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Sequence characterization and expression pattern analysis of six kinds of IL-17 family genes in the Asian swamp eel (*Monopterus albus*)

Dongdong Tang[a,b], Shipei Wu[a], Kai Luo[a], Hanwen Yuan[a], Weihua Gao[a], Dashi Zhu[a], Wenbing Zhang[a], Qiaoqing Xu[a,b,*]

[a] School of Animal Science, Yangtze University, Jingzhou, 434020, China
[b] Key Laboratory of Aquaculture Disease Control, Ministry of Agriculture, China

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ABSTRACT

Interleukin-17 (IL-17) is an important cytokine that plays a critical role in the inflammatory response and host defense against extracellular pathogens. In the present study, six novel IL-17 family genes (*MaIL-17*) were identified by analyzing Asian swamp eel (*Monopterus albus*) genome. Sequence analysis revealed that the *MaIL-17* family genes shared similar features, comprising a signal peptide, an IL-17 superfamily region, and four conserved cysteines. Phylogenetic analysis showed that the *MaIL-17* genes were clustered together with their corresponding IL-17 genes from other species. The similarity and identity of all IL-17 family genes indicated that the MaIL-17 genes are conserved among teleosts, while *Ma-IL-17D* is more conserved than the other Ma-IL-17s. Except for *MaIL-17A/F3* and *MaIL-17D*, all *MaIL-17s* shared the same genomic structure as the genes from other fish, namely three exons and two introns. The *MaIL-17s* showed conserved synteny among fish, and we found that the *MaIL-17D* locus has a more conserved syntenic relationship with the loci from other fish and humans. These results demonstrated that *MaIL-17D* and human IL-17D might have evolved from a common ancestral gene and subsequently diverged. The analysis of swamp eel reference genes revealed that *EEF1A1* (encoding eukaryotic translation elongation factor 1 alpha 1) was an ideal reference gene for accurate real-time qRT-PCR normalization in the swamp eel. The *MaIL-17* genes are widely distributed throughout tissues, suggesting that *MaIL-17s* carry out their biological functions in immune and non-immune tissues compartments. The transcript of Ma-IL17s exhibited different fold changes in head kidney cells in response to *Aeromonas veronii* phorbol 12-myristate 13-acetate (PMA) and polyinosinic:polycytidylic acid (poly I:C) challenge, showing that *MaIL-17A/F1* has stronger antiviral activities compared with other *MaIL-17* family genes, and that *MaIL-17A/F3* and *MaIL-17A/F2* possess stronger effects against extracellular pathogens compared with the others; however, *MaIL-17C2* and *MaIL-17D* may play vital roles during pathogen infection. The differential immune responses of these genes to *Aeromonas veronii*, PMA and poly I:C implied distinct mechanisms of host defense against extracellular pathogens.

1. Introduction

Cytokines are key modulators of T cell maturation, proliferation, and activation. To date, many cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-6, and IL-17 have been identified [1,2]. Interleukin-17 (IL-17) is a key pro-inflammatory cytokine that has diverse biological effects in the pathogenesis of inflammatory and autoimmune diseases via activating neutrophils and granulocyte-attracting chemokines, and affects intracellular signaling pathways in innate immunity [3,4].

Recently, IL-17 family genes have emerged as a novel family that is widely distributed in teleost's and mammals, and comprises six family members (IL-17A to IL-17F) [5–7]. Among IL-17 family members, IL-17A shares 16–50% amino acid identity with each additional member of the IL-17 family. Moreover, IL-17A and IL-17F share the highest amino acid sequence identity (50%), whereas IL-17E is the most divergent, with 16% identity to IL-17A [8]. The protein structures of IL-17 family members share common features, with four conserved cysteines critical for forming intrachain disulfide bridges [9]. Notably, IL-17D is largest protein, with an extended C-terminal domain, which might regulate a unique receptor interaction [10]. IL-17A and IL-17F can form either disulfide-linked homodimers or heterodimers (IL-17AF)

* Corresponding author. School of Animal Science, Yangtze University, Jingzhou, 434020, China.
E-mail address: xuqiaoqing@163.com (Q. Xu).

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that synergize with the expression of pro-inflammatory cytokines, chemokines, and antimicrobial proteins to augment the induction of pro-inflammatory responses [11]. Compared with IL-17A and IL-17F, IL-17B and IL-17C share similar biological functions in stimulating the release of TNF-α and IL-1β in immune-related cells [12]. IL-17D was first cloned from humans and induces the expression of IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in endothelial cells [10,13]. Uniquely among the IL-17 family, IL-17E is involved in the TH2-associated immune response. Moreover, IL-17 family members also participate in the stimulation of various cellular activities, such osteoclastogenesis [14], granulopoiesis [15], and T-cell proliferation [16]. During the cellular immune response, the recognition of pathogens stimulates intracellular signaling pathways that lead to the transcription of soluble mediators (such as IL-17) through their corresponding receptors, resulting in recruitment of ACT1 (nuclear factor kappa B (NF-κB) activator 1), which subsequently interacts with TNF receptor-associated factor 6 (TRAF6) to activate the NF-kB, CCAAT/enhancer-binding protein (C/EBP) and mitogen activated protein kinase (MAPK) pathways, which induces the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and chemokines involved in pro-inflammatory response [17–19]. Among these cytokines, IL-17s have attracted significant interest because of the special relationship with various pro-inflammatory cytokines in immune response system.

In teleosts, IL-17A and IL-17F are difficult to distinguish because of their high homology and are recognized as IL-17A/F1 and IL-17A/F2 [20]. Interestingly, IL-17A/Fs are identified to be split into three subgroups (IL-17A/F1 to IL-17A/F3) in zebrafish [21]. Meanwhile, IL-17Cs could also be divided into two subclades (IL-17C1 and IL-17C2) in Japanese puffer fish [22]. Extensive comparative genomics studies have shown that teleost fish have undergone genome duplication, which has affected the gene evolution of the IL-17 family [23]. To date, research has shown that IL-17s are present in rainbow trout [24], salmon [25], and the large yellow croaker [13]. The swamp eel is emerging as a new model for biological studies because of its small genome, and its enormous economic and potential medical value [26] (it is widely farmed in Asia [27]). In recent years, a series of illness caused by microorganisms, such as bacteria and parasites, have occurred under artificial breeding, which resulted in serious economic losses in swamp eel aquaculture [28,29]. However, research into function of the swamp eel IL-17 family might be beneficial to expand our knowledge concerning the immune defense against pathogens in swamp eels. In the present study, six IL-17 family genes (IL-17A/F1, IL-17A/F2, IL-17A/F3, IL-17C1, IL-17C2, and IL-17D) from the swamp eel were characterized, and their expression patterns and associations with immune regulation upon pathogen infection were analyzed to offer insights into the function of the MaIL-17 family in the immune response.

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2. Materials and methods

2.1. Fish

Swamp eels (50–80 g) were obtained from the research center base for swamp eels at Yangtze University (Jinzhou, China) and maintained in laboratory tanks for two weeks before experimental processing. All experiments followed the experimental animal management law of China and were approved by the Animal Ethics Committee of Yangtze University.

2.2. Gene sequences

The sequences of swamp eel IL-17 family genes were obtained from the Asian swamp eel genome (Accession No: AONE00000000.1). The sequences of IL-17 family members from other species were obtained from the NCBI. Open reading frame (ORF) primers were used to amplify the coding region of the cDNA. by PCR. Specific primers for quantitative real-time PCR (qPCR) were designed using Primer Premier 5.0 and pre-tested to ensure that all primers could amplify the cDNA. The swamp eel IL-17 family genes were named as *MaIL-17s*.

2.3. Sequence characterization of swamp eel IL-17 family genes

Alignments for each pair of amino acid sequences were performed using the Clustal W server (http://www.ebi.ac.uk/Tools/msa/clustalo/). Multiple sequence alignment was performed using the BioEdit software. The similarity and identity between IL-17 family genes and IL-17 genes from other species were analyzed using software MatGAT (version 2.0). An ORF search tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict the ORFs of the IL-17 family genes and to deduce the encoded protein sequence. The presence of a signal peptide in the deduced amino acid sequence was predicted using the Signal 4.1 program (http://www.cbs.dtu.dk/services/signal/). To identify the location and length of introns and exons in the IL-17 family genes DNA sequence, the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/spideyweb.cgi) was used to align the mRNA and genomic sequences. The molecular weight and theoretical isoelectric point (pI) were predicted using the online Protparam tool (http://web.expasy.org/protparam).

The secondary structure and specific domains of the IL-17 family members were predicted using the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) and Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/), respectively. A phylogenetic tree of the IL-17 family genes of swamp eel and those from other species was constructed using the neighbour-joining (N-J) method in the MEGA 7.0 software, with 1000 bootstrap replicates. The intron phase was calculated and labeled based on their sizes. The gene synteny analysis was performed using the Ensemble and NCBI databases.

2.4. Tissue expression of IL-17 family genes in healthy swamp eels

To determine the tissue expression pattern of MaIL-17 family genes and identify the differences in their mRNA expression, muscle, liver, kidney, spleen, intestine, skin, heart, and blood were isolated from four healthy swamp eels and used for total RNA extraction using the Trizol reagent (Invitrogen). Total RNA was used for cDNA synthesis using PrimeScript RT Reagent Kit with gDNA Eraser (Takara).

The expression levels of MaIL-17s were analyzed using KAPA SYBR® FAST qPCR Master Mix (KAPA BIOSYSTEMS) and a Step-one Plus real-

Table 1
Primers used in the study.

