory cells can be found in fish skin, including but not limit to goblet cells, malpighian cells, sacciform cells and club cells (Salinas et al., 2011), suggesting various biological activities could be potential involved in the immune

activities in fish skin. Thus, combing all the characteristics described above, along with the fact that fish skin is directly contacted with the environment, the roles of the fish skin involved in immunity, especially for scaleless fish species, become certain interest for scientists.

Skin-associated immune system has undergone great evolutionary changes from the invertebrate to the vertebrates. The invertebrates adopt a non-specific humoral immune system, completely different from the system adopted by the vertebrates. The first biggest difference between these two immune systems is the emerging of dendritic cells. Before that, the invertebrate mainly has antimicrobial peptides, oxidase, lysozyme, agglutinins and other proteins to keep its immune system functional (Schempp et al., 2009). However, the dendritic cells in the vertebrates can link the innate immune system and the adaptive immune system, an advanced and efficient way to enhance the capability of the whole immune system. Furthermore, the invertebrate has evolved with a more efficient immune system, the specific immunity, which first found in the fish species. This system consists of antibodies and T-cell receptors, and also possesses the ability of cell memory to enhance the immune defense when facing the same threat again (Schempp et al., 2009). Over the evolutionary course, the immune system has become an efficient and complexity system, starting from the invertebrate to the vertebrate, and the development of fish immune system has been a critical turning point for organism immunity. Of the fish species, the development of teleost immune system has played a major and pivotal role. The landmark of the adaptive immunity, which is the IgM positive

lymphocytes, was first found in the skin of the teleost (Schempp et al., 2009). Amazingly, further study indicated that the pattern of the teleost skin immunity resemble that of the teleost gut immunity (Xu et al., 2013). That's mainly based on theobservation that IgT, an antibody mainly found in the gut immunity, is expressed extensively in the teleost skin (Xu et al., 2013). To our knowledge, the studies of teleost skin immunity mainly focus on two fields, one is the biology of mucosal T cells and another is the mechanism of the cell memory (Salinas, 2015). All these two aspects have become certain interests for scientists specialized in the fish skin immunity.

Comparing to the systematic immunity, mucosal immunity is more vulnerable to the environmental changes. This is particularly critical for the fish species, since they live in pathogen rich waters. Indeed, the antibody secreting cells (ASCs), which has distribution in the fish skin (Zhao et al., 2008), are directly facing the challenges caused by the invasion of pathogens (Delamare-Deboutteville et al., 2006). Thus, the environmental factors, especially for the diversity of the environmental factors, could have considerable impacts on the fish mucosal immunity. One of these factors is hyperosmotic pressure, which can increase the expression of antibodies (Wu et al., 2004), leading to the enhancement of the immune system. Even though this study was conducted on mammalian cell lines, similar observation can be expected in fish species as well, which is still need further experiments to validate. Also, the active degree of the mucosal antibodies are totally different for a fish species, which higher expression of antibodies was observed in the seawater that that in the fresh water

(Delamare-Deboutteville et al., 2006). For fish species, seasonality plays prevailing roles in several aspects, like spawning, homing and most importantly, the immunity. However, less effort has been put on this subject with only few studies has been focused on the relation between seasonal changes and fish immune activities. A correlation was observed between the mucosal immunoglobulin and seasonal changes, which is a mechanism adopted by flounder to protect itself when higher water temperature incubates more pathogens (Jung et al., 2012). So other than studies of the mucosal or skin immunity, external environmental factors, and their effects on the mucosal immunity, should be deserved more effort from scientists.

There are several pathogens can cause severe fish diseases, including virus, bacteria, prion and fungus, leading to enormous economic losses in aquaculture industry. Among these, one virus, IPNV, can escape from the cytolytic activities of macrophages, thus causing fish diseases (Ellis, 2001). Also, interferons (IFNs) can initiate innate immune response in fish through producing dsRNA during their replications (Ellis, 2001). Also, the innate immune systems of fish species are effective against a broad spectrum of bacterial pathogens. This is achieved by producing anti-microbial substances, and cytokines. Besides, a series of immune biological activities, including non-classical complement activation, inflammation and phagocytosis, are induced after infections. Amazingly, some fish without a functional immune system, still can co-exist with some high virulence bacterial pathogens, and have no sign of morbidity (Ellis, 2001). *Flavobacterium psychrophilum,* a bacterial disease that has extensive impacts on a broad range of

cold water living fish species, can cause severe coldwater disease (CWD) (Starliper, 2011). *Aeromonas hydrophila*, a Gram-negative bacterium, can cause severe lethal disease in catfish, leading to a enormous losses in the US catfish industry (Li et al., 2013a, 2013b). More than that, *Edwardsiella ictaluri*, a bacterium can invade the catfish through the intestinal epithelium, as well as other mucosal surfaces, were found that it can induce severe diseases too (Li et al., 2012). It is also a common phenomenon that, *Flavobacterium columnare*, are responsible for the outbreaks of columnaris disease in both cultured and wild catfish (Sun et al., 2012).

Previous studies revealed that several proteins, which can be found in the fish skin, have significant roles for skin immunity. Foremost, mucus is fundamental to fish skin immunity far than any other immune-related substances. It covers the epidermis of fish, and heavily involved in the skin immunity, a unique characteristic very different form that of mammals (Salinas et al., 2011). In the larvae stage, the fish already has mucus cells in its skin, even though the skin is thin with only two cell layer (Varsamos et al., 2005). For the adult, the mucus layer covers the whole external body surfaces, along with the epidermis and dermis (Ángeles Esteban, 2012). Thus, because of the main function of mucus secreted by the fish skin, it could act like the first line of immune defenses against the pathogen infections (Easy and Ross, 2009). Also, other cells, which can produce watery and serous fluid, were found in the skin epidermis (Ángeles Esteban, 2012). The number of mucous cells in fish species varies, and is mainly affected by many stress factors. Now, it is confirmed that the skin mucous cells can monitor stress (Vatsos et al., 2010). The

mucus found in the fish skin is slipperiness, as a result of the presence of high molecular weight watery gels, most importantly, the glycoprotein (Magnadottir, 2010). After injuries caused by the predators, or human activities, the mucus secretion cells can serve as a quick response to help the skin healing from wounds, through mucous secretion and accumulation on the surface of adjacent epithelial cells (Gostin et al., 2011). Among all types of mucus, cutaneous mucus is the one that be considered as the first line of immune defense against the infections (Mestecky et al., 2005). It plays pivotal roles in fish skin immunity mainly as a physical and biological barrier (Raj et al., 2011; Subramanian et al., 2008, 2007). Over the evolutionary course, the fish skin has become a robust mechanism that has several immune-related functions, including trapping and immobilizing pathogens (Cone, 2009). Moreover, the secretion of mucus in most fish species is continuously, further preventing the stable colonization of potential infectious microorganisms and parasites (Nagashima et al., 2003). The mucus protects the fish skin mainly through the prevention of the pathogen adhesion to the skin (Cone, 2009). Another dynamic ability of mucus is that it can maintain an unstirred layer adjacent to the epithelial surfaces (Cone, 2009). It is complex, and its composition varies. As it is completely exposed to the environment in which the fish live, so there is a high demanding that all the proteins in the fish skin mucus should be able to deal with extremely conditions, such as high temperature and hydraulic pressure (Ogawa et al., 2002). Besides, lipids in the fish skin mucus, including covalently attached fatty acids, can contribute to the interactions between fibers, thus increasing the viscoelasticity of

the gel (Ángeles Esteban, 2012). Taken together, the mucus secreted in the fish skin not only biologically, but also physically serves as the first line of immune defense against a variety of pathogens.

As a complex immune system, the fish skin involve in immunity based on a lot of effective functional substances. They can exhibit their immune-related functions independently, or work together to eliminate the pathogens. One of the most abundant components is mucin, a type of glycoproteins (Phillipson et al., 2008). It is a strong adhesion protein that can form a matrix with a broad spectrum of antimicrobial molecules, further affecting the viscoelastic and rheological characteristics of the fish skin mucus layer (McGuckin et al., 2011; Thornton et al., 2008; Yan, 2009). Previously, scientists most dedicated their effort to the fish mucins, however, the nature of the glycoproteins remains largely unknown (Kumari et al., 2009). Innate immune components have been previously reported that extensively involved in the fish skin immunity (Fast et al., 2002; Salinas et al., 2011; Whyte, 2007). These substances include complement, C-reactive proteins, protease, lectin, lysozyme, haemolysin, agglutinin, proteolytic enzymes, antimicrobial peptides and immunoglobulin. All of these have significant roles for the fish skin immunity, and constitute a series of strong innate immune activities against the bacterial infections.

Lysozyme, also named as N-acetylmuramide glucanohydrolase, was found that expressed ubiquitously across a broad chordate phyla. It exists in mucus, lymphoid tissue, and serum of several fish species, and contributes the fish skin immunity

against the pathogen infections (Bergsson et al., 2005; Ellis, 1999; Magnadottir et al., 2005; Nigam et al., 2012; Saurabh and Sahoo, 2008). However, the degree of lysozyme involved in the fish skin immunity varies in different fish species (Nigam et al., 2012). For instance, in the skin mucus of Atlantic salmon, the expression level of lysozyme was higher in the fresh water species than that in the seawater species (Fast et al., 2002). Two of the lysozyme enzymes, acid and alkaline phosphatases, all have significant roles involved in the fish skin innate immunity (Nigam et al., 2012). Similar expression patterns were found for these two lysozymes, as their expression in fish skin is species-specific. They mainly used for wound healing for carp and prevention of parasite invasion in Atlantic salmon (Dutta and Rai, 1994; Ross et al., 2000). Cathepsins, which were found in the early stages like egg and larvae, also play pivotal roles in the fish skin immunity, including Japanese eel and catfish (Cho et al., 2002; Jung et al., 2003).

Taste Receptor

Taste sensation, as a primary sensory system, is mainly responsible for sensing food and corresponding food sources. After vision check and smell, taste sensation occurred in the mouth helps organisms to make the final recognition of food and thus make the selection. There are five types of taste sensation in vertebrates, including sweet, umani, sour, bitter, and salty. Each of them was detected through different groups of receptors, constituting a complete taste sensation system for organisms, which is essential for living. To nowadays, much effort is now being

made on the identification and characterization of taste receptors in various organisms, from invertebrates to vertebrates (Lindemann, 2001). Sweet sensation is mainly used for the detection of nutrients, umani is mainly used for the recognition of amino acids, salty sensation was mainly used for dietary electrolyte balance, and sour and bitter were mainly used for warning of potentially noxious and poisonous chemicals (Chandrashekar et al., 2006).

The detection of sweet taste sensation is mainly mediated by three subfamilies of taste receptors, including T1R1, T1R2, and T1R3 (Bachmanov et al., 2001; Hoon et al., 1999; Kitagawa et al., 2001; Li et al., 2002; Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2002, 2001; Sainz et al., 2001). T1R3 is co-expressed with T1R1 for the sensation of umani, and T1R3 is co-expressed with T1R2 for the sensation of sweet. The roles of T1Rs in the taste sensation is the perception of tastants, including like natural sugars, D-amino acids, sucrose, saccharin, dulcin, sweet proteins, L-amino acids and monosodium L -glutamate. Another taste receptor family, T2R family (taste receptor, type 2), which has the most members in taste receptor gene family (Adler et al., 2000; Chandrashekar et al., 2000), was found mainly function for the bitter sensation even though the functions of several members within T2R family are not clear (Bachmanov and Beauchamp, 2007; Ishimaru et al., 2005; Lindemann, 2001; Mombaerts, 2004). Another interesting finding is that several T2Rs co-expressed with each other in the same TRCs, along with the facts that the number of bitter tasted chemical compounds in the nature are larger than that of T2R taste receptors (Jaggupilli et al., 2016), indicating that taste

receptor in vertebrates are more dedicated for sensing rather than distinguishing for bitter sensation, a characteristic unlike with the olfactory receptors (Adler et al., 2000; Bachmanov and Beauchamp, 2007; Caprio, 1975; Lindemann, 2001).

