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Detection of non specific toll-like receptor 3 in the marine and freshwater fishes

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Abstract. The non-specific immune encoding genes was found in different species of fishes which is Toll-like receptor 3 (TLR3) genes. This gene was known to be used to protect from disease attack in the development of aquaculture fisheries that decreases their production. The aim of this study is to detect the TLR3 gene in disease-resistant freshwater fish not only in the catfish (*C. gariepinus*) but also in marine and freshwater fishes. The research method was conducted by designing TLR3 genes primer from *C. batrachus* catfish species to be applied to *C. gariepinus* catfish following by homology analysis with other fishes. The results of the study have obtained TLR3 gene in *C. gariepinus* catfish. Phylogenetic analysis with other fishes showed that TLR3 gene from catfish was closely related to *Ictalurus punctatus* (96%) and *C. batracus* (87%) as well as some freshwater and seawater fishes.

1. Introduction

Immunity is the primary mechanism of defense of the body of a living being consisting of two parts, a non-specific immune system and a specific immune system. The Pisces class as a vertebrate is believed to have an immune (non-specific immune) immune function that serves to preserve their lives against pathogenic attacks in their environment. Such mechanisms are physical, physiological, cellular and molecular defense [1]. Non-specific immune mechanisms have always been readily available, immediate and strong response to pathogens in healthy individuals. This immune system is not specific because it is not intended for certain pathogens or microbes. Response of non-specific immune system to fish has begun since the phase of embryogenesis [2].

There are various forms of non-specific immune responses in the fish such as: physical barrier (skin, scales, and mucus), cytotoxic cells, antimicrobial proteins, ascocytes, necrosis factor (INF), Interleukin (IL), and cytokines such as Toll-Like Receptors (TLR), and Chemokine (CXCL) [3]. Toll-Like Receptors (TLR) are believed to play an important role in the defense of living organisms to prevent and inhibit pathogenic invasion in non-specific immune systems [4].

The development of technology and the development of science led to the genomic era and great effort in the research, identification and molecular characterization of the main components of the non-specific immune genes of mammals including Toll-like Receptors (TLR) [3]. Attention to fish disease issues developed in line with the increased fish cultivation system very high economical. Many studies on conventional and molecular fish diseases are performed, ranging from disease detection to isolation of bacterial and pathogenic DNA, bacterial filtration to certain immune gene detectors in fishery and marine aquaculture products [5-7].



The PCR primer is short oligonucleotide which acts as the initiation of amplification of DNA molecules. The existence of the PCR primer, the target gene will be amplified throughout the course of the PCR reaction. Specific primer PCR analysis is the best step for the purpose of detecting of specific region because it can produce rapid determination of the presence of target genes, quite sensitive and easy to use [8,9]. The purpose of the research is to obtain a specific and accurate primer it is necessary to design the appropriate primer for detecting the presence of TLR3 immune gene in *C. gariepinus* catfish from the same immune gene that has been found and confirmed to its presence in *C. batrachus* catfish. The primer which has detected will be use to search the TLR3 gene in the *C. batrachus* following by their phylogenetic analysis.

2. Material and Method

This research was part of in the project of Research and Development of Fish Disease Control (IP4L), and conducted on Research Center and Freshwater Cultivation Development (BPPBAT). Depok. The DNA extraction was performed using sample from the lymphocytoid tissue organs of the fish consisting of spleen tissue, upper kidney and liver.

2.1. Material

Material which was used as a sample was catfish species *C. gariepinus* from the wet laboratory of IP4L Depok.

2.2. DNA Extraction

Extraction was conducted using the Promega kit. Extraction with the Promega kit is done by: stored leptoiloid tissue cut into pieces of rice beans, put in microtube 1.5 ml added 0.5 M EDTA 120 µl, mixed with 500 µl nucleid lye, then rested in ice gel box for 5 minutes. The sample was then crushed pastel until became porridge. Sample added with Proteinase K by 17.5 µl and incubated in thermomixer eppendorf for 3 hours at 55°C and homogenized every hour, then taken and incubated at room temperature for 10 minutes. The sample was added with 3 µl RNase enzyme solution. Sample was re-incubated in thermomixer for 15-20 minutes at 37°C temperature and keep at room temperature. The sample was added 200 µl protein precipitation solution, vortexed for 20 seconds and then left in ice gel box for 5 minutes. Samples were centrifuged at 16000 rpm for 4 minutes. The supernatant formed was transferred into a new tube and added 600 µl isopropanol then homogenized by flipping the tube and centrifuged at 16000 rpm for 1 minute. The supernatant is removed. The pellet was washed using 70% ethanol of 600 µl, then sentrifugated on speed 16000 rpm for 1 minute. The supernatant is discarded and the pellet was dried in the room temperature. The dried DNA pellet was added with 100 µl DNA Rehydration Solution. The DNA senome samples was incubated for 30 min at 65 ° C in DNA Rehydration solution to work optimally. The sample is ready for use and then stored at -20°C in the freezer.