Primer	Sequence (5′-3′)	Application
IL17A/F1-F/R	ATGTTTCCAGCATCAAACTCCT/TCACTGCTGGCGCTGAACAATG	Amplification of complete ORF
IL17A/F2-F/R	ATGAAGCTGAGACTCTGCACTCT/GTCTTGGTTGGTTTTGGCCCTGA	Amplification of complete ORF
IL17A/F3-F/R	ATGCTACTGTTAGTTTTTCCACAT/CTGTGGTATGACGGTAGGCCTG	Amplification of complete ORF
IL17C1-F/R	CATGGGTTAGTCTGCAAATGATC/TCACCTATTGGACTTGGGCACAAC	Amplification of complete ORF
IL17D-F/R	ATGCTGCGTCGTATTCGCGTCC/CACTTTCTTGCCTGCAGGGATG	Amplification of complete ORF
IL17A/F1-F/R	CAAAGTGACGGCTGCCTGCACTG/TCGTGGGAGATGTTGTATGTCCA	qPCR
IL17A/F2-F/R	GGAGCTGGAGGTCGTCCACAGTG/GTCTTGGTTGGTTTTGGCCCTGA	qPCR
IL17A/F3-F/R	ATGCTACTGTTAGTTTTTCCACAT/CAACAGCAGTTTCACTGTCTTGC	qPCR
IL17C1-F/R	CATGGGTTAGTCTGCAAATGATC/CTCGGTCTAGGCTGTACTTCCA	qPCR
IL17C2-F/R	CATGAAGCAGATTCTCATATTTGG/CTAGGTGCTGGTGCTGGTGCTG	qPCR
IL17D-F/R	ATGCTGCGTCGTATTCGCGTCC/TGGGTCATACGAAATCCTGTAG	qPCR
EF-1α-F/R	CGGTGTGAAGCAGCTCATCGT/GCAGAGTGGTTCCAGTGGCATT	qPCR

Table 2
Summary of Ma- IL-17 family members sequence characterization.

Sequence feature	Ma-IL-17A/F1	Ma-IL-17A/F2	Ma-IL-17A/F3	Ma-IL-17C1	Ma-IL-17C2	Ma-IL-17D
GenBank Accession No.	XM_020602687.1	XM_020602664.1	XM_020616982.1	XM_020624574.1	XM_020585628.1	XM_020618899.1
Length of amino acid	165	145	183	163	163	210
ORF (bp)	498	428	552	492	492	633
Signal peptide	31	21	42	20	24	26
Molecular weight (KDa)	18.32	15.76	19.93	18.39	18.94	23.5
Theoretical pl	8.66	8.93	10.18	9.11	8.81	9.49
Numbers of Intron	3	3	4	3	3	2

Table 3
List of selected candidate reference genes.

Gene symbol	Primer pair sequence	Gene name	GenBank Accession No.
ACTB-F/R	GGCTACTCCTTCACCACCACAG/GATTCCGCAGGACTCCATACCAA	Beta actin	XM_020621264.1
GAPDH-F/R	GCCGCATCATCCACATTCTCCT/CTCTCACACCTTGACGCCATCTC	Glyceraldehyde- 3-phosphate dehydrogenase	XM_020597211.1
EF-1α-F/R	CCTCGGTGTGAAGCAGCTCATC/GCAGAGTGGTTCCAGTGGCATT	Elongation factor-1-a	XM_020592430.1
RPL13-F/R	GTTCAACCAGCCAGCCAGGAAG/CGGCACGAACCTTGGTGTGATAC	Ribosomal protein L13	XM_020605381.1
18sRNA-F/R	CGTATTGTGCCGCTAGAGGTGAA/GGTGCCCTTCCGTCAATTCCTTT	18S ribosomal RNA	XM_020624505.1

Table 4
Significant difference of reference genes expressed in 6 tissues ($P < 0.05$).

Tissues	ACTB	18S	GAPDH	RPL13	EF-1α
L	18.23 ± 0.46^{a}	14.65 ± 0.08^{ac}	16.26 ± 0.16^{b}	17.44 ± 0.32^{ab}	15.83 ± 0.38^{a}
SP	13.09 ± 0.24^{e}	10.02 ± 0.28^{e}	23.14 ± 0.72^{a}	15.19 ± 0.12^{c}	14.10 ± 0.15^{c}
I	15.27 ± 0.65^{c}	13.06 ± 0.73^{bd}	12.03 ± 0.56^{d}	13.87 ± 0.74^{d}	12.36 ± 0.86^{d}
H	17.06 ± 0.1^{ab}	12.89 ± 0.16^{c}	12.54 ± 0.21^{b}	16.96 ± 0.22^{b}	15.71 ± 0.3^{b}
K	13.91 ± 0.52^{d}	11.19 ± 0.57^{bd}	21.61 ± 0.28^{a}	15.39 ± 0.25^{c}	13.64 ± 0.45^{c}
M	17.27 ± 0.12^{b}	12.16 ± 0.21^{d}	12.55 ± 0.13^{c}	17.56 ± 0.24^{a}	16.65 ± 0.23^{a}
Level	a-e	a-e	a-d	a-d	a-d

time PCR system (ABI). For comparison of the relative expression levels of different genes, a standard curve was established using a series of 10-fold dilutions of purified PCR products of each gene amplified from cDNA. A serial of dilution of the standard was run along with the cDNA samples in the same 96-well PCR plate and served as reference for quantification. According to the alignment between the mRNA of IL-17 family genes and genomic sequence, specific primers were designed on Table 1, and *EEF1A1* (encoding eukaryotic translation elongation factor 1 alpha 1) was tested and selected as a reference gene. The qPCR amplification conditions were as follows: 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 33 s. Each sample measurement was repeated in triplicate. The expression level of swamp eel IL-17 family genes was analyzed using the comparative cycle threshold method ($2^{-\triangle\triangle Ct}$) [30].

2.5. Analysis of several swamp eel reference genes

To explore the expression patterns of the target MaIL-17s gene, it was necessary to determine the best reference gene for swamp eel. Therefore the five swamp eel candidate genes *ACTB* (encoding beta actin), *GAPDH* (encoding glyceraldehyde-3-phosphate dehydrogenase), *EEF1A1*, *RPL13* (encoding ribosomal protein L13) and the 18S rRNA gene, were examined for their potential as reference genes in the study of gene expression in different tissues (spleen, intestine, heart, liver, muscle, and kidney). The specific primers of each gene were designed using Primer Premier 5.0 (Table 1). RNA extraction and cDNA synthesis of these tissues from health swamp eel was performed as described in section 2.4 [30]. The expression levels of swamp eel reference genes were analyzed using qPCR. qPCR was performed using quantified cDNA templates (about 0.3–0.4 mg/mL) and their corresponding primers, respectively, on an ABI 7500 real-time PCR system (Applied Biosystems, USA). The SYBR Premix ExTaq (Takara, Japan) was used according to the manufacturer's protocol with a primer concentration of 200 nM. The stability of the Ct values was analyzed using the comparative cycle threshold method, GeNorm, and NormFinder software

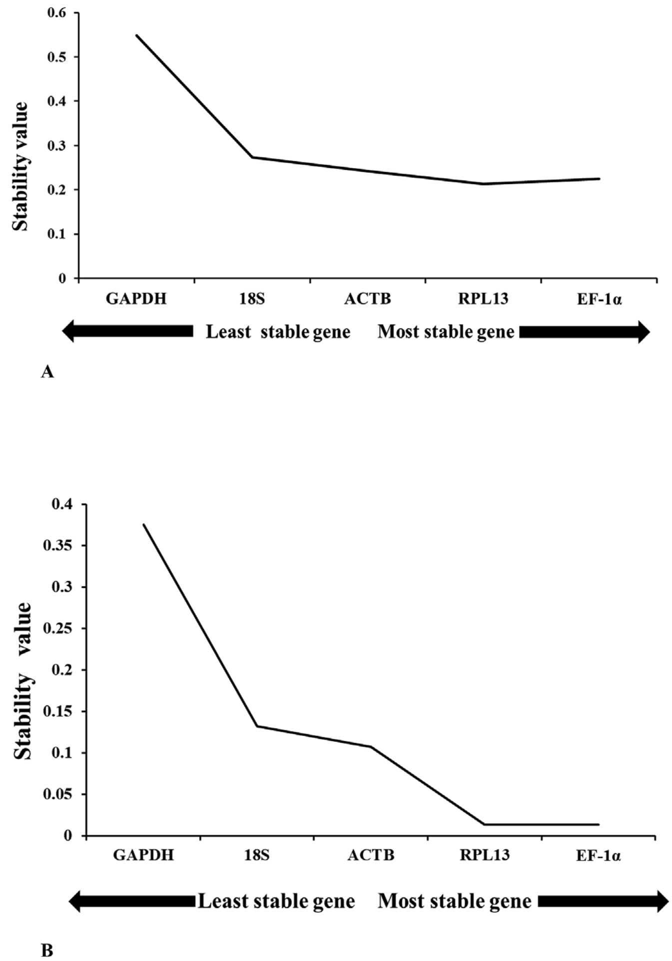

Fig. 1. Gene expression stability of the reference genes calculated by GeNorm software and NormFind software. 1A represents GeNorm software and 1B represents NormFind software. M value (y-axis) is referred as a measure of gene expression stability, with a decreasing M value correlating with more stability. The most stable genes are displayed on the right, and the least stable genes are displayed on the left.

analysis [31]. GeNorm and NormFinder software were used to analyze the optimal reference gene for swamp eel. The stability of the expression of each gene was shown as the M value calculated using the geNorm software.

2.6. Preparation of primary cultures

Head kidneys cells were collected aseptically from four fish, and cells were pushed through a 100-μm nylon mesh (John Stanier) in incomplete Leibovitz L-15 medium (Gibco), i.e., supplemented with penicillin/streptomycin (P/S) at 100 units/mL and 100 μg/mL respectively (Gibco), 0.5% fetal bovine serum (FBS; Life Technologies, USA), and 10 U/mL of heparin (Sigma-Aldrich). The suspensions were then centrifuged at $200 \times g$ for 5 min, and washed once with complete medium (same constituents as the incomplete medium but with 10% FBS). Cells were then counted and seeded into a fresh 6-well plate at a density of 1.5×10^6 cells per well and were maintained at 24 °C in M199 media supplemented with 10% FBS.

2.7. qPCR analysis of the induction IL-17 family gene expression upon pathogen infection

Head kidney primary cultures, prepared as described in section 2.6, were incubated with known stimulatory concentrations of *Aeromonas veronii* (2.56×10^6 colony forming units (cfu)/mL) polyinosinic:polycytidylic acid (poly I:C, 150 μg/mL), phorbol 12-myristate 13-acetate (PMA, 1 μg/mL), for 4, 24, and 48 h. Control groups were treated with an equal volume of phosphate-buffered saline (PBS). After incubation, the cells were harvested and lysed with 1 mL of Trizol reagent (Invitrogen) to extract total RNA. The RNA extraction and cDNA synthesis was performed as described in section 2.4 [30]. *EEF1A1* was used as the reference gene. The expression level of swamp eel IL-17 family genes was analyzed using the $2^{-\triangle\triangle Ct}$ method.

