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Comparative study of pro-inflammatory cytokine expression among two common freshwater Cichlids

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Abstract

Although there is little information on the non-mammalian cytokines due to insufficient recombinant molecules and specific antibodies, this study was conceived to access the variation exhibited by different fish species using *Coptodon zillii* being one of the most stress-tolerant fish species, which was compared with *Sarotherodon melanotheron* found in same water bodies. We examined these species' peptide and nucleotide sequences of tumour necrosis factor α to determine its biological role in fish production in Nigeria water. The study was initiated to evaluate the survival strategy of these cichlid groups with varying degrees of physicochemical and organic stressors in their environment. This study also aimed to reveal the underlying genes responsible for these species' tolerance to various environmental conditions. Molecular and bioinformatics techniques were employed to carry out a comparative expression study of the proinflammatory cytokine gene of these two common freshwater tilapia species. TNF-α expression in the tilapia species studied was confirmed because of the single specific DNA band of all the PCR amplificons generated without a non-specific amplification, and 21 SNP variants were recorded on the sequence, which would have significant effects in their protein TNF-α primary sequences and subsequently have an effect in their protein structure as well as Pro-inflammatory responses.

Kew Words: Cytokine, *Coptodon zillii* ***(CZ)*****, *Sarotherodon melanotheron* (SM), Sequence, Gene expression, TNF-α, RT-PCR**

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Introduction

Many aquatic ecosystems have undergone several changes over the years, with attendant changes in fish distribution and life history traits (Gueye *et al*., 2013). Aside from these environmental pressures caused by global warming, aquatic ecosystems are also subjected to anthropogenic alterations, such as drain discharges from domestic and industrial sources that increase pollutants, which can interact with physiological processes and disturb community structures. These environmental variations stimulate the fish's immune response, enhancing innate responses (Lluis, 2011).

Fish illnesses pose a significant challenge in aquaculture, leading to substantial financial losses and often necessitating the use of antibiotics for treatment. However, the emergence of drug-resistant bacteria and concerns about food hygiene have prompted a shift away from antibacterial medications. In this context, the potential benefits of modifying genetic components of the immune system come to the fore, making an in-depth study of cytokines imperative. The group of protein cytokines that we focus on in our research plays a pivotal role in the innate immune response of fish. Zou and Secombes (2016) argued that innate immunity is superior to acquired immunity in preventing infectious

diseases in fish. These findings, therefore, hold promise for revolutionizing fish disease control and shaping the future of aquaculture, offering a beacon of hope in the face of these challenges.

This innate immune protein includes the pro-inflammatory cytokine tumor necrosis alpha (TNF-α), which is part of a class of small proteins involved in immune system cell-to-cell communication and is typically described as a crucial trigger of inflammatory processes following the identification of infections. (Aristizábal B. and González, A. (2013).; Kany *et al*., 2019). This cytokine has also been known to activate and migrate leukocytes, initiating fever, acute phase reactions, and apoptosis. It is responsible for systemic and cellular reactions (Baud & Karin, 2001). It has been reported that TNF-α mRNA transcription occurs in various cells and is heavily controlled post-transcriptionally, promoting the release of various other cytokines (Zhao *et al*., 2021).

Because of their various adaptive phenotypic immune response features, cichlid fishes exhibit remarkable phenotypic diversity. As a result, they offer a fascinating chance to research the genetics of speciation and adaptation through sexual and natural selection (Fan *et al*., 2012). Since the immune system is essential to every individual, many changes have occurred throughout evolution to produce diversity and specialization among cichlid species from different environments, even though the immune system has retained some significant traits that are shared by all species over millions of years of evolution (Kaufman, 2010).

Innate immunity protects against pathogens, foreign bodies, chemical agents, or environmental changes (Dawood *et al*., 2020). In this study, we evaluated the expression of the pro-inflammatory cytokine involved in innate immunity tumor necrosis factor-alpha (TNF-α) in two tilapia species (*Coptodon* and *Sarotherodon melanotheron*) known to have physiological characteristics of interest in culture from two water bodies.