2.3. Design of PCR Primer

The PCR primer was designed from TLR3 immune genes of *C. gariepinus* catfish species by aligning sequences of *C. batrachus* TLR3 mRNA partial cds accession number KC907862 using the BioEdit Sequence Alignment Editor software version 7.2.5.0. to get a conserved area of the TLR3 gene sequences. The conserved area is the primary candidate (reverse and forward). The sequences were analyzed in silico using selected program (<http://sg.idtdna.com/>) to determine the base length, T_m (°C), GC percentage (% GC), secondary structure such as self-dimer, cross homology, hair pin and hetero-dimer to obtained primary candidates.

2.4. Amplification of Toll-Like Gen Receptors (TLR3) gene

The PCR process uses two primer consist of reverse primer TLR3-R1 (3'CAAGCTTCGTTCCA CCCAAA5') and forward primer TLR3-F1 (5'GCACAATAATTTGGCCAGGG3'). The program used in the PCR process consists of 2 processes, optimization and amplification. The optimization process

is made using the PCR gradient technique. The PCR gradient technique allows the occurrence of several annealing temperatures in a PCR process. The program used for amplification is obtained after the optimization process is completed. The PCR program for the amplification process uses optimal annealing temperature which has been obtained from the optimization results, ie 53°C. PCR stages consist of a pre-denaturation of 92 °C, 2 minutes; denaturation phase of 92 °C, 30 seconds; stage annealing phase 53 °C, 30 second, phase elongation 72 °C for 1 minute. The PCR process consists of 30 cycles. Then post PCR at 72 °C for 7 minutes and step PCR stop at 4 °C. PCR results are stored at -20 °C. Electrophoresis was performed by agarose gel electrophoresis. The gel concentrate used was 1.5% agarose gel dissolving with TBE 1X solution The electrophoresis is operated with a 100 volt voltage for 25 minutes. The DNA bands formed on the gel are seen with UV-Transilluminator, and then documented for analysis.

2.5. Sequencing and Analysis

Sequencing was carried out by amplifying TLR3-R1 and TLR3-F1 primers of 50 µl and supplying 30 µl reverse TLR3-R1 primers and 30 µl primers TLR3-F1 in the PCR-specific microtube. Samples are then sent to Integrated DNA Technology Singapore for sequencing process. The sequencing results of the TLR3 immune gene sample of catfish (*C. gariepinus*) were confirmed using the Blastn program at GenBank (www.ncbi.nlm.nih.gov). The homologues sequences results was shown in Table 1 below. Gene sequences that have been obtained are aligned using the Clustalx program using bootstrapping 1000x for optimizing the degree of confidence in constructing representative phylogenetic tree.

Table 1. Sequences of TLR3 gne in fish on GenBank Data Resources

Species	Accession Number	Species	Accession Number
<i>C. carpio</i>	DQ885910.1, JN194200.1	<i>S. salar</i>	BK008646.1
<i>C. carassius</i>	KC816576.1	<i>C. gariepinus</i>	-
<i>S. richardsonii</i>	JX855121.2	<i>O. mykiss</i>	DQ459470.1
<i>L. rohita</i>	JN886779.1	<i>T. rubripes</i>	AC156436.1
<i>C. idella</i>	DQ864497.1	<i>N. furzeri</i>	HADY01023702.1
<i>G. rarus</i>	DQ885909.1	<i>S. maximus</i>	KX216854.1, KJ194173.1 KJ194174.1
<i>D. rerio</i>	NM_001013269.3	<i>E. coioides</i>	HQ880667.1
<i>C. batrachus</i>	KC907862.1	<i>L. crocea</i>	HQ585077.1, NM_001303313.1 HQ589262.1
<i>I. punctatus</i>	HQ677715.1, NM_001200068.1	<i>M. miiuy</i>	KR709247.1
<i>B. bendelisis</i>	JX566893.2	<i>C. griseum</i>	HG964661.1