2.8. Statistical analysis

All data are expressed as the mean ± the standard error of the mean (SEM). All data were analyzed using one-way analysis of variance (ANOVA) with the SPSS software (version 22). $^*p < 0.05$, $^{**}p < 0.01$ were considered statistically significant.

3. Results

3.1. Sequence characterization of IL-17 family members

The full length ORFs of the *MaIL-17* family members were confirmed by PCR amplification. The data for the sequence characterization of the *MaIL-17* family members are shown in Table 2. The lengths of the ORFs of *MaIL-17A/F1*, *MaIL-17A/F2*, *MaIL-17A/F3*, *MaIL-17C1*, *MaIL-17C2*, and *MaIL-17D* were 498, 428, 552, 492, 492, and 633 bp, respectively, which encoded putative proteins of 165, 145, 183, 163, 163, and 210 amino acids, respectively. The theoretical pIs and molecular weights of *MaIL-17A/F1, MaIL-17A/F2, MaIL-Ma17A/F3, Ma IL-17C1, MaIL-17C2* and *MaIL-17D* were 8.66/18.32, 8.93/15.76, 10.18/19.93, 9.11/18.39, 8.81/18.94, and 9.49/23.5 kDa, respectively. All IL-17 family members were predicted to have a signal peptide (Table 2).

3.2. Selection of a swamp eel reference gene

The expression levels of the five swamp eel reference genes (Table 3) in tissues were examined using their Ct values. The Ct values analysis indicated that *EEF1A1*, *GAPDH*, and *RPL13* were divided into four levels (a-d) to confirm the stability reference genes in six tissues (Table 4). GeNorm analysis was used to assess the stability values of all the reference genes in the different tissues examined (Fig. 1A). The results indicated that the reference genes pair of *EEF1A1* and *RPL13* showed the lowest M value (highest stability) in different tissues. By contrast, *GAPDH* demonstrated low stability in the different tissues (Fig. 1A). NormFinder was used to calculate the stability ranking of the five reference genes in the different swamp eel tissues. The stability ranking was *EEF1A1/RPL13* > *ACTB* > *18S* > *GAPDH* (Fig. 1B). These results demonstrated that *EEF1A1* could be selected as the best reference gene for the Asian swamp eel.

3.3. Phylogenetic relationship, amino acid features, and sequence analysis

To clarify the evolutionary phylogenetic relationships among IL-17 members, a phylogenetic tree was constructed using MEGA 7.0 with the NJ-method. The tree indicated that the swamp eel IL-17 family proteins were grouped closely with their counterparts from other species (Fig. 2). Within the vertebrate IL-17s, five groups were formed based on their phylogenetic relationships, including IL-17A/F, IL-17B, IL-17C, IL-17D, and IL-17E. Surprisingly, *MaIL-17A/F1* and *MaIL-17A/F3* were clustered into a branch that was separated from *MaIL-17A/F2*. *MaIL-17C1* and MaIL-17C2 were clustered into a branch with fugu IL-17C1 and fugu IL-17C2, respectively. *MaIL-17D* and miiuy croaker IL-17D were divided into a group.

Analysis of the amino acid sequences indicated that *MaIL-17A/F1-3*, *MaIL-17C1*, *MaIL-17C2*, and *MaIL-17D* have putative signal peptides comprising 31, 21, 42, 20, 24, and 26 amino acids, respectively

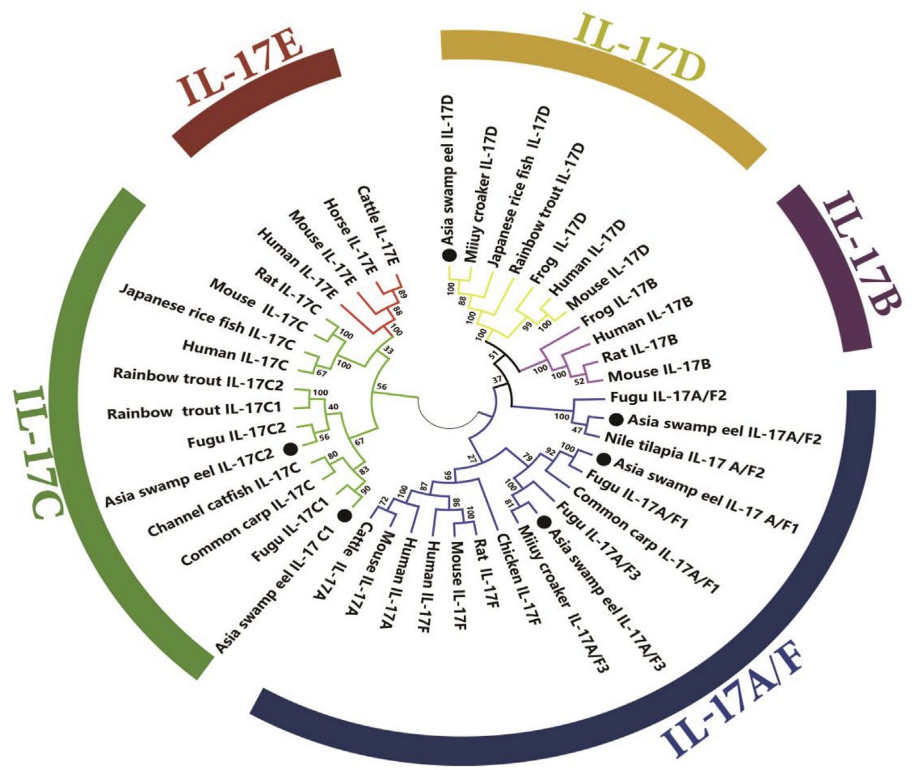

Fig. 2. Phylogenetic tree of IL-17 family genes from swamp eel and other species. Deduced amino acid sequences of IL-17 family members were aligned and the tree was constructed with the neighbour-joining method using the MEGA (version 7.0) software. The tree is bootstrapped 10,000 times, and the bootstrap values of the major branches are shown as percentages. The MaIL-17s are shown with the symbol ● and distinguished by different colors. The GenBank accession numbers of IL-17R amino acid sequences used here are as follow Fugu IL17C1 D4AHP4, Rainbow trout IL-17C1 D4HTR8, Common carp IL-17C A0A173M1X1, Channel catfish IL-17C W5UD15, Fugu IL-17C2 D4AHP5, Rainbow trout IL-17C2 NP_001171959.1, Human IL-17C NP_037410.1, Mouse IL-1717C NM_145834.4, Rat IL-17C NM_001191115.1, Dog IL-17C XM_846163.3, Miiuy croaker IL-17D A0A0U3ULH4, Japanese rice fish IL-17D E3WEB1, Human IL-17D NP_612141.1, Mouse IL-17D NM_145837.3, Rainbow trout IL-17D NM_001124399.1, Frog IL-17D NM_001114247.1, Fugu IL-17A/F1 D4AHP1, Common carp IL-17A/F1 A0A173M1X4, Miiuy croaker IL-17 A/F2 A0A0U3THV3, Nile tilapia IL- 17A/F2 I3J5T2, Fugu IL-17A/F2 D4AHP2, Miiuy croaker IL-17A/F3 A0A0U3SCX5, Fugu IL-17A/F3 BAI82580, Rat IL-17F Q5BJ95, Human IL-17A NP_002181.1, Human IL-17F NP_443104.1, Mouse IL-17A NP_034682.1, Mouse IL-17F NM_080729.3, Cattle IL-17F NM_001008412.2, Chicken IL-17F NM_204460.1, Rat IL-17B NP_446241, Human IL-17B NP_055258.1, Chicken IL-17B XM_425192.4, Mouse IL-17B NM_019508.1, Frog Il-17 B NM_001006698.1, Horse IL-17E XM_001918325, Cattle IL-17E XM_605190.4, Human IL-17E NP_073626.1, Mouse IL-17E NM_080729.3. The accession number of MaIL-17s are described in Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

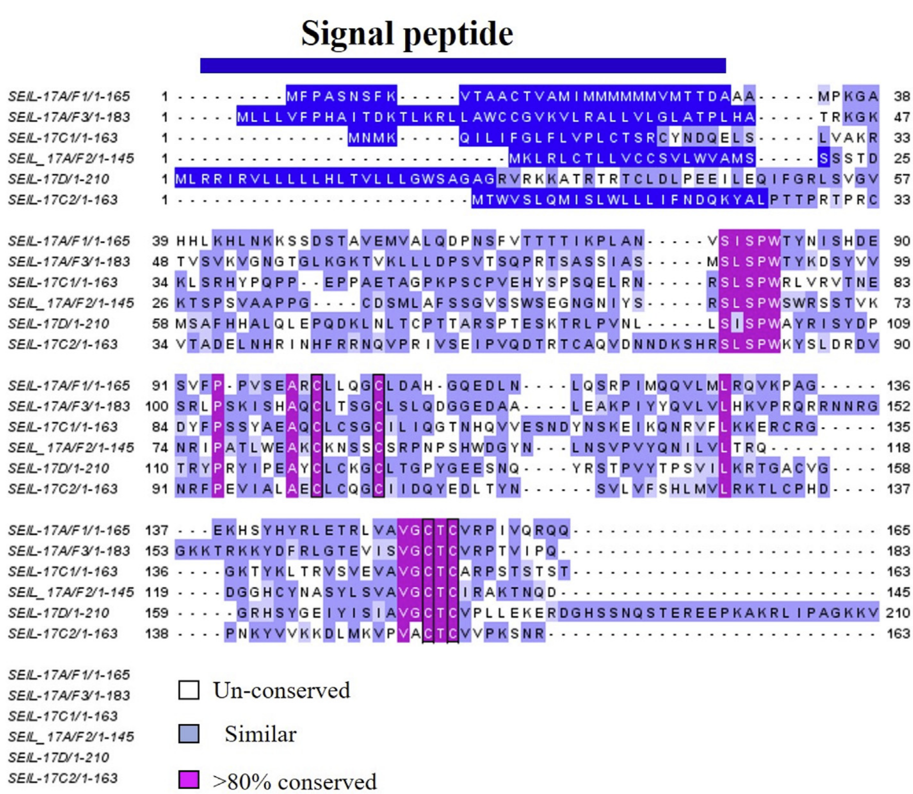

Fig. 3. Multiple alignment of Ma-IL-17s amino acid sequence. The signaling peptide are shown by underlined. The four conversed cysteines are displayed with red frame. The abbreviations are swamp eel (SE) or *Monopterus albus* (Ma). Conserved region was showed with different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