Coptodon zillii is a tough fish that is seen as a possible competitor with other Tilapia species. Among the many desirable traits for aquaculture, *C. zillii* is known for its high tolerance to environmental stress (Geletu *et al*., 2024). *C. zillii* is a highly adaptable fish that can thrive in various water quality and environmental conditions (Froese & Pauly, 2022; Adham *et al*., 1997). It is naturally found in lakes, rivers, wetlands, estuaries, and, in some cases, marine habitats. *Sarotherodon melanotheron*, like C. Zillii, is another Tilapia species with a wide range of environmental stress tolerance, such as salinity (Tine *et al*., 2008). They have adapted to diverse habitats, including permanent and temporary rivers, large equatorial lakes, tropical and subtropical rivers, open and closed estuaries, lagoons, swampy lakes, deep lakes, and coastal brackish lakes (Usman *et al*., 2017). Understanding the molecular basis for stress adaptability in these species is crucial to harnessing their potential. This study, therefore, utilized molecular and bioinformatics techniques to conduct a comparative expression study of the pro-inflammatory cytokine gene of the two common tilapia species in the studied water bodies. It assesses the expression pattern of cichlids by analyzing the links of environmental factors with the sequence variation in SNPs in *C. zillii* and *S. melanotheron* species from Omobala River and Onitsha end of River Niger in Southeastern Nigeria.

Materials and Methods

Sample Collection, Identification and Storage

Live adult Tilapia samples, consisting of 20 pieces each, were collected from fishermen at two water bodies in the eastern region of Nigeria: The Omabala River (N60 34.72'; E60 83.77') and Onitcha end of the River Niger (N60 10.3'; E60 46.25'). Following the protocol of Cordero et al. (2017), tissue samples were taken from the ventral region of the fish with an average body weight of 100 g. The excised tissues were immersed in RNA preserve buffer and stored at -20°C until further use.

RNA Isolation and Quantification

Following the manufacturer's protocol of the JENA mini kit (Jena Bioscience, Germany), total RNA was extracted from 50 mg of Tilapia tissue sample stored earlier and collected into a microcentrifuge tube while the remaining were stored for further use. The potency, purity, and concentration of the nucleic acids were determined using the ThermoFisher 2000C nanodrop spectrophotometer.

cDNA generation and Quantification

Following the manufacturer's instructions, a reverse transcription polymerase chain reaction (RtPCR) was carried out for 20 reaction samples using Fire script RT cDNA synthesis kits (FIREScript®, Estonia), using

Oligo-dt primer (with the thermal profile: 65° C for 5 mins for denaturation, followed by Primer annealing at 25° C for 10 mins and 85° C for 5 min to inactivate RT enzyme).

The Reverse Transcription Profile procedures were completed after the following steps: primer annealing at 25 °C for 10 minutes, reverse transcription at 50 °C for 50 minutes, enzyme inactivation at 55 °C for 5 minutes, and reaction holding at 10 °C for 4 minutes. The housekeeping Gene, Glyceraldehyde 3 Phosphate dehydrogenase (GAPDH), was used to quantify complementary DNA (Primer sequence shown in Table 1.0).

Table 1. Primers used for GAPDH and TNF-α expression study

S/n	Primer	Forward	Reverse
1	GAPDH	GCCCTCTGGTAAAATGTGGA	ATTCCCTTCATGGGTCCTTC
2	TNF-α	GGTTAGTTGAGAAGAAATCACCTGCA	GTCGTCGCTATTCCCGCAGATCA

Tumor Necrosis Factor Gene Amplification, quantification, and Sequencing

Tumour Necrosis Factor (TNF-α) was amplified using Eppendorf Nexus Gradient Thermal Cycler in which 2µl of reverse-transcribed cDNA (RTcDNA) was mixed in a 1.5 µl PCR tube with 18 µl reaction cocktail, PCR was done with an initial 2 min denaturation at 94 °C followed by 25 cycles of denaturation at 94 °C for 30 s annealing at 57 °C for 30 s, extension at 72 °C for 30 s. A total of 2 µl of TNF-α amplicon was used for quantification on 2% Agarose Gel electrophoresis. PCR amplicons (1.5 µl) of each sample contained in 1.5 ml PCR tubes were labelled and shipped for sequencing. Sanger Sequencing of TNF-α amplicons was performed by Inqaba Biotech West Africa Ibadan, Oyo State, Nigeria.

