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

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

Frans Oktavianus Siregar, Hermin Pancasakti Kusumaningrum and Rejeki Siti Ferniah

Biology Department, Faculty of Science and Mathematics, Diponegoro University Semarang 50275

E-mail: herminsakti@gmail.com

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 freswater 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. Pphylogenetic 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), cytotoxin cells, antimicrobial proteins, ascocytes, necrosis factor (INF), Interlinsin (IL), and cytokines such as Toll-Like Receptors (TLR), and Chemocine (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 spesific 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 lymphocyloid 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 leftoiloid 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 55ºC 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 37ºC 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 -20ºC 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 accesion 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, Tm (ºC), 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 53ºC. PCR stages consist of a pre-denaturation of 92 ºC, 2 minutes; denaturation phase of 92 ºC, 30 seconds; stage annealing phase 53 ºC, 30 second, phase elongation 72 ºC for 1 minute. The PCR process consists of 30 cycles. Then post PCR at 72 ºC for 7 minutes and step PCR stop at 4 ºC. PCR results are stored at -20 ºC. 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
C. carpio	DQ885910.1, JN194200.1	*S. salar*	BK008646.1
C. carassius	KC816576.1	*C. gariepinus*	-
S. richardsonii	JX855121.2	*O. mykiss*	DQ459470.1
L. rohita	JN886779.1	*T. rubripes*	AC156436.1
C. idella	DQ864497.1	*N. furzeri*	HADY01023702.1
G. rarus	DQ885909.1	*S. maximus*	KX216854.1, KJ194173.1 KJ194174.1
D. rerio	NM_001013269.3	*E. coioides*	HQ880667.1
C. batrachus	KC907862.1	*L. crocea*	HQ585077.1, NM_001303313.1 HQ589262.1
I. punctatus	HQ677715.1, NM_001200068.1	*M. miiuy*	KR709247.1
B. bendelisis	JX566893.2	*C. griseum*	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(bp)	Tm (ºC)	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 / μL 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.

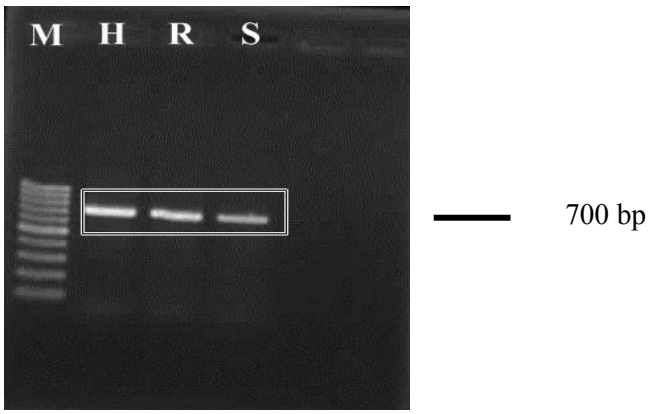

Figure 1. Amplification result of DNA Imun TLR3-F1 dan TLR3-R1 (M = Marker DNA 100 bp, H-hepar tissue of *C. gariepinus*, R = kidney tissue *C. gariepinus,* S= spleen tissue of *C. gariepinus*)

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 CTGTGCTGTTCCTTCACGATGCTCAGAACCTTACCGTACTTGAGTTGGACTATAATGGTC 78
                 ||||||||||||||  |||||||||||||||||||| |||||||||||||| |||||||
C. gariepinus 104 CTGTGCTGTTCCTTCGTGATGCTCAGAACCTTACCGTTCTTGAGTTGGACTACAATGGTC 163

C. batrachus   79 TTGATGAGATTCCAACGATGGCCTTTCAAGGCCTCTCACAACTAAGATACCTCAGCATTA 138
                 |||||||||||||||||||||||||| || ||||||||||||||||||||||||||||
C. gariepinus 164 TTGATGAGATTCCAACGATGGCCTTTCGAGACCTCTCACAACTAAGATACCTCAGCATTA 223
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C. batrachus  139 GTGGGAATCTCCTGAATTTTCTCTACGACTCCATTTTTGACGATTTGCGGTCTTTGCGCT 198
                  |||||||||||||  ||  ||||||  |||||||||||||  ||||||||||||||||||||||
C. gariepinus 224 GTGGGAATCTCCTCAACTTTCTCCACGACTCCATTTTCGACGATTTGCGGTCTTTGCGCT 283

C. batrachus  199 TCCTGTCGCTGCAGAAGAATCTTTTGACCTCGGTGAGGCCGGAGACGTTCAAAGTGCCGC 258
                  ||||   |  |||||||||||||| ||||||||||||||||||||||||||||||||||||||
C. gariepinus 284 ACCTGCGGATGCAGAAGAATCTTCTGACCTCGGTGAGGCCGGAGACGTTCAAAGTGCCGC 343