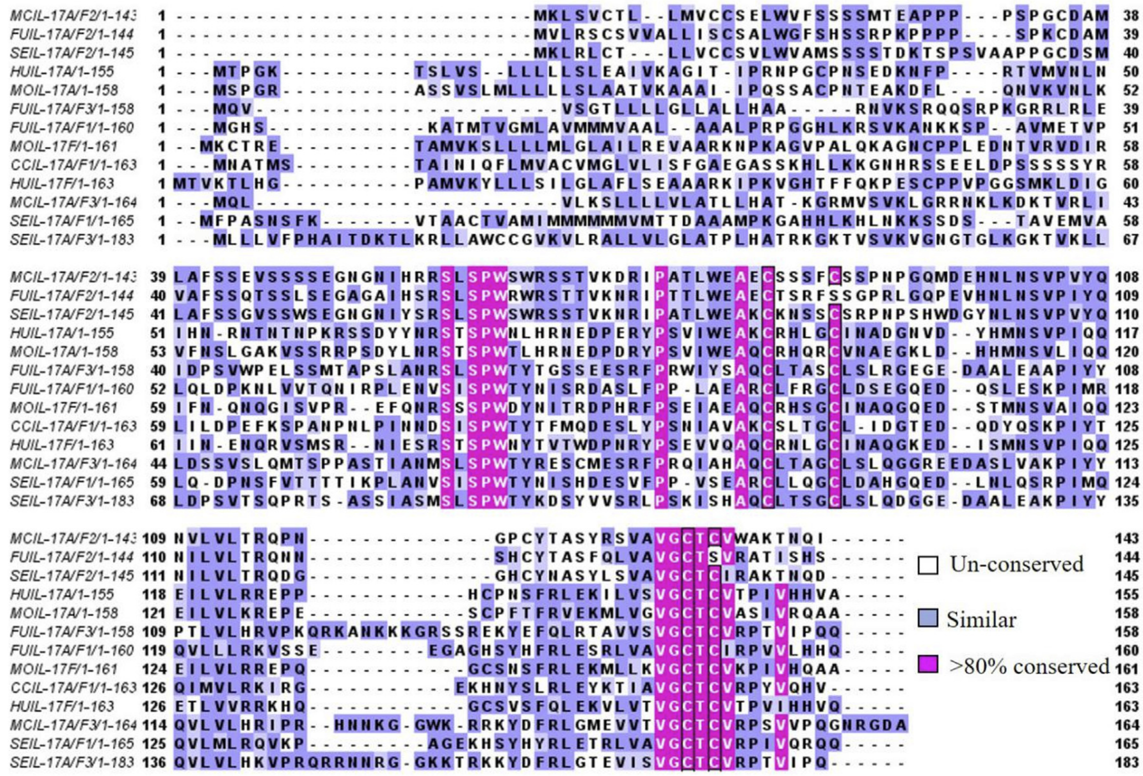

Fig. 4. Multiple alignment of MaIL-17 A/Fs with IL-17A/Fs sequences from other species. The four conversed cysteines are displayed with red frame. Conserved regions were showed with different colors (light blue and red). The abbreviations are Fugu (FU), swamp eel (SE), Miiuy croaker (MC), Common carp (CC), Human (HU), Mouse (MO). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Table 2, Fig. 3). All IL-17 family proteins have the IL-17 superfamily region, which is located the C-terminal region in all IL-17 family members. A multiple alignment with IL-17 members from other species indicated that all IL-17 family members possess four cysteine residues that form two disulfide bridges (Figs. 4–6). In addition, IL-17 family members share important features and have a highly conserved C-terminal region (Figs. 3–6). These results demonstrated that IL-17 family genes were highly conserved in vertebrates.

The deduced amino acid sequence homologies of IL-17 family members were analyzed using the MatGat software (Table 5). The swamp eel IL-17 family members share amino acid similarity ranging from 45 to 89.6% compared to those of other species. *MaIL-17A/F1* shares high similarity with members from other species, ranging from 43 to 72.7%, and *MaIL-17A/F2* shares higher similarity with members from other species, ranging from 40.9 to 80%, while *MaIL-17A/F3* shares similarity ranging from 36.6 to 73.2%. Among the *MaIL*-17A/F proteins, *MaIL-17A/F1*, *MaIL-17A/F2*, and *MaIL-17A/F3* share relatively low similarity (49.7%, 52.3%, and 41%, respectively) with human IL-17A and human IL-17F.

MaIL-17C1 and *MaIL-17C2* share high similarity (66.3% and 57.7%) compared to other their counterparts from fish such as fugu, while MaIL-17C1 and MaIL-17C2 share low similarity (40.6% and 48.2%) compared with human IL-17C. However, the amino acid similarity of swamp eel IL-17D was the highest in the various IL-17 family members with that of miiuy croaker (Table 5), and shared the highest similarity (69%) with human IL-17D.

3.4. Genomic structure and synteny analysis of IL-17 family genes

The DNA structure of the vertebrate IL-17 genes was analyzed using the NCBI and Ensemble database. The result showed that the genomic structures of the swamp eel IL-17 family genes were similar to the human and miiuy croaker IL-17 family genes. Swamp eel IL-17 family members A/F1, A/F2, C1, and C2 comprised three exons and two introns (Fig. 7A and B). The swamp eel *IL-17A/F3* genes has four exons and three introns (Fig. 7A), whereas *IL-17D* has two exons and one intron (Fig. 7B).

To elucidate the evolutionary relationship of these genes, gene synteny analysis was performed using the NCBI and Ensemble databases. Six swamp eel IL-17 family genes on the scaffold were analyzed to confirm the gene order and orientation compared with those in other fish. The swamp eel I*L-17A/F1* and *IL-17A/F2* genes were linked tightly to the same scaffold (Fig. 8A). The same phenomenon was observed in tilapia, zebrafish, and fugu. However, the upstream genes in the scaffold of *MaIL-17A/F1* and *MaIL-17A/F2* were *GTF2A2*, *FOXB1B*, and *ANXA2B*, which is different from other fish. The downstream genes of *MaIL-17A/F1* and *F2* were conserved (*STMN4* and *GPN1*). However, the remaining the MaIL-17 family genes were observed in different scaffolds of swamp eel chromosome. The upstream gene of *MaIL-17A/F3* is conserved as *MCM3*, while the downstream gene of *IL-17C1* is conserved as *CHTF8*, which is the same as that for zebrafish IL-17s (Fig. 8B). The *MaIL-17A/F3* and *MaIL-17C1* loci were syntenically conserved among fish species (Fig. 8B and C); however, the gene order for *MaIL-17C1* is different from that of its human counterpart. Moreover, the *MaIL-17D* region lacks *N6AMT2* and *MPHOSPH8* compared with that in the other fish (Fig. 8C). *MaIL-17D* is more syntenically conserved among fish and human IL-17 family genes (Fig. 8D). Interestingly, *MaIL-17C2* has a unique gene order in the chromosome compare with that of fugu.

3.5. Expression pattern variation of IL-17 family genes in swamp eel

The expression levels of IL-17 family member transcripts were detected using qPCR. IL-17 family genes showed constitutive expression in various tissues (Fig. 9), including the spleen, muscle, blood, heart, brain, liver, intestine, skin, and kidney. *Ma-IL17A/F1* exhibited highest

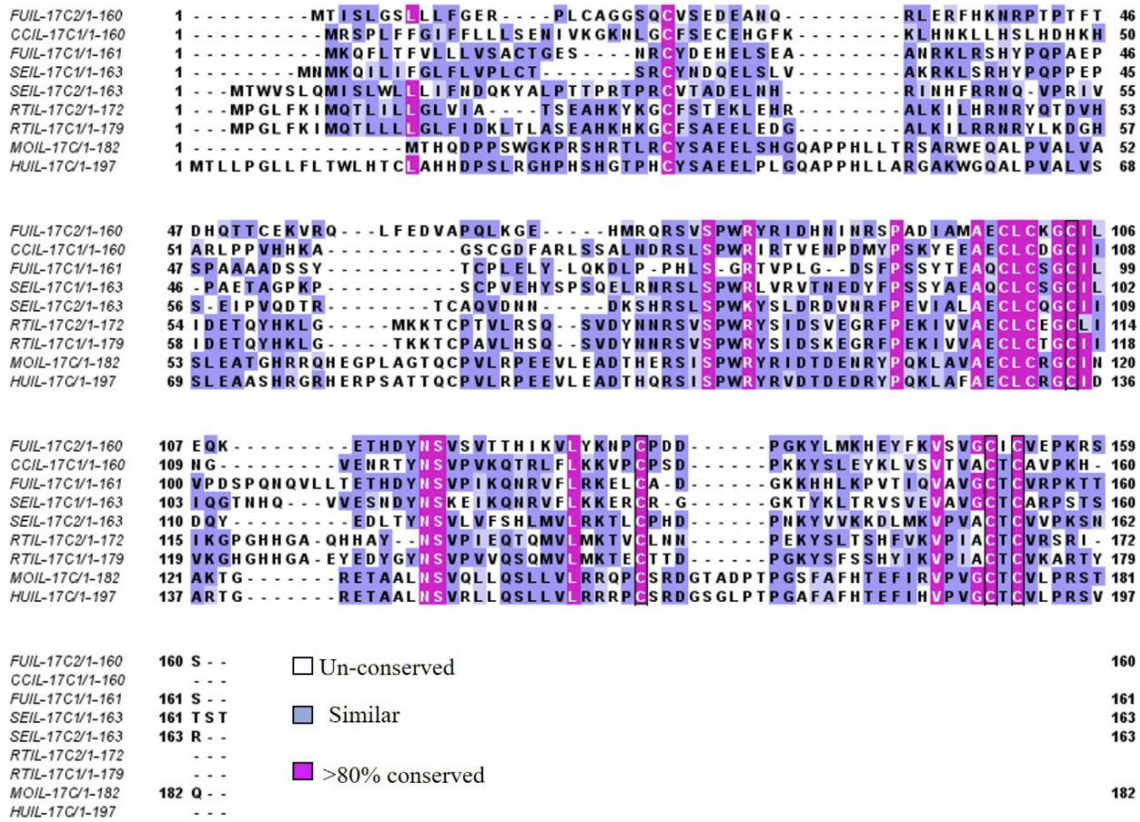

Fig. 5. Multiple alignment of MaIL-17C1 and C2 with IL-17C sequences from other species. The four conversed cysteines are displayed with red frame. Conserved regions were showed with different colors (light blue and red). The abbreviations are Fugu (FU), Rainbow trout (RT), Common carp (CC), Human (HU), Mouse (MO). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

expression level in the intestine and kidney. High expression levels of *MaIL-17A/F2* and *MaIL-17C2* were observed in the blood and spleen, and the baseline expression levels of *IL-17A/F3*, *IL-17C1*, and *IL-17D* were highest in the heart, kidney, and brain, respectively, and followed by skin and intestine. Swamp eel IL-17 family genes were highly expressed in most tissues.

