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Evaluation of Genetic Variation in Three Tilapias of Oreochromis Genus in the South Zone of Nigeria Using Mitochondrial DNA Hypervariable Region

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Abstract

The understanding of genetic variation within and between species is the preliminary requirement for proper selection and breed improvement. This research aimed to evaluate the genetic variation in three species of tilapia (Oreochromis niloticus, Oreochromis aureus and Oreochromis mossambicus) in some rivers of South-South, Nigeria. A total of 300 samples of tilapia across the three species were used for this research. Blood samples were collected from all the fishes for DNA extraction, amplification and sequencing of the mitochondrial (mt) control region. From mt DNA, O. aureus had the highest polymorphism with 225 polymorphic sites followed by O. niloticus and O. mossambicus with 129 and 84 polymorphic sites, respectively. Haplotype numbers were five in O. niloticus and three in both O. aureus and O. mossambicus. The highest haplotype diversity was recorded in O. niloticus (0.796), while the highest nucleotide diversity was recorded in O. aureus (0.139). The highest genetic distance was between O. aureus and O. mossambicus (0.388) while the lowest genetic distance was between O. niloticus and O. mossambicus (0.217). Selective breeding of tilapia fish from Itu, Ethiope and New Calabar Rivers that showed high variation will enhance tilapia production in Nigeria.

Significance

Considering the upsurge in human population especially in Africa, the rate of food insecurity remains at a high. Cheaper and readily available food sources such as tilapia are advocated to mitigate food insecurity. Genetic diversity studies can help to fully utilize this fish as a sustainable food source. Information from such studies can be utilized in breeding programmes for genetic improvement. Tilapia from Itu, Ethiope and New Calabar Rivers showed more genetic variation in all the parameters considered. By sourcing broodstock from these populations, breeders can enhance genetic improvement efforts and contribute significantly to overall food security.

Introduction

In Sub-Saharan Africa, diet shift, economic uncertainty, insecurity and population growth have placed a higher demand for fish, which has outstripped supply. There are more than 200 million people in Africa who eat fish regularly as a source of protein, mineral and micronutrients [1] and there are about 20 African countries where fish accounts for more than 20% of animal protein supplies [2]. Globally, there has been a recent shift in diet toward animal protein sources such as meat and fish [3-4]. Population growth, accessibility, affordability and associated health benefits derived from fish consumption are key factors for their high demand [5-6].

The production rate of fish in Africa has been very slow despite its increasing demands [7-9]. Indiscriminate fishing, lack of effective management, water and land abuse have caused a decline in African fish stock [1] and their important species may soon face genetic erosion. In recent times, scholars have raised concern about the need to utilize a data-driven approach for the characterization and measurement of aquatic food systems to enhance short and long-term sustainability [10-13] Information from such robust data will play a crucial part in decision-making [14], as well as support investments in aquabusiness [15-16] which can result in a sustainable, equitable, inclusive and resilient food system [17]. Given this, there is an urgent need for the government, policymakers and research institutions to put measures on the ground to explore, exploit and

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conserve fish in Nigeria. One of the most sought-after fishes is tilapia due to its good aquaculture characteristics. Tilapia fish farming is becoming more popular as demand has continually increased [18]. Tilapia is the second most farmed fish in the world after carp [18-19]. In countries such as China, Egypt, Philippines, Brazil, Thailand and Bangladesh where they are commercially produced, tilapia has contributed substantially to food security [18]. Aquaculture production of tilapia in the world is estimated at 4.2 million tonnes with an estimated monetary value of 3.5 billion dollars [18]. The fact that tilapia fish feed on omnivores' diets and simple mode of reproduction makes tilapia farming the easiest and the most profitable fish farm. In 2018, the total sales of tilapia were estimated to reach 12 billion dollars [20]. The largest global production of tilapia is from China with 1,800,000 tonnes followed by Indonesia with 1,200,000 tonnes and Egypt with 1,000,000 tonnes [17,20,21]. Other countries that have made significant contributions in the production of tilapia fish are Thailand, Bangladesh, Brazil, Vietnam, Myanmar, Mexico, Ecuador, Costa Rica, Honduras, Uganda and Kenya. The highest production in Africa is from Egypt. It has been reported that 80% of tilapia production in Africa is from Egypt while 20% is contributed by the rest of Africa [21-22]. Nigeria is lagging behind in tilapia fish production and depends almost wholly on the importation of fish with an estimated 125.38 billion naira per annum [23]. This unfortunate situation places a wake-up call on the Nigerian government, private organizations and researchers to put up reliable measures to boost fish production, which will contribute significantly to food security in Nigeria.

Understanding the genetic variation of tilapia and the different populations where they are domiciled will constitute a key advantage for selection, breeding improvement and conservation. Genetic diversity study is a key step to assessing the genetic richness in populations and it is used as an indicator for the selection of individuals with good genetic potential for breeding programmes [24]. Although it has been reported that the commonest methods to study these genetic diversities is through meristic counts and morphometric measurements [27-28], however, the advent of molecular markers has also greatly facilitated genetic diversity studies with promising results that are utilized in animal improvement and enhanced agricultural productivity [6]. Sequence variation in mitochondrial DNA has been used in related studies to discriminate tilapia species [29-32]. Therefore, unveiling the genetic diversity of tilapia species using mt D-loop will grant an in-depth understanding of tilapia stock variations which is a preliminary requisite in selection for breeding improvement and conservation.

Materials & methods

Location and sample collection

Three hundred matured tilapia [*Oreochromis niloticus* (n=100); accession number: MF385