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The expression and function of *gpr54a* and *gpr54b* in allotriploid crucian carp and diploid red crucian carp (*Carassius auratus* red var.)

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ABSTRACT

The kisspeptin receptor (GPR54), a member of the G-protein-coupled receptors, plays a central role in regulating reproduction. However, research on GPR54 in allotriploid fish remains limited. In this study, we reported the full-length cDNAs, tissue expression, and localization of *gpr54a* and *gpr54b* in allotriploid crucian carp (ACC) and diploid red crucian carp (*Carassius auratus* red var., RCC). The full-length cDNAs of *gpr54a* in ACC and RCC were 2224 bp and 2218 bp, respectively. The full-length cDNAs of *gpr54b* in ACC and RCC were 1413 bp and 1182 bp, respectively. qRT-PCR revealed that ACC *gpr54a* and RCC *gpr54a* were highly expressed in the brain but had lower expression in peripheral tissues. Unlike *gpr54a*, ACC *gpr54b* and RCC *gpr54b* were highly expressed in the brain, liver, and muscle. During the breeding season, the expression of *gpr54a* in ACC was significantly lower than in RCC, but *gpr54b* had significantly higher expression in ACC than in RCC in the HPG axis. This finding suggested that *gpr54a* might promote ovarian development, while *gpr54b* might inhibit ovarian development. During the breeding season, ISH indicated abnormal ovarian development in ACC and found signals of *gpr54a* and *gpr54b* in several regions of the brain, pituitary, and ovary of ACC and RCC. These regions of the brain, pituitary, and ovary are associated with the secretion of several hormones related to reproductive regulation. Hence, we hypothesized that the lower expression of *gpr54a* and higher expression of *gpr54b* in ACC might be involved in sterility by regulating the secretion of reproductive hormones. This study suggested that *gpr54a* and *gpr54b* might function oppositely in regulating reproduction. In conclusion, these findings will aid in further investigating the functions of GPR54 and provide a theoretical basis for studying ACC sterility.

1. Introduction

The reproductive function in teleost is primally regulated by the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH), the first hormone of the HPG axis, is secreted from the hypothalamus in a pulsatile manner [1,2]. GnRH stimulates the anterior pituitary to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [3]. FSH mainly activates the synthesis of estrogens and the maturation of gametes in gonads. LH is involved in ovulation and secretion of progesterone [3,4].

Kisspeptin, encoded by the *kiss1* gene, is initially known as a metastasis suppressor in melanoma and breast cancer cells [5,6]. G protein-coupled receptor 54 (GPR54), the kisspeptin receptor, was isolated from the rat in 1999 [7]. A growing body of evidence supports the fact that the Kisspeptin/GPR54 system plays a crucial role in regulating gonadal development and pubertal maturation. The kisspeptin/GPR54 system stimulates the secretion of GnRH from the hypothalamus [8]. In addition, the *gpr54* gene is mainly expressed in the central nervous system [9]. Studies have shown that the mutations in GPR54 are associated with hypogonadotropic hypogonadism (IHH) in human and mice, which fail to release the FSH and LH [10,11]. The alteration of the GPR54 balance might result in abnormal reproduction and metabolism of rats [12].

The kisspeptin/GPR54 system is conserved in vertebrates. GPR54 has been identified in many vertebrates, such as tilapia (*Oreochromis niloticus*) [13], grey mullet (*Mugil cephalus*) [14], cobia (*Rachycentron canadum*) [15], goldfish (*Carassius auratus*) [16], swamp eel (*Monopterus albus*) [17], and zebrafish (*Danio rerio*) [18]. Most fish have more than

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Table 1
Primers used in the study.

Primer names	Primer sequences (5′–3′)	Purpose
gpr54a-S	GATGAGGACCGCCACTAA	PCR
gpr54a-A	CTGGGATAGAAAGACTGGAACA	PCR
gpr54b-S	ATCTCAAAGCCCTGTCCCG	PCR
gpr54b-A	AGCAGCACCATTACAACCA	PCR
3′-site adaptor	CTGATCTAGAGGTACCGGATCC	3′ RACE
3′- *gpr54a*-1	ATGTCCTATGCCAACTCCTC	3′ RACE
3′- *gpr54a*-2	CAAAGCCAACTACGCAACAT	3′ RACE
3′- *gpr54b*-1	TGAGTTACCCACCAAGCCTAC	3′ RACE
3′- *gpr54b*-2	TAAAGTGGCAGTGAAAGGAAC	3′ RACE
5′- *gpr54a*-1	GAGGAGTTGGCATAGGACAT	5′ RACE
5′- *gpr54a*-2	ATGTTGCGTAGTTGGCTTTG	5′ RACE
5′- *gpr54b*-1	TACTCCTTCTTGGCTGTTTACC	5′ RACE
5′- *gpr54b*-2	GAGTATCCAGAATGGTGGTTGT	5′ RACE
UPM	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT (long)	5′ RACE
NUP	AAGCAGTGGT AACAACGCAGAGT	5′ RACE
gpr54a-Q-F	GCCTCTGTGGAACCAGTGGATAA	qRT-PCR
gpr54a-Q-R	GGACCCCAGCAGATGGTGAAG	qRT-PCR
gpr54b-Q-F	AAAGCCCAATCCCACTGACA	qRT-PCR
gpr54b-Q-R	AGGAACACAGCAAACCAGGA	qRT-PCR
β-actin-Q-F	GGAAGGGCCAGACTCATCGTA	qRT-PCR
β-actin-Q-R	TCCCTTGCTCCTTCCACCA	qRT-PCR
gpr54a-ISH-F	TTACGGCAATGAGTGGAGAC	ISH
gpr54a-ISH-R	GGGATAGAAAGACTGGAACA	ISH
EX + T7+*gpr54a*-ISH-R	CAGTGAATTGTAATACGACTCACT ATAGGGAGAGGGATAGAAAGACTGGAACA	ISH
gpr54b-ISH-F	TGTTCCTTTCACTGCCACTT	ISH
gpr54b-ISH-R	CACCATCACAACCACCATTC	ISH
EX + T7+*gpr54b*-ISH-R	CAGTGAATTGTAATACGACTCACT ATAGGGAGAAAGAGCAGAGCCACCACCATCA	ISH

F, Forward primer; R, Reverse primer.

one copy of the *gpr54* gene compared to mammals [17]. Two forms of GPR54 (GPR54-1 and GPR54-2) involved in initiating puberty have been identified in zebrafish, the first report of multiple GPR54 in this species [18]. Parhar et al. have cloned GPR54 in cichlid fish and found its functions in regulating reproduction and the onset of puberty [13]. The GPR54 co-localizes with GnRH1, GnRH2, and GnRH3 in tilapia brain, and the GPR54 has significantly higher expression in the mature fish compared to the immature fish, suggesting GPR54 is associated with the gonadal development and pubertal maturation [13]. A previous study has shown that there is a potential relationship between GPR54 and GnRH [15]. Nocillado et al. indicate that GPR54 may play a role in regulating pubertal development in grey mullet [14]. Therefore, a deeper understanding of the role of GPR54 in regulating reproductive function is vital for the breeding and farming of economic species.

Allotriploid crucian carp ($3n = 150$) with sterility, rapid growth, and strong resistance, which is obtained by mating the male allotetraploids ($4n = 200$) and female red crucian carp (*Carassius auratus* red var., $2n = 100$) [19,20]. Allotriploid crucian carp has three kinds of gonads (testis, ovary, and fat type), but none produce normal gametes [21]. Hence, the allotriploid crucian carp is a great model for exploring reproductive function. In this study, we cloned the full-length cDNAs, investigated the tissue distribution, and studied the localization of *gpr54a* and *gpr54b* in sterile allotriploid crucian carp and fertile diploid red crucian carp.

2. Materials and methods

2.1. Animal and ethics statement

Allotriploid crucian carp and diploid red crucian carp were collected from the Engineering Research Center of Polyploid Fish Reproduction and Breeding of the Ministry of Education at Hunan Normal University in May (breeding season) and December (non-breeding season), severally. The animals were anesthetized with MS222 (1:10,000, Sangon Biotech, China) before decapitation, and the tissues were immediately excised. All tissues were stored at −80 °C for the subsequent experiments. All animal experiments were approved by the Animal Care Committee of Hunan Normal University and the Administration of Affairs Concerning Experimental Animals of China.

2.2. RNA extraction and gene cloning

Total RNA was purified with the Trizol™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of total RNA was detected using gel electrophoresis, showing clear 28S, 18S, and 5S rRNA bands. The concentration and A260/280 ratio of total RNA were evaluated using the NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA), in which the concentration ranged between 500 and 1300 ng/μL and the A260/280 ratio ranged between 1.8 and 2.0. The first-strand cDNAs for amplifying the partial cDNA fragments of coding sequence (CDS) were synthesized using PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). The first-strand cDNAs for amplifying the 5′ - untranslated region (UTR) and 3′ - UTR were synthesized with SMART™ RACE cDNA Amplification Kit (Clontech, San Francisco, CA, USA) and 3′-Full RACE Core Set (Takara, Tokyo, Japan), respectively. Then, the first-strand cDNAs were stored at −20 °C for the subsequent experiments.