3.6. Modulation of IL-17 family members' expression upon pathogen infection

To explore how the expression of IL-17 family genes are modulated in response to poly I:C and PMA (Fig. 10), primary head kidney cells are challenged with poly I:C (1.83×10^6 cfu/mL) and PMA (1.2×10^6 cfu/mL) for 4, 24, and 48 h *in vivo*. In PMA-stimulated head kidney cells, *MaIL-17A/F2* and *MaIL-17C1* showed similar expression patterns and were significantly upregulated at 4 h. Both of them reached their highest expression levels at 4 h with a 6.61-fold ($P < 0.05$) increase and a 7.35-fold ($P < 0.05$) increase, respectively. *MaIL-17A/F2* and *MaIL-17C1* expression levels were restored to normal levels at 48 h. By contrast, *MaIL-17A/F1* expression was significantly upregulated by 4.64-fold ($P < 0.05$) and 4.45-fold ($P < 0.05$) at 24 and 48 h, respectively, reaching its maximum expression at 24 h. *MaIL-17A/F3* showed its highest expression at 24 h. *MaIL-17C2* and *MaIL-17D* expression levels were significantly upregulated by 7.07-fold and 4.35-fold change at 24 h, respectively, and reached their maximum expression at 24 h. After poly I:C treatment, *MaIL-17C1*, *MaIL-17A/F2*, *MaIL-17D*, and *MaIL-17A/F3* showed the same expression patterns, reaching their the maximum expression at 24 h. *Ma IL-17C1* expression levels were restored to normal levels at 48 h. *MaIL-17A/F2* and *MaIL-17A/F3* expression levels were strikingly upregulated at 24 h, by 13.95-fold ($P < 0.01$) and 31.1-fold ($P < 0.01$), respectively. *MaIL-17D* expression was remarkable upregulated at 24 h by 6.22-fold ($P < 0.01$) at peak and significantly upregulated at 48 h by 4.22-fold change ($P < 0.05$). The expression levels of *MaIL-17A/F1* were upregulated dramatically at 24 and 48 h. *MaIL-17C2* expression was significantly and maximally upregulated at 4 h (by 7.92-fold). After *Aeromonas veronii* treatment (Fig.11), *MaIL-17A/F2* and *MaIL-17C1* reached their highest level at 24h (10.89 fold-change and 4.16 fold-change, respectively, $P < 0.05$). *MaIL-17A/F2* and *MaIL-17C1* expression level were returned to basal level at 48h. *MaIL-17C2* was strikingly upregulated from 4h to 48h ($P < 0.05$), with its highest expression at 24h (8.02 fold-change). *MaIL-17A/F3* and *MaIL-17D* were significantly upregulated at 48h (6.49 fold-change and 4.51 fold-change, respectively, $P < 0.05$). *MaIL-17A/F1* expression was significantly upregulated at 48h by 66.84-fold ($P < 0.01$) and showed its highest fold change compared with that of other *MaIL-17s*. Curiously, *MaIL-17A/F3* was only downregulated at 4h with a relatively low fold-change (0.542 fold). The expression levels of *MaIL-17A/F1* and *MaIL-17D* kept increasing from 4h to 48h compared to the control group.

4. Discussion

IL-17 is an important cytokine that mediates cell–cell communication in many biological processes, particularly host defense and inflammatory responses [32]. The IL-17 family comprises several members with diverse roles in the immune response [33]. However, the function and characteristics of IL-17 family genes have not been characterized well in invertebrates [34]. In the present study, the swamp eel *IL-17A/F1*, *IL-17A/F2*, *IL-17A/F3*, *IL-17C1*, *IL-17C2*, and *IL-17D* genes were identified from the swamp eel genome (but not IL-17B and IL-17E). The situation were observed in some fish such as zebrafish [21] and fugu [22]. While IL-17 family members (*IL-17A–IL17E*) were

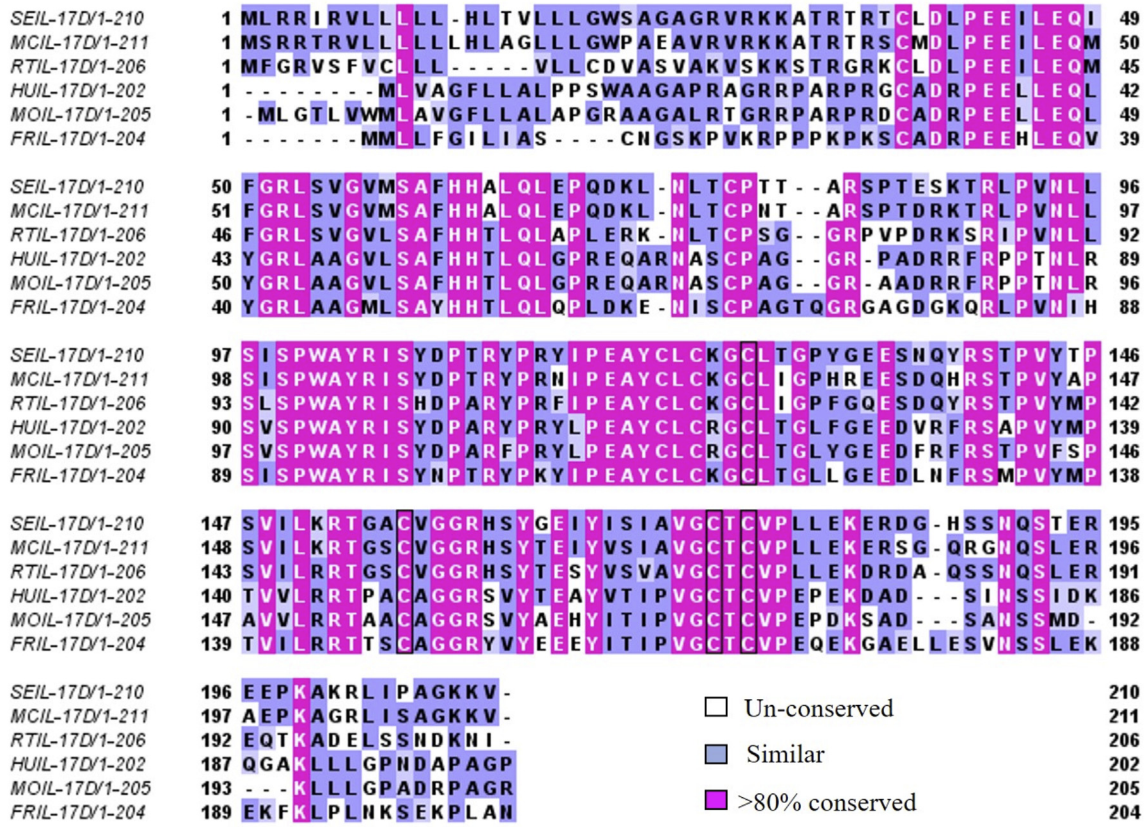

Fig. 6. Multiple alignment of MaIL-17D with IL-17D sequences from other species. The four conversed cysteines are displayed with red frame. Conserved regions were showed with different colors (light blue and red). The abbreviations are Miiuy croaker (MC), Fugu (FU), Common carp (CC), Frog (FR), Human (HU) and Mouse (MU). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

identified in human [35]. so the types of IL-17 vary greatly among different species [4,6,21,22], which may reflect their unusually high evolutionary rate. Surprisingly, our analysis showed that *IL-17C* and *IL-17D* exist widely exist in fish and mammals, while *IL-17B* and *IL-17E* do not exist in most teleosts. This may be because the *IL-17C* and *IL-17D* genes could help animals adapt to diverse aquatic environment via an immune response. Structural analysis of the swamp eel IL-17 family members via multiple alignments revealed that these proteins share typical features, such as the IL-17 superfamily domain. The IL-17 family is thought to represent a distinct signaling system that appears to be highly conserved across vertebrate evolution [36]. All *MaIL-17* family members can participate in the intracellular transport of G protein-coupled receptors via signal peptide [37]. The results suggested the intracellular MaIL-17 proteins are secreted out of cell and are involved in the proinflammatory response mediated by IL-17 receptors. IL-17 proteins may participate in intracellular activities that combat pathogen infection. In addition, the C-terminal region of the swamp eel IL-17 family members showed high similarity with each other, whereas little conservation was observed in the N-terminal regions. This phenomenon has been observed in human IL-17 [38], suggesting that IL-17 family members have similar biological functions.

Before assessing the tissue expression patterns of MaIL-17 members in swamp eels, it was necessary to determine a suitable reference gene. Typical reference genes are highly and stably expressed in most cells. General, the expression stability of a reference gene is estimated using qPCR and related software analyses. In addition, the reference genes need to be validated under the specific species and experimental conditions to be used subsequently. In this study, the five reference genes tested (*ACT*, *GAPDH*, *EEF1A1*, *RPL13*, and 18S) showed different expression levels in different tissues. Similar results were observed in zebrafish [39] and channel catfish [40]. Our results demonstrated that *ACTB*, *18S*, and *GAPDH* exhibited the least stability in swamp eel tissues, and were not suitable as reference genes. However, *EEF1A1* and *RPL13* expression was stable in the tissues tested. Our results agreed with those of previous research showing that *EEF1A1* was a suitable reference gene in 13 tissues and different stages of gonads during sex reversal in the swamp eel [41]. So far, *EEF1A1* has been reported as a common reference gene in amphioxus [42], Atlantic salmon [43], Atlantic halibut larvae, and Senegalese sole [44]. Importantly, *EEF1A1* is an appropriate internal reference for studies of gene expression regulation in some species because it is one of the most stable genes when expressed under different experimental conditions [44]. Traditionally, *ACTB*, 18S, and *GAPDH* have been considered as highly expressed housekeeping genes in certain animals. However, accumulating data from vertebrates revealed that their expression could vary in development and after external treatment [45–47]. They may be related to diverse roles in cells, specific species, and external conditions Moreover, *ACTB* is widely used as a reference gene in some species, such as large yellow croaker [13], because the protein plays a key role in determining cellular shape and anchorage where transmembrane glycoproteins link fibronectin in the extracellular matrix with actin microfilaments on the cytoplasmic side of the membrane [48]. However, it was rejected as a reference gene because of high-level fluctuation compared with the other candidate genes in the swamp eel. The 18S rRNA gene was reported to be a common reference gene in medaka [49] and zebrafish [50]. However, its expression was affected by experiment conditions, such as bacterial challenge, in turbot [51] and showed a high expression level compared with the target genes, suggesting that 18S might be not suitable as a reference gene. In earlier studies, the expression of *GAPDH* varied according to its diverse functions [48,52], suggesting that *GAPDH* might be unsuitable as a reference gene. The results of the present study supported the results of these previous