3. Result and Discussion

3.1. Primer design

Primer design were performed using sequence TLR3 immunized genes where partial open reading frames were cloned and known to have an immune gene TLR3. The pairs of primer forward and reverse was gained the primer design as axhibited on Table 2. The gene sequence used was obtained from Gen TLR3 *C. batrachus* with accession number KC907862. The two types of fish have a very high resemblance, so it is predicted that the DNA of the resulting immune gene encoding has a very high homology in the conserved area. The forward and reverse primer annealing sites were created using the Bio Edit Sequence Alignment Editor version 7.2.5.0 software. The primer pair obtained in the table above is selected from several pairs of primer candidates that have been designed using Bio Edit Sequence Alignment System software.

Table 2. Primer PCR *TLR3 C. gariepinus*

Primer	Sequence	Primer Position (*)	Product sizek(b p)	Tm (°C)	GC Content (%)
TLR3-F1	5'GCACAATAATTTGGCCAGG3'	57-76	699	56.3	50
TLR3-R1	3'CAAGCTTCGTTCCACCCAAA5'	774-755		56	50

3.2. Amplification of Toll-Like Receptor (TLR-3) Gene in *C. batrachus*

Target of DNA amplification using this technique requires some protocols, including DNA prints, primer forward, reverse primary, Green taq master mix, RNA free water, and DNA polymerase enzymes. The DNA molding used for this amplification process is the previous isolated DNA. A primer pair is required to initiate the occurrence of DNA amplification or multiplication. In this phase, the primer pairs of design results are TLR3-F1 and TLR3-R1. Concentrations used from a pair of primers are also certain. In this study used primer forward and reverse primer with concentration 10 pmol / μ L with each 20 nucleotide length. The PCR cycle used in the DNA amplification target of the TLR3 immune gene was 35 times. The TLR3 of amplification on Figure 1. was showing different amplification result which showing different concentration of the gen in the tissues. The DNA amplification with PCR with TLR3-F1 and TLR3-R1 primer pair was analyzed qualitatively using electrophoresis with 20 mg of 1.5% agarose gel concentration. Primer which has been designed based on homology results of TLR3 *C. batrachus* immune with accession number KC907862 is expected to amplify about 700 pb. This is evidenced by the image of electrophoresis.

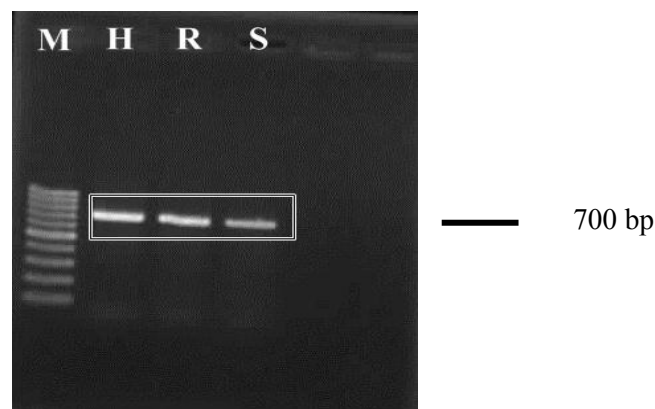


Figure 1. Amplification result of DNA Imun TLR3-F1 dan TLR3-R1 (M = Marker DNA 100 bp, H-hepar tissue of *C. gariepinus*, R = kidney tissue *C. gariepinus*, S= spleen tissue of *C. gariepinus*)