Primer Premier 5.0 was used to design the primers (Table 1). The full-length cDNAs of these genes were obtained by the rapid amplification of cDNA ends (RACE) as previously reported [22]. The PCR products were separated using 1.2 % agarose gels, and the target fragments were purified using a SanPrep Column DNA Gel kit (Sangon Biotech, Shanghai, China). Next, the purified products were subcloned into the pMD18-T vector (TaKaRa) and sequenced in Sangon.

2.3. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to explore the relative expression of *gpr54a* and *gpr54b* in the medulla, mesencephalon, cerebellum, olfactory bulb, telencephalon, hypothalamus, pituitary, spleen, muscle, ovary, testis, gill, heart, liver, kidney, head kidney, and skin, which obtained from ACC (54.50 ± 7.24 g, 3 females and 3 males) and RCC (47.47 ± 6.40 g, 3 females and 3 males), respectively. The specific primers were designed by AlleleID 6, and the *β-actin* was used as the internal control (Table 1). The specific primers were selected with a single melting curve and 95%–105 % amplification efficiency. qRT-PCR was carried out using the Prism 7500 Sequence Detection System (ABI, Foster City, CA, USA). The reaction mixture consisted of 5 μl SYBR green PCR Master Mix, 3 μl ddH_2O, 1 μl cDNA, and 0.5 μl each primer. The condition of qRT-PCR was established according to the procedure described previously [22]. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression of the genes [23].

2.4. In situ hybridization

In situ hybridization (ISH) was performed in the RNase-free environment. Primer Premier 5.0 was used to construct the specific primers for ISH (Table 1). The MAXIscript™ T7 In Vitro Transcription Kit (Invitrogen, USA) and DIG RNA labeling kit (Roche, Germany) were used to synthesize the antisense digoxigenin (DIG)-labeled probes according to the manufacturer's instructions. Probes were purified using LiCl precipitation and stored at −80 °C.

The brain, pituitary, and ovary were excised from ACC and RCC and fixed in 4 % paraformaldehyde (PFA, Sangon) at 4 °C for 24 h. Subsequently, the tissues were dehydrated with a series of graded sucrose solutions (15 %, 20 %, and 30 %) and then embedded in Tissue-Tek O.C.T. Compound® (Sakura, USA). The embedded tissues were cut into 20

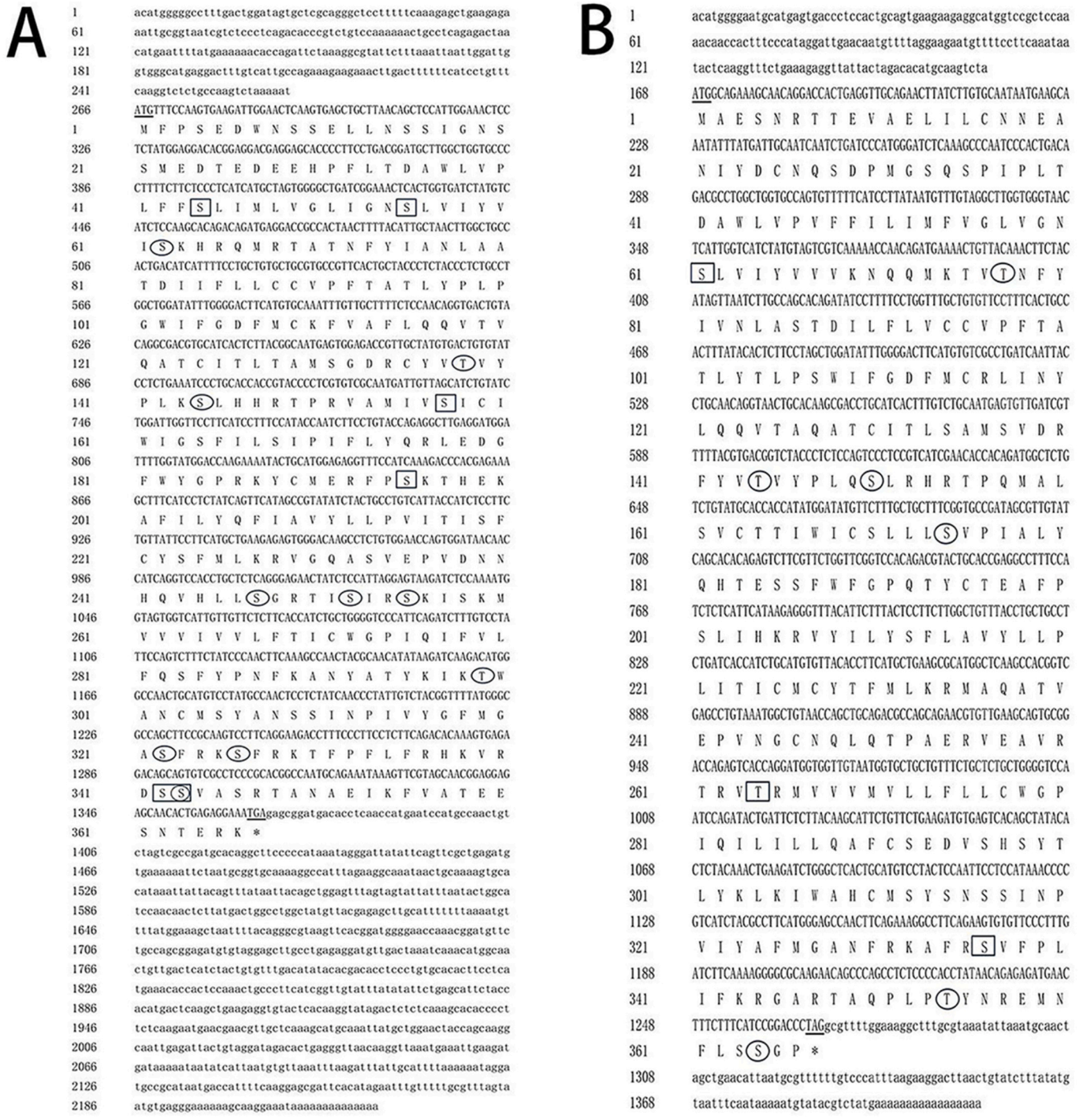

Fig. 1. Nucleotide and deduced amino acid sequences of GPR54a (A) and GPR54b (B) in allotriploid crucian carp. Lowercase letters represent the non-coding regions of the nucleotide sequence, uppercase letters represent the coding regions of the nucleotide sequence, underline denotes the start codon, Asterisk shows the stop codon, rectangular boxes refer protein kinase A (PKA) phosphorylation site, oval boxes show protein kinase C (PKC) phosphorylation site.

μm thick coronal sections with a freezing microtome (Leica CM3050 S Cryostat, Germany).

Prior to hybridization, the sections were washed 3 times with 1 × PBST at room temperature (RT) for 5 min each time, fixed with 4 % PFA at RT for 10 min, and prehybridized at 60 °C for 3 h. The prehybridization solution was mixed as follows: 50 ml of deionized formamide, 25 mL of 20 × SSC, 920 μl of 1M citric acid, 1 ml of 0.5M EDTA, 500 μl of 20 % Tween-20, 100 μL of 50 mg/ml heparin, 100 μL of 50 mg/ml tRNA, and 22.38 mL of DEPC-H_2O.

The hybridization solution was prepared by adding the RNA probe into the prehybridization solution. The hybridization solution was denatured at 80 °C for 10 min and then immediately chilled on the ice for 5 min before use. Next, the sections were incubated with the hybridization solution at 60 °C for 16 h. The negative control was incubated with PBS instead of the hybridization solution.

After hybridization, the sections were washed with 50 % deionized formamide at 60 °C for 15 min and 2 × SSCT at RT for 15 min. Then, they were incubated with 2 μg/mL RNase A buffer at RT for 30 min and 50 % deionized formamide at 60 °C for 30 min. Subsequently, they washed with 2 × SSCT, 0.2 × SSCT, and 1 × PBST at 60 °C for 15 min