Despite the functions of taste receptors are similar in mammals and fish species, distributions of taste receptors (or distribution of taste buds) in mammals and fish species are not exactly the same. Taste receptors were found in several common organs with the epithelium for both mammals and fish species, while some distinct fish organs were also found to harbor taste receptors. For example, in mammals, taste receptors were found mainly in epithelia of lips, oral and oropharyngeal cavity, including the tongue, palate and pharynx (Lindemann, 2001). In fish species, especially in the catfish, distributions of the taste receptors are more diversified (Caprio and Derby, 2008). Besides all the organs and tissues aforementioned, the entire external body surfaces in fish species, including the barbel and skin, were also considered as the density regions covered by taste buds (Caprio, 1975; Raji and Norozi, 2010). In addition, the taste receptors were also identified in fish gill, a fish specific organ for breathing (Caprio and Derby, 2008; Hansen et al., 2014; Hansen et al., 2002). Also, solitary chemosensory cells, a type of cells distributed with high density in teleost external body surface, shared similar functions with TRCs (Caprio and Derby, 2008; Hansen et al., 2014; Hansen et al., 2002).

Beyond TRs, some G proteins were believed to be involved in gustatory activities. They co-expressed with T1Rs for sweet sensing in the gut of mammals (Dyer et al., 2005; Margolskee et al., 2007). These proteins, especially the α subunit,

were found involved in transduction signal pathways for both bitter and sweet in the taste buds of rat tongues (Shen et al., 2005). These studies indicating that G proteins may play significant roles involved in gustatory than previously expected (Shigemura et al., 2008). However, expression analysis of gustatory associated G proteins remains largely unknown in fish species. Thus, expression profiling of these G proteins, along with the expression profiling of taste receptors, becomes necessary for the study of taste receptor in fishes.

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Chapter 2

Comparative Transcriptomic Analyses of the Channel Catfish Skin Suggest its Roles Involved in Immunity

Abstract

Fish scales are physical barriers protecting them from various external stresses, in most cases, pathogen infections. However, vast majority of catfishes are evolutionarily scaleless. How scaleless fish respond to external environment, particularly against pathogen infections, is of great interest to biologist. In present study, we conducted the intra-species transcriptomic comparison between catfish skin and four other organs (including barbell, gill, liver, intestine) upon the RNA-Seq datasets generated from Illumina sequencing platform. Also, skin transcriptomes of four other fish species (including zebrafish, rhodeus, notothenia and stickleback), along with catfish skin transcriptome, were used to conduct the inter-species comparison. Our results indicate that the channel catfish skin is more involved in the host immunity than previous expected through expression comparison between skin and the other tissues and organs. Inter-species transcriptomic comparison suggests that not only the percentage of catfish-specific genes involved in the immunity is the highest among all fish species used in our study, but also the percentage of catfish-specific GO term directly involved in the

immunity is the highest, too. This might be related to the scaleless characteristic of the channel catfish skin. As the pathogen diseases become a main reason of the severe economic losses in aquaculture around the world, our study could facilitate the development of fish disease precaution strategies and thus support the sustainable growth of the catfish industry.

Introduction

Comparing to other armored fish species, channel catfish is scaleless, a characteristic leaving its skin as the most external physical barrier facing the microorganisms in the environments, especially a broad spectrum of pathogens causing lethal diseases. According to the anatomical classification, the catfish skin belong to the mucosa-associated lymphoid tissue (MALT), a group consists of several other tissues, including gut- and gill-associated lymphoid tissue (Ángeles Esteban, 2012). All of these MALTs form the first line of defense against pathogen invasions, but only skin constitute the largest and the most exposed area when facing potential pathogen infections (Wilson and Laurent, 2002). Considering catfish live in an environment fulfilled of various pathogens (Magnadottir, 2010), and the wounds caused by its wide range of predators (Power, 1984), the study on the roles of catfish skin involved in immunity has become a certain interest for researchers.

Previous studies, based on various gene expression techniques, identified several associated genes that were significantly up regulated after the bacterial

challenge in catfish skin, highlighting its roles involved in immunity. These genes include lectins, chemokines, and interleukins (Li et al., 2013a, 2013b). Also, several bacteria were used to challenge the catfish, including *Edwardsiella ictaluri*, *Flavobacterium columnare* and *Aeromonas hydrophila* (Li et al., 2013a, 2013b, 2012; Sun et al., 2012; Zhang et al., 2014), all of which will cause serious disease in the catfish, further leading to the severe economic losses in the catfish industry. However, the concentrations of bacteria used in previous studies were so high, unlikely resemble the challenges catfish facing in natural environments, leaving a big void for the study of catfish skin immune mechanisms. Besides, varieties of bacteria exist in the living environment of channel catfish and may evoke the skin immune reactions simultaneously, a complicated situation that cannot be simulated only using one type of bacteria for challenging. Thus, an experiment designed for clarifying the exact status of the catfish skin against the pathogens under normal status, like the expression levels of immune related genes identified in aforementioned studies, becomes necessary for the research of catfish skin.

Mucus, a gel like protein secreted from the mucosa of fish skin, plays pivotal roles involved in immunity (Salinas et al., 2011). It protects the catfish skin from the attachment, then the invasion of environmental pathogens. Basically, the mucus contacted the pathogens first and then trapped them, constructing a physical barrier isolated the pathogens from the fish external body surfaces (Cone, 2009). More than that, the components of the mucus change as the environmental factors vary, accordingly, allowing the fish to make suitable adaptations to a new or changing

living environment (Easy and Ross, 2009; Jung et al., 2012). Beyond the roles it served directly against the pathogen infection, the mucus also comprise of several immune associated proteins, including lysozymes, lectins, antimicrobial peptides and most importantly, the mucins, a high molecular weight filamentous protein that its roles involved in immunity has been well addressed (Ellis, 2001; Palaksha et al., 2008; Rajan et al., 2011; Rombout Jan et al., 2011). Therefore, the biological features of fish skin mucus can effectively delay the invasion of various pathogens, or even eliminate them before they invade into fish, a unique characteristic that has drawn extensive interests from scientists.

Upon the completion of the catfish reference genome, along with several organ-specific RNA-Sequencing experiments conducted in our lab, the comparative transcriptomic study of catfish skin with other tissues and organs become feasible. Furthermore, several fish skin RNA-Seq databases generated from Illumina sequencing platform were published, allowing us to conduct the comparative analysis among skin transcriptomes of various fish species. In present study, we highlight the significant roles of catfish skin involved in immunity, including both innate and adaptive immune responses, through intra-species and inter-species transcriptomic comparisons. Our study provides a comprehensive picture of catfish skin involved in immunity, as well as its transcriptome. This will facilitate the development of catfish disease precaution strategies or vaccines, further supporting the fast growth of the catfish industry across the world.

Materials and methods

Transcriptomic comparison of catfish skin with other organs

RNA-Seq datasets of channel catfish skin, barbel, gill, liver, and intestine were pooled together and assembled using Trinity (Grabherr et al., 2011; Haas et al., 2013). The *de novo* assembled transcriptome was annotated through blast against zebrafish reference protein database. Expression values of all genes were identified using RSEM (Li and Dewey, 2011) while the alignment method is bowtie2 (Ding et al., 2013). We used edgeR (Robinson et al., 2009) to identify DEGs. FPKM values were log transformed before heatmap was generated using R. The overlap of differential expressed genes identified between skin and each of other four tissues was extracted representing skin-specific expressed genes comparing to other four tissues (FDR < 0.01). Besides, up-regulated overlap DEGs identified between skin and mucosal tissues (gill and intestine) as well as skin and non-mucosal tissues (barbel and liver) in order to confirm more genes associated with immunity may listed as DEGs in catfish skin versus non-mucosal tissues. Up-regulated genes in channel catfish skin were selected at least two-fold change from group of genes described above. Finally, overall expression pattern of all the tissues and organs were examined using Cufflinks and CummeRbund (Trapnell et al., 2012).

We took a stepwise procedure to identify DEGs, starting from identify DEGs through comparing expression levels of all genes in the catfish skin with each of other four organs separately, and then extract the shared DEGs among the four

groups of comparison. Besides, in order to distinguish the degree of relevance and importance of DEGs involved in skin immunity, we identified three groups of DEGs based on their expression levels in catfish skin comparing to other tissues and organs, including group 1 (16X fold change), group 2 (8X fold change, excluding genes in group 1), and group 3 (4X fold change, excluding genes in group 1 and 2) (Figure 1). Each of these groups is highlighted using different colors in Supplemental Table 1.

Comparative transcriptomic analysis of various fishes skin

RNA-Seq datasets of four fishes skin (zebrafish, rhodeus, notothenia, stickleback) were downloaded from NCBI SRA database and assembled using Trinity (Grabherr et al., 2011; Haas et al., 2013), respectively. Each of the transcriptome plus channel catfish transcriptome was annotated through blast against zebrafish reference protein database. Transcripts from all five fish species were considered orthologous if they have best hits with same zebrafish protein. Distribution of orthologous was displayed in Venn diagram. External numbers delineates list of genes unique to certain species as well as numbers in overlapping region represents groups of orthologous. GO annotation for each gene of all five fish transcriptomes was conducted using Blast2GO (Conesa and Gotz, 2008; Conesa et al., 2005; Gotz et al., 2008) based on three categories: cellular component, molecular function, and biological process. Shared and unique GO terms between five fish transcriptomes displayed in Venn diagram. After GO term functional

annotation, we performed annotation augmentation and GO slim summarization to extract the sequences annotated with level 2 GO term "immune system process".

Results and discussion

Transcriptomic comparison among tissues and organs

To provide insights into the characteristics of the channel catfish skin transcriptome, we compared transcriptomes of several tissues of channel catfish available including that of barbels, gill, liver, and intestine. A total of 372 genes were expressed at least four times higher (FDR < 0.01) in the skin than in all other four organs, of which 200 genes and 86 genes were expressed at least 8 times and 16 times higher than in all other analyzed tissues (Figure 1). A heat map is used to display the expression patterns of these genes and similarities among the tissues (Figure 2). In addition, 32 genes were expressed at least four times lower (FDR < 0.01) in the skin than in all four other tissues (Figure 3). The descriptions, FPKM values, and fold changes across tissues and organs for all these genes significantly differentially expressed in the skin are summarized in Supplemental Table 1, following the descending order of FPKM values in the catfish skin.