Expression study

The results obtained from sequencing all the amplicons were saved as chromatogram files in ab1 format and then exported to FASTA format. These exported FASTA files were then analyzed using the Basic Local Alignment Search Tool (BLAST) on the NCBI platform, following the method described by Li *et al*. (2019. This analysis aimed to ensure that the sequence file corresponded to the gene of interest amplified and sequenced during the PCR and sequencing steps. This was verified by examining the BLAST report's expected value (E-value). Furthermore, the TNF-α FASTA sequences of Tilapia species in this study were aligned using the Bioedit tool to identify any point of variations between the two species of interest, which may account for differences in the expression of the proinflammatory cytokine.

Only African Cichlids were considered in the BLAST search hits in this study. This was because enhancing the immune regulatory system of Tilapia species from this specific region is required.

Cichlids with significant sequence similarity to TNF-α of Species in this study were downloaded and subsequently used for a comparative expression study.

Results

RNA/cDNA Quantification and TNF-α alpha Amplification

The concentration and absorbance values of RNA isolates in this study (Table 2) had a range of 5.9– 93.8 ng/ul and 1.70– 2.88, respectively, meeting the optimum concentrations that are required for downstream. The quantified cDNA gel capture (Figure 1) shows GAPDH PCR amplicons on 2 % agarose gel electrophoresis. Because all amplification products had a sole specific DNA band without apparent non-specific amplification, this confirms that cDNA synthesis was positive. Subsequently, TNF-α was amplified from cDNA with the expected band size of 800 bp compared to the DNA ladder with 50 bp and 800 bp markers, as shown in Figure 2. TNF-α expression in the tilapia species studied was confirmed because

of the single specific DNA band of all the PCR amplificons generated without a non-specific amplification.

Table 2. The concentration and absorbance values of RNA isolates

Sample	Concentration (ng/ul)	A260/280	Factor
1	16.5	2.88	40
2	93.8	1.91	40
3	12.1	1.70	40
4	27.2	1.95	40
5	15.8	1.89	40
6	5.9	1.84	40
7	47.8	1.75	40

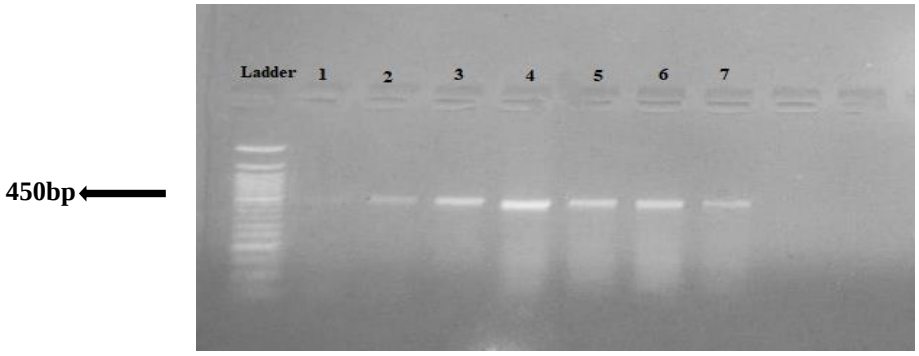

Fig 1: Gel Image capture of cDNA, quantification of samples was done in triplicate, using GAPDH, with an expected amplicons band size of 450 bp