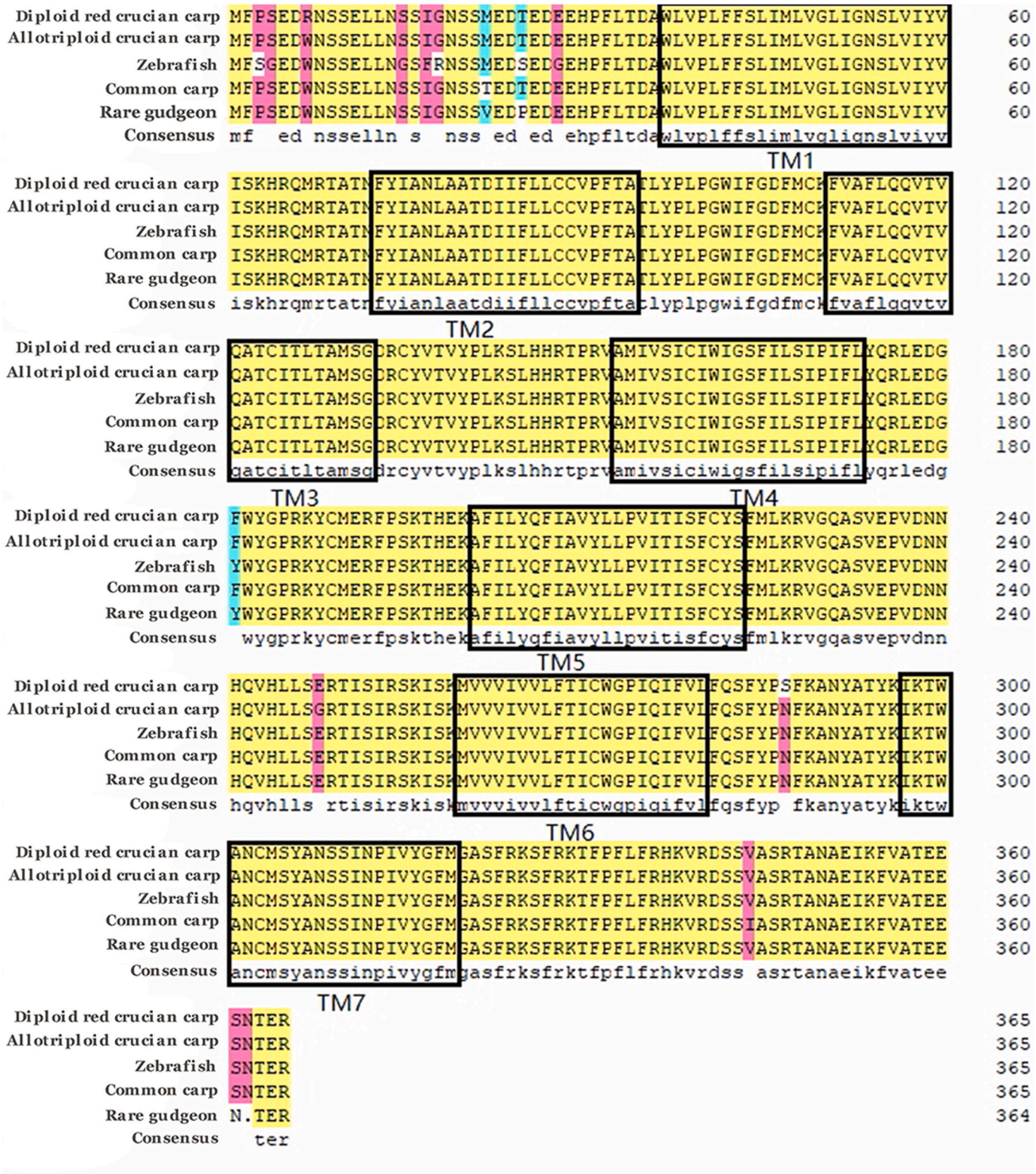

Fig. 2. Amino acid sequence alignment of GPR54a in allotriploid crucian carp, diploid red crucian carp, and other species. Lowercase letters represent identical amino acids, yellow parts refer highly conserved amino acids, purple parts refer moderately conserved amino acids, blue parts refer lowly conserved amino acids, rectangular boxes denote transmembrane domains (TM1-7). Allotriploid crucian carp (OR946413), diploid red crucian carp (*Carassius auratus* red var. OR946412), common carp (*Cyprinus carpio* GPR54a: AFM08411.1), zebrafish (*Danio rerio* KISS1 receptor a: NP_001099149.2), rare gudgeon (Gobiocypris rarus GPR54a: AHH83759.1).

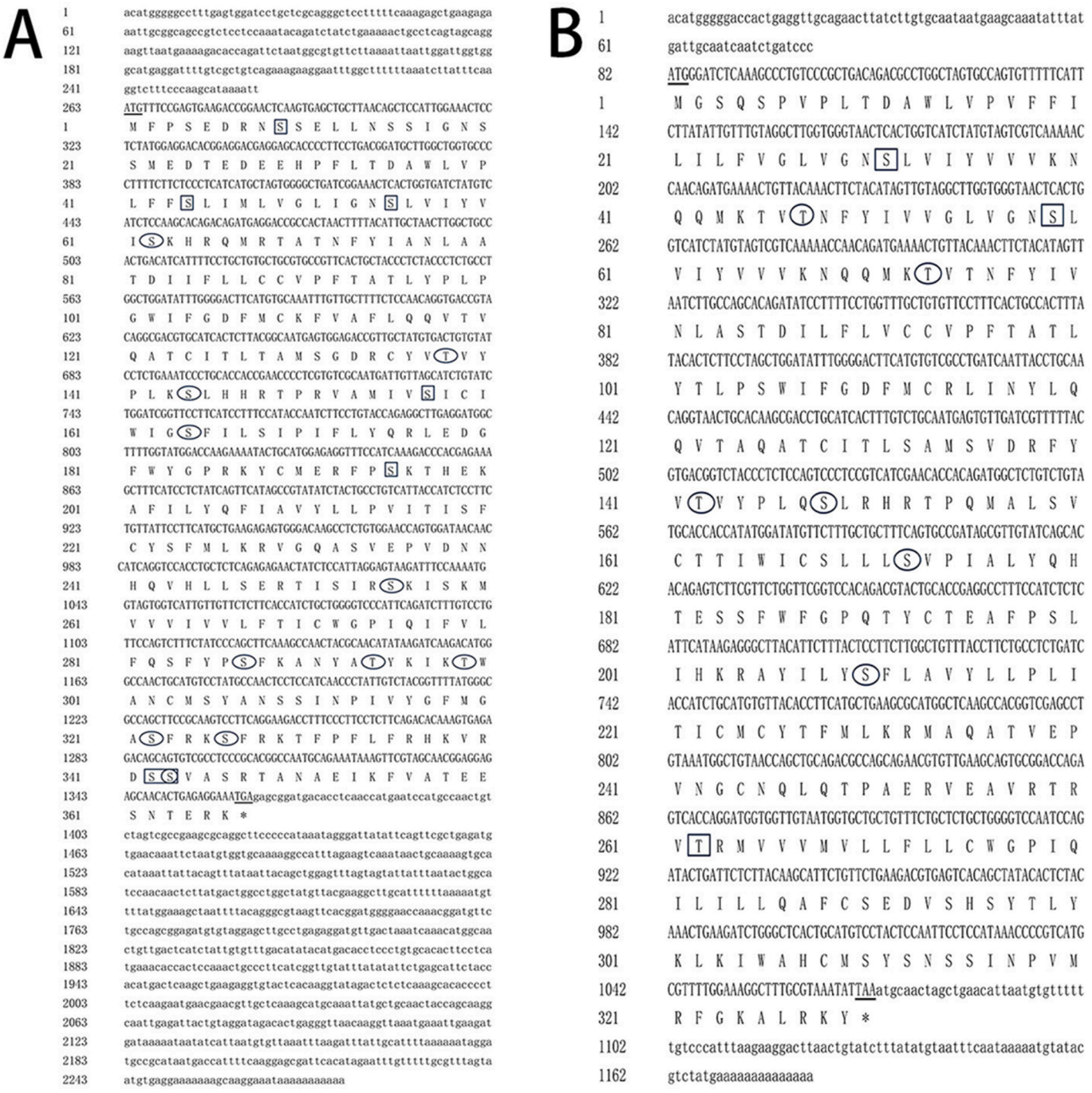

Fig. 3. Nucleotide and deduced amino acid sequences of GPR54a (A) and GPR54b (B) in diploid red crucian carp. Lowercase letters represent the non-coding regions of the nucleotide sequence, uppercase letters represent the coding regions of the nucleotide sequence, underline denotes the start codon, Asterisk shows the stop codon, rectangular boxes refer protein kinase A (PKA) phosphorylation site, oval boxes show protein kinase C (PKC) phosphorylation site.

each time. After that, the sections were blocked with blocking buffer at RT for 3 h and then incubated with AP-conjugated anti-DIG antibody (Roche, Germany, 1:1500 diluted in the blocking buffer) overnight at 4 °C.

Finally, the sections were stained with NBT/BCIP stock solution (Roche, Germany), and the hybridization signals were captured with light microscopy (Olympus, Japan).

2.5. Statistical analysis

All data were shown as mean ± SEM. DNAMAN version 5.0 was used to analyze multiple sequence alignment. The phylogenetic tree was constructed using MEGA 5 software with the neighbor-joining (NJ) method. SPSS 19.0 software was used to calculate the significant differences. Student's t-test was used to analyze statistically significant differences in gene expression between multiple groups.

