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Evolutionary Analysis of the TLR Genes in Ten Fishes

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Abstract

Fish are a commonly available type of vertebrate that rely on both congenital and acquired immunity to protect against pathogens. Congenital immunity acts as their first line of defense, while acquired immunity is crucial in defending against specific pathogenic infections. Toll-like receptor (TLR) genes play a significant role in their innate immunity and are essential to their immune system. To better understand the evolutionary status of TLRs in fish and their adaptive immune defense characteristics, researchers analyzed ten fish species' TLR7 and TLR13 gene sequences using the NCBI and Ensembl databases. The analysis included primary sequence, phylogenetic, and selective pressure analyses. Results showed variations in base composition and amino acid composition in TLR7 and TLR13 genes among different fish species, with adenine being the most frequent and guanine the least frequent base. Leucine was the most frequently used amino acid, while tryptophan was the least among the 20 amino acids comprising TLR7 and TLR13 genes. The phylogenetic analysis divided TLR7 and TLR13 into two branches, each with two parts, and the clustering structure within the branches was consistent with the divergence of species. The selective pressure analysis indicated that adaptive evolution had occurred through positive selection sites in TLR7 and TLR13. The analysis of TLR7 and TLR13 in fish provides significant implications for understanding immune-related genes and the evolution of TLR genes in fish.

Keyword: Fish; TLR; receptor gene; Amino acid

Background

Fishes are an incredible type of aquatic vertebrate known for their unique characteristics[1]. They are ectothermic, possess bony scales, breathe through gills, and feed using upper and lower jaws. Fish use the swing of their tail, body, and fins to move through the water[2]. Belonging to the subphylum of vertebrates in the phylum Chordata, fish is one of five categories of vertebrates, with reptiles, birds, amphibians, and mammals being the others. There are over 20,000 fish species worldwide[3], including around 800 species of Chondrichthidae and roughly 20,000 species of teleost[2]. Contrary to popular belief, chondrichthys did not give rise to primitive fish. Instead, chondrichthyans and teleost are two parallel groups closely related to the ancestor of the Asterostomus[4]. Fish play a vital role in the overall evolutionary landscape. Toll-like receptors (TLRs) play a crucial role in the immune system of both invertebrates and vertebrates[5]. These transmembrane proteins have three distinct parts: the extracellular, transmembrane, and cytoplasmic domains. TLRs are non-catalytic and have the unique ability to recognize the structural molecules of microorganisms[6]. They can identify the corresponding PAMPs to resist and kill pathogens, essential in innate immunity. TLRs also bridge non-specific and specific immunity[7]. Fish have at least 20 different types of TLRs, with TLR7 and TLR13 being particularly important for intracellular infection and immunity mediated by the intracellular pathogen MTB[8]. The TLR7 gene is in Xp22 and consists of three exons and 1050 amino acids. The murine TLR7 protein is 1049 amino acids in length, and the TLR7 belongs to the subfamily of innate pattern recognition receptors, which was necessary for fish[9].

TLR13 receptor is a neglected receptor of the TLR family that specifically recognizes bacterial 23S ribosomal RNA[10]. The hairpin structure of the TLR13 SSRNA ligand is the structural basis of TLR13-specific recognition[11]. HMGB1, a high mobility group box B-1 protein, recognizes TLR13ssRNA ligands with high affinity[12]. HMGB1 binding disrupts the hairpin structure of TLR13 ligands, which negatively regulates TLR13 signaling. The REDOX state of HMGB1 is vital for its recognition and regulation of the TLR13 signaling pathway[13]. Fish TLR13 may be involved in recognizing a variety of PAMPs in bacterial RNA and viruses. To evaluate the immune adaptability of fish, we selected ten different fish species and downloaded the published genome data of TLR7 and TLR13 from the NCBI and Ensembl databases, respectively. We then sorted and analyzed the downloaded genome data, constructed a local gene data screening database, and performed selection pressure and phylogenetic analyses on the acquired data.

Material and Method

1.2 Acquisition and Comparison of TLR Gene Family Sequences in Fish

The NCBI database (NCBI, http://www.ncb.nlm.govi) and Ensembl database (http://www.ensembl.org) were used to obtain the gene sequences of 10 fish species, including the Cypriniformes, including Danio rerio, Sinocyclocheilus anshuiensis, and Sinocyclocheilus anshuiensis. The order Percidae includes Larimichthys crocea, Parambassis ranga and Larimichthys crocea.