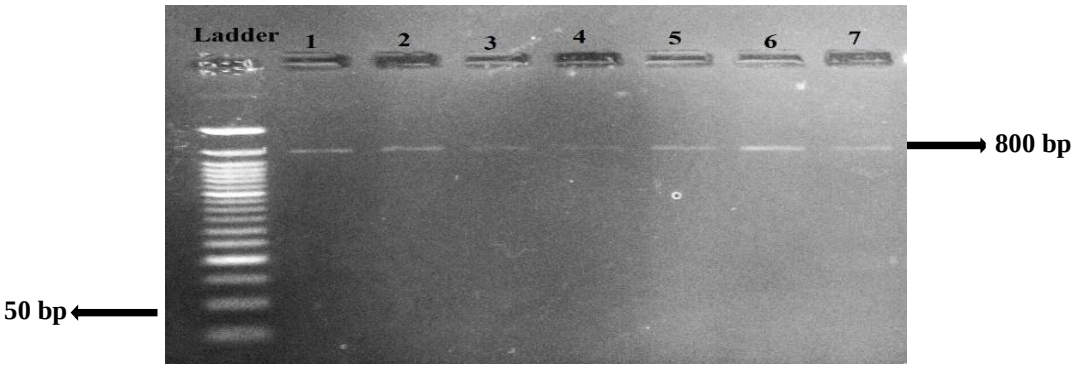

Fig 2: Gel image capture of TNF-α PCR amplicons ran on 2 % agarose gel electrophoresis, with expected bands size 800 bp. Samples were loaded in triplicate from left to right, *Sarotherodon melanotheron* (SM) and *Coptodon zillii* (CZ)

Tilapia species TNFα coding sequence

Below are the confirmed BLAST searches for the pro-inflammatory cytokine TNFα using the *Coptodon zillii* and *Sarotherodon melanotheron* sequences. The sequences with a nucleotide sequence of 566 bp were exported from the chromatogram sequencing result. The confirmed sequences are similar to other TNF sequences deposited in the NCBI database.

>*Coptodon* ~~Tilapia~~ zillii

CGAGAAGAAATCACCCGCAGAGTGGATATGGAA
GGTGTGCGCCGTCCTCGTCATCGTGGCTCTTTG
TTTAGCAGGTGTCCTGCTGTTTGCCTGGTACTG
GAATACAAGGCCAGAAAGGAGACACAATTAGGA
CAGCCAGAAGCACTAAAGGCGAAGAACACTGGC
GACAAAACAGAGCCCCACTCCACGCTAAAACGG
ATCAGCAGCAAAGCCAAGGCAGCCATCCATCTA
GAAGGTGAGTCACCGCTTCCCTTGATTTGGTCC
TCAGCAAACAATAGGCTCATAATCTCGGCCTTCA
GAGGAGCTTTTCTGAGTTTTTCTTCTCCCTCTGT
GGTATTGGTAGCTCTCTTCTTTTTTGAAACTAAG
AGCGCTATAAGAAAGTCCCATAAGTTTACTGGCT
TGTCAATACTTCATTCTGCTCTCCAAATAACAAA
CCTGTCAGTAATTCACACTCAGGTATTTCAAGGC
TGTAACGTTTCTGATCTTTACTTGCAGGCAGGAC
TCAAAAGGTCATCTGGAGTGGAGGGATGGTCAA
GGCCAGGCGTTCACTCAGGGGAGGCCAACA

>*Sarotherodon melanotheron*

TGAGAAGATCTCACCCGCAGAGCACATATGGAA
GGTGCGCCCCCTCCTCGTCATCGTGTCTCTCTG
TTTCACAGGTGTCCCCCTGTCCGCCCGGTTCCG
GTACACGAGCACAGAGAGGAGACACAATTAGGA
CAGCCAGAAGCACTAAAGGCGAAGAACACTGGC
GACAAAACAGAGCCCCACTCCACGCTAAAACGG
ATCAGCAGCAAAGCCAAGGCAGCCATCCATCTA
GAAGGTGAGTCACCGCTTACCTTGATTTGGTCC
TCAGCAAACAATAGGCTCATAATCTCGGCCTTCA
GAGGAGCTTTTCTGAGTTTTTCTTCTCCCTCTGT
GGTATTGGTAGCTCTCTTCTTTTTTGAAACTAAG
AGCGCTATAAGAAAGTCCCATAAGTTTACTGGCT
TGTCAATACTTCATTCTGCTCTCCAAATAACAAA
CCTGTCAGTAATTCACACTCAGGTATTTCAAGGC
TGTAACGTTTCTGATCTTTACTTGCAGGCAGGAC
TCAAAAGGTCATCTGGAGTGGAGGGATGGTCAA
GGCCAGGCGTTCACTCAGGGGAGGCCAACA