3. Results

3.1. Nucleotide and deduced amino acid sequences of GPR54a and GPR54b

The full-length cDNAs of ACC *gpr54a* (GenBank: OR946413) and RCC *gpr54a* (GenBank: OR946412) were 2224 bp and 2218 bp,

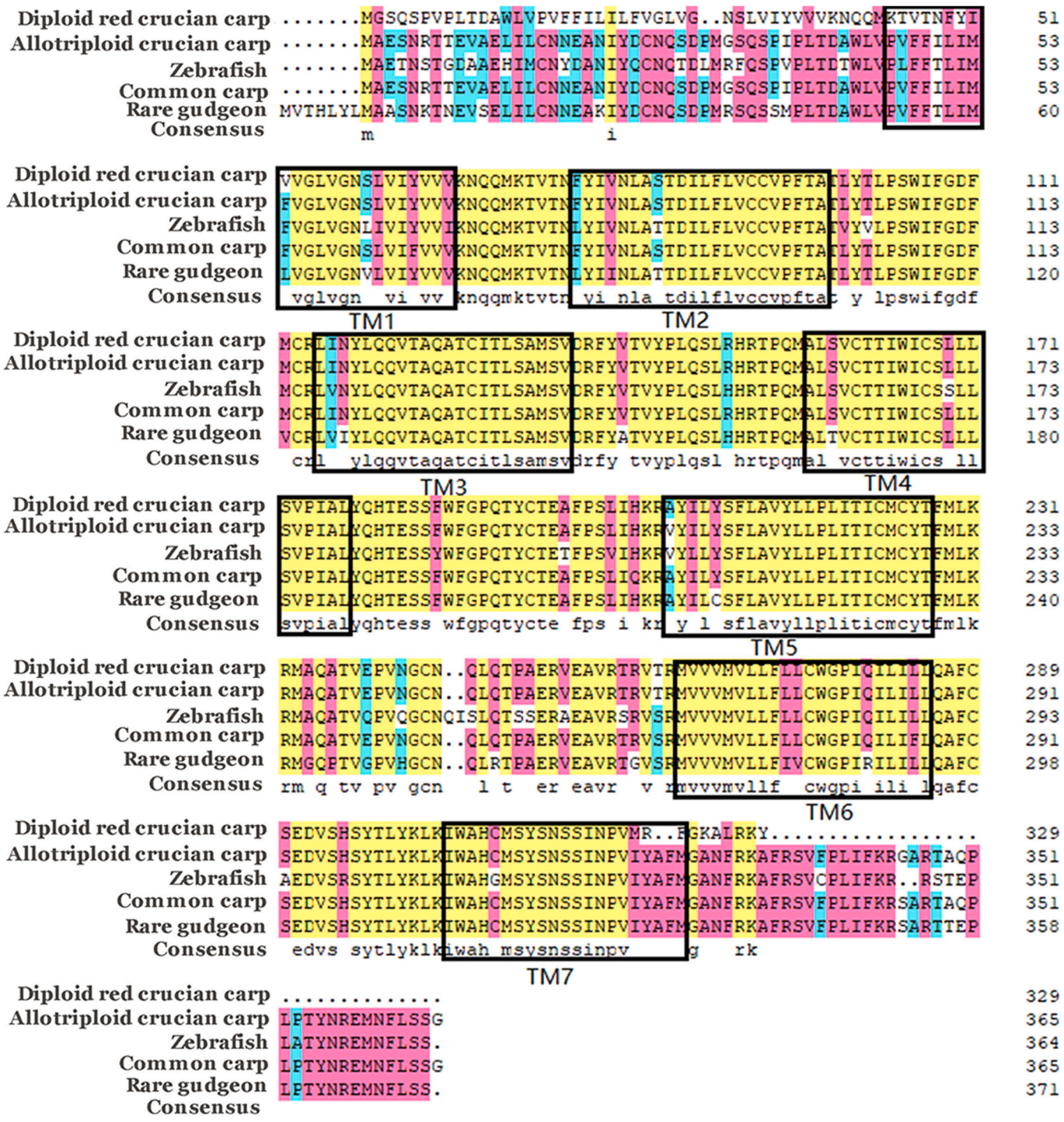

Fig. 4. Amino acid sequence alignment of GPR54b in allotriploid crucian carp, diploid red crucian carp, and other species. Lowercase letters represent identical amino acids, yellow parts refer highly conserved amino acids, purple parts refer moderately conserved amino acids, blue parts refer lowly conserved amino acids, rectangular boxes denote transmembrane domains (TM1-7). Allotriploid crucian carp (OR946414), diploid red crucian carp (*Carassius auratus* red var. OR946415), common carp (*Cyprinus carpio* GPR54b: AFM08412.1), zebrafish (*Danio rerio* KISS1 receptor b: NP_001104001.1), rare gudgeon (Gobiocypris rarus GPR54a: AHH83760.1).

respectively (Figs. 1A and 3A). ACC *gpr54a* had 265 bp 5′ UTR, 858 bp 3′ UTR, and 1101 bp open reading frame (ORF) that encoded a protein of 366 amino acids (Fig. 1A). RCC *gpr54a* had 262 bp 5′ UTR, 855 bp 3′ UTR, and 1101 bp ORF that encoded a protein of 366 amino acids (Fig. 3A). The analysis of phosphorylation sites indicated that ACC *gpr54a* had six protein kinase A (PKA) phosphorylation sites and eleven protein kinase C (PKC) phosphorylation sites (Fig. 1A), RCC *gpr54a* had seven PKA phosphorylation sites and eleven PKC phosphorylation sites (Fig. 3A). We found that the ACC and RCC GPR54a had seven transmembrane domains (TMDs) (Fig. 2), which is a typical characteristic of the G-protein-coupled receptor family. Multiple sequence alignment analysis showed that GPR54a shared high identities of 99.2 % between ACC and RCC, as well as to other fish with higher identities (Fig. 2).

The full-length cDNAs of ACC *gpr54b* (GenBank: OR946414) and RCC *gpr54b* (GenBank: OR946415) were 1413 bp and 1182 bp, respectively (Figs. 1B and 3B). ACC *gpr54b* had 167 bp 5′ UTR, 145 bp 3′ UTR,

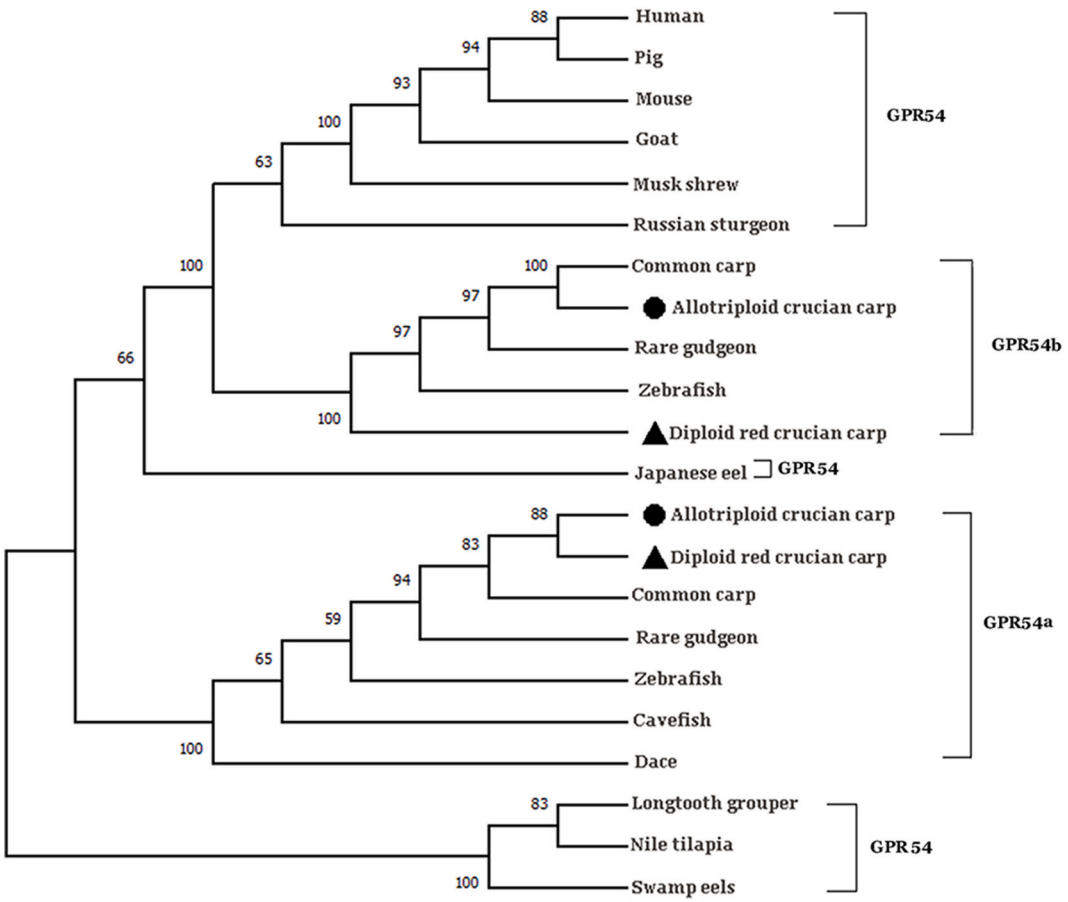

Fig. 5. Phylogenetic tree of GPR54a and GPR54b. The phylogenetic tree was constructed with the neighbor-joining (NJ) method. Numbers at nodes denote the bootstrap percentages, obtained for 1000 replicates. Allotriploid crucian carp (GPR54a: OR946413, GPR54b: OR946414), diploid red crucian carp (*Carassius auratus* red var. GPR54a: OR946412, GPR54b: OR946415), common carp (*Cyprinus carpio* GPR54a: AFM08411.1), zebrafish (*Danio rerio* KISS1 receptor a: NP_001099149.2), cavefish (Triplophysa rosa GPR54a: KAI7809676.1), rare gudgeon (Gobiocypris rarus GPR54a: AHH83759.1), dace (Tribolodon brandtii GPR54a: ASU91840.1), common carp (*Cyprinus carpio* GPR54b: AFM08412.1), zebrafish (*Danio rerio* KISS1 receptor b: NP_001104001.1), rare gudgeon (Gobiocypris rarus GPR54b: AHH83760.1), human (*Homo sapiens* GPR54: AAK83235.1), pig (*Sus scrofa* GPR54: ABE73453.1), mouse (*Mus musculus* GPR54: AAK83236.1), goat (*Capra hircus* GPR54: ACY91951.1), Japanese eel (Anguilla japonica GPR54: BAM72049.1), swamp eels (*Monopterus albus* GPR54: AVZ65972.1), musk shrew (Suncus murinus GPR54: BAL04095.1), Russian sturgron (*Acipenser gueldenstaedtii* GPR54: AYD88374.1), longtooth grouper (Epinephelus bruneus GPR54: AEN14599.1), Nile tilapia (*Oreochromis niloticus* GPR54: NP_001266708.1).