The Killiformes include Cyprinodon variegatus, Poecilia mexicana, Xiphophorus maculatus, Xiphophorus couchianus, Salmo salar, and Gouania willdenowi. A local database was constructed, and the TLR genes of fish were screened using Blast software.

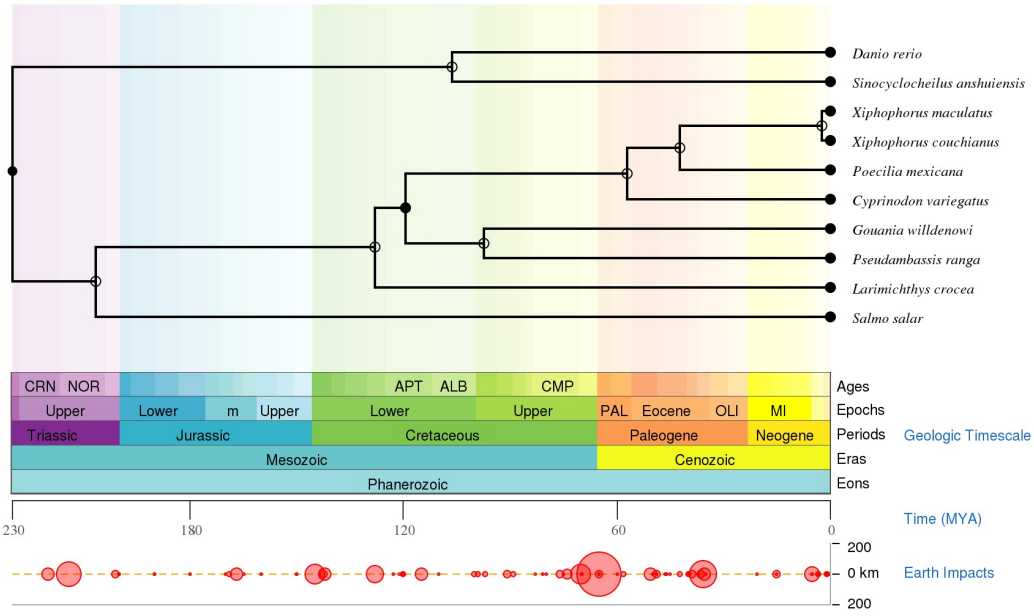

Figure 1: Tree of Ten Fish Species

1.3 Selective Pressure Analysis of TLR Gene Families

The selective pressure on a genetic test during long-term evolution is generally expressed as the ratio ω (ω = dN/dS) of nucleotide non-synonymous substitution (dN) to synonymous substitution (dS)[14]. ω > 1, ω < 1, and ω=1 denote positive, purifying, negative, and neutral selection, respectively. PAML is a software for selection pressure and phylogenetic analysis of nucleotide and amino acid sequences based on the maximum likelihood method[15].

1.4 Phylogenetic Analysis of TLR Gene Family

In order to clarify the relationship between TLR genes in ten fish species and improve the reliability of the results, we used several methods to construct phylogenetic trees. Commonly used are the UPGM method, maximum likelihood, maximum parsimony, and adjacency method[16]. In this study, Muscle software was used to compare the amino acid sequences of the TLR gene[17]. The phylogenetic tree was constructed using the proximity method and the sum maximum likelihood method based on the distance.

1.5 Structural Modeling

Each forecast model of TLR genes by CPHmodels 3.0 build (available CPH models/http://www.cbs.dtu.dk/services/. We then used TMHMM to predict the transmembrane regions of TLRs[18].

Results

2.1 Sequence Analysis of TLR Genes In Fish

This research delved into the TLR gene request numbers of ten fish species in Table 2.1. Mega and MUSCLE software were utilized to analyze these genes' base sequences, as outlined in Table 2.2. TLR7 exhibited a similar number of different fish genes, averaging approximately 3163.8. C. variegatus had the highest thymine count at 29.2, while O. salmon had the lowest at 27.5 below the mean. Conversely, O. salmon had the highest cytosine count. G. willdenowi had the highest adenine count, while O. salmon had the lowest. Regarding guanine, the highest amount was found in large yellow croaker while the lowest amount was in G. willdenowi, 19.4 below the mean.