Table 5
Amino acid identity and similarity of MaIL-17A/Fs and MaIL-17C1-2, MaIL-17D putative peptides compared with that of other teleost fish.

species	Ma-IL-17A/F1	Ma-IL-17A/F2	Ma-IL-17A/F3	species	Ma-IL-17C1	Ma-IL-17C2	species	Ma IL-17D
	identity/similarity(%)	identity/similarity(%)	identity/similarity(%)		identity/similarity(%)	identity/similarity(%)		identity/similarity(%)
Fugu IL-17A/F1	55.4/72.7	24.7/45	33.9/50.8	Fugu IL-17C1	50.9/66.3	28.6/46.6	Miiuy croaker IL-17D	84.8/89.6
Fugu IL-17A/F2	24.4/43	56.8/69.7	23.8/41.5	Fugu IL-17C2	31.4/49.7	38.8/57.7	Common carp IL-17D	66.7/80
Fugu IL-17A/F3	30.7/50.3	26.4/44.9	46.8/61.7	Rainbow trout IL-17C1	31.1/50.3	37.2/53.6	Frog IL-17D	50.9/67.6
Common carp IL-17A/F1	36.5/56.4	24.7/41.1	34.4/49.2	Rainbow trout IL-17C2	30.7/49.4	34.8/53.5	Mouse IL-17D	51.9/69.5
Miiuy croaker IL-17A/F2	28.1/47.3	71.9/80	25.5/36.6	Common carp IL-17C	37.5/49.7	30.4/49.7	Human IL-17D	52.8/69
Miiuy croaker IL-17A/F3	28.5/50.9	25.3/40.9	55.5/73.2	IL-17C	28/40.6	29.4/48.2		
Human IL-17A	28.3/49.7	28.5/52.3	25.7/41	Mouse IL-17C	28.3/46.2	29.8/50.5		
Human IL-17F	28.1/55.8	25.3/46.6	30.4/49.7	Ma-IL-17C1		23.9/44.2		
Mouse IL-17A	28.7/50.9	26.2/48.1	24.9/40.4					
Mouse IL-17F	32.9/55.8	26.2/45.3	29.4/47					
Ma-IL-17A/F1		24.3/42.4	33.7/55.2					
Ma-IL-17A/F2			27.7/39.3					

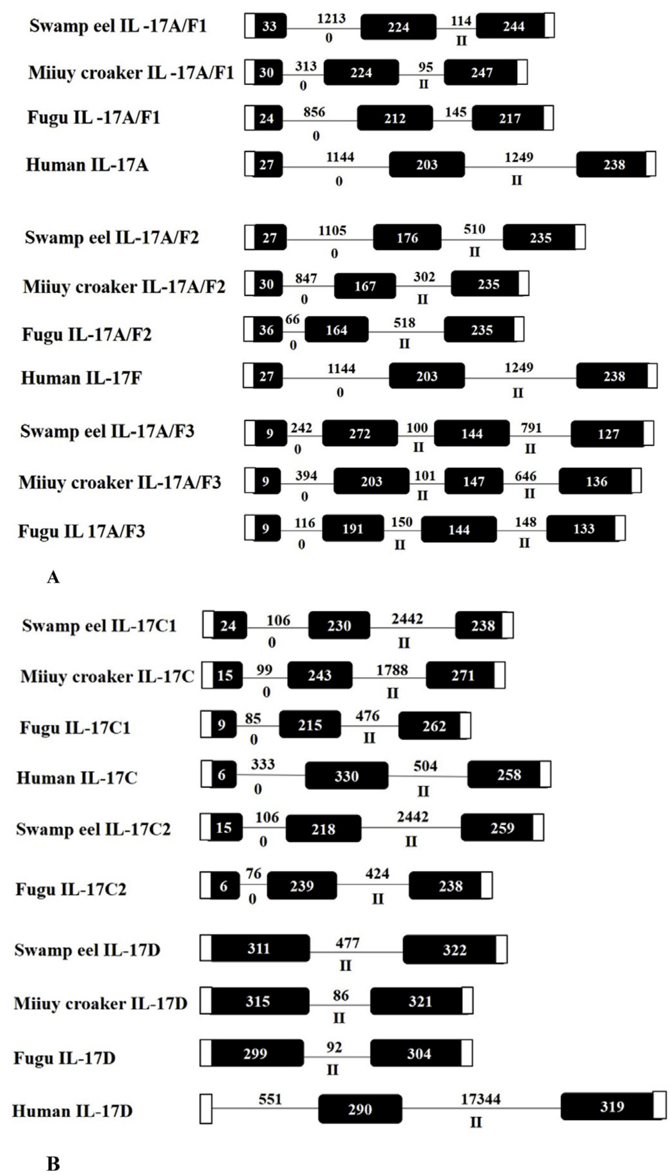

Fig. 7. Genomic gene organization of Ma-IL-17s, compared with other animals IL-17s, including Fugu, Miiuy croaker and human. Exons are shown in solid boxes, and black boxes represent the encoding region. Introns are shown by bold lines. The sizes of exons and introns in base pairs are displayed in the top of black boxes and black lines. 7A represents genomic gene organization of MaIL-17A/F1-3 and other animals IL-17A/F. 7B represents genomic gene organization of MaIL-17C1-2, MaIL-17D and other animals IL-17C/D.

reports. Another suitable swamp eel gene in our study, *RPL13*, showed high stability when tested by different analytical methods. However, *RPL13* has not been reported as a common reference gene for the normalization of real-time RPCR. Consequently, *EEF1A1* could be considered as an ideal reference gene for accurate real-time PCR normalization in swamp eel.

Our phylogenetic analysis showed that the IL-17 proteins were clustered into their corresponding IL-17 families, termed IL-17A/F, IL-17B, IL-17C, IL-17D, and IL-17E group. The phenomenon is the same as that in fugu [22]. The IL-17A/F proteins are divided into three subclades: IL-17A/F1, IL-17A/F2, and IL-17A/F3. IL-17C proteins are split into two subclades: IL-17C1 and IL-17C2. These results demonstrated that swamp eel IL-17A/Fs are homologous to fugu and miiuy croaker IL-17A/Fs. Fugu IL-17C1 and IL-17C2 form a clade with high homology with swamp eel IL-17C1 and IL-17C2. We speculated that swamp eel is closely related with fugu and miiuy croaker. These findings indicated

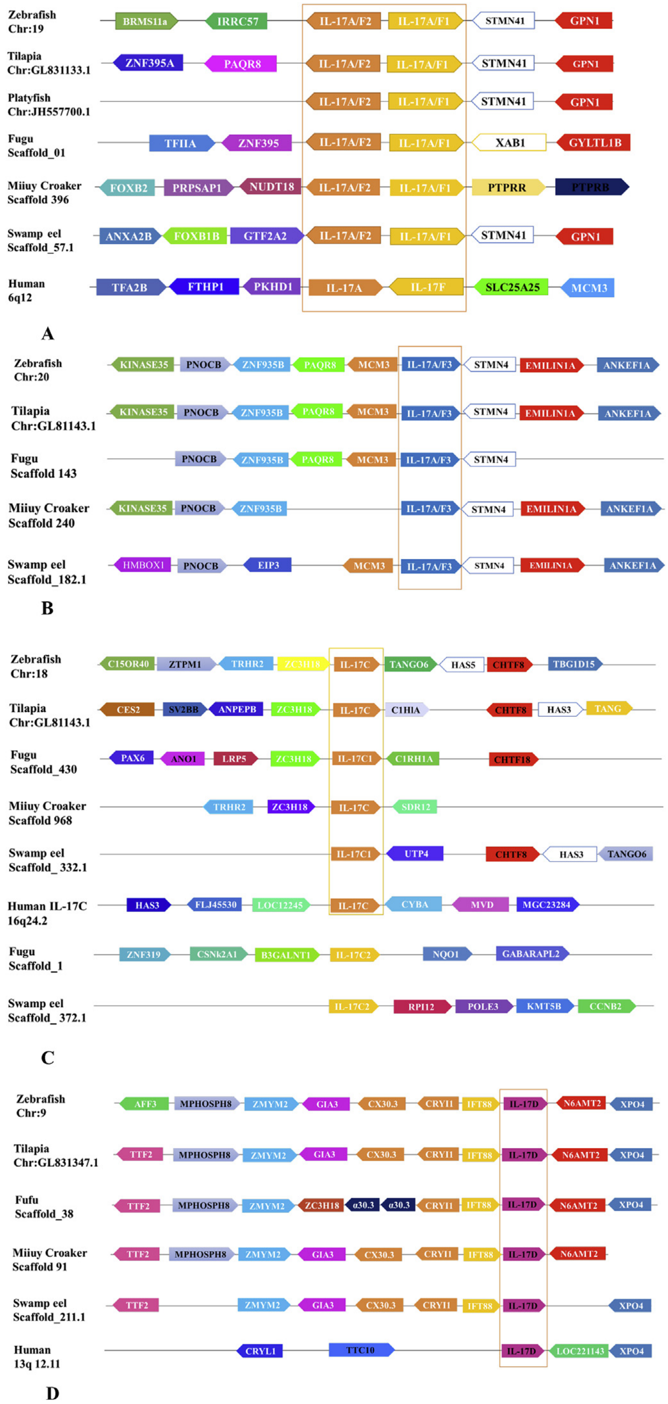

Fig. 8. Schematic representation of gene synteny at the IL-17s loci in swamp eel and other species. Same genes are represented in same colors, and the MaIL-17 genes is highlighted with big frames. Different genes are showed with different colors. The arrows indicated the transcriptional direction. The arrow of frame indicates the transcriptional orientation. The abbreviations of these genes are taken from the Ensembl and NCBI database. 8A indicates gene synteny analysis at the IL-17A/F1 and IL-17A/F2. 8B indicates gene synteny analysis at the IL-17A/F3 loci in vertebrates. 8C indicates gene synteny analysis at the IL-17C loci in vertebrates. 8D indicates gene synteny analysis at the IL-17D loci in vertebrates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

MaIL-17 family genes and other known fish IL-17s evolved from the same ancestral gene and that genomic duplication led to the diversification of IL-17 family genes during evolution. Multiple sequence alignments demonstrated that all species IL-17 family members share important features, with a highly conserved C-terminal region. Among these features, the four cysteine molecules required for the formation of a classical cysteine knot were strictly conserved in the IL-17 family. Crystal structure analysis of human IL-17 revealed that these four cysteine residues are involved in the formation of a typical cysteine knot that comprises two disulfide bridges [53]. The four cysteines that comprise two disulfide bridges are preserved on most species, suggesting that disulfide linkages are important in the evolutionary process of animals. We found the other cysteine positions were not conserved between human and swamp eel IL-17A, which may indicate differences in protein structure among invertebrate IL-17s. Human IL-17A and IL-17F can form a homodimer or heterodimer and bind to the complex of IL-17 receptor A (IL-17RA) and IL-17RC [54]. However, the tertiary structures of IL-17 proteins are unclear in fish. A future protein structure analysis of IL-17 family members will explain the differences of IL-17s' function in animals.