and 1101 bp ORF that encoded a protein of 366 amino acids (Fig. 1B). RCC *gpr54b* had 81 bp 5′ UTR, 111 bp 3′ UTR, and 990 bp ORF that encoded a protein of 329 amino acids (Fig. 3B). The analysis of phosphorylation sites indicated that both ACC and RCC *gpr54b* had three protein kinase A (PKA) phosphorylation sites and six protein kinase C (PKC) phosphorylation sites (Figs. 1B and 3B). Seven TMDs were found in ACC and RCC GPR54b (Fig. 4). Multiple sequence alignment analysis showed that GPR54b shared high identities of 82.7 % between ACC and RCC and had higher identities among other fish (Fig. 4). Phylogenetic tree analysis showed that ACC GPR54a was nested with RCC GPR54a, ACC GPR54b and RCC GPR54b were localized in a clade, and GPR54a and GPR54b were clustered into two separate clades (Fig. 5).

3.2. Tissue expression of *gpr54a* and *gpr54b*

qRT-PCR was performed to verify the relative expression of *gpr54a* and *gpr54b* in different tissues and stages of ACC and RCC (Figs. 6–7). In both the breeding season and non-breeding season, the female ACC and RCC *gpr54a* were mainly expressed in the brain but expressed at low levels in periphery tissues (Fig. 6A–C). The male ACC and RCC *gpr54a* were highly expressed in the brain but had lower expression in peripheral tissues (Fig. 6B–D). In addition, it is noted that the expression of *gpr54a* had significant differences between ACC and RCC in most tissues especially in the brain, pituitary, muscle, and gonads (Fig. 6).

Unlike *gpr54a*, ACC and RCC *gpr54b* were widely expressed in the brain and periphery tissues (Fig. 7). In the breeding season, the female ACC *gpr54b* was highly expressed in the brain, liver, muscle, skin, pituitary, and ovary (Fig. 7A). The female RCC *gpr54b* was highly in the brain, spleen, heart, kidney, and head kidney (Fig. 7A). The higher expression of male ACC *gpr54b* was found in the liver, muscle, and testis (Fig. 7B). The male RCC *gpr54b* was highly expressed in the pituitary and mesencephalon and moderately expressed in muscle, spleen, and testis (Fig. 7B). In the non-breeding season, the female ACC *gpr54b* was highly expressed in the brain, heart, and liver (Fig. 7C). The female RCC *gpr54b* was mainly expressed in the brain, muscle, and liver (Fig. 7C). The higher expression of male ACC *gpr54b* was detected in the brain, liver, skin, muscle, and pituitary (Fig. 7D). The male RCC *gpr54b* was primarily expressed in the brain, muscle, and pituitary (Fig. 7D). Similar to *gpr54a*, the expression of *gpr54b* had significant differences between female ACC and RCC in most tissues, especially in the brain, pituitary, spleen, muscle, liver, sky, and gonads (Fig. 7).

3.3. Relative expression of *gpr54a* and *gpr54b* in HPG axis

To further understand the physiological function, we analyzed the relative expression of *gpr54a* and *gpr54b* in the HPG axis of ACC and

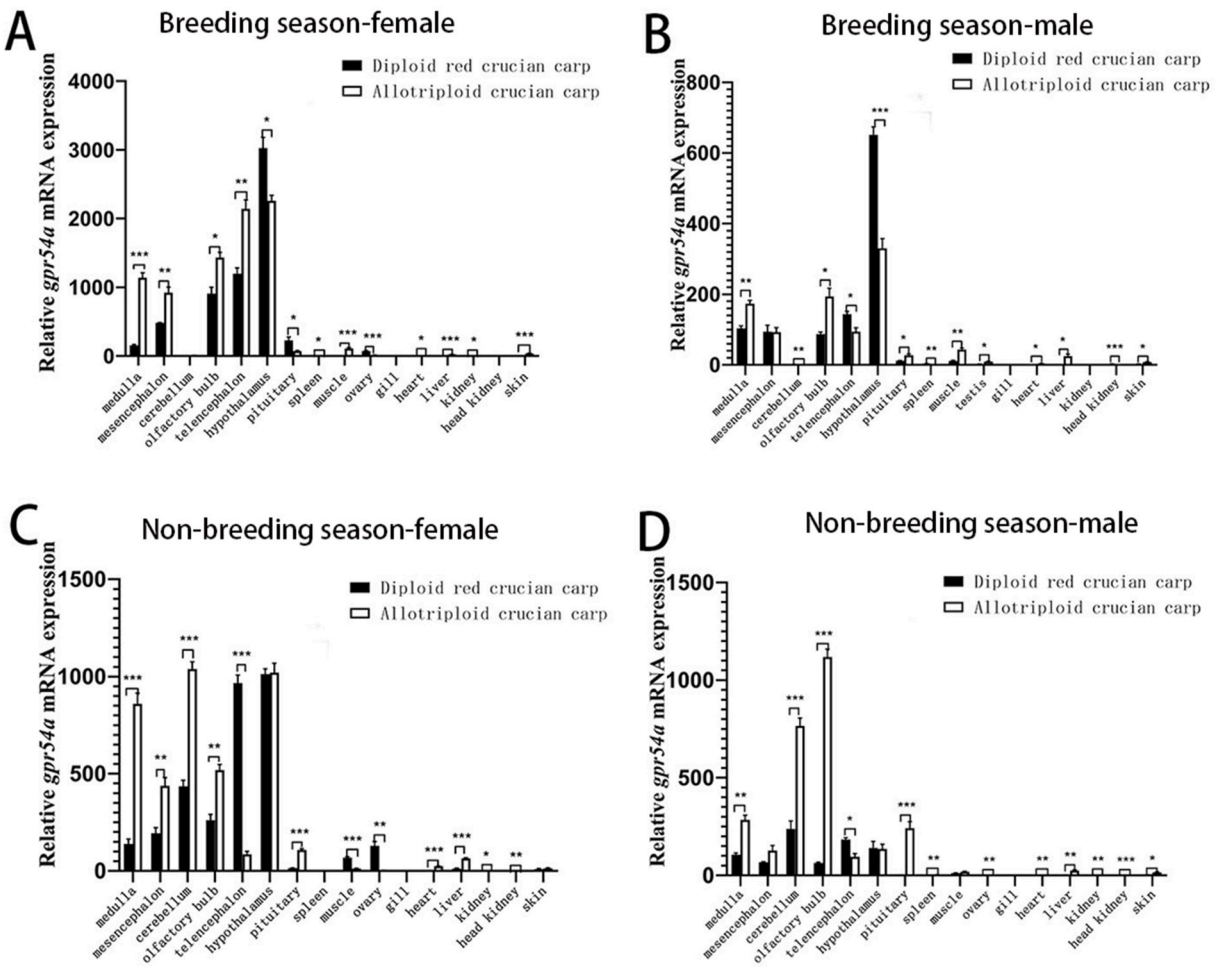

Fig. 6. Relative expression of gpr54a in allotriploid crucian carp and diploid red crucian carp. (A) Relative expression of gpr54a in female allotriploid crucian carp and diploid red crucian carp during the breeding season. **(B)** Relative expression of gpr54a in male allotriploid crucian carp and diploid red crucian carp during the breeding season. **(C)** Relative expression of gpr54a in female allotriploid crucian carp and diploid red crucian carp during the non-breeding season. **(D)** Relative expression of gpr54a in male allotriploid crucian carp and diploid red crucian carp during the non-breeding season. The β-actin was used as the internal control. Data were shown as the mean ± SEM (n = 3). Asterisk indicates a significant difference ("*" indicates $P < 0.05$, "**" indicates $P < 0.01$, "***" indicates $P < 0.001$).

RCC, specially (Fig. 8). In the breeding season, the expression of *gpr54a* in the hypothalamus, pituitary, and ovary of ACC was significantly lower than in RCC (Fig. 8A). In the non-breeding season, the expression of *gpr54a* in the ovary of ACC was significantly lower than in RCC, ACC *gpr54a* was higher expressed in the pituitary compared to RCC, and there was no significant difference between ACC and RCC in the hypothalamus (Fig. 8B). In addition, we found that the expression of *gpr54a* in the hypothalamus was higher in the breeding season than in the non-breeding season (Fig. 8A and B).

In sharp contrast to the expression of *gpr54a* during the breeding season, the expression of *gpr54b* in the hypothalamus, pituitary, and ovary of ACC was higher than in RCC, significantly (Fig. 8C). In the non-breeding season, *gpr54b* had a higher expression in the hypothalamus and ovary of ACC than in RCC, but *gpr54b* had a lower expression in the pituitary of ACC than in RCC (Fig. 8D). However, it is worth noting that the expression of *gpr54b* in the breeding season was higher than in the non-breeding season (Fig. 8C and D).