Name	Gene ID	Sequence Number
Danio rerio	TLR7	XM_021479060.1
	TLR13	XM_005167756.4
Xiphophorus maculatus	TLR7	XM_005799326.3
	TLR13	XM_023326109.1
Cyprinodon variegatus	TLR7	XM_015390713.1
	TLR13	XM_015370152.1
Xiphophorus couchianus	TLR7	XM_028022887.1
	TLR13	XM_028036652.1
Poecilia mexicana	TLR7	XM_015013976.1
	TLR13	XM_014982174.1
Sinocyclocheilus anshuiensis	TLR7	XM_016489773.1
	TLR13	XM_016457077.1
Gouania willdenowi	TLR7	XM_028436047.1
	TLR13	XM_028474807.1
Parambassis ranga	TLR7	XM_028410705.1
	TLR13	XM_028425453.1
Larimichthys crocea	TLR7	XM_010743042.3
	TLR13	NM_001303396.1
Salmo salar	TLR7	XM_014174491.1
	TLR13	XM_014150555.1

Table 2.1: Fish TLR Gene Request Number

We have uncovered fascinating findings through our analysis of the TLR13 gene in ten distinct fish species (as outlined in Table 2.3). The zebrafish boasts the highest thymidine content, while the Ontario salmon has the lowest content - even lower than the average of 28.3. The Ontario salmon boasts the highest cytosine content, while the zebrafish has the lowest. Furthermore, we found that the variolated Oryza boasts the highest adenine content, while the Gouania willdenowi has the lowest - a staggering 29.5 below the average. Finally, the Ontario salmon has the highest guanine content at 21.1, while the pied swordtail has the lowest.

	T(U)	C	A	G	Total	Pos #1	Pos #2	Pos #3
Danio rerio	27.6	24.0	29.3	19.1	3111.0	1037.0	1034.0	1040.0
Xiphophorus maculatus	27.9	22.4	30.3	19.4	3156.0	1051.0	1050.0	1055.0
Cyprinodon variegatus	29.2	22.6	29.3	18.8	3180.0	1062.0	1059.0	1059.0
Xiphophorus couchianus	28.1	22.2	30.4	19.3	3156.0	1051.0	1050.0	1055.0
Poecilia mexicana	27.7	22.5	30.4	19.4	3177.0	1059.0	1057.0	1061.0
Sinocyclocheilus anshuiensis	26.6	24.9	28.5	20.0	3150.0	1050.0	1048.0	1052.0
Gouania willdenowi	27.9	22.3	32.1	17.8	3159.0	1052.0	1051.0	1056.0
Parambassis ranga	28.8	22.9	28.8	19.5	3195.0	1065.0	1063.0	1067.0
Larimichthys crocea	25.9	24.3	29.0	20.8	3162.0	1053.0	1052.0	1057.0
Salmo salar	25.3	26.3	28.4	20.0	3192.0	1064.0	1062.0	1066.0
Avg.	27.5	23.4	29.7	19.4	3163.8	1054.4	1052.6	1056.8

Table 2.2: TLR7 Gene Sequence Analysis

	T(U)	C	A	G	Total	Pos #1	Pos #2	Pos #3
Danio rerio	32.1	19.4	29.0	19.5	2856.0	953.0	952.0	951.0
Xiphophorus maculatus	26.6	24.3	30.2	19.0	2880.0	961.0	961.0	958.0
Cyprinodon variegatus	28.7	21.7	31.4	18.2	2859.0	955.0	953.0	951.0
Xiphophorus couchianus	29.0	22.4	29.2	19.4	2817.0	943.0	939.0	935.0
Poecilia mexicana	28.3	22.7	30.2	18.9	2877.0	961.0	959.0	957.0
Sinocyclocheilus anshuiensis	27.8	23.3	29.5	19.4	2862.0	955.0	955.0	952.0
Gouania willdenowi	28.5	24.0	27.7	19.8	2904.0	970.0	973.0	961.0
Parambassis ranga	28.4	23.2	29.1	19.2	2934.0	978.0	979.0	977.0
Larimichthys crocea	27.5	23.1	30.0	19.4	2862.0	956.0	954.0	952.0
Salmo salar	25.9	25.0	27.9	21.1	2097.0	698.0	701.0	698.0
Avg.	28.3	22.9	29.5	19.3	2794.8	933.0	932.6	929.2