Nucleotide Sequence Pairwise Sequence Alignment

Pairwise alignment of TNF-α sequences analyzed using the BioEdit sequence alignment editor tool elucidated the point of sequence divergence (SNPs) between CZ and SM, highlighted in bold and underlined. This study confirmed 21 coding sequence sequences (SNPs) between the two tilapia species (Table 3).

```
          ....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
            5        15        25        35
45        55
TZ          CGAGAAGAAA TCACCCGCAG
AGTGGATATG GAAGGTG**T**GC **G**CC**G**TCCTCG
TCATCGTG**G**C
SM          TGAGAAGATC TCACCCGCAG
AGCACATATG GAAGGTG**C**GC **C**CC**C**TCCTCG
TCATCGTG**T**C

          ....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
            65        75        85        95
105       115
TZ          TCT**T**TGTTT**A** **G**CAGGTGTCC
**TG**CTGT**TT**GC C**T**GGT**A**C**T**GG **A**A**T**AC**A**AG**GC**
CAGA**A**AGGAG
SM          TCT**C**TGTTT**C** **A**CAGGTGTCC
**CC**CTGT**CC**GC C**C**GGT**T**C**C**GG **T**A**C**AC**G**AG**CA**
CAGA**G**AGGAG

          ....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
            125       135       145       155
165       175
TZ          ACACAATTAG GACAGCCAGA
AGCACTAAAG GCGAAGAACA CTGGCGACAA
AACAGAGCCC
SM          ACACAATTAG GACAGCCAGA
AGCACTAAAG GCGAAGAACA CTGGCGACAA
AACAGAGCCC

          ....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
            185       195       205       215
225       235
TZ          CACTCCACGC TAAAACGGAT
CAGCAGCAAA GCCAAGGCAG CCATCCATCT
AGAAGGTGAG
SM          CACTCCACGC TAAAACGGAT
CAGCAGCAAA GCCAAGGCAG CCATCCATCT
AGAAGGTGAG

          ....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
            245       255       265       275
285       295
TZ          TCACCGCTT**C** CCTTGATTTG
GTCCTCAGCA AACAATAGGC TCATAATCTC
GGCCTTCAGA
SM          TCACCGCTT**A** CCTTGATTTG
GTCCTCAGCA AACAATAGGC TCATAATCTC
GGCCTTCAGA

          ....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
            305       315       325       335
345       355
TZ          GGAGCTTTTC TGAGTTTTTC
TTCTCCCTCT GTGGTATTGG TAGCTCTCTT
CTTTTTTGAA
SM          GGAGCTTTTC TGAGTTTTTC
TTCTCCCTCT GTGGTATTGG TAGCTCTCTT
CTTTTTTGAA
```

....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
365 375 385 395
405 415
TZ ACTAAGAGCG CTATAAGAAA
GTCCCATAAG TTTACTGGCT TGTCAATACT
TCATTCTGCT
SM ACTAAGAGCG CTATAAGAAA
GTCCCATAAG TTTACTGGCT TGTCAATACT
TCATTCTGCT

....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
425 435 445 455
465 475
TZ CTCCAAATAA CAAACCTGTC
AGTAATTCAC ACTCAGGTAT TTCAAGGCTG
TAACGTTTCT
SM CTCCAAATAA CAAACCTGTC
AGTAATTCAC ACTCAGGTAT TTCAAGGCTG
TAACGTTTCT

....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
485 495 505 515
525 535
TZ GATCTTTACT TGCAGGCAGG
ACTCAAAAGG TCATCTGGAG TGGAGGGATG
GTCAAGGCCA
SM GATCTTTACT TGCAGGCAGG
ACTCAAAAGG TCATCTGGAG TGGAGGGATG
GTCAAGGCCA