3.4. *The localization of gpr54a and gpr54b in brain, pituitary, and ovary*

To investigate the involvement of *gpr54a* and *gpr54b* in regulating reproduction, ISH was performed to detect the localization of the two mRNAs in the brain, pituitary, and ovary of ACC and RCC during the breeding season. In the brain, cell group expressing *gpr54a* mRNAs were detected in several parts, such as lateral tuberal nucleus (NLT), nucleus anterior periventricularis (NAPv), parvocellular part of the parvocellular preoptic nucleus (NPO), and posterior periventricular nucleus (NPP) of ACC and RCC (Fig. 9A–C). In the pituitary, the positive *gpr54a*-labeled cells were evident in rostral pars distalis (RPD), proximal pars distalis (PPD), pars intermedia (PI), and neurohypophysis (NH) of ACC and RCC (Fig. 9E–G). The results of ISH showed that the ovary of RCC had three oocyte phases (phases II, III, and IV), but the ovary of ACC consisted of a few oocytes of stage III and some small oogonium-like cells. The signals of *gpr54a* were detected in the yolk granule, nucleus, cortical alveolus, zona radiata, and follicle cell of RCC. Signals of *gpr54a* were also found in the nucleus, zona radiata, follicle cell, and yolk vesicle of ACC (Fig. 9I–K). It is noted that the signals of RCC *gpr54a* were stronger in the brain, pituitary, and ovary than in ACC. No signals were found in negative control (Fig. 9B–D, F, H, J, and L).

Similar to the *gpr54a*, a large number of signals of *gpr54b* were detected in the brain, pituitary, and ovary of ACC and RCC. In contrast, the signals of *gpr54b* in ACC were stronger than in RCC. In the brain, cell groups expressing *gpr54b* mRNAs were also detected in NLT, NAPv, NPO, and NPP of ACC and RCC (Fig. 10A–C). In the pituitary, the positive *gpr54b*-labeled cells were evident in the RPD, PPD, PI, and NH of ACC and RCC (Fig. 10E–G). In the ovary, signals of *gpr54b* were found in the nucleus, zona radiata, follicle cell, and yolk vesicle of ACC and RCC (Fig. 10I–K). No signals were found in negative control (Fig. 10B–D, F,

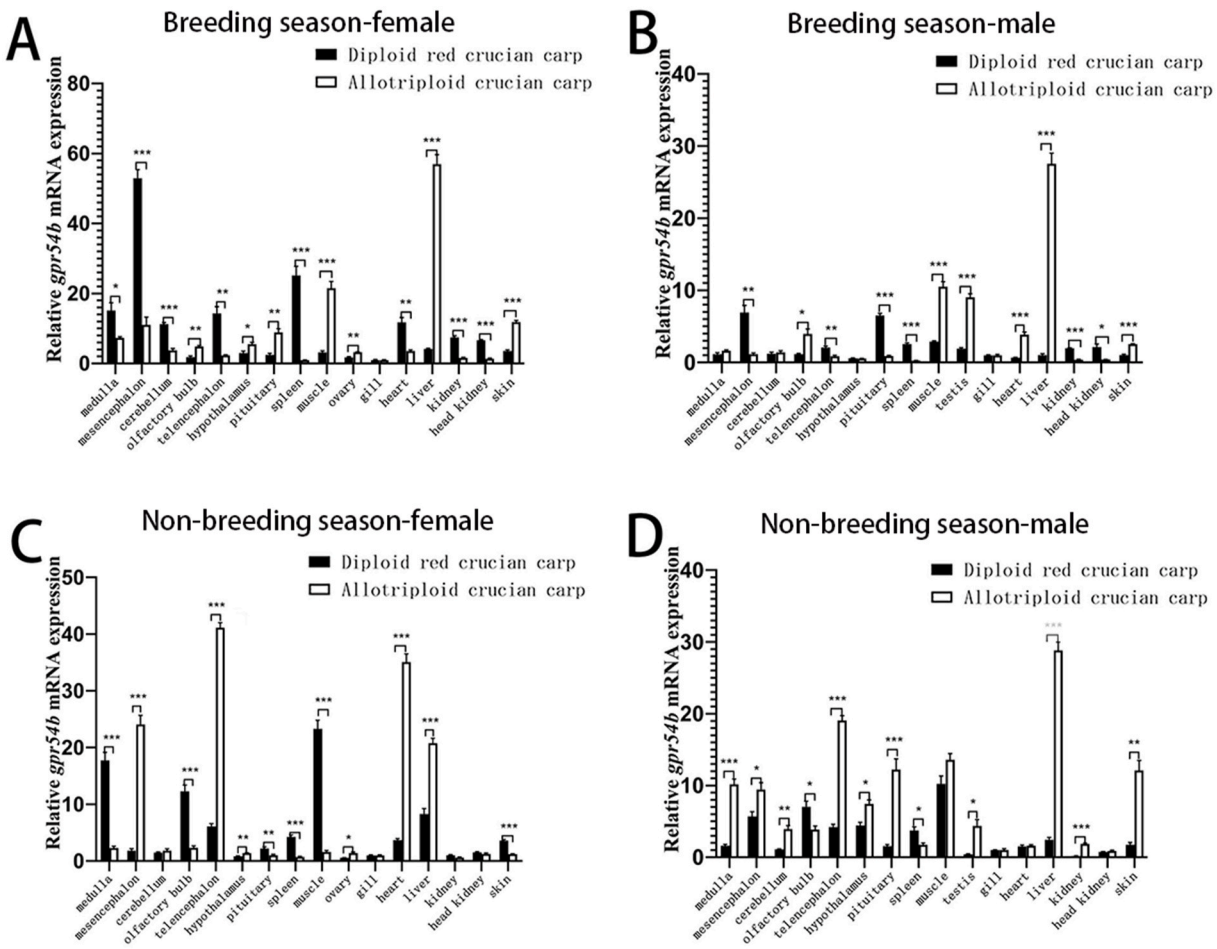

Fig. 7. Relative expression of gpr54b in allotriploid crucian carp and diploid red crucian carp. (A) Relative expression of gpr54b in female allotriploid crucian carp and diploid red crucian carp during the breeding season. **(B)** Relative expression of gpr54b in male allotriploid crucian carp and diploid red crucian carp during the breeding season. **(C)** Relative expression of gpr54b in female allotriploid crucian carp and diploid red crucian carp during the non-breeding season. **(D)** Relative expression of gpr54b in male allotriploid crucian carp and diploid red crucian carp during the non-breeding season. The β-actin was used as the internal control. Data were shown as the mean ± SEM (n = 3). Asterisk indicates a significant difference ("*" indicates $P < 0.05$, "**" indicates $P < 0.01$, "***" indicates $P < 0.001$).

H, J, and L).

4. Discussion

In the present study, we cloned the full-length cDNA of *gpr54a* and *gpr54b* genes in ACC and RCC. ACC and RCC *gpr54a* were predicted to encode a putative protein of 366 amino acids. ACC and RCC *gpr54b* encoded putative proteins of 366 amino acids and 329 amino acids, respectively (Figs. 1 and 3). Two genes in ACC and RCC had similar structural characteristics and high identity with other teleosts (Figs. 2 and 4), consistent with previous research [13,16,18,24–28]. Phylogenetic tree analysis showed that ACC GPR54a was grouped with RCC and other teleost GPR54a, and ACC GPR54b was also grouped with RCC and other teleost GPR54b (Fig. 5).

The distribution of *gpr54b* was wider than *gpr54a* in ACC and RCC (Figs. 6 and 7), which coincided with the previous reports in zebrafish and European sea bass (*Dicentrarchus labrax*) [18,27]. We observed that ACC and RCC *gpr54a* were highly expressed in the brain but expressed at low levels in peripheral tissues (Fig. 6). Unlike *gpr54a*, ACC and RCC *gpr54b* were highly expressed in the brain and widely expressed in peripheral tissues, such as muscle, liver, heart, sky, and gonads (Fig. 7). These data suggested that *gpr54a* and *gpr54b* had different roles in regulating the physiological functions of ACC and RCC. Moreover, the high expression of the *gpr54a* and *gpr54b* in the brain of ACC and RCC suggested that they had an essential role in regulating gonadal function via autocrine/paracrine mechanisms.