Table 2.3: TLR13 Gene Sequence Analysis

2.2 TLR Codon Usage Frequency in Fish

The results indicated that leucine was the most commonly used amino acid in TLR7 among all ten species. Specifically, X. couchianus and X. maculatus had the highest frequency of leucine usage (15.8), while P. ranga and L. crocea had the lowest (14.8). Conversely, tryptophan had the most minor frequency of usage, with K. swordtail, spotted swordtail, O. brevifinta, and O. salmon having the lowest frequency of tryptophan (1.1). Additionally, the analysis of TLR14 showed that leucine was the most frequently used amino acid among the 20, with G. willdenowi having the highest frequency of leucine use (17.4). In contrast, sizeable yellow crocea had the lowest frequency of leucine use (15.0). Intriguingly, S. anhydrinus had the lowest usage frequency of TLR13 (0.9), followed by serine and aspartic acid.

	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Danio rerio	3.0	2.6	4.5	4.9	4.9	3.1	1.9	6.4	5.5	15.7	1.8	8.6	4.0	3.5	4.2	9.8	5.7	4.6	1.3	3.9	1036.0
Xiphophorus maculatus	3.2	2.6	4.6	5.1	5.1	3.6	2.1	5.7	6.7	15.8	2.2	7.7	3.6	2.8	4.6	10.0	5.3	4.5	1.1	3.7	1051.0
Cyprinodon variegatus	3.9	2.5	4.2	4.1	5.9	4.2	2.7	4.8	4.8	15.2	2.3	8.8	4.6	5.1	3.7	9.9	4.7	4.2	1.2	3.1	1059.0
Xiphophorus couchianus	3.1	2.6	4.7	5.1	5.1	3.6	2.1	5.7	6.6	15.8	2.1	7.8	3.6	2.8	4.6	10.0	5.3	4.6	1.1	3.7	1051.0
Poecilia mexicana	3.2	2.6	4.9	4.9	5.5	3.7	2.0	5.2	7.2	15.6	2.0	7.7	3.8	2.9	4.5	9.3	5.8	4.6	1.1	3.6	1058.0
Sinocyclocheilus anshuiensis	3.0	2.4	4.6	5.1	5.0	3.0	2.8	6.3	5.7	15.4	1.8	8.6	3.8	3.9	3.9	9.6	5.8	4.2	1.4	3.7	1049.0
Gouania willdenowi	2.7	2.5	4.3	5.4	5.1	3.2	2.9	6.5	7.3	15.7	1.9	8.5	4.0	3.2	3.2	9.5	5.1	3.7	1.2	4.0	1052.0
Parambassis ranga	3.8	2.5	4.9	3.9	6.4	3.6	2.8	4.5	4.6	14.8	2.3	9.2	4.2	4.6	3.9	8.6	4.3	5.4	1.6	4.0	1064.0
Larimichthys crocea	3.1	2.8	4.5	5.4	5.1	3.5	2.2	5.1	6.4	14.8	2.6	7.6	4.5	3.0	4.7	9.7	5.1	4.5	1.2	4.3	1053.0
Salmo salar	3.2	2.4	5.1	4.9	4.9	3.1	2.3	6.4	5.6	15.6	1.9	7.9	4.1	3.6	4.9	8.9	5.6	4.6	1.1	4.0	1063.0
Avg.	3.2	2.5	4.6	4.9	5.3	3.5	2.4	5.7	6.0	15.5	2.1	8.2	4.0	3.5	4.2	9.5	5.3	4.5	1.3	3.8	1053.6