Sequences of TLR3 gene that was used for homology analysis had found high similarities and close genetic relationship between TLR3 gene between catfish *C. batrachus* and *C. gariepinus* as revealed on Figure 2. The research result had identified a conserved region of 640 in 666 there was a base difference with 26 bases of 666 base pairs.

```

C. batrachus   19 CTGTGCTGTTCCCTTCACGATGCTCAGAACCTTACCGTACTTGAGTTGGACTATAATGGTC 78
                |||
C. gariepinus 104 CTGTGCTGTTCCCTTCGTGATGCTCAGAACCTTACCGTTCTTGAGTTGGACTACAATGGTC 163
                |||
C. batrachus   79 TTGATGAGATTCCAACGATGGCCTTTCAAGGCCTCTCACAACCTAAGATACCTCAGCATT 138
                |||
C. gariepinus 164 TTGATGAGATTCCAACGATGGCCTTTTCGAGACCTCTCACAACCTAAGATACCTCAGCATT 223
  
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C. batrachus 139 GTGGGAATCTCCTGAATTTTCTCTACGACTCCATTTTACGATTTGCGGTCTTTGCGCT 198
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 224 GTGGGAATCTCCTCAACTTTCTCCAGACTCCATTTTCGACGATTTGCGGTCTTTGCGCT 283
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 199 TCCTGTGCGTGCAGAAGAATCTTTTACCTCGGTGAGGCCGAGACGTTCAAAGTGCCGC 258
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 284 ACCTGCGGATGCAGAAGAATCTTCTGACCTCGGTGAGGCCGAGACGTTCAAAGTGCCGC 343
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 259 TGTCCAACCTGACTGATCTCTACATGGATCATAACCCGTTTGATTGCACCTGCGAGAGCA 318
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 344 TGTCCAACCTGACTGATCTCTACATGGATCATAACCCGTTTGATTGCACCTGCGAGAGCA 403
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 319 TAATGTGGTTCACAAATGGCTCAATACCAACACTAGCGTACCTGATGTAGAAAGCT 378
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 404 TAATGTGGTTCACAAATGGCTCAATACCAACACTAGCGTACCTGATGTAGAAAGCT 463
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 379 ACATCTGCAACTCCATCCTTGTACTTTAATCGCTCGTTTGTGGACTTCAACCCAGAAT 438
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 464 ACATCTGCAACTCCATCCTTGTACTTTAATCGCTCGTTTGTGGACTTCAACCCAGAAT 523
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 439 CCTGTAAGGACCTGGTACCCCTTTCAGGCTGCATATATAATCTCCAGCACTTAGTACTGG 498
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 524 CATGTAAGGACCTGGTACCCCTTTCAGGCTGCATATATAATCTCCAGCACTTAGTACTGG 583
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 499 GTCTAATGGTTATAGCCTTCCTGGTGCCTACCAGGGCTGGAGGATTTCATTTCTATTGGA 558
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 584 GTCTAATGGTTATAGCCTTCCTGGTGCCTACCAGGGCTGGAGGATTTCATTTCTATTGGA 643
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 559 ACATTCTAGTCAGTCGCACCCTGGATATAAATGAGTTTCGCTATAAAAGGTTGAAACGAGT 618
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 644 ACATTTTAGTCAGTCGCACCCTGGATATAAATGAGTTTCGCTATAAAAGGTTGAAACGAGT 703
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. batrachus 619 ---GCAGATACAAATACAAAGCCTACATTGTCCATTCTCAGGAGGATACACTTTGGGTGGA-CGAA 680
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 704 TGAGCAGATACAAATACAAAGCCTACATTGTCCATTCTCAGAAGGATACACTTTGGGTGGAACGAA 769
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
    
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Figure 2. Homologies of TLR3 Gene between *C. batrachus* and *C. gariepinus*

Homology analysis between *C. gariepinus* and channel catfish *I. punctatus* exhibited conserved region values in 577 in 664 base pairs (Figure 3). There are base and gap differences as much as 87 base pairs from 664 base pairs. This result also supported by other researcher in founding 13 of BAC clones of the catfish BAC library that were positively detected by hybridization method [1].

3.3. Phylogenetic analysis

Multiple alignment was performed using ClustalX2 software with comparable sequences of about 100 species of fish that have a TLR3 sequence found in the GenBank with outgroup *C. griseum* outgrowth, phylogenetic tree analysis results can be seen in Figure 3 below. The alignment results show 20 species of fish that have been selected which show a high enough homogeneity value of TLR3 gene. Multiple alignment results indicate that samples to 20 fish species have identical (conserved) domains and different domains (variables) with other species.

```

I. punctatus 21 GTGCTGTTTCCTTACGATGCTCAGAACCTTACCGTACTTGAGTTGGACTATAATGGTCTT 80
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 3726 GTGCTGATCCTTCGCGATGCTCAGAACCTTACTGTTCTCGAGTTGGACTACAATGGTCTC 3785
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
I. punctatus 81 GATGAGATTCCAACGATGGCCTTTCAAGGCTCTCACAACTAAGATACCTCAGCATTAGT 140
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 3786 GATGAGATTCCAATGAAGGCCCTTCCAAGGCTCTCAAACTAAAATACCTCAGTATTAGT 3845
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
I. punctatus 141 GGAATCTCCTGAATTTTCTCTACGACTCCATTTTACGATTTGCGGTCTTTGCGCTTC 200
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 3846 GGAATCTCCTGAATTTTCTGCATGACTCCATTTTGACAACTGCGGTCTCTGCGCTTC 3905
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
I. punctatus 201 CTGTCGCTGCAGAAGAATCTTTTACCTCGGTGAGGCCGAGACGTTCAAAGTGCCGCTG 260
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
C. gariepinus 3906 CTGCGGCTGCAGAAAACCTCTTGACCTCAGTGCAGGCGGGAGACGTTGCGGTGCGCTG 3965
      ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
    