Among these genes that are expressed significantly higher (Group 1, > 16X) in the skin, the ten genes that are expressed highest in the skin were: four collagens, two periostins, thread biopolymer filament, mimecan, troponin and myosin. Using expression patterns of these genes, liver is most similar to the intestine, and barbel is

Table 1. Expression levels of immune-related genes expressed significantly higher in the skin (FPKM, FDR < 0.01)

Group	Gene Description	Barbel	Gill	Intestine	Liver	Skin
Group 1 (> 16X)	myosin, light chain 10, regulatory	0.42	6.45	0.00	0.10	316.83
	myosin-7-like	0.22	4.75	0.04	0.00	222.31
	slow myosin heavy chain 3	0.13	6.26	0.09	0.00	159.33
	myosin-9-like	6.06	7.72	0.09	0.00	142.14
	hemoglobin subunit alpha	0.00	0.00	0.00	0.00	40.84
	myosin light chain 4	0.00	1.42	0.00	0.00	39.54
	myosin-binding protein C, cardiac-type isoform X1	0.00	0.19	0.00	0.00	7.09
	WAP, Kazal, immunoglobulin, Kunitz and NTR domain-containing protein 2	0.11	0.18	0.00	0.00	5.35
	immunoglobulin-like and fibronectin type III domain containing 1, isoform X2	0.00	0.00	0.06	0.00	2.56
	mucin-4	0.06	0.08	0.02	0.00	2.55
	CD109 antigen isoform X2	0.02	0.04	0.01	0.00	2.31
	periostin isoform 1 precursor	0.97	47.09	10.30	0.03	1779.40
	periostin isoform X4	0.48	8.42	1.86	0.05	373.63
	periostin isoform X2	0.53	2.35	0.33	0.07	49.68
	myosin-binding protein C, cardiac-type isoform X2	0.06	0.60	0.00	0.00	12.57
	myosin-binding protein C, cardiac-type isoform X5	0.08	0.48	0.00	0.00	12.52
Group 2 (> 8X)	slow myosin heavy chain 1	1.99	53.35	0.40	0.34	912.73
	cytochrome P450 26A1	5.56	4.73	3.42	1.26	83.33
	myosin-binding protein C, cardiac-type	0.08	0.58	0.00	0.00	9.15
	myosin-binding protein C, cardiac-type isoform X3	0.07	0.56	0.00	0.00	7.85
	myosin-binding protein C, cardiac-type isoform X4	0.06	0.26	0.00	0.00	4.41
	myosin-7 isoform X2	0.00	0.26	0.00	0.00	4.15
	immunoglobulin-like and fibronectin type III domain containing 1, isoform X1	0.00	0.04	0.14	0.00	2.26
	leukocyte tyrosine kinase receptor isoform X1	0.11	0.12	0.06	0.00	1.49
Group 3 (> 4X)	myosin heavy chain, fast skeletal muscle-like	0.14	107.79	0.00	1.05	632.30
	leukocyte receptor cluster member 8 homolog	10.01	13.52	11.20	2.29	109.58
	myosin binding protein Hb	2.89	8.17	0.11	0.64	48.76
	myosin-binding protein C, slow-type isoform X6	1.22	5.95	0.03	0.07	40.19
	probable E3 ubiquitin-protein ligase MID2 isoform X2	2.26	4.36	2.03	0.38	24.39
	mucin-2 isoform X1	2.73	3.49	0.00	0.00	23.17
	myosin-binding protein C, slow-type isoform X5	0.34	3.32	0.03	0.01	22.36
	myosin-binding protein C, slow-type isoform X2	0.07	2.49	0.01	0.03	15.01
	serine protease HTRA3-like	1.90	1.84	0.22	0.09	13.48
	immunoglobulin superfamily member 10	0.05	1.28	0.13	0.00	9.18

most similar to the gill, and the skin is most different from the other four tissues (Figure 2). Several collagens were among the highest expressed genes in the catfish skin in all three groups, consistent with previous findings that they represent more than half of the fish skin compositions (Nagai and Suzuki, 2000; Nagai et al., 2002). Also, collagen can contribute to wound healing (Rho et al., 2006) , an effective manner to prevent pathogen invasion. Actually, the expression of collagen genes in catfish skin is diversely and ubiquitously. Even we used a vey stringent threshold (16X higher in skin), we still identified a lot of collagen genes among genes expressed significantly higher in the skin, from different types of collagens genes to different isoforms of a certain collagen gene, further highlights the fundamental roles of collagen in catfish skin upon the RNA-Seq experiment. Also, periostin is found to be associated with several biological activities related to collagens, including fibrillogenesis and crosslinking (Yamaguchi, 2014). This gene has several isoforms among genes expressed higher in the skin, similar to collagens. Another gene product we listed here is myosin, which plays pivotal roles in the skin formation and maintenance through taking part in biological processes, like cellular proliferation, differentiation and movement (Connell, 1961; Morioka et al., 2006). The products of all the genes mentioned above are the main structure components of the catfish skin or they have supporting functions in the skin regular and persistent biological activities. Taken together, we succeed to identify genes that are fundamental to catfish skin's basic biological activities and structure maintenance,

which indicating our further analysis of genes associated with the catfish skin immunity is robust.

Table 2. Expression levels of immune-related genes expressed significantly higher in the skin through comparisons with non-MALTs organs (FPKM, FDR < 0.01)

Gene Description	Barbel	Liver	Skin
C-type lectin domain family 12 member B-like	2.20	0.35	26.33
lectin, galactoside-binding, soluble, 2a	10.54	1.81	126.66
plectin isoform X14	0.21	0.02	2.85
plectin isoform X5	0.89	0.08	10.69
immunoglobulin-like and fibronectin type III domain-containing protein 1	0.00	0.00	2.26
immunoglobulin-like and fibronectin type III domain-containing protein 1	0.00	0.00	3.96
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3Ga precursor	5.04	0.19	52.73
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3Ga isoform X1	0.56	0.53	13.98
mucin-2 isoform X2	0.97	0.00	11.71
mucin-2 isoform X4	0.34	0.00	12.02
chemokine-like receptor 1	0.08	0.00	4.01

Among these genes that are expressed significantly higher in ten genes that were expressed lowest in the skin were Nipsnap, LOC100135433, lymphocyte antigen 86, paramyosin, LOC568167, prolactin receptor, myosin-2 heavy chain, indian hedgehog protein and two CMPs. Using expression patterns of these genes, liver is most similar to the intestine, and barbel is most similar to the gill, and the skin is most different from the other four tissues (Figure 3).

Particular attention was paid to the detection of immune related genes because catfish skin does not have scales. The immune-related genes expressed significantly higher in the skin are summarized in Table 1. These included hemoglobin (> 16X),

immunoglobulins (> 16X), CD109 (> 16X), periostin (> 16X) and mucins (> 16X), and cytochrome P450 26A1 (> 8X), ubiquitin (> 4X), serine proteases (> 4X). These genes' roles involved in immunity have been well studied (Ellis, 2001; Gorman and Paskewitz, 2001; Lin et al., 2002; Majetschak, 2011; Palaksha et al., 2008; Rajan et al., 2011; Rombout Jan et al., 2011; Ullal et al., 2008; Yamaguchi, 2014; Zhang et al., 2014; Zilberg and Klesius, 1997). The significantly higher expression of these immune-related genes in the skin may have reflected the significant functions of the catfish skin in immune responses. Some of these immune-related genes such as mucins and myosins were also found to be induced after bacterial infection (Li et al., 2013a, 2013b), further supporting their roles in immune response within the catfish skin. Although it is possible that the fish samples in this study could have been exposed to the pathogens, we believe that these genes expressed differentially highly in the skin are homeostatically expressed at high levels. This belief is partially supported by the observation that many disease-induced genes were not among these highly expressed genes in the skin. For instance, chemokines, lectins, and interferons were found drastically induced after bacterial infection (Bao et al., 2006; Li et al., 2013a, 2013b; Peatman et al., 2006; Takano et al., 2008).

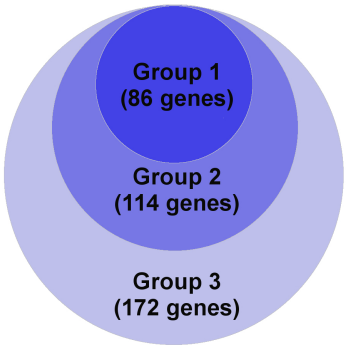

Figure 1. Schematic representation of genes expressed significantly higher in the skin. Group 1 includes all genes expressed 16X higher in the skin (FDR < 0.01); Group 2 includes all genes expressed 8X higher in the skin and exlcuding all genes from group 1 (FDR < 0.01); Group 3 includes all genes expressed 4X higher in the skin (FDR < 0.01) and exlcuding all genes from group 2 and group 3.

Table 3. Statistics of five fish skin transcriptomes

Species	No. of contigs assembled	Contig N50 (bp)	No. of contigs annotated	Annotated contig N50 (bp)	No. of total reads	RNA-Seq library type
Catfish	296,359	1,611	24,999	2,877	169,843,454	Pair end
Zebrafish	215,096	1,352	28,653	2,191	403,042,559	Single end
Notothenia	80,456	2,175	19,893	2,869	81,957,428	Pair end
Stickleback	99,816	2,243	19,918	3,010	58,853,956	Pair end
Rhodeus	118,861	2,198	24,925	3,004	87,001,500	Pair end

Immune-related genes were also found among the genes that were expressed significantly lower in the catfish skin including GTPase (IRG), lymphocyte antigen 86 and T-lymphocyte activation antigen CD80. IRG is mainly activated for the expansion of $CD4^+$ T cell, and it is ineffective for intracellular pathogens (Feng et al., 2008). Thus we infer that even though the catfish skin is actively involved in immunity under normal status, but it cannot express all genes involved in the host immunity, only genes specific to skin immunity might be expressed. The two

self-antigens are produced to introduce autoimmune in host (Overwijk et al., 1999), and high expression level of these self-antigens can cause damage to host cells (Hauben et al., 2001). Therefore, the lower expression of these two self-antigens in catfish skin may be beneficial to the catfish.

Expression comparison between skin and non-MALTs

Mucosal immune system is a critical component of the entire fish immune system, and it acts as the first line against the pathogen invasion (Ángeles Esteban, 2012). This system comprises of skin-associated lymphoid tissue (SALT), gut-associated lymphoid tissues (GALT) and gill-associated lymphoid tissues (GIALT) (Salinas et al., 2011). Together, these tissues are named as mucosa-associated lymphoid tissue (MALT). In the section above, the differentially expressed genes were obtained by comparison with transcriptome from four other tissues including the gill and intestine that are also members of MALTs. However, previous studies reported that there are some similarities shared between the immune mechanisms of the SALTs and other MALTs (Rombout et al., 2014; Salinas, 2015). Therefore, we compared the catfish skin transcriptome with that of the liver and barbels, both of which are non-MALTs. This comparison allowed identification of lectins and chemokines (Table 2), which are differentially expressed post bacterial challenges (Li et al., 2013a, 2013b), along with plectins (Hijikata et al., 2008), to be highly expressed in the skin, suggesting additional significance of the skin in immune responses.