C. batrachus  259 TGTCCAACCTGACTGATCTCTACATGGATCATAACCCGTTTGATTGCACCTGCGAGAGCA 318
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C. gariepinus 344 TGTCCAACCTGACTGATCTCTACATGGATCATAACCCGTTTGATTGCACCTGCGAGAGCA 403

C. batrachus  319 TAATGTGGTTCCACAAATGGCTCAATACCACCAACACTAGCGTACCTGATGTAGAAAGCT 378
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C. gariepinus 404 TAATGTGGTTCCACAAATGGCTCAATACCACCAACACTAGCGTACCTGATGTAGAAAGCT 463

C. batrachus  379 ACATCTGCAACACTCCATCCTTGTACTTTAATCGCTCGTTGTTGGACTTCAACCCAGAAT 438
                  |||||||||||||||||||||||||||||||||||  ||||||||||||||||||||
C. gariepinus 464 ACATCTGCAACACTCCATCCTTGTACTTTAATCGCTCTGTGTTGGACTTCAACCCAGAAT 523

C. batrachus  439 CCTGTAAGGACCTGGTACCCTTTCAGGCTGCATATATAATCTCCAGCACTTTAGTACTGG 498
                  | ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
C. gariepinus 524 CATGTAAGGACCTGGTACCCTTCCAGGCTGCATATATAATCTCCAGCACTTTAGTACTGG 583

C. batrachus  499 GTCTAATGGTTATAGCCTTCCTGGTGCACTACCAGGGCTGGAGGATTCATTTCTATTGGA 558
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
C. gariepinus 584 GTCTAATGGTTATAGCCTTCCTGGTGCACTACCAGGGCTGGAGGATTCATTTCTATTGGA 643

C. batrachus  559 ACATTCTAGTCAGTCGCACCCTGGATATAAATGAGTTTCGCTATAAAAGGTTGAACGAGT 618
                  |||||  |||||||||||||||||||||||||||||||||||||||||||||||||| |||||
C. gariepinus 644 ACATTTTAGTCAGTCGCACCCTGGATATAAATGAGTTTCGCTATAAAAGGTTGAGCGAGT 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 homogeny 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.

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I. punctatus     21  GTGCTGTTCCTTCACGATGCTCAGAACCTTACCGTACTTGAGTTGGACTATAATGGTCTT  80
                     ||||||  ||||||  ||||||||||||||||||  ||  ||  ||||||||||  ||||||||
C. gariepinus  3726  GTGCTGATCCTTCGCGATGCTCAGAACCTTACTGTTCTCGAGTTGGACTACAATGGTCTC  3785

I. punctatus     81  GATGAGATTCCAACGATGGCCTTTCAAGGCCTCTCACAACTAAGATACCTCAGCATTAGT  140
                     ||||||||||||  ||  ||||||  ||||||||||||  ||||||  |||||||||  ||||||
C. gariepinus  3786  GATGAGATTCCAATGAAGGCCTTCCAAGGCCTCTCAAAACTAAAATACCTCAGTATTAGT  3845

I. punctatus    141  GGGAATCTCCTGAATTTTCTCTACGACTCCATTTTTGACGATTTGCGGTCTTTGCGCTTC  200
                     ||||||||||||||||||||  |  ||||||||||||||  |  |||||||||  ||||||||
C. gariepinus  3846  GGGAATCTCCTGAATTTTCTGCATGACTCCATTTTTGACAACTTGCGGTCTCTGCGCTTC  3905

I. punctatus    201  CTGTCGCTGCAGAAGAATCTTTTGACCTCGGTGAGGCCGGAGACGTTCAAAGTGCCGCTG  260
                     |||  |||||||||  ||  ||  ||||||||  |||  |||  ||||||||||   |||||||||
C. gariepinus  3906  CTGCGGCTGCAGAAAAACCTCTTGACCTCAGTGCGGCGGGAGACGTTCGGCGTGCCGCTG  3965
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I. punctatus     261  TCCAACCTGACTGATCTCTACATGGATCATAACCCGTTTGATTGCACCTGCGAGAGCATA  320
                      ||||||||||| || || | ||||||||||||||| || |||||||||| ||||||||
C. gariepinus   3966  TCCAACCTGACCGAGCTTTTCATGGATCATAACCCCTTCGATTGCACCTGTGAGAGCATT  4025

I. punctatus     321  ATGTGGTTCCACAAATGGCTCAATACCACCAACACTAGCGTACCTGA---TGTAGAAAGC  377
                       | |||||| |||||||||||||||| |||||||| ||||||||||   ||   |||||
C. gariepinus   4026  CTATGGTTCAACAAATGGCTCAATACAACCAACACCAGCGTACCTGACCGTGCCAAAAGC  4085