The amino acid homology of IL-17 family members revealed that MaIL-17 family members share different similarities and identities with their counterparts from other species. MaIL-17D shares the highest similarity with those from other fish, while the similarity/identity of MaIL-17C1 and MaIL-17C1 are low compared with those from other fish. Based on the phylogenetic analysis, Ma-l7C1 and Ma-ILC2 are different types of IL-17 family members, with high similarity/identity with their counterparts from fugu, suggesting that they are closely related. MaIL-17A/F1, MaIL-17A/F3, and MaIL-17A/F2 share low similarity with human IL-17A. The result was higher than the similarity of between zebrafish and human IL-17A [21], which indicated that MaIL-17A/Fs are more conserved than that of zebrafish. MaIL-17D has the highest similarity/identity among the MaIL-17 family members to its counterparts in the miiuy croaker and human, suggesting that MaIL-17D is more conserved among the MaIL-17 family members.

In terms of the genomic organization of the MaIL-17 family genes, *Ma-IL-17A/F1*, *MaIL-17A/F2*, *MaIL-17C1*, and *IL-17C2* share similar structures, comprising three exons that separated from two introns, which is the same pattern as human and other fish *IL-17A/F* and *IL-17C* genes [21,35]. Moreover, the genomic organization of *MaIL-17D* and *MaIL-17A/F3* are similar to fugu *IL-17D* and *IL-17A/F3*, with two exons and four exons, respectively. All these intron-containing MaIL-17s were separated by one to two phase 0 introns. The results suggested that intron loss or duplication from IL-17 family genes mainly depended on retrotransposition. A substantial number of new duplicates are inserted far from the original locus by retrotransposition, as studied in humans and mammals [55]. These findings might explain the IL-17 family genes evolution process, comprising gene insertion and deletion from invertebrates to mammals.

MaIL-17A/F1 and *MaIL-17A/F2* are linked tightly to the same scaffold, which is the same as fugu, miiuy croaker, zebrafish, and tilapia. Human *IL-17A* and *IL-17F* are tandemly linked to same location on the same chromosome [56]. The chromosomal location of *MaIL-17A/F1* and *MaIL-17A/F2* was similar to the chromosomal locations of *IL-17A* and *IL-17F* in humans. Combined with the amino acid similarity and phylogenetic relationships, we hypothesized that human *IL-17A* and *IL-17F* may have common ancestors with fish *IL-17A/F1* and *IL-17A/F2*. In comparison, the mini-chromosome maintenance deficient 3 (*MCM3*) gene is conserved upstream of the *MaIL-17A/F3* genes, while the stathmin-like 4 (*STMN4*) gene is conserved in zebrafish and Fugu *IL-17A/F1* and *IL-17A/F2* genes [21,22]. This demonstrated that human and fish IL-17s may share common ancestral gene duplications [57]. The genomic loci of *MaIL-17C1* and *MaIL-17A/F3* were conserved syntenically among fish species. This result indicated that the genomic regions of three IL-17 family genes are conserved in fish, suggesting evolutionary conservation. *MaIL-17D* was more syntenically conserved among fish species and human, suggesting that human *IL-17D* and fish *IL-17D* may have a common ancestral gene. However, *MaIL-17C2* showed no syntenic conservation compared with that of fugu. The

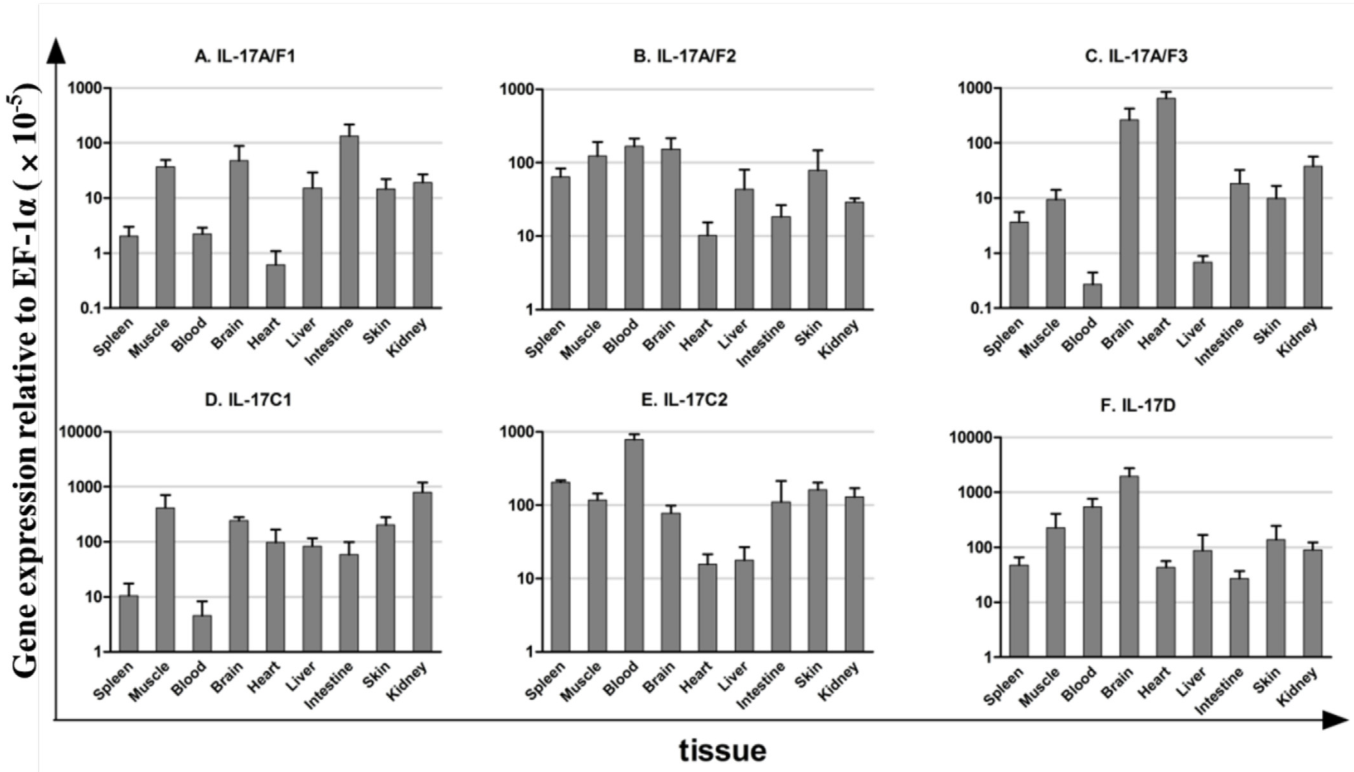

Fig. 9. Expression pattern of MaIL-17 family genes (MaIL-17A/F1, MaIL-17A/F2, MaIL-17A/F3, MaIL-17C1, MaIL-17C2 MaIL-17D) in various tissue in healthy swamp eel. The MaIL-17s expression in mRNA level was detected by qRT-PCR. where gene expression was normalized to that of the elongation factor 1 alpha (EF-1α). Data are shown as mean ± SD.

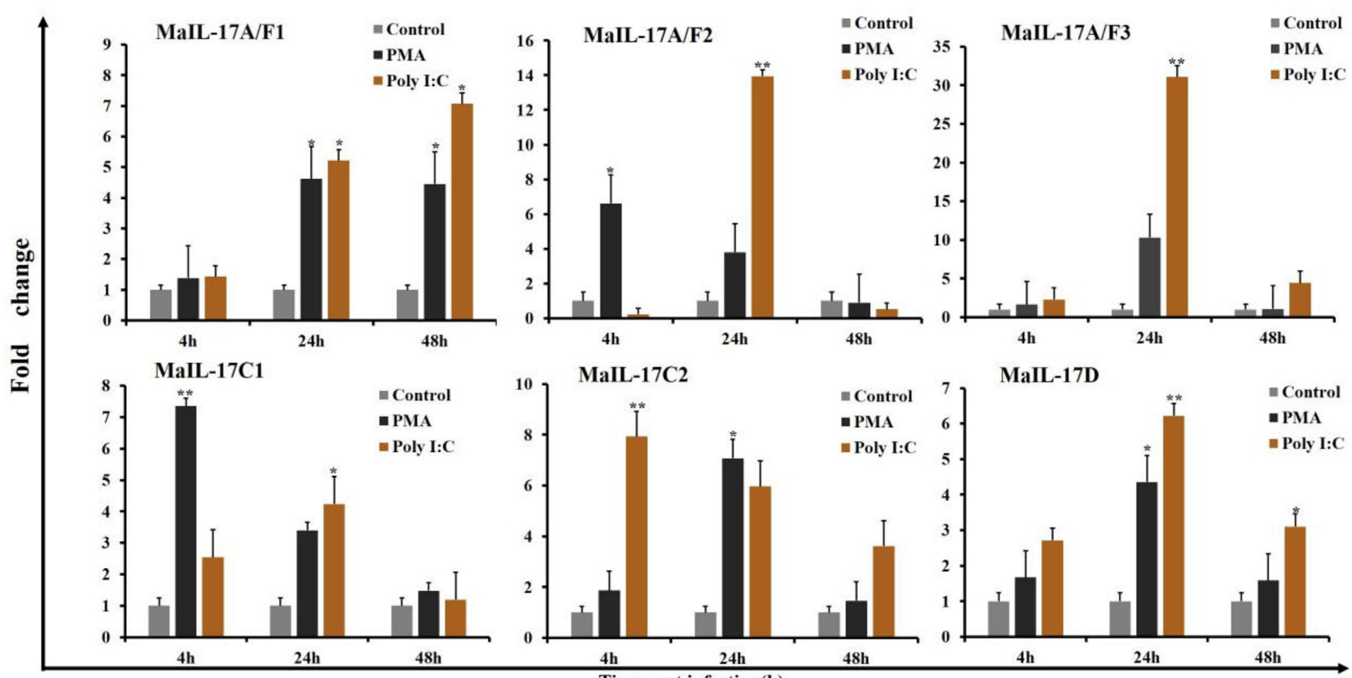

Fig. 10. Expression modulation of MaIL-17 family genes in head kidney cell after PMA and poly I:C injection, and PBS as control. The expression levels of MaIL-17s are detected by qRT-PCR at different time points and presented as the fold-change compared with the respective control group (which was set to 1). Data are shown mean ± SEM(N = 4). Significances difference between pathogen and control was indicated with asterisks as ***P* < *0.01*, **P* < *0.05.*