Inter-species transcriptomic comparison

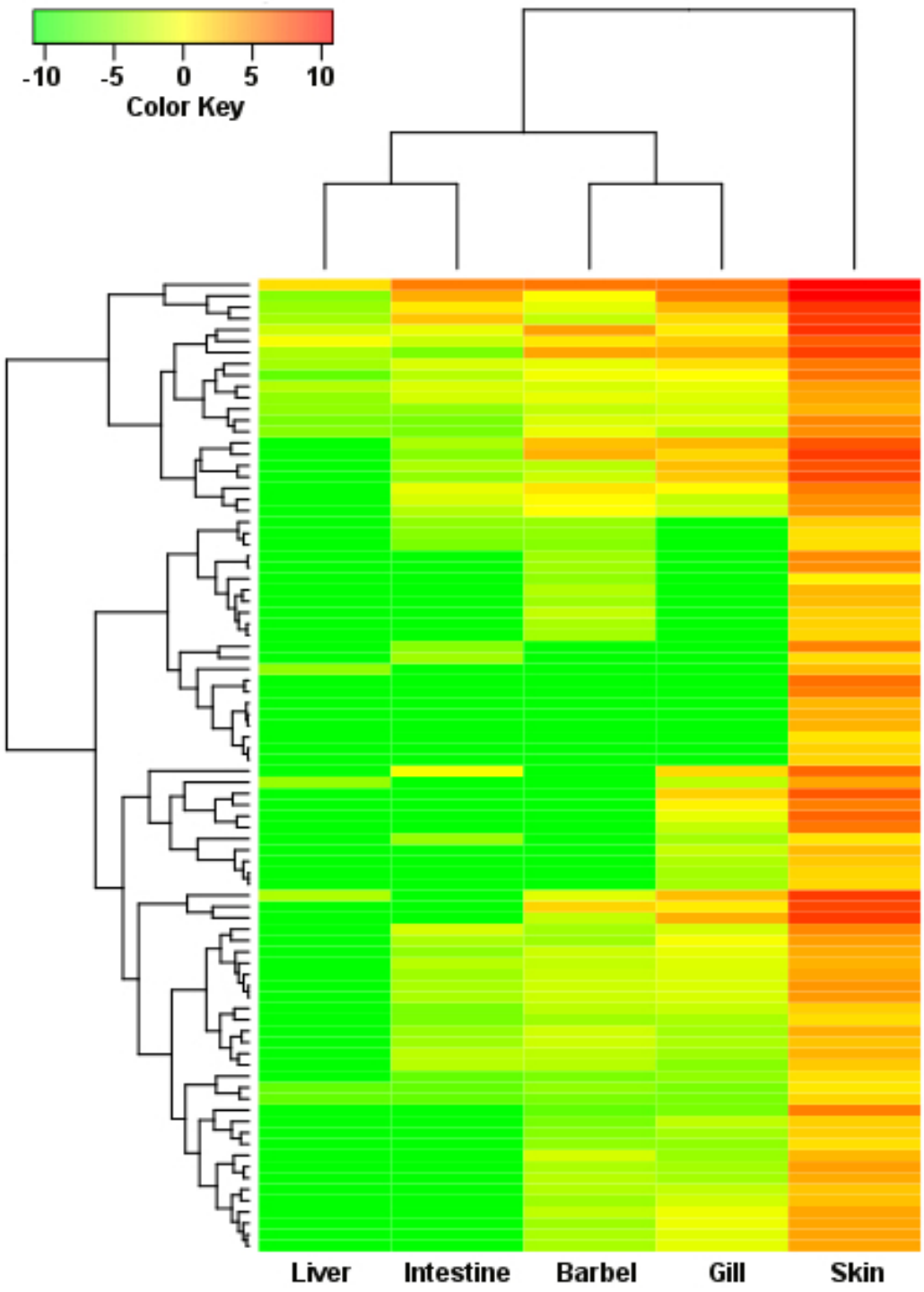

Figure 2. A heatmap generated for the group 1 genes. FPKM values were log-transformed and genes that have similar expression patterns were clustered together.

The channel catfish is scaleless, a character that makes its skin as the most external tissue facing the challenges of the environmental pathogens comparing to other armored fish species. However, to our knowledge, the similar immune roles shared between the skins of scaled and scaleless fish species, as well as the unique immune roles that only could be found in the skin of scaleless fish, remains largely unknown. Recently, the skin transcriptomes of several scaled and scaleless fish species, which generated from Illumina sequencing platforms, were published (Jones et al., 2012; Kong et al., 2015; Petzold et al., 2013; Shin et al., 2014). This allows us to perform an inter-species transcriptomic comparison to address the problems aforementioned.

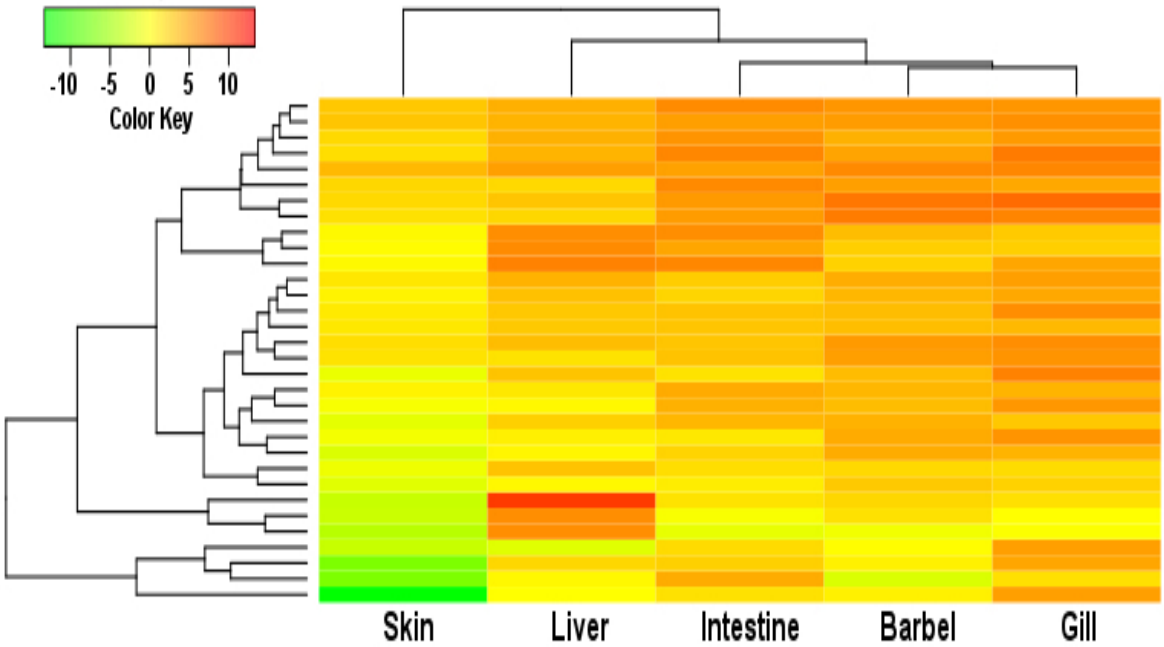

Figure 3. A heatmap generated of the genes expressed significantly lower in the skin. FPKM values were log-transformed and genes that have similar expression patterns were clustered together.

Table 4. Shared and species-specific immune-related GO terms

Species-specifc/ Shared	GO ID	Name
Shared	GO:0002250	adaptive immune response
	GO:0002312	B cell activation involved in immune response
	GO:0006955	immune response
	GO:0002757	immune response-activating signal transduction
	GO:0002376	immune system process
	GO:0045087	innate immune response
	GO:0002233	leukocyte chemotaxis involved in immune response
	GO:0002522	leukocyte migration involved in immune response
	GO:0002281	macrophage activation involved in immune response
	GO:0002275	myeloid cell activation involved in immune response
	GO:0002283	neutrophil activation involved in immune response
	GO:0052033	pathogen-associated molecular pattern dependent induction by symbiont of host innate immune response
	GO:0045089	positive regulation of innate immune response
	GO:0002682	regulation of immune system process
	GO:0045088	regulation of innate immune response
	GO:0002286	T cell activation involved in immune response
	GO:0006959	humoral immune response
	GO:0042092	type 2 immune response
	GO:0030153	bacteriocin immunity
	GO:0002446	neutrophil mediated immunity
Catfish	GO:0050778	positive regulation of immune system process
	GO:0002520	immune system development
	GO:0002285	lymphocyte activation involved in immune response
	GO:0050776	regulation of immune response
Zebrafish	GO:0002456	T cell mediated immunity
Stickleback	GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains

The skin transcriptomes of zebrafish, rhodeus, stickleback, notothenia, and catfish were first *de novo* assembled using Trinity, annotated through blast against the zebrafish RefSeq protein database, and then compared (Figure 4). Statistics of these five fish skin transcriptomes were summarized in Table 3. A total of 11,843 genes were shared by skin transcriptomes of the five species. A total of 3,083, 1,536,

560, 577, and 1,209 genes were specifically identified from the skin transcriptome of zebrafish, catfish, stickleback, notothenia, and rhodeus, respectively.

Gene Ontology analysis was conducted with these species-specific genes and shared genes in the skin, and particular attention was devoted to immune-related genes. As shown in Figure 3, the number of immune-related genes specifically expressed in the skin of these fish varied from 6-51 genes, with the largest number of species-specific expression of immune genes in zebrafish (51), followed by catfish (33), rhodeus (20), stickleback (8), and notothenia (6). Obviously, the ability of gene identification from transcriptome data depends on the depths of transcriptome sequencing and the quality of transcriptome analysis. In this case, the number of reads available for the transcriptome assembly was largest with zebrafish (~400 million reads), followed by catfish (~170 million reads), rhodeus (~87 million reads), notothenia (~82 million reads), and stickleback (~59 million reads). However, the percentage of immune-related genes among these species-specific transcripts was the highest in catfish (2.15%), followed by zebrafish (1.65%), rhodeus (1.65%), stickleback (1.43%), and notothenia (1.04%), suggesting that the catfish skin is more enriched with immune-related genes. Therefore, we conclude that the scaleless characteristic of the catfish skin makes itself more involved in immunity than other armored fish species. Lacking of an external physical barrier forces the channel catfish to possess more lineage-specific genes involved in immunity, an effective way protect itself from pathogen rich environments. Also, 294 genes (2.48%), which shared by all these five fish species, were involved in the skin immunity. Even

though the percentage of lineage-specific genes involved in immunity is highest in the catfish skin transcriptome, it is still lower than the percentage of genes involved in immunity in the orthologs. This might suggests that, even after millions years of divergence from each other (Volff, 2005), fish species still harbor a large number of same genes involved in the skin immunity, regardless of they are scaled or not. However, for the subsets of lineage-specific genes, the percentage of genes involved in skin immunity varies among fish species, possibly caused by the scaled and scaleless characteristic of the fish skin.

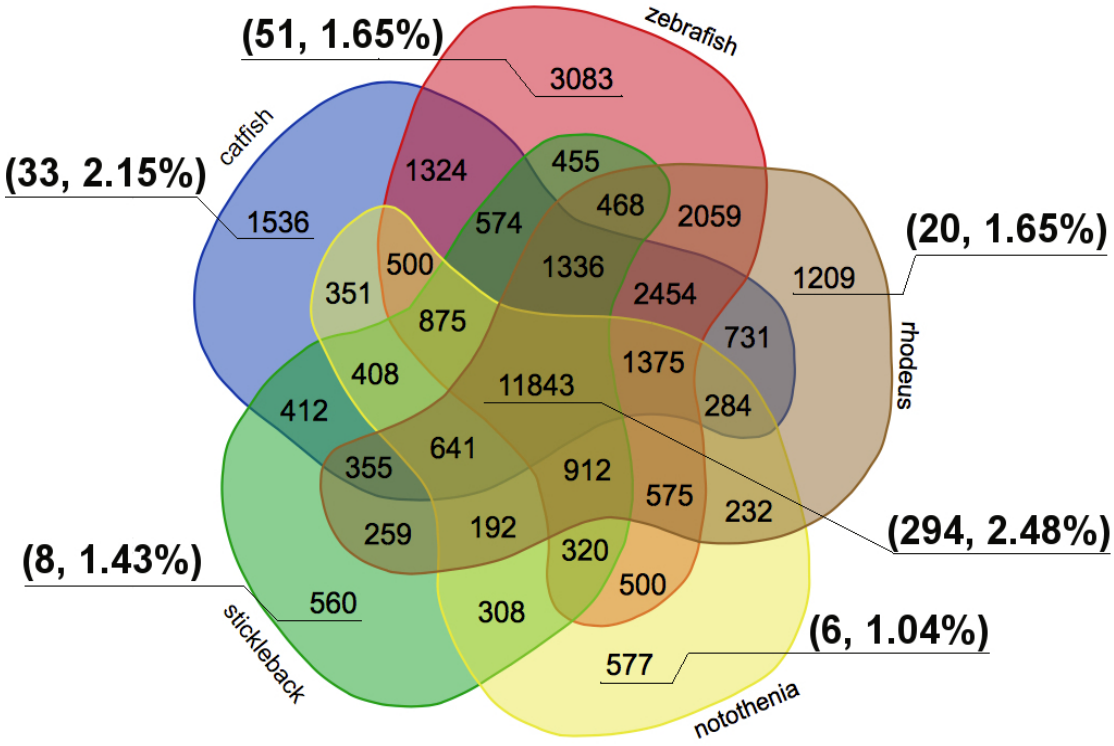

Figure 4. A venn diagram originated from inter-species transcriptomic comparisons. External numbers delineates list of genes unique to certain species as well as numbers in overlapping region represents groups of orthologous. The number of immune-related species-specific genes and the corresponding percentage is displayed in parentheses.