I. punctatus     378  TACATCTGCAACACTCCATCCTTGTACTTTAATCGCTCGTTGTTGGACTTCAACCCAGAA  437
                      ||| |||||||||||||||| ||||||| || || ||| ||||||||||||||||||||
C. gariepinus   4086  TACGTCTGCAACACTCCATCCATGTACTTCAACCGTTCGGTGTTGGACTTCAACCCAGAA  4145

I. punctatus     438  TCCTGTAAGGACCTGGTACCCTTTCAGGCTGCATATATAATCTCCAGCACTTTAGTACTG  497
                      ||||||||||||||||| ||||| ||||||   ||| ||||| ||||| | ||||||||
C. gariepinus   4146  TCCTGTAAGGACCTGGTCCCCTTCCAGGCTTTGTATGTAATCACCAGCTCCTTAGTACTA  4205

I. punctatus     498  GGTCTAATGGTTATAGCCTTCCTGGTGCACTACCAGGGCTGGAGGATTCATTTCTATTGG  557
                      ||||| |||||| |||||||||||||||| |||||||||||||||| ||||| |||
C. gariepinus   4206  GGTCTGATGGTTCTAGCCTTCCTGGTGCACTTCCAGGGCTGGAGGATTCAATTCTACTGG  4265

I. punctatus     558  AACATTCTAGTCAGTCGCACCCTGGATATAAATGAGTTTCGCTATAAAAGGTTGAACGAG  617
                      |||||| |||||| ||||||||| |||||||| || | ||| ||||||||| ||  ||||
C. gariepinus   4266  AACATTTTAGTCAATCGCACCCTAGATATAAACGACTCTCGTTATAAAAGGGTGGCCGAG  4325

I. punctatus     618  TGCAGATACAAATACAAAGCCTACATTGTCCATTCTCAGGAGGATACACTTTGGGTGGA-CGAA  680
                      ||||||||  |||| ||||||||| |||||||| |||||||||||| |||||||||||| ||||
C. gariepinus   4326  TGCAGATATGAATATAAAGCCTACGTTGTCCATGCTCAGGAGGATAGACTTTGGGTGGAACGAA  4389
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Figure 3. Homologies of TLR3 gene between *C. gariepinus* and *I. punctatus*

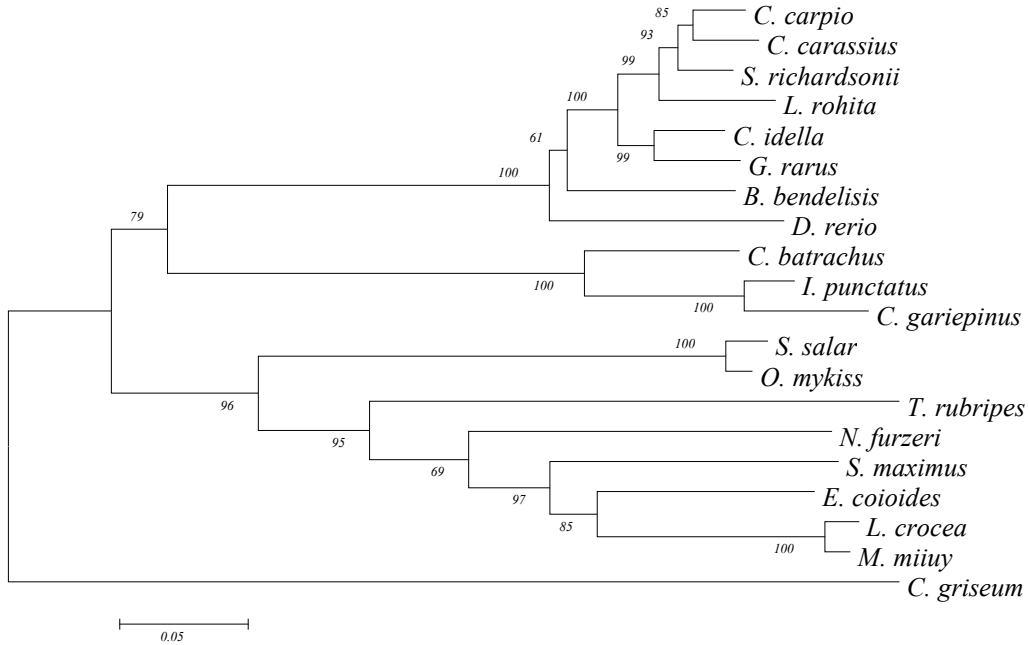

Figure 4. Phylogenetic analysis of fish which was having TLR3 gene.

4. Discussion

4.1. Primer Design for TLR3 Gene on fish

Primer TLR3-F1 and TLR3-R1 have a smaller Tm (ºC) 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 53ºC - 56ºC, and this study used annealing temperature of 53ºC. 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 3ºC. The best annealing temperature is usually 2-5 ° C below Tm (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 ares still fourteen nominal *Clarias* species have been reported in literature as naturally occurring inIndonesia [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 became 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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