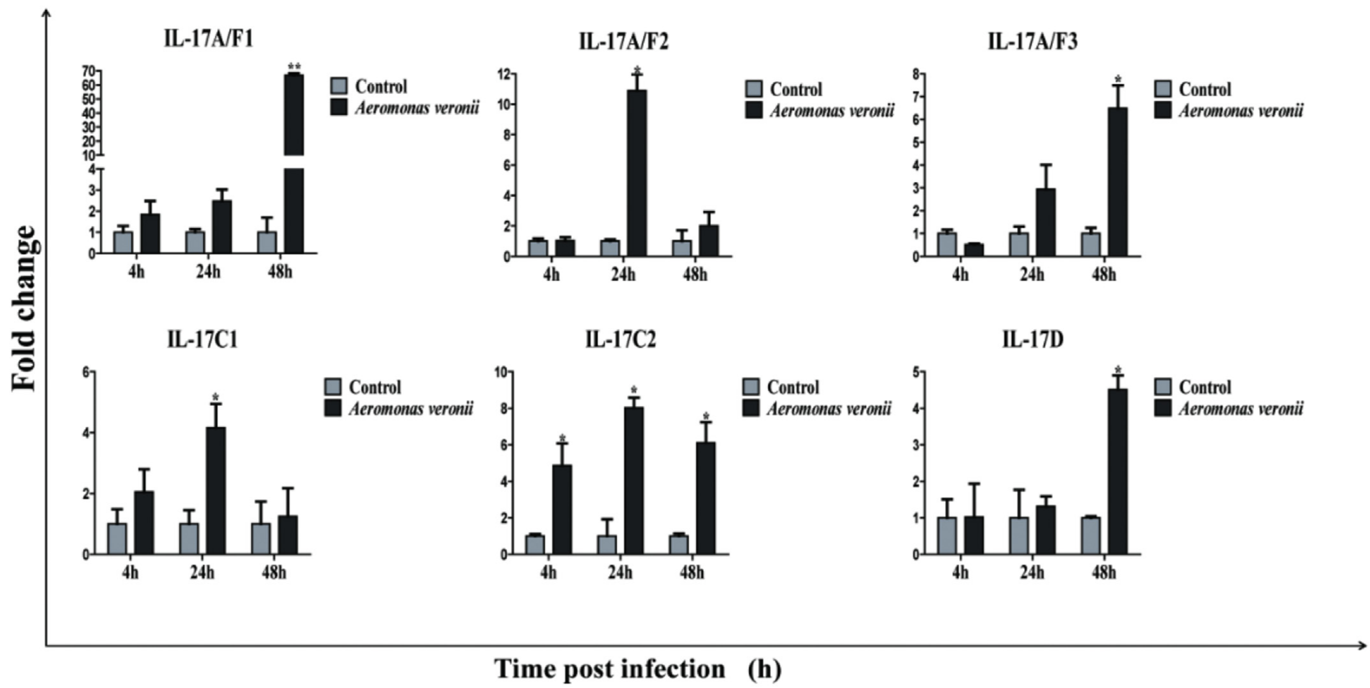

Fig. 11. Expression modulation of MaIL-17 family genes in head kidney cell after *Aeromonas veronii* injection, and PBS as control. The expression levels of MaIL-17s are detected by qRT-PCR at different time points and presented as the fold-change compared with the respective control group (which was set to 1). Data are shown mean ± SEM (N = 4). Significances difference between pathogen and control is indicated with asterisks as $^{**}P < 0.01$, $^{*}P < 0.05$

phylogenetic tree analysis indicated that *MaIL-17C2* and fugu *IL-17C2* were clustered into a group; however, their synteny analyses were not identical. It has been hypothesized that specific gene lineage duplication events might have occurred in fish, resulting in the retention of more genes in one taxon for chromosomal rearrangement [58]. Therefore, further study of the IL-17 family gene synteny in various organisms is required.

MaIL-17 family gene expression in a wide range of tissues was examined. *MaIL-17A/F2* and *MaIL-17C2* showed the highest expression in blood and spleen. *Ma-IL17C1* and *MaIL-17A/F1* are highly expressed in the intestines and kidney, respectively. All MaIL-17 family genes were highly expressed in the spleen, skin, and kidney, which suggested their involvement in the immune response, which is the same as that in the miiuy croaker [12]. *MaIL-17A/F1* was highly expressed in the intestines, which is in agreement with a previous study performed in zebrafish [21] and suggested that it may play a role in mucosal immunity. Especially for the high expression level of *MaIL-17C1* and *MaIL-17C2* in the skin among all *MaIL-17s*, suggesting that skin may be the first line of defense against potential pathogens in innate immunity [59]. *MaIL-17A/F2* and *MaIL-17C2* may have biological activities in blood-related immunity [60,61]. By contrast, *Ma-IL17A/F3* and *MaIL-17D* were highly expressed on brain. The structure of the IL-17 proteins highlighted four to six functionally conserved cysteines that adopt a cysteine knot conformation, similar to a common structural motif found in nerve growth factor [62]. Ma-IL-17A/F2 and MaIL-17C2 may take part in nerve regulation to control the immune response. In general, the immune tissues and mucosal sites have a relatively high expression level, with tissues such as the heart and liver being relatively low. Curiously, *MaIL-17A/F3* had a high expression level in the brain, suggesting a role in the nervous system. *MaIL-17D* possessed a high expression level of the heart, suggesting a role in the blood circulatory system. Thus, the high expression levels of MaIL-17s in these tissues may play an important roles in innate immunity.

IL-17 has been reported to play an important role in the host defenses against extracellular pathogens [63,64]. Considered its pro-inflammatory properties, it is not surprising that there are interaction of transcription factors events mediated by IL-17 to make accurate reaction against pathogens in inflammatory response [65]. In the current study, *MaIL*-17A/Fs, *MaIL*-17Cs, and *MaIL-17D* expression was triggered rapidly by PMA and poly I:C stimulation in head kidney cells, which is similar to IL-17 expression after exotic pathogen stimulation in *Crassostrea gigas* [66] and pearl oyster [67]. After *Aeromonas veronii* treatment, all MaIL-17s were upregulated. The expression levels of *MaIL-17A/F2* and *MaIL-17C1* ascended at 4h and decreased at 48h to the basal level of the control group. However, *MaIL-17A/F1*, *MaIL-17D* and *MaIL-17C2* were upregulated from 4h to 48h. This result indicated that *MaIL-17A/F1, MaIL-17D* and *MaIL-17C2* had longest effect than *MaIL-17A/F2* and *MaIL-17A/F1*, and that *MaIL-17A/F2* and *MaIL-17A/F1* had a faster antibacterial activities against pathogen. Despite MaIL-17A/F1's response to *Aeromonas veronii* at 24h to 48h, its fold-change was higher than other MaIL-17s, suggested that *MaIL-17A/F1* had strongest antibacterial activities. *MaIL-17A/F3* showed stronger antibacterial response against pathogen. Downregulation levels of *MaIL-17A/F3* at 4h indicated that MaIL-17A/F3 may be regulated by other cytokines as a protective mechanism. Poly I:C is a double-stranded RNA and is used to mimic viral infection [68]. PMA is a potent tumor inducer that can facilitate activation of the transcription factors TNF-α, NF-κB, and activator protein 1 (AP1) [69,70]. *MaIL-17A/F2* and *MaIL-17C1* were quickly upregulated at 4 h by PMA stimulation, and the expression levels of these genes decreased at 48 h, suggesting they participate in the acute immune response upon pathogen infection and have longer effect. The expression levels of *MaIL-17A/F3* and *MaIL-17D* were prominently upregulated at 24 h, suggesting that they would exert notable effects against pathogens. *MaIL-17A/F2* and *MaIL-17A/F3* were strikingly upregulated with highest fold changes at 24 h after poly I:C administration, and this upregulation was maintained at 48 h. This suggested that *MaIL-17A/F2* and *MaIL-17A/F3* exerts a strong antiviral function and would have the longest effect on the extracellular response. *MaIL-17A/F1* was gradually upregulated from 4 to 48 h, suggesting that *MaIL-17A/F1* would have longer lasting effects against viruses compared with the other MaIL-17s. *MaIL-17D* was significantly upregulated at from 24 to 48 h after poly I:C administration, suggesting that *MaIL-17D* has long lasting effects in the extracellular response. Comprehensive analysis of *MaIL-17s* showed that *MaIL-17A/F1* had stronger antibacterial function compared with its antiviral function. *MaIL-17A/F3* possessed stronger antiviral function compared with its antibacterial activity. These findings suggested that MaIL-17 family members have different roles in response to different pathogen stimulations, and that MaIL-17 family members participate in the related pathways of the innate immune system to protect the eels against extracellular pathogens.

In summary, six MaIL-17 family genes were identified and characterized from the Asian swamp eel genome, and their genomic organizations and expression patterns were analyzed. MaIL-17 family genes are involved in the pro-inflammatory response to PMA and poly I:C stimulation. However, the function and regulation mechanism of MaIL-17 proteins in extracellular signaling pathways should be the subject of further study.

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