It has been confirmed that the HPG axis plays a role in regulating reproductive functions. There is a growing body of evidence indicating that GPR54 plays a crucial role in maintaining the normal function of the HPG axis [12,29–33]. We compared the relative expression of *gpr54a* and *gpr54b* in the HPG axis between female ACC and RCC during the breeding season and non-breeding season (Fig. 8). The data showed that *gpr54a* had significantly higher expression in RCC than in ACC during the breeding season. Moreover, the expression of *gpr54a* in hypothalamus of ACC and RCC during the breeding season was significantly higher than in the non-breeding season (Fig. 8A and B). Our results were consistent with previous studies showing temporal expression of gpr54 in some species. The expression level of *gpr54* was increased in the hypothalamus of rats at puberty [34]. In Nile tilapia (*Oreochromis niloticus*), the expression of *gpr54* in the mature stage was significantly higher than in the immature stage [35]. In addition, the *gpr54* had the highest expression at the onset of ovarian development of fathead minnow (*Pimephales promelas*) and zebrafish [18,36]. Hence, these data further verified the function of *gpr54a* in reproduction. It is noted that the expression of *gpr54a* was higher in HPG axis of RCC than in ACC during breeding season, which might relate to the sterility of ACC. In addition, the expression of *gpr54a* in the breeding season was higher than in the non-breeding season, indicating the function in regulating the reproduction. However, *gpr54b* had higher expression in ACC than in RCC, suggesting that *gpr54b* had a different role in promoting fertility

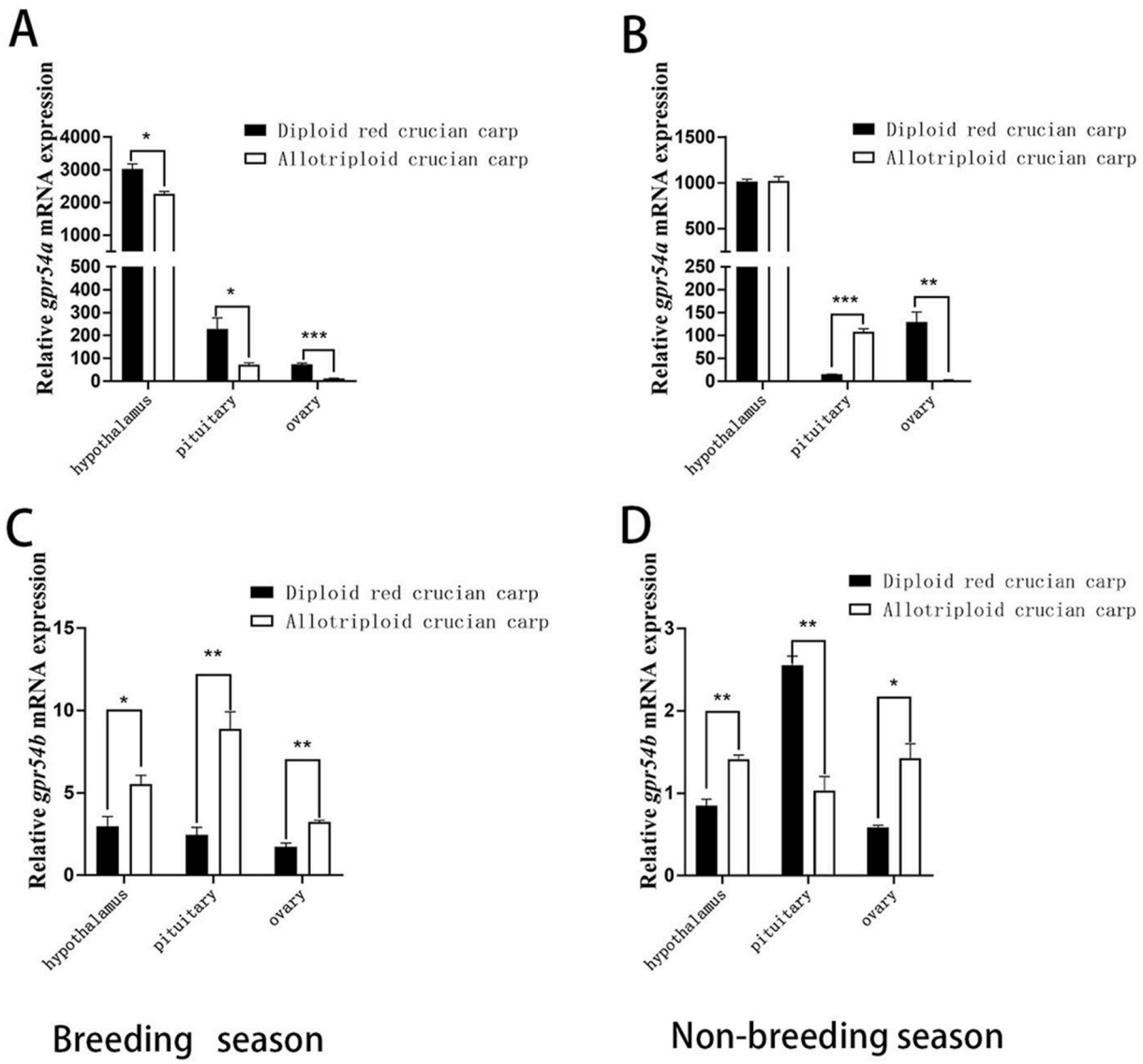

Fig. 8. Relative expression of gpr54a and gpr54b in HPG axis of female allotriploid crucian carp and diploid red crucian carp. (A) Relative expression of gpr54a in HPG axis of female allotriploid crucian carp and diploid red crucian carp during the breeding season. **(B)** Relative expression of gpr54a in HPG axis of female allotriploid crucian carp and diploid red crucian carp during the non-breeding season. **(C)** Relative expression of gpr54b in HPG axis of female allotriploid crucian carp and diploid red crucian carp during the breeding season. **(D)** Relative expression of gpr54b in HPG axis of female allotriploid crucian carp and diploid red crucian carp during the non-breeding season. The β-actin was used as the internal control. Data were shown as the mean ± SEM (n = 3). Asterisk indicates a significant difference ("*" indicates $P < 0.05$, "**" indicates $P < 0.01$, "***" indicates $P < 0.001$).

compared to *gpr54a*, and the mechanism of reproductive function of *gpr54b* was worth further exploration.

We investigated the localization of *gpr54a* and *gpr54b* in the brain, pituitary, and ovary of ACC and RCC during the breeding season (Figs. 9 and 10). The results of ISH in the brain showed that the signals of *gpr54a* and *gpr54b* were detected in NLT, NAPv, NPO, and NPP of the brain in ACC and RCC (Fig. 9A–C and Fig. 10 A, C), coincided with the previous reports in goldfish, zebrafish, medaka (*Oryzias latipes*), chub mackerel (*Scomber japanicus*), European seabass, and grass puffer (*Takifugu niphobles*) [27,28,37–42]. Neuropeptide Y (NPY) plays a role in the reproduction of fish and is highly expressed in the ventral and lateral telencephalon of sea bass and goldfish [43,44], and *gpr54* is detected in GnRH neurons [13], so we speculated that the *gpr54a* and *gpr54b* were highly expressed in regions with high expression of NPY and GnRH might involve in reproduction by regulating the secretions of NPY and GnRH. However, the signals of *gpr54b* in ACC were stronger than in RCC, suggesting that *gpr54b* might inhibit the secretions of NPY and GnRH to influence ACC sterility. In the pituitary, the signals of *gpr54a* and *gpr54b* were detected in RPD, PPD, PI, and NH (Fig. E, G and Fig. 10 E. G). The pituitary secretes the FSH and LH to stimulate the maturation of gametes, progesterone secretion, and ovulation [3,4]. Several studies have investigated the localization of FSH and LH in RPD, PPD, PI, and NH [45–49]. Hence, we suggested that *gpr54a* and *gpr54b* were highly expressed in the regions with high expression of FSH and LH might relate to the secretion of FSH and LH to influence reproduction. A study has proved that GPR54 is associated with the development of oocytes [35]. We found the massive signals of *gpr54a* and *gpr54b* in the nucleus, zona radiata, follicle cell, and yolk vesicle of ACC and RCC (Fig. 9I–K and Fig. 10 I, K). We speculated that *gpr54a* and *gpr54b* play a role in ovarian development. In conclusion, we hypothesized that *gpr54a* might promote the secretions of NPY and GnRH in the brain, leading to the production of FSH and LH by the pituitary, ultimately promoting ovarian development. However, the wide expression of *gpr54b* in the brain, pituitary, and ovary of ACC might inhibit the secretions of NPY and GnRH