```

<i>I. punctatus</i>	261	TCCAACCTGACTGATCTCTACATGGATCATAACCCGTTTGTATTGCACCTGCGAGAGCATA	320
<i>C. gariepinus</i>	3966	TCCAACCTGACCGAGCTTTTCATGGATCATAACCCCTTCGATTGCACCTGTGAGAGCATT	4025
<i>I. punctatus</i>	321	ATGTGGTTCCACAAATGGCTCAATACCACCAACTAGCGTACCTGA---TGTAGAAAGC	377
<i>C. gariepinus</i>	4026	CTATGGTTCAACAAATGGCTCAATACAACCAACACCAGCGTACCTGACCGTGCCAAAAGC	4085
<i>I. punctatus</i>	378	TACATCTGCAACTCCATCCCTGTACTTTAATCGCTCGTTGTGGACTTCAACCCAGAA	437
<i>C. gariepinus</i>	4086	TACGTCTGCAACTCCATCCATGTACTTCAACCGTTTCGGTGTGGACTTCAACCCAGAA	4145
<i>I. punctatus</i>	438	TCCTGTAAGGACCTGGTACCCTTTCAGGCTGCATATATAATCTCCAGCACTTTAGTACTG	497
<i>C. gariepinus</i>	4146	TCCTGTAAGGACCTGGTCCCCTTCAGGCTTTGTATGTAATCACCAGCTCCTTAGTACTA	4205
<i>I. punctatus</i>	498	GGTCTAATGGTTATAGCCTTCCTGGTGCCTACCAGGGCTGGAGGATTCATTTCTATTGG	557
<i>C. gariepinus</i>	4206	GGTCTGATGGTTCTAGCCTTCCTGGTGCCTACCAGGGCTGGAGGATTCATTTCTACTGG	4265
<i>I. punctatus</i>	558	AACATTCTAGTCAGTCGCACCCTGGATATAAATGAGTTTCGCTATAAAAGGTTGAACGAG	617
<i>C. gariepinus</i>	4266	AACATTTTAGTCAATCGCACCCCTAGATATAAACGACTCTCGTTATAAAAGGTTGGCCGAG	4325
<i>I. punctatus</i>	618	TGCAGATACAAATACAAAGCCTACATTGTCCATTCTCAGGAGGATACACTTTGGGTGGA-CGAA	680
<i>C. gariepinus</i>	4326	TGCAGATATGAATATAAAGCCTACGTTGTCCATGCTCAGGAGGATAGACTTTGGGTGGAACGAA	4389

Figure 3. Homologies of TLR3 gene between *C. gariepinus* and *I. punctatus*

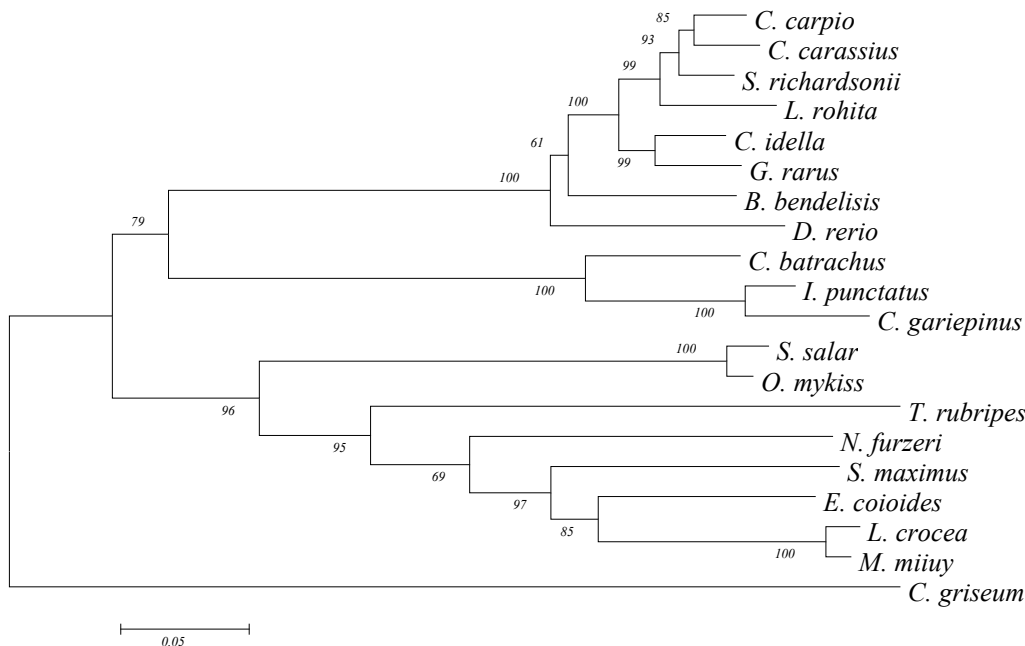


Figure 4. Phylogenetic analysis of fish which was having TLR3 gene.

4. Discussion

4.1. Primer Design for TLR3 Gene on fish

Primer TLR3-F1 and TLR3-R1 have a smaller *Tm* (°C) difference compared to other primer pairs. This is to anticipate primary primer failure during PCR amplification. Secondly, it has 50% GC components. In accordance with the primer design results, the sequence of TLR3-F1 and TLR3-R1 primer base sequences amounted to 20 bp each. The primer length ranges from 18 to 30 bases based

on the consideration of random combinations that may be found in one sequence of genomes[8]. The conserved area of homologous area of TLR3 sequences was used for primer construction.

4.2. Amplification of Toll-Like Receptor (TLR-3) Gene in *C. batrachus*

Melting temperature range used in TLR3-F1 and TLR3-R1 primer PCR optimization processes ranged from 53°C - 56°C, and this study used annealing temperature of 53°C. This refers to the comparison of melting temperature values obtained in this study then annealing temperature reference is obtained from temperature melting temperatures reduction of 3°C. The best annealing temperature is usually 2-5 ° C below T_m (results