To better understand the specific genes of the channel catfish skin involved in immunity comparing to other fish species, extensive GO functional annotation was conducted for all the skin transcriptomes. The results are summarized in Figure 5. Species-specific and shared GO terms related with immunity are summarized in Table 4. The vast majority (20 out of 26) of GO terms involved in immunity were shared by all the five skin transcriptomes, suggesting that the core functions of the fish skin in immunities remain highly conserved, regardless of the presence or absence of the scales. Only six species-specific GO terms for immunity were found, four of which were found in catfish, while one was found from zebrafish, and one was found from stickleback. The four immune GO terms of catfish were: positive regulation of immune system process, immune system development, lymphocyte activation involved in immune response and regulation of immune response. These results suggest that the channel catfish skin may have more significant roles in immune responses than in other fish skins.

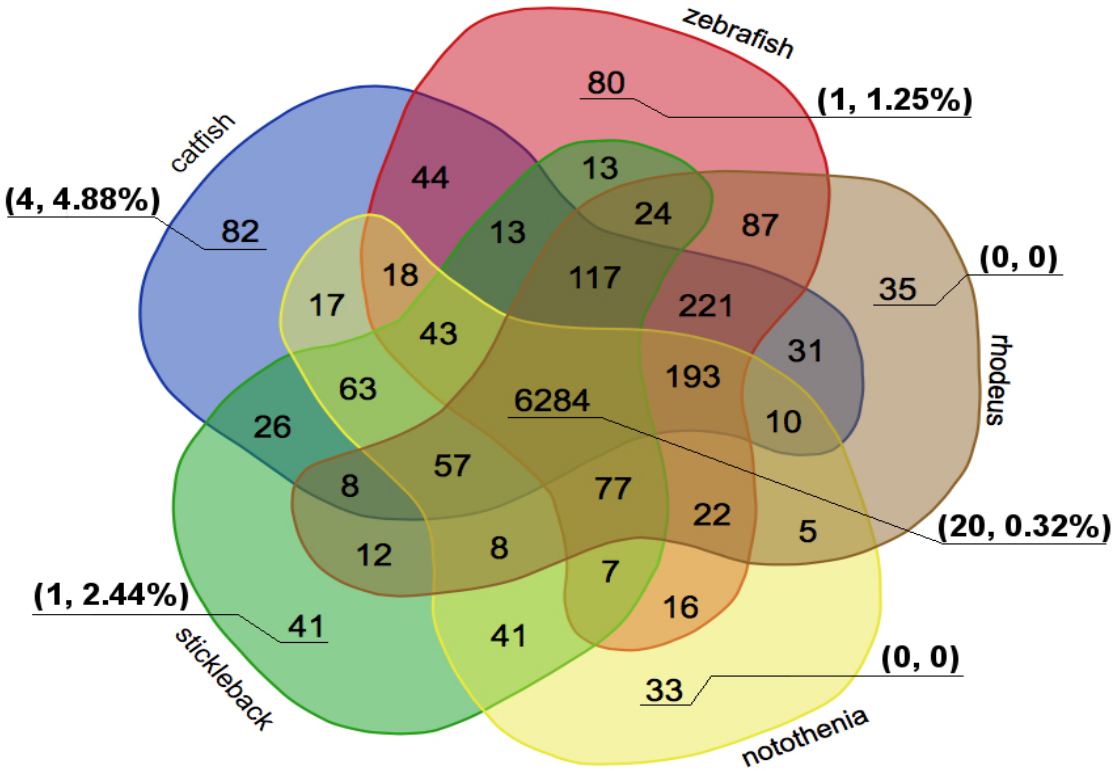

Figure 5. A venn diagram originated from inter-species GO term comparisons. External numbers delineates list of terms unique to certain species as well as numbers in overlapping region represents groups of shared GO terms. The number of immune-related species-specific GO terms and the corresponding percentage is displayed in parentheses.

Conclusions

In conclusion, in this study, we generated the channel catfish skin transcriptome that is a valuable resource for genome annotation and transcriptome analysis. Through intra-species tissue comparisons and inter-species comparison of skin transcriptomes, we identified a set of genes differentially expressed in the skins. Specifically, the catfish skin transcriptome contained the highest percentage of immune-related genes and GO terms for immunity from the species-specifically expressed transcripts, suggesting that the catfish skin is more significant in immune

functions as compared to skin of other species. This feature may have been related to the lack of scales in channel catfish.

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Chapter 3

Taste Receptors and Gustatory Associated G Proteins in Channel Catfish

Abstract

Taste sensation plays pivotal roles for nutrient identification and acquisition of channel catfish, which lives in turbid waters with limited vision. This biological process, along with olfaction, are mediated by taste receptors expressed in taste buds distributed in several organs and tissues including the barbels and skin. In the present study, we identified a complete repertoire of taste receptor and gustatory associated G proteingenes in the catfish genome. A total of eight taste receptor genes were identified, including five type I taste receptor genes and three type II genes. Addition four genes for gustatory associate G protein genes were also identified. Their genomic location, copy numbers, phylogenetic relations, orthologies, and expression were determined. Phylogenetic and syntenic analysis allowed determination of their evolution dynamics of these gene families. Furthermore, motif and dN/dS analyses allowed the inference of selection pressure imposed on these receptors. Expression patterns of catfish taste receptors and gustatory associated G proteins across organs are similar to the distribution of taste buds across organs. Expression comparison between catfish and zebrafish skin

transcriptome provided evidence for potential roles of catfish skin in taste sensation.

Introduction

Taste sensations, along with the other chemosensory system of olfaction, play pivotal roles for food searching (Bachmanov and Beauchamp, 2007; Bruch et al., 1988) and thereby for their survival. Taste sensations are mediated by the taste receptors expressed on the taste buds located within gustatory papillae. Five basic taste sensations, including sweet, bitter, umani, salty and sour, can be detected by these receptors (Chandrashekar et al., 2006). Three of these (sweet, bitter and umani) can be detected by the taste receptors in vertebrates; and the bitter sensation is considered as being the most important for vertebrates' survival, because usually toxic and harmful substances are bitter (Bachmanov and Beauchamp, 2007). Taste receptors, identified as seven trans-membrane G protein-coupled receptors, can detect soluble stimuli and initiate signal transduction pathways for taste sensation (Mombaerts, 2004).

Two families of the taste receptors that expressed on different subsets of taste receptor cells (TRCs), are adopted by vertebrates for taste sensation. T1R family (type 1taste receptor), first discovered for sweet sensation, has three subfamilies, including T1R1, T1R2 and T1R3 (Bachmanov and Beauchamp, 2007). Further analyses indicated that T1R3 is usually co-expressed with T1R2 for sweet sensation in responding to tastants like natural sugars, D-amino acids, sucrose, saccharin, dulcin and sweet proteins (Montmayeur et al., 2001; Nelson et al., 2001; Zhao et al.,

2003); or co-expressed with T1R1 for umani sensation in responding to L-amino acids and monosodium L -glutamate (Bachmanov and Beauchamp, 2007; Chandrashekar et al., 2006; Mombaerts, 2004). T2R family (type 2 taste receptor,) has the most members among the taste receptor gene family (Adler et al., 2000; Chandrashekar et al., 2000). It was found to mainly function for bitter sensation even though the functions of several members within T2R family are not clear (Bachmanov and Beauchamp, 2007; Ishimaru et al., 2005; Lindemann, 2001; Mombaerts, 2004). Another interesting finding is that several T2Rs co-expressed with each other in the same TRCs. Along with the facts that the number of bitter tasted chemical compounds in the nature are larger than that of T2R taste receptors (Jaggupilli et al., 2016), such co-expression indicated that taste receptor in vertebrates are more dedicated for sensing rather than distinguishing for bitter sensation, a characteristic dissimilar to that of olfactory receptors (Adler et al., 2000; Bachmanov and Beauchamp, 2007; Caprio, 1975; Lindemann, 2001).

Although the functions of taste receptors are similar in mammals and fish species, distributions of taste receptors (or distribution of taste buds) in mammals and fish species are quite different. Taste receptors were found in several common organs with the epithelium for both mammals and fish species, while some distinct fish organs were also found to harbor taste receptors. For example, in mammals, taste receptors were found mainly in epithelia of lips, oral and oropharyngeal cavity, including the tongue, palate and pharynx (Lindemann, 2001). In fish species, especially in catfish, taste receptors are distributed in diverse organs and tissues

(Caprio and Derby, 2008). In addition to all the organs and tissues with taste receptors as in mammals, the entire external body surfaces in fish species, including the barbel and skin, were also considered as regions covered by taste buds (Caprio, 1975; Raji and Norozi, 2010). Taste receptors were also identified in fish gill, a fish-specific organ for breathing (Caprio and Derby, 2008; Hansen et al., 2014; Hansen et al., 2002). Solitary chemosensory cells, a type of cells distributed with high density in teleost external body surface, shared similar functions with TRCs (Caprio and Derby, 2008; Hansen et al., 2014; Hansen et al., 2002).

Additional to TRs, some G proteins were also believed to be involved in gustatory activities. They are co-expressed with T1Rs for sweet sensing in the gut of mammals (Dyer et al., 2005; Margolskee et al., 2007). These proteins, especially the α subunit, were found to be involved in signal transduction pathways for both bitter and sweet in the taste buds of rat tongues (Shen et al., 2005). These studies indicated that G proteins may play significant roles involved in gustation than previously expected (Shigemura et al., 2008). However, expression analysis of gustatory associated G proteins remains largely unknown in fish species. Thus, expression profiling of these G proteins, along with the expression profiling of taste receptors, becomes necessary for the study of taste receptor in fishes.

Upon the completion of reference genome assembly (Liu et al., 2016) and availability of RNA-Sequencing datasets from various tissues, the identification, annotation, and analysis of their expression profiles for the full set of channel catfish taste receptor genes, as well as gustatory associated G protein genes, becomes

feasible. Here we report our work for the identification, annotation, phylogenetic analysis, orthogrouping, collinearity analysis, motif and dN/dS analyses of the catfish taste receptors and gustatory associated G protein genes, and illustrate the evolutionary dynamics of the taste receptor gene family across a broad range of chordate phyla.