Table 2.4: Frequency of TLR7 Codon Use

	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr	Total
Danio rerio	3.5	2.9	5.8	4.7	6.4	3.3	3.5	6.7	5.7	15.4	1.9	6.1	2.7	2.9	3.3	9.3	4.5	7.4	1.2	2.9	951.0
Xiphophorus maculatus	3.6	1.9	4.6	4.1	5.2	3.5	2.5	5.5	6.5	15.4	1.1	8.2	4.0	5.5	4.4	8.7	6.8	4.3	1.1	3.0	959.0
Cyprinodon variegatus	3.7	2.3	5.1	3.6	4.9	3.7	2.6	6.1	6.7	16.2	1.5	8.2	3.2	5.0	4.6	8.6	5.8	4.1	1.1	3.0	952.0
Xiphophorus couchianus	2.9	2.5	5.5	4.8	6.3	3.3	2.5	5.9	5.4	15.6	2.5	7.2	3.6	4.5	4.6	7.8	5.4	5.1	1.4	3.3	938.0
Poecilia mexicana	3.8	2.3	5.3	4.0	5.4	3.1	3.0	6.4	6.8	15.2	2.1	7.8	3.4	4.4	4.7	9.5	5.4	3.5	1.3	2.5	958.0
Sinocyclocheilus anshuiensis	4.2	2.1	5.2	4.2	5.2	2.8	3.0	5.8	5.9	16.4	1.4	8.8	3.1	4.3	4.2	7.8	5.8	4.9	0.9	3.9	953.0
Gouania willdenowi	3.5	2.2	5.8	5.0	5.4	3.3	3.3	5.4	4.9	17.4	2.1	6.5	3.5	4.1	3.3	9.2	5.4	5.8	1.2	2.8	967.0
Parambassis ranga	3.5	2.5	4.8	4.4	5.3	3.6	3.3	5.4	5.3	16.2	1.6	8.9	3.1	3.5	5.0	8.4	5.1	5.1	1.0	4.0	977.0
Larimichthys crocea	3.9	1.7	5.0	4.4	5.9	3.9	2.6	6.6	5.7	15.0	2.3	8.1	3.8	4.0	5.1	8.4	6.2	4.1	1.0	2.3	953.0
Salmo salar	4.0	2.1	4.9	5.3	5.6	5.0	2.7	6.2	5.4	15.8	1.1	6.0	4.0	4.4	4.4	7.7	6.9	4.2	1.3	2.9	698.0
Avg.	3.6	2.2	5.2	4.4	5.6	3.5	2.9	6.0	5.8	15.8	1.8	7.6	3.4	4.3	4.4	8.6	5.7	4.9	1.1	3.1	930.6

Table 2.5: TLR13 Codon Frequency

2.3 Diverging Prediction of TLR Gene Evolution in Fish

In order to examine the development of TLR genes across multiple fish species, we utilized the sophisticated Mega 7.0 software to

generate Table 2.6. This comprehensive table effectively illustrates the variations in amino acids present at each site of TLR7 sequences across ten distinct fish species. Following our meticulous analysis, we omitted any locations with gaps or incomplete data, resulting in a final dataset of 1015 locations.

	1	2	3	4	5	6	7	8	9
Danio rerio									
Xiphophorus maculatus	0.359								
Cyprinodon variegatus	0.645	0.632							
Xiphophorus couchianus	0.359	0.007	0.633						
Poecilia mexicana	0.359	0.046	0.63	0.046					
Sinocyclocheilus anshuiensis	0.155	0.329	0.633	0.329	0.328				
Gouania willdenowi	0.377	0.252	0.633	0.251	0.252	0.343			
Parambassis ranga	0.654	0.636	0.303	0.635	0.637	0.643	0.641		
Larimichthys crocea	0.365	0.207	0.636	0.206	0.201	0.326	0.241	0.637	
Salmo salar	0.327	0.286	0.631	0.284	0.28	0.297	0.286	0.641	0.278

Table 2.6: Estimates of Evolutionary Divergence in TLR7 Genes

Estimates of evolutionary divergence show the number of base substitutions per site between TLR13 sequences. The maximum integrated likelihood model was used for analysis. Rate variation between sites was modeled using a GAMMA distribution with shape parameter = 1, and an evolutionary analysis was performed in MEGA7.0 involving 10 nucleotide sequences. Codon positions included 1 +2 +3 + noncoding. All locations containing gaps and missing data were removed. There were 1818 locations in the final dataset.