....|....| ....|....| ....|
545 555 565
TZ GGCGTTCACT CAGGGGAGGC
CAACA
SM GGCGTTCACT CAGGGGAGGC
CAACA

Table 3. Location of TNF-α coding sequence variation among two Tilapia species

Sample	Locations of sequence variation																			
	38	41	44	59	64	70	71	81	82	87	88	92	96	98	101	103	106	109	110	150
TZ	T	G	G	G	T	A	G	T	G	T	T	T	A	T	A	T	A	G	C	A	C
SM	C	C	C	T	C	C	A	C	C	C	C	C	T	C	T	C	G	C	A	G	A

Nucleotide Sequence Translation and Pairwise Alignment

The ExPASY translation tool (Singewar *et al.*, 2020) translated the TNF-α nucleotide sequence, which is 565 base pairs, resulting in 179 varied protein sequences from both tilapia species, inferring the presence of non-synonymous sequence variation. The TNF-α protein sequences are indicated in amino acid pairwise alignment, which confirmed the protein sequence variation between the two tilapia species arising from the non-synonymous sequence variation. The regions of sequence variations between the two species are indicated in Table 4.

....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
5 15 25 35
45 55
TZ MEG**VRR**PRHR **G**SLF**S**RCPAV
CLVL**EY**K**A**RK ETQLGQPEAL KAKNTGDKTE
PHSTLKRISS
SM MEG**APP**PRHR **V**SLF**H**RCPPV
RPVP**VH**E**H**RE ETQLGQPEAL KAKNTGDKTE
PHSTLKRISS

....|....| ....|....| ....|....| ....|....|
....|....| ....|....|
65 75 85 95
105 115
TZ KAKAAIHLEG ESPL**P**LIWSS
ANNRLIISAF RGAFLSFSSP SVVLVALFFF
ETKSAIRKSH
SM KAKAAIHLEG ESPL**T**LIWSS
ANNRLIISAF RGAFLSFSSP SVVLVALFFF
ETKSAIRKSH

....|....| ....|....| ....|....| ....|....|
....|....| ....|....
125 135 145 155
165 175

TZ KFTGLSILHS ALQITNLSVI HTQVFQGCNV SDLYLQAGLK RSSGVEGWSR PGVHSGEAN

SM KFTGLSILHS ALQITNLSVI HTQVFQGCNV SDLYLQAGLK RSSGVEGWSR PGVHSGEAN

Table 4. Location of TNF-α Protein sequence variation among Tilapia species

	locations of sequence variation									
Sample	**4**	**5**	**6**		**11**	**15**	**19**	**21**	**22**	
	24	**25**	**28**	**75**						
TZ	V	R	G			C	L	E	P	
		R	S		A	L	A			
SM	A	P	V			R	P	V	T	
		P	H		P	P	H			

TNF-α Expression Study

A comparative TNF-α expression pattern among common African cichlids with significant sequence similarity with tilapia species used in this study elucidated diverse expression patterns, as shown in the phylogenetic analysis in Figure 3. In this study, *Sarotherodon melanotheron* and *Coptodon zillii* species clustered from other common African cichlids seen in node 1, and the two common tilapia species used globally clustered in node 2 from other cichlids.

All species in nodes have similar TNF-α expression patterns.