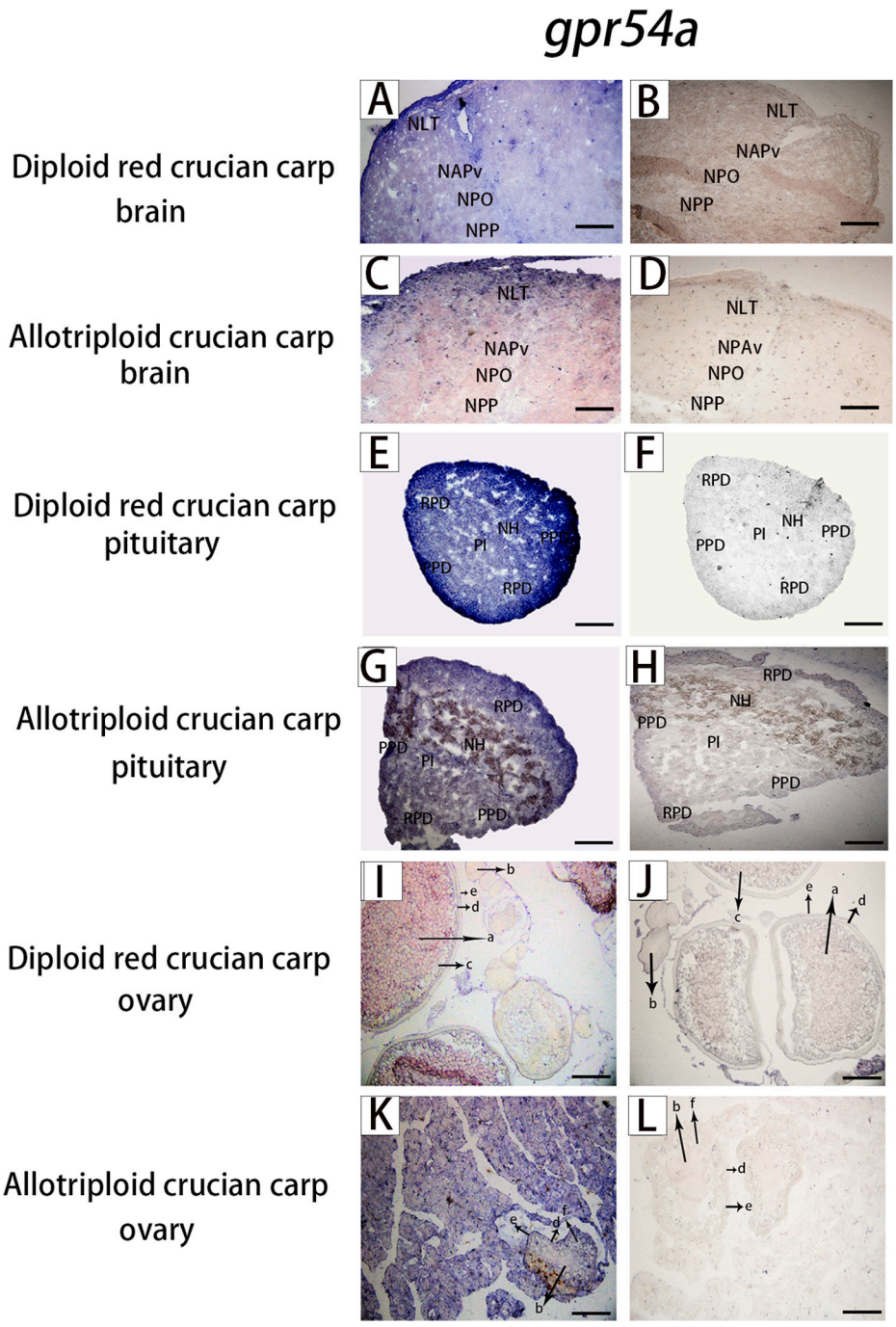

Fig. 9. Localization of gpr54a in the brain, pituitary, and ovary of diploid red crucian carp and allotriploid crucian carp. (A, C) Localization of gpr54a in the brain of diploid red crucian carp and allotriploid crucian carp. **(E, G)** Localization of gpr54a in the pituitary of diploid red crucian carp and allotriploid crucian carp. **(I, K)** Localization of gpr54a in the ovary of diploid red crucian carp and allotriploid crucian carp. **(B, D, F, H, J, L)** Negative control of gpr54a in the diploid red crucian carp and allotriploid crucian carp. Posterior periventricular nucleus (Npp), Parvocellular part of the parvocellular preoptic nucleus (Npo), Lateral tuberal nucleus (NLT), Nucleus anterior periventricularis (NAPv). Rostral pars distalis (RPD), Proximal pars distalis (PPD), Pars intermedia (PI), Neurohypophysis (NH). a represents yolk granule, b represents nucleus, c represents cortical alveolus, d represents zona radiata, e represents follicle cell, and f represents yolk vesicle. Scale bars: 100 μm.

in the brain, which could inhibit the pituitary to produce FSH and LH, eventually leading to abnormal gonadal development and gametogenesis. Therefore, the lower expression of *gpr54a* and higher expression of *gpr54b* in ACC might be related to ACC sterility. However, the complex mechanisms of *gpr54b* require further study.

In summary, these data suggested that *gpr54a* and *gpr54b* might play different roles in regulating the reproduction of ACC and RCC, *gpr54a* might promote ovarian development, while *gpr54b* might inhibit ovarian development. These findings provide new theoretical guidance for the molecular mechanisms of ACC sterility.

CRediT authorship contribution statement

Lu Huang: Writing – original draft, Data curation, Conceptualization. **Qiubei Wang:** Data curation. **Shuxin Zhang:** Data curation. **Faxian Yu:** Conceptualization. **Shengnan Li:** Data curation. **Huan Zhong:**

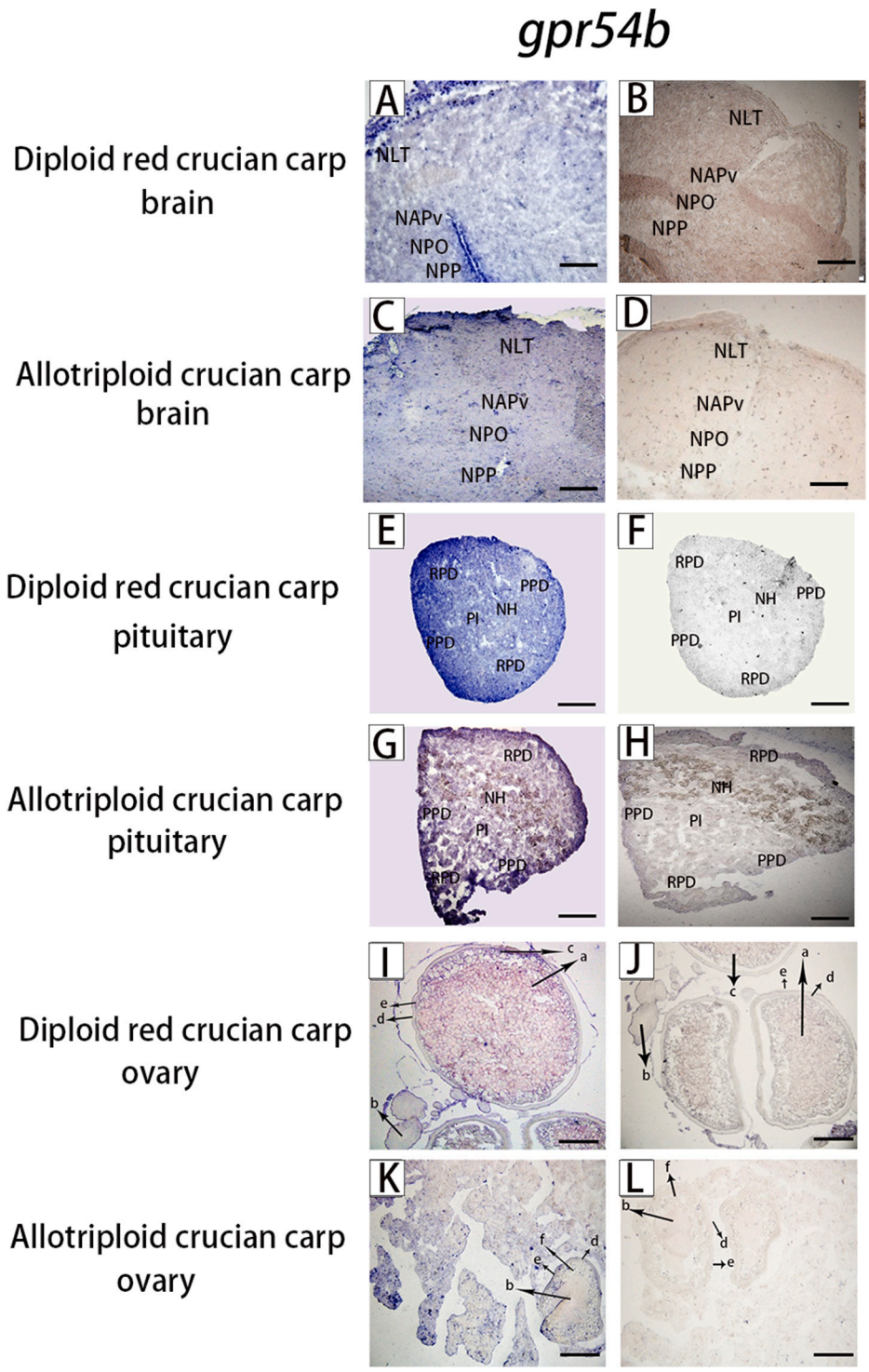

Fig. 10. Localization of gpr54b in the brain, pituitary, and ovary of diploid red crucian carp and allotriploid crucian carp. (A, C) Localization of *gpr54b* in the brain of diploid red crucian carp and allotriploid crucian carp. **(E, G)** Localization of *gpr54b* in the pituitary of diploid red crucian carp and allotriploid crucian carp. **(I, K)** Localization of *gpr54b* in the ovary of diploid red crucian carp and allotriploid crucian carp. **(B, D, F, H, J, L)** Negative control of *gpr54b* in the diploid red crucian carp and allotriploid crucian carp. Posterior periventricular nucleus (Npp), Parvocellular part of the parvocellular preoptic nucleus (Npo), Lateral tuberal nucleus (NLT), Nucleus anterior periventricularis (NAPv). Rostral pars distalis (RPD), Proximal pars distalis (PPD), Pars intermedia (PI), Neurohypophysis (NH). a represents yolk granule, b represents nucleus, c represents cortical alveolus, d represents zona radiata, e represents follicle cell, and f represents yolk vesicle. Scale bars: 100 μm.

Conceptualization. **Rurong Zhao:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Min Tao:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

Min Tao is an editorial board member for Reproduction and Breeding and was not involved in the editorial review or the decision to publish this article. All authors declare that they have no conflict of interest.

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