	1	2	3	4	5	6	7	8	9
Danio rerio									
Xiphophorus maculatus	1.709								
Cyprinodon variegatus	1.625	0.311							
Xiphophorus couchianus	2.177	1.308	1.325						
Poecilia mexicana	1.811	0.333	0.388	1.27					
Sinocyclocheilus anshuiensis	2.191	1.29	1.304	1.474	1.307				
Gouania willdenowi	2.066	1.229	1.286	1.002	1.254	1.425			
Parambassis ranga	2.368	1.738	1.65	1.985	1.666	1.995	1.963		
Larimichthys crocea	1.871	0.573	0.595	1.228	0.538	1.277	1.187	1.689	
Salmo salar	2.047	0.872	0.94	1.109	0.944	1.105	1.023	1.929	0.827

Table 2.7: Estimates of evolutionary divergence in TLR13 genes

2.4 Construction of Fish TLR Gene Phylogenetic Tree

Based on the phylogenetic tree constructed using Mega 7.0, the model test determined the optimal model. The TLR7 and TLR13 genes from ten fish species were utilized to create the tree, with 1000 self-test replicates. As Figure 2 showed that the gene tree separates into TLR7 and TLR13. TLR7 has two significant branches. TLR13 is divided into two major branches, including the D. rerio and S. anshuiensis are closely related, whereas the remaining eight fish species are on the other branch. P. mexicana and G. will-

denowi are one clade and closely related. X. maculatus and X. couchianus belong to the Oryziforma order are closely related.