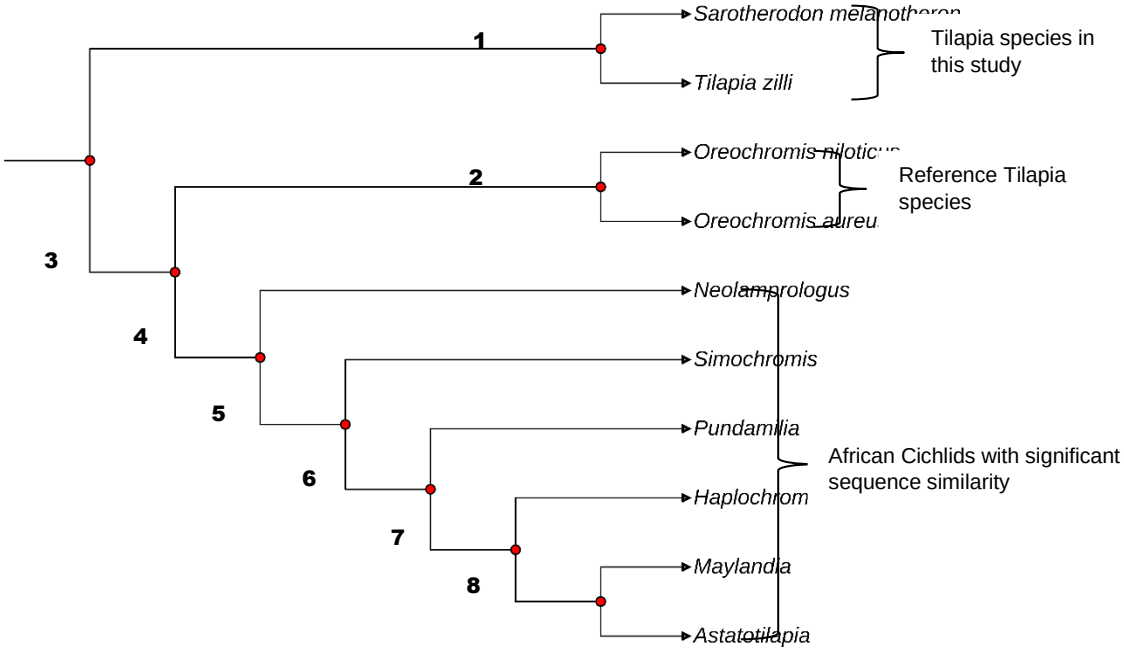

Fig 3: Phylogeny of African Cichlids TNF- α having significant sequence similarity with Tilapia species.

Discussion

Sarotherodon melanotheron and *Coptodon zillii* are dominant freshwater cichlids with varying physiological features of interest endemic to Africa. The pro-inflammatory cytokine TNF-α expressed by both species in this study signified that the innate immunity of both species was active for immunologic response to environmental pathogens, confirming the earlier submission of Ambika and Kaliyamurthi (2019).

The sequence variations recorded among these two species could be said to have confirmed the previous studies of Saeij *et al*. (2003) and Savan and Sakai (2004), where high sequence homology among species has varying gene expression patterns, as seen in

these tilapia species. Their TNF-α gene has a high sequence identity but with varying expression patterns. This could be attributed to the disparity in the coding sequence of the species TNF-α with single nucleotide base variation at 21 locations. The varying TNF-α expression observed in this study among the two tilapia species confirmed the effect of genetic variation on their immunologic response to environmental pathogens, which varies among these species, as earlier reported by Peterson *et al*. (2012) and Zhang *et al*. (2019). These authors emphasized the significance of variations in SNP among tilapia species, as reported in their study, where one species could have a physiological innate immune response advantage over the other (Tarkan, 2022). The varying TNF-α expression, as shown by the pairwise sequence alignment of CZ (*Coptodon zillii*) and SM (*Sarotherodon melanotheron*) in this study with 21 regions of the sequence, had significant effects in their protein TNF-α primary sequences, which will, in turn, have an effect in their protein structure as well as pro-inflammatory responses.

TNF-α expression, as seen from the expression study of the TNF-α protein sequence of SM and CZ with a similar increase in transcript level, shows that the two environments are probably laden with contaminants that elicited an immune response like that previously expressed in rainbow trout during the early phases of infection with *I. multifiliis* (Sigh *et al*., 2004)

Coptodon zillii is regarded as a highly tolerant species among several other known Tilapia species, with its ability to survive and thrive well in environmentally challenged environments (Tarkan, 2022). This study, therefore, used its innate immunity gene (TNFα) to confirm one of the possible genetic bases of its varying tolerance to different environments and water courses by pairing with another species (*Sarotherodon melanotheron*) that has a similar physiological and economic feature of interest in Nigeria. The variation in the amino acid sequences of the two species is an indication of varying expression patterns. This variation justifies the crucial role of cytokines and tumor necrosis factors in combating stress-induced activities in their respective environments. This is similar to the observed cytokine-based immunoreactivity in *Cyprinus carpio* (Baloch *et al*., 2022).

Therefore, these two species' suspected immune tolerance ability makes it feasible to consider them for aquaculture within the studied area.

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