of amplification of the immune gene TLR3-F1 and TLR3-R1 Joshi and Despande, 2010). This result was in accordance with other researcher which is stated that TLR are type I transmembrane proteins associating with the innate immune response [4]. This TRL are involved in the sensing of microbial specific structures and danger-associated molecular patterns that recognized by spesific protein receptors that may occur in various cellular compartments.

4.3. Detection of Toll-Like Receptor (TLR-3) Gene in Freshwater and Marine Fishes

The phylogenetic tree construction using the Neighbor Joining (NJ) method shows that *C. gariepinus* catfish species with Channel catfish *I. punctatus* are in the same clade. These two species form the same clade with *C. batrachus*. These result has strong indication that *C. batrachus* was an ancestor of *I. punctatus* and *C. gariepinus*, which both *C. gariepinus* and *I. punctatus* gain their survival ability in the TLR3 gene form from *C. batrachus*. This result also have implication that TLR3 gene of *Clarias* was quite different from the other fish genus in the time of evolution. The result also revealed that the fish TLRs were having distinct features and large of diversity.

The *C. batrachus* was also known as Southeast Asian walking catfish. Both fishes also showing a close genetic relationship with African sharp-toothed catfish (*C. gariepinus*) according to their Cytochrome Oxidase I (COI) gene in their mitochondria [10]. The *C. batrachus* is a native fish of Indonesia, while there are still fourteen nominal *Clarias* species have been reported in literature as naturally occurring in Indonesia [11]. Based on the research result, since *C. batrachus* is detected to have the TRL 3 gene, it provide an opportunity to obtain the gene TLR 3 on various types of *Clarias* in Indonesia. Moreover, based on the TLR3 phylogenetic analysis on freshwater and marine fishes, it was exhibited high polymorfisms among them. This result became important since Toll-like receptors have become a crucial sensors and as a defense mechanisms against pathogen organisms like fungi, Bacteria, viruses, and protozoa [12]. The high polymorphisms founded in TLRs will be adressed with immunity and resistance distribution on fishes which is interesting for further investigation .

5. Conclusion

The results of phylogenetic analysis showed that the TLR3 gene of the catfish was closely related to *I. punctatus* and *C. batracus* as well as some freshwater fish and seawater.

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