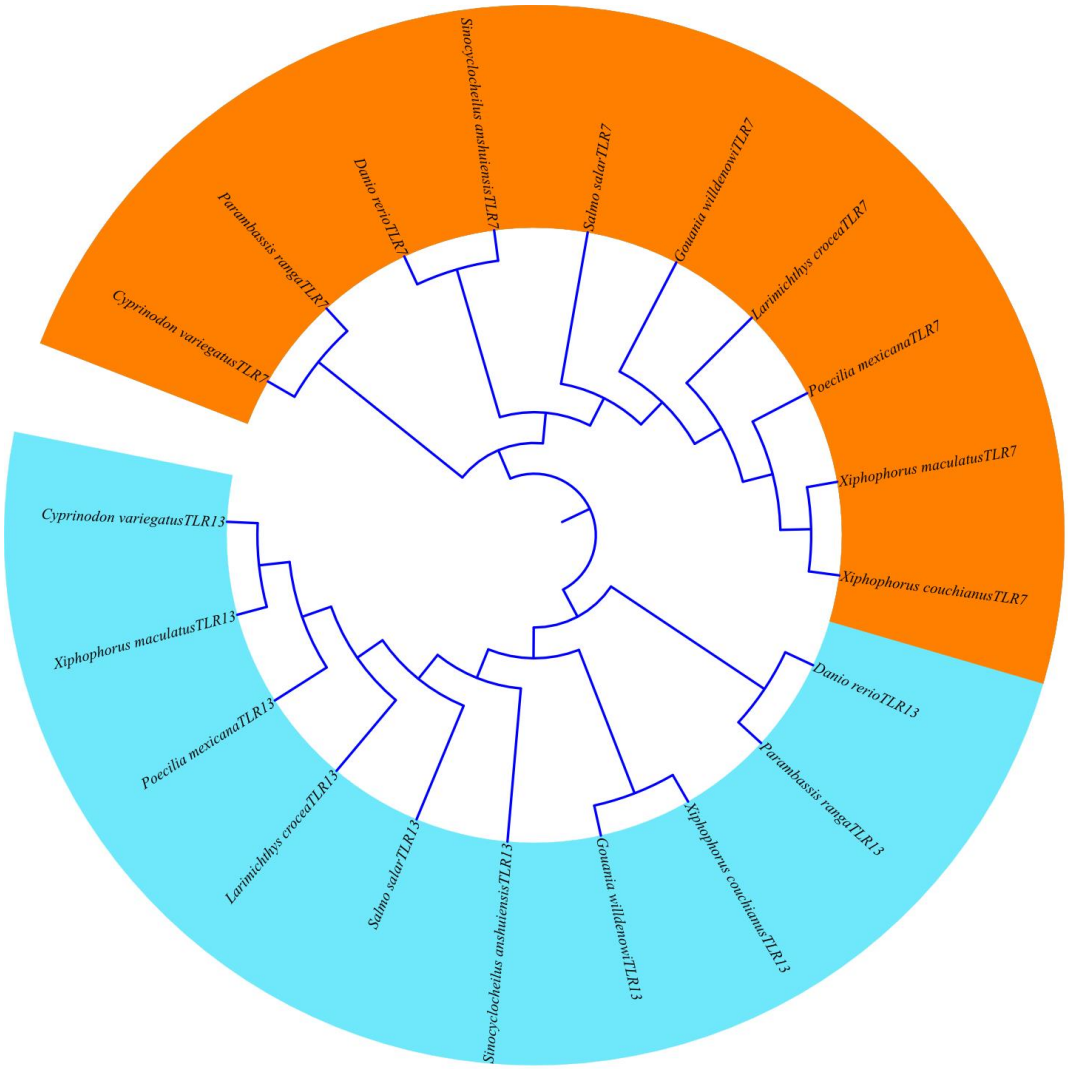

Figure 2: Phylogenetic Relationships of TLR Gene In Fish

2.5 Analysis of Fish TLR Gene Selection Pressure

The selective pressure on a genetic test during long-term evolution is generally expressed as the ratio ω (ω = dN/dS) of nucleotide non-synonymous substitution (dN) to synonymous substitution (dS). PAML is a software for selection pressure and phylogenetic analysis of nucleotide and amino acid sequences based on the maximum likelihood method [5]. This table shows the selection pressure analysis of TLR genes in fish. TLR7 was detected to have three common positive selection sites, which were 46, 650, and 686. TLR13 had two positive selection sites, 183 and 741, respectively.

	No. of species	**LRT** P-value	**M8**	REL	IFEL	Common sites
TLR7	10	0.0000041	19, 46, 61, 67,198, 201, 339, 341, 343, 371, 449, 450,650, 686, 691, 694, 716,	none	7, 10, 14, 15, 46, 68, 173, 299, 340, 372, 432, 593, 650, 686, 705, 708, 737,	3
TLR13	10	0.00000021	183, 212, 741	none	77, 125, 163, 169, 183, 193, 264, 294, 319, 390, 425, 477, 491, 506, 547, 548, 607, 694, 705, 708, 741, 823, 890, 961,	2

Table 2.5: Selective Pressure Analysis of TLR Genes in Fish

2.6 TLR Protein Structure Prediction

The function of a protein is closely linked to its spatial structure. Take, for example, the TLR7 gene protein, which has a relative molecular weight of 121.70kD and a theoretical isoelectric point of 7.88. Its horseshoe-like shape is made up of 28 α helices and 26 β folds, as depicted in Figure 3A. Meanwhile, TLR13 is a vital immune gene in Epinephelus plagioris's innate immune response. It plays a critical role in recognizing Vibrio para-hemolytic RNA and triggering the production of inflammatory cytokines, thus strengthening the immune response. The predicted structure of the TLR13 protein was shown in Figure 3B.

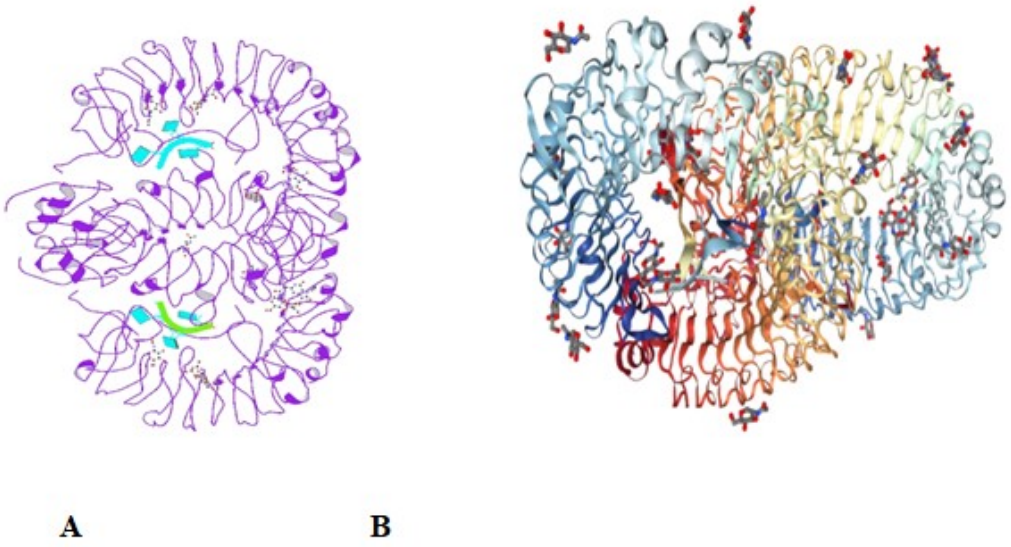

Figure 3: Prediction of TLR Protein Structure in Fish (A: TLR13, B: TLR7)

2.7 TLR Gene Structure of Fish

Our team utilized SMART software to analyze the LRR composition of the TLR7 and TLR13 genes in fish, with a focus on their structural makeup. The results of our analysis indicate that TLR7 is a glycoprotein receptor that features a transmembrane structure, including an external domain responsible for ligand binding, a transmembrane region, and an intracellular Toll/interleukin-1 receptor domain (TIR) that's involved in signaling. The TIR shape is bent into a horseshoe pattern with 13 leucine-rich repeats (LRR). Additionally, we observed that two LRR-TYPs are formed, with each end covered by a specific structure of LRR-NT and LRR-CT. Meanwhile, TLR13 consists of four LRRs, 1 LRR-TYP, and TIR (as shown in Figure 4a), while TLR7 comprises 12 LRRS, 2 LRR-TYP, and TIR (as shown in Figure 4b).

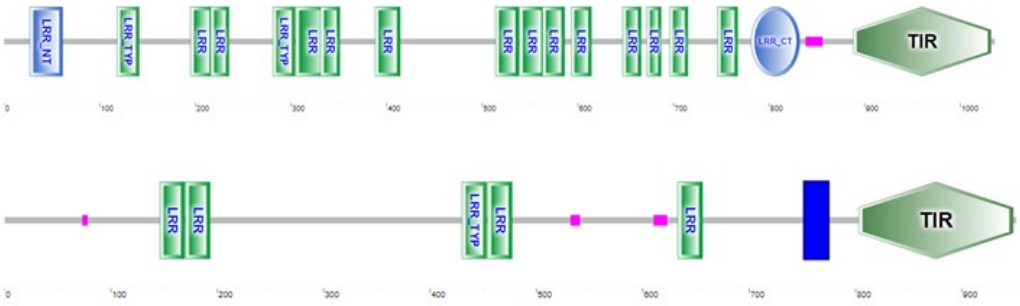

Figure 4: TLR7 and LR13 Gene Structure Map

Discussion

Fish have developed a robust immune defense system through their struggle against harmful microorganisms in their aquatic environment[19]. Their innate immune system is their first line of defense against external pathogens, and it can be activated quickly. This system recognizes and eliminates pathogens with a conserved molecular structure through pattern recognition receptors such as Toll-like receptors[20]. TLR7 and TLR13 are vital members of the TLR family, particularly in antiviral immune responses. A recent study analyzed the gene structure, sequence, codon usage frequency, and evolutionary divergence of TLR7 and TLR13 in ten different fish species[21]. The researchers found that these receptors consist of an extracellular LRR domain, transmembrane domain, and intracellular TIR domain, with varying compositions[22]. The configuration of TIR affects pathogen recognition, and different TLRs recognize different PAMPs, possibly due to their recognition of various immune sources[23].

The TLR bases show a striking similarity across different fish species. TLR7 and TLR13 exhibit adenine as the most common base, while guanine is the least common. The amino acid usage in TLR indicates leucine as the most frequently used and tryptophan as the least frequent. Phylogenetic analysis of TLR7 and TLR13 sequences reveals 1015 amino acid differences in ten fish species, indicating high conservation and functional stability. Positive selection sites suggest adaptive evolution for both TLR7 and TLR13. Due to unique evolutionary methods, the diversity in TLR family structure in fish suggests significant differences in immune recognition, activation, and regulation compared to other species.

This study provides a foundation for further research on Toll-like receptors in fish, including evolutionary status and adaptive characteristics, disease resistance, and selection and breeding of new disease-resistant varieties.

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