Materials and methods

Retrieval of taste receptors gene sequences of other vertebrates

We downloaded the full sets of taste receptor genes from sixteen vertebrate species, including amazon molly (*Poecilia formosa*), cave fish (*Astyanax mexicanus*), cod (*Gadus morhua*), fugu (*Takifugu rubripes*), medaka (*Oryzias Latipes*), platyfish (*Xiphophorus maculatus*), spotted gar (*Lepisosteus oculatus*), stickleback (*Gasterosteus aculeatus*), green spotted pufferfish (*Tetraodon nigroviridis*), tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), anole lizard (*Anolis carolinensis*), chicken (*Gallus gallus*), mouse (*Mus musculus*), cow (*Bos taurus*) and human (*Homo sapiens*) from the ENSEMBL database. Then, we carefully selected query genes based on the following three standards: 1) only full-length taste receptor protein sequences were used in our study; 2) only the longest sequences were selected when genes have multiple isoforms using custom script; 3) genes labeled with "pseudogene" were removed. Besides, sequence descriptions were also downloaded using BioMart and were combined with protein sequences aforementioned using custom script. The amino acid sequences for all

species used in the present project, including the sequences from channel catfish, are provided in File S1.

Identification of taste receptor genes in channel catfish

We used an extensive computational method to identify candidates for taste receptors in the channel catfish genome. First, the draft genome sequences of channel catfish (Liu et al., 2016) were masked using RepeatMasker (Smit et al., 2014), and the masked sequences were used to predict putative protein sequences using FGENESH embedded in MolQuest (Solovyev et al., 2006). All the predicted protein sequences were annotated through BLAST against the NCBI non-redundant database. The catfish amino acid sequences with hits to well annotated taste receptor proteins sequences were then selected, and used for further analysis in the project. At the same time, mRNA sequences, corresponding to the annotated protein sequences, were generated from FGENESH and further used as reference sequences for mapping of RNA-Seq reads from various organs and tissues.

Phylogenetic, orthogroup and collinearity analyses

To provide a comprehensive understanding of the evolutionary dynamic of taste receptor in vertebrates, we conducted phylogenetic, orthogroup and syntenic analyses. First, all taste receptor protein sequences from all the species used in the study, including newly identified taste receptor protein sequences from the catfish genome, were aligned using MUSCLE (Edgar, 2004) before the construction of

phylogenetic tree using FastTree (Price et al., 2009, 2010) with default settings, based on the JTT model of amino acid evolution and the approach of Bayesian. Olfactory receptors were used as outgroups to root the phylogenetic tree. The local support values were computed as well. The phylogenetic tree was used as input into MEGA6 for visualization (Tamura et al., 2013). Second, all protein sequences from genomes of the catfish and other 16 species were used for the all-versus-all comparative analysis using OrthoFinder (Emms and Kelly, 2015) without gene length bias, and orthogroups harboring taste receptors were extracted. Only orthogroup consists of at least two orthologs or two paralogs were retained. Third, chromosomal locations of all the genes in both zebrafish and catfish, along with results of all-versus-all blast searches between genomes of zebrafish and catfish, were used to conduct collinearity analysis using MCScanX (Wang et al., 2012) Tandem duplicated genes for these two species were identified based on their genome position coodinates.

Identification of conserved motifs

To figure out the conserved regions for fish TRs, we conducted motif analysis. We only selected amino acid sequences possessing the seven trans-membrane topology, and then aligned them using MUSCLE (Edgar, 2004). Then the gaps were removed using trimAl (Capella-Gutierrez et al., 2009) for each taste receptor subfamily. Finally, we collected all taste receptor amino acid sequences and conducted analysis for conserved motifsfor Type I and Type II TRs, respectively,

using MEME (Bailey et al., 2009). The length of the conserved motif can be up to fifty, but only the top five are listed.

dN/dS Analysis

In order to measure the selection pressure imposed on each subfamily of fish TRs, we conducted natural selection analysis based on the relative rates of synonymous and non-synonymous substitutions. The coding sequences, which corresponding to the amino acid sequences used for motif identification, were collected to calculate the global and dN/dS ratios for each subfamily of fish taste receptors using Datamonkey (Delport et al., 2010). Only subfamilies that have sequences originating from at least two species were used.

Expression profiling of catfish TRs and G proteins

RNA-Seq datasets from five organs of channel catfish, including barbel, skin, gill, liver, and intestine were downloaded from NCBI SRA database. Sequencing adaptors, low quality reads and reads with length lower than 36 bases were removed using Trimmomatic (Bolger et al., 2014). Clean reads were mapped to mRNA sequences of the TR receptors and the gustatory associated G proteins mentioned above, and FPKM (fragments per kilobase per million mapped reads) for each gene was calculated using RSEM (Li and Dewey, 2011).

We also compared the expression levels of taste receptors between catfish and zebrafish skin. RNA-Seq dataset generated from the skin of zebrafish were downloaded from the NCBI SRA database. The results are summarized in Table 5.

Results and Discussion

Identification and characterization of TR genes in channel catfish

We have identified eight TR genes in the genome of channel catfish, with one gene falls into subfamily T1R1, three falls into subfamily T1R2, one falls into subfamily T1R3 and three falls into subfamily T2R201. Each subfamily resides on a different chromosome, and their genomic locations are summarized in Table 1.

The copy numberof TR subfamily varied, T1R1 and T1R3 each has a single copy, as what was in mammals.However, T1R2 has three copies in the catfish genome while it is a single copy gene in mammals. Of the three copies of T1R2 in catfish, T1R2a and T1R2b are present as tandem duplicated genes. The situation with T2Rs is different in fish versus that in mammals. In mammals, multiple copies of T2R were found belonging to three subfamilies (Adler et al., 2000; Chandrashekar et al., 2000; Ishimaru et al., 2005). However, only three T2R genes were found in catfish, and they all belong to subfamily 201. There are also three copies in zebrafish, and generally one or two copies in other fish species.

Table 1. Genomic organization of TR genes in the catfish genome

Gene	Chromosome	Staring	Ending
T1R1	15	1582298	1585481
T1R2a	5	8954348	8959672
T1R2b	5	8963481	8972227
T1R2c	5	8974294	8979582
T1R3	21	1021838	1024216
T2R201a	6	5333686	5335131
T2R201b	6	5343501	5344343
T2R201c	6	5369269	5370202

Phylogenetic, orthogroup and collinearity analyses of TR genes

We constructed a phylogenetic tree to delineate the evolution history of taste receptors in mammals and fish species (Fig. 1). Type I taste receptors are well divergent from type II taste receptors, regardless of separation of mammals and fish species, indicating that the fish T1Rs and the mammalian T1Rswere derived from the same ancestral gene. Same is true for T2Rs. However, within each clade of T1Rs and T2Rs, the mammalian TR genes are well divergent from their fish counterparts. This may be related to the different living environments of these two taxa, with mammals mainly living on land while fish living in waters. The different living environments present different ligands to organisms (Caprio et al., 2014; DeSimone et al., 2001; Lin et al., 2004; Lyall et al., 2001; Sakurai et al., 2009), leading to the divergence of necessary receptors among taxa.

Orthogroup analysis was performed to further validate the evolution relationships within the TR genes. Our results indicate that, in most occasions, the orthogroup analysis is well consistent with the phylogeny analysis. For instance,

each subfamily of fish T2Rs not only harbors a single clade in the phylogenetic tree, but also contains all members that from the same orthogroup. However, there is one exception, with T1R2s failing to cluster within one single clade in the phylogenetic tree (Fig. 1). Interestingly, all these T1R2s are listed in the same orthogroup, implicating orthogroup analysis can be complimentary with phylogeny analysis, providing more reliable options when interpreting evolution dynamics for gene families.

Synteny analysis has been extensively used to support orthology relationships beyond phylogeny analysis. In the present study, we used collinear relation, a special form of synteny to illustrate the orthologies for certain catfish TR subfamilies. Also, combining the tandem duplication relationships identified in our analysis, we can further infer the relative emerging time point for each member of these subfamilies. There are two pairs of conserved homologous regions among the genome of catfish and zebrafish, each of them contains one pair of taste receptor genes listed as collinear genes. Figure 2 illustrates the situation for T1R2. The catfish and zebrafish T2Ra can be listed as pair of collinear genes, while their copies cannot be listed as pair of genes. Between the T1R2a and next collinear gene, there are two other copies for catfish and one copy for zebrafish, but they are not co-linear (not orthologous) because they originated from tandem duplications. Thus we infer that T1Ra is the most ancient gene for catfish and zebrafish, T1R2b abd T1R2c in catfish, as well as the T1R2b in zebrafish were descended from T1R2a after speciation. In other words, they were derived from lineage-specific gene duplication

in the form of tandem duplication. Similarly, T2R201a was the most ancient gene, and T2R201b and T2R201c were derived from lineage-specific tandem duplications (Fig. 3).

Table 2. A summary description of selection pressure for each subfamily of catfish TR

Gene	Global	No. of Positive Sites	No. of Negative Sites
T1R1	0.253856	1	224
T1R2	0.315728	0	135
T1R3	0.314331	1	285
T2R2	0.405283	0	9
T2R2	0.480796	0	15
T2R2	0.258602	0	33

Conserved motifs

Despite there are discrepancies among all three subfamilies of fish type I taste receptors, such as sequence similarity and molecular functions, five best-conserved motifs among these subfamilies can still be detected. The logo presentation and distribution of the five motifs of the catfish T1R2a is displayed in Fig. 4. As expected, there are two motifs reside on the extracellular N-terminal domain, which are usually the binding sites for ligands (Nie et al., 2006), indicating that even though the ligands binding to fish T1Rs are not the same, they sharedthe same binding sites or domains. Other conserved motifs identified in our study mainly reside on the trans-membrane domains, which can interact with sweetener to initiate

enhancing effects for sweet sensation (Fujiwara et al., 2012). However, little is known about whether similar enhancing effects can be induced for umani sensation. Based on the conserved motifs, we infer that there are likely some tastants inducing similar promotion effects for umani sensation, just like sweetener to sweet sensation, but this is purely speculation.

Similarly, we conducted the motif analysis for fish type II taste receptors that is displayed in Fig. 5. Unlike the type I taste receptors, type II taste receptors have a much shorter extracellular N-terminal domain (Chaudhari and Roper, 2010). Thus, all the conserved motifs identified for type II receptors reside on the trans-membrane domains, intracellular and extracellular loops. Strikingly, when we collected all the fish TRs from this family and tried to identify the five best-conserved motifs, three of them were missing in all members of subfamilies 200 and 201. In the section above, all three subfamilies of fish T2Rs are well divergent from each other, as indicated by their cluster into three different sub-clades in the phylogenetic tree, or into three different groups withthe orthogroup analysis (Fig. 1). While the phylogeny and orthogroup analyses show the occurrence of divergences among these T2R subfamilies, motif analysis displays the locations of these divergences occur in the two-dimensional topology structure. The three missing motifs mainly reside on trans-membrane domains and extracellular loops, regions could contain potential binding sites for T2Rs (Roper, 2007), implying that these subfamilies of fish T2Rs tended to separate themselves from each other for the discrimination of structurally distinct bitter tastants.

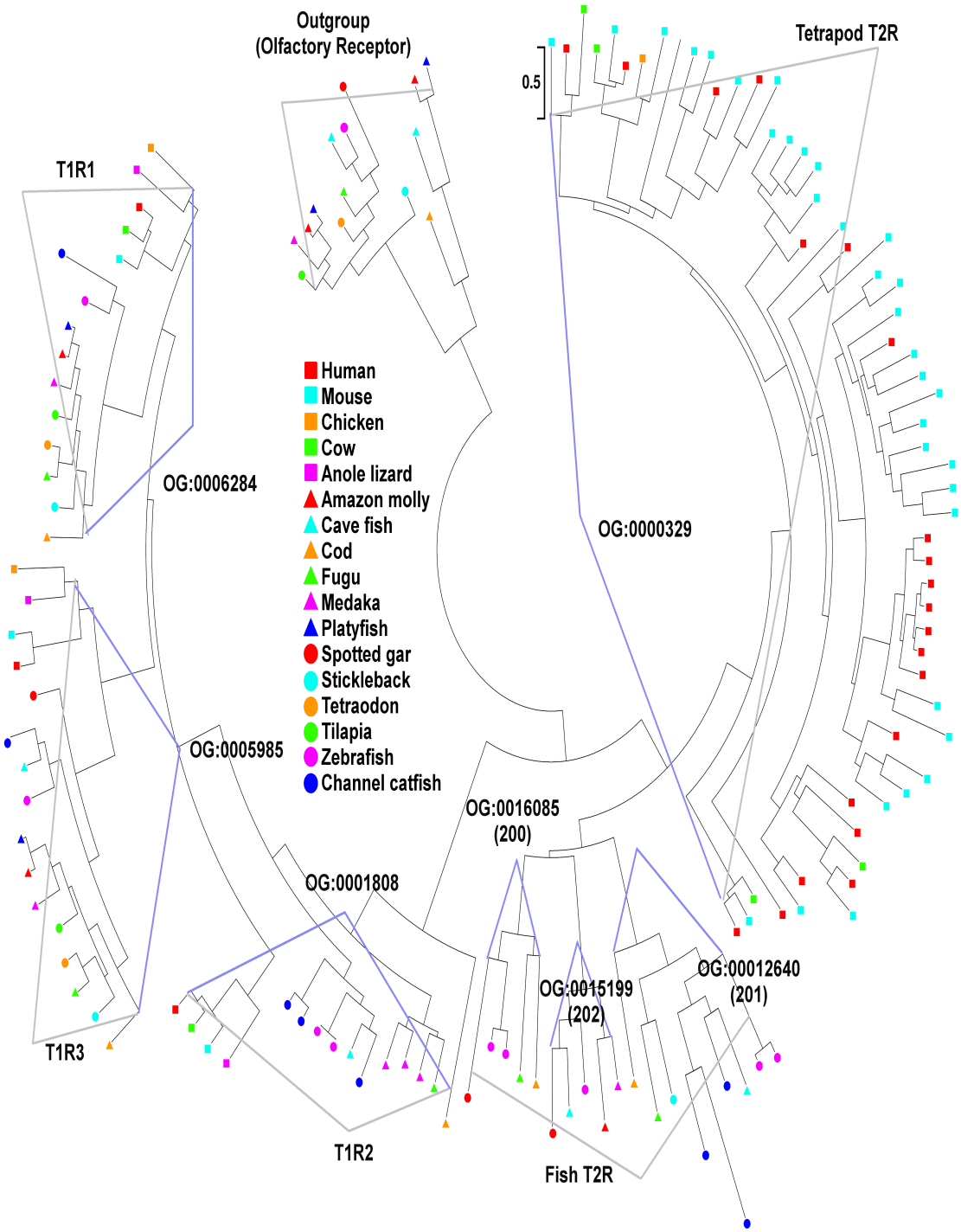

Figure 1. A phylogenetic tree of TRs constructed using amino acid sequences of 139 TR genes from 17 vertebrate species (legends are indicated on the center of the figure). Members from the same family are coverd with grey fold lines with their family names indicated outside of the lines, while members from the same orthogroup are coverd with blue fold lines with their family names

indicated outside of the lines. For example, orthogroup OG:0006284 shares the same group of members with subfamily T1R1.

Table 3. Expression profiling of TR genes across five different organs in catfish (FPKM)

Gene	Barbel	Skin	Gill	Liver	Intes
T1R1	3.54	1.17	2.22	0.16	0.17
T1R2a	2.22	0.11	0.36	0.00	0.04
T1R2b	2.99	0.03	0.06	0.00	0.00
T1R2c	0.14	0.03	0.06	0.00	0.00
T1R3	3.07	0.24	0.71	0.98	0.11
T2R20	0.25	0.00	0.00	0.00	0.00
T2R20	0.00	0.06	0.00	0.00	0.00
T2R20	0.00	0.00	0.00	0.00	0.00

Ratios of dN/dS

Natural selection is the major force behind the frequency fluctuation of alleles within a group of taxa, which can be measured by the relative ratios of synonymous (dS) and non-synonymous (dN) substitutions. Here, we conducted the global and site-by-site dN/dS ratios for all subfamilies of fish taste receptors to delineate the evolution dynamics. Overall, the average dN/dS ratio of T1Rs is smaller than that of T2Rs, mainly caused by more negative selection sites found in T1Rs than T2Rs (Table 2). Besides, a few positive selection sites found in T2Rs, however, this is too few to lift the global dN/dS ratio of T2Rs. Interestingly, subfamilies 200 and 201 possess the top two highest dN/dS ratios among all the fish subfamilies. As we mentioned in the last section, these two subfamilies lost three conserved motifs comparing to subfamily 202. Therefore, we conclude that different degrees of selection pressures imposed on different fish TR subfamilies, in turn, probably

caused further divergence between subfamilies, a phenomenon especially significant in type II taste receptors.

Table 4. Expression profiling of gustatory associated G protein genes across five different organs in catfish (FPKM)

Gene	Barbe	Skin	Gill	Live	Intestine
gna14	54.15	6.95	12.1	0.00	7.54
gnaia	4.65	35.7	22.7	0.38	3.00
gnb1	25.47	35.4	42.4	4.09	20.22
gng13	13.43	1.27	1.03	0.00	0.37

Expression profiling of TRs in channel catfish

We characterized the expression patterns of all TRs in channel catfish using RNA-Seq datasets from various tissues (Table 3). Overall, Type I TRs, a group of receptors mainly expressed for sensation of umani and sweet, was expressed at higher levels than Type II TRs, a group of receptors mainly expressed for sensation of bitter, in all analyzed organs/tissues. Actually, in most cases, Type II TRs were not detected in the RNA-Seq datasets, suggesting no expression or extremely low expression. For Type I TR receptors, their expression levels were highest in the barbell, followed by that in the gill, and then by that in the skin. Very low or no expression was detected in the intestine and liver. This pattern is in line with the distribution/density of taste buds in catfish, with barbels harboring the most taste buds, followed with gill and skin (Iwai, 1963; Northcutt, 2005; Raji and Norozi, 2010). There is no expection of taste buds in the intestine or liver although no

literature is available. Taken together, the channel catfish mainly use TRs for the sensation of umani and sweet rather than for bitter, and the barbel is the primary organ for taste sensation.

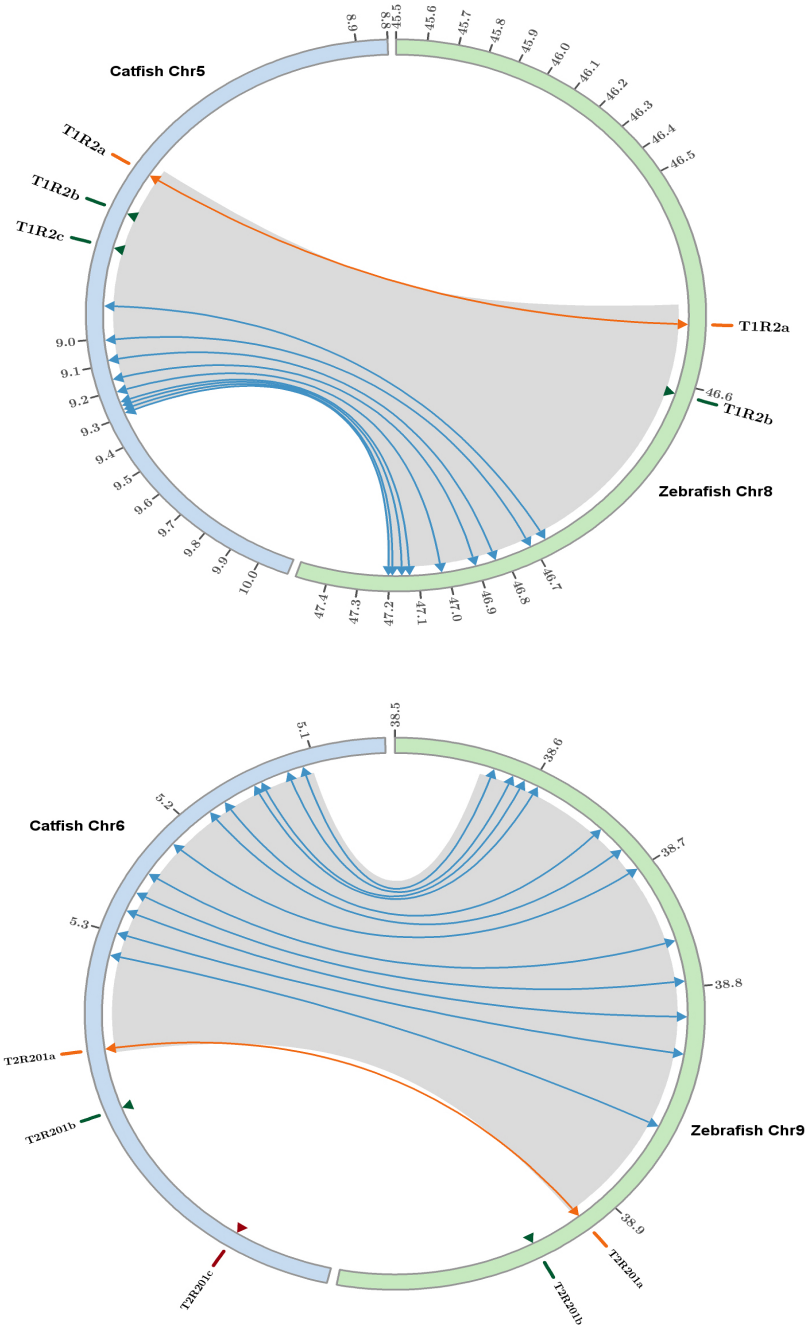

Figure 2. Identification of two pairs of homologous chromosomal regions between channel catfish and zebrafish. The first pair of regions is between zebrafish chromosome 8 and catfish chromosome 5, and the second region is between zebrafish chromosome 9 and catfish chromosome 6.

Anchor genes are linked with arrowed lines. TRs are linked with orange arrowed lines while other genes are linked with blue arrowed lines. Genomic positions for both fishes are indicated with numbers (in Mb) along its own chromosome. Tandem duplicated genes are indicated with green triangles. Catfish T2R201c is listed in dark red because it is not originated form tandem duplication.

Table 5. Expression comparison of TR genes between catfish and zebrafish skin (FPKM)

Gene	Catfish	Zebrafish
T1R1	1.17	0.17
T1R2	0.17	0.00
T1R3	0.24	0.01
T2R200	-*	0.04
T2R201	0.06	0.00
T2R202	-*	0.18

* That gene doesn't exist in the catfish genome

Identification and expression of gustatory associated G protein genes

Previous studies have reported that TR associated genes, like G proteins (including subunits α, β and γ), play pivotal roles in gustation based on the observations that they are co-expressed or coupled with TRs to initiate the gustatory signal cascade (Spielman, 1998). However, the canonical alpha subunit (gnat3) of G protein, which coordinates the gustatory process in mammals, is missing in fish species, presumably owing to the lineage-specific gene losses (Lagman et al., 2012). Some scientists proposed that gna14 and gnaia in fish might share the same gustatory associated roles with gnat3 in mammalian (Oka and Korsching, 2011). We identified the catfish gustatory associated G proteins, and characterized their expression pattern using the channel catfish RNA-Seq datasets (Table 4). We first

identified these genes, including gna14 and gnaia, gnb1 (beta subunit), and gng13 (gamma subunit), in the genome of channel catfish.

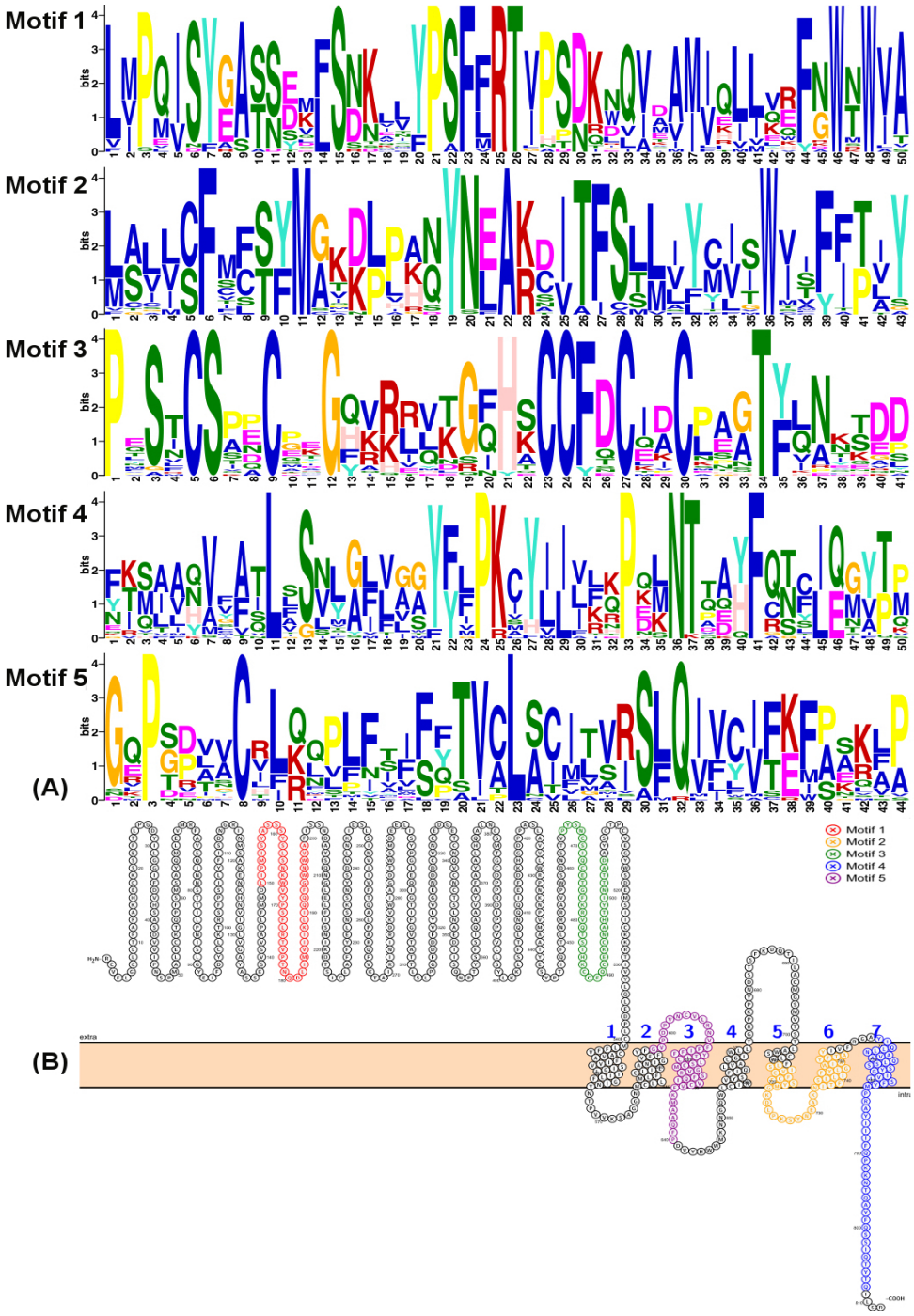

Figure 3. Logo representation and distribution of the five best conserved motifs identified for teleost type I taste receptors. (A) Sequence logos of the conserved motifs, as the degree of conservation is indicated by the height of amino acid code. (B) The distribution of these motifs as

displayed in the two-dimensional topology structure of type I taste receptors. The blue numbers represent the number of each trans-membrane domain.

The expression levels of all G protein genes are significantly higher than that of all TR genes as revealed by RPKM in the RNA-Seq datasets. Among all the G proteins, only catfish gna14 exhibits the similar expression pattern with that of taste receptors across catfish organs, following the descending order of barbel, gill, skin, intestine and liver. This might suggest that fish gna14, at least for catfish, has functions involved in gustatory activities similar to gnat3 in mammals.

Expression comparison between the channel catfish skin and zebrafish skin for the TRs

Comparing to other armored fish species, the scaleless characteristic of catfish makes it more external body surfaces, mostly the skin, exposing to the environment and thus increasing their ability for gustatory sensing (refs). To validate this hypothesis, we compared the expression profiles of taste receptors between the channel catfish skin and zebrafish skin (Table 5). Our results indicate that all Type I taste receptors were expressed at higher levels in the catfish skin than in the zebrafish skin. This could mean that the catfish skin is involved in the gustation (refs), as one would expect because Type I taste receptors are mainly for sensation of sweet and umani, which are rich within the food sources. However, Type II taste receptors were expressed at similar levels between catfish skin and zebrafish skin, although more genes of type II taste receptors existed in the zebrafish genome.

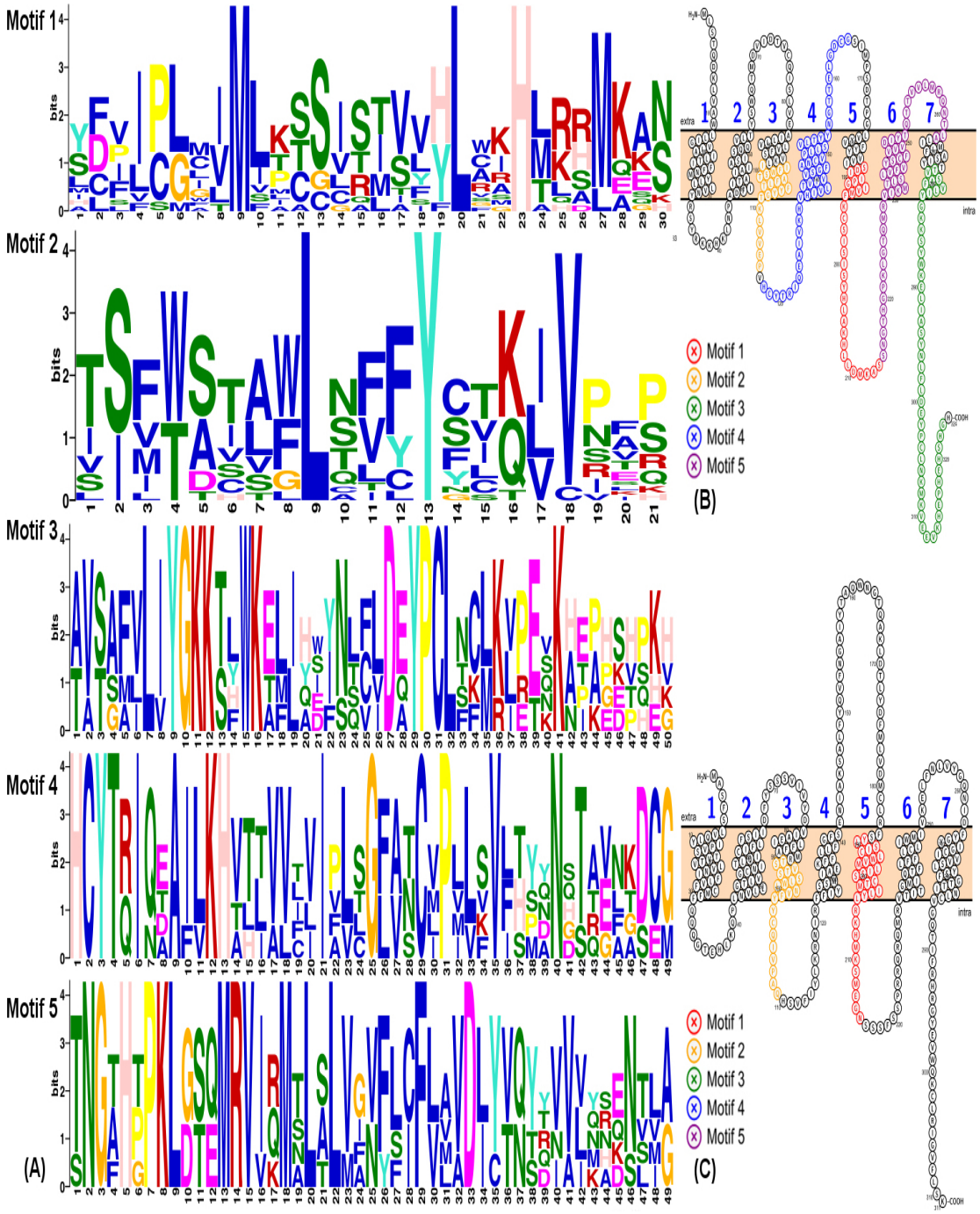

Figure 4. Logo representation and distribution of the five best conserved motifs identified for teleost type II taste receptors. (A) Sequence logos of the conserved motifs, as the degree of conservation is indicated by the height of amino acid code. (B) The distribution of these motifs as displayed in the two-dimensional topology structure of type II taste receptors from subfamily 202. The blue numbers represent the number of each trans-membrane domain. (C) The distribution of

these motifs as displayed in the two-dimensional topology structure of type II taste receptors from subfamilies 200 and 201.

Conclusions

Here, we report a complete repertoire of channel catfish taste receptors, and gustatory associated G proteins, which mediate gustatory activities that are essential for animal feeding. . Phylogenetic and orthogroup analyses allow us to elucidate the evolution dynamics of taste receptors across a broad range of chordate phyla. The identification of two conserved homologous regions between catfish and zebrafish, which contain TRs as anchor genes, reveals the relative emerging time for members of catfish subfamilies T1R2 and T2R201, and tandem gene duplication within each lineage. Motif analysis indicated that three conserved motifs are missing in fish T2R subfamilies 200 and 201, which could reflect the divergence of fish T2Rs', as revealed in phylogenetic and orthogroup analyses. The mechanism underlying this phenomenon is further uncovered by dN/dS analysis, which indicated that fish T2R subfamilies 200 and 201 were under greater selection pressure than any other TR subfamilies. Finally, expression patterns of catfish TRs across different organs are in line with distribution of taste buds in catfish, with the highest expression in the barbels, followed with gill and skin tissues.

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Chapter 4

Conclusions

In the present study, we generated the channel catish skin transcriptome that is a valuable resource for genome annotation and transcriptome analysis. Through intra-species tissue comparisons and inter-species comparison of skin transcriptomes, we identified a set of genes differentially expressed in the skins. Specifically, the catfish skin transcriptome contained the highest percentage of immune-related genes and GO terms for immunity from the species-specifically expressed transcripts, suggesting that the catfish skin is more significant in immune functions as compared to skin of other species. Also, we report a complete repertoire of channel catfish taste receptors, and gustatory associated G proteins, which mediate gustatory activities that are essential for animal feeding. Phylogenetic and orthogroup analyses allow us to elucidate the evolution dynamics of taste receptors across a broad range of chordate phyla. Collinear analysis, motif analysis and dN/dS analysis indicated that fish T2R subfamilies 200 and 201 were under greater selection pressure than any other TR subfamilies. Finally, expression patterns of catfish TRs across different organs are in line with distribution of taste buds in catfish, with the highest expression in the barbels, followed with gill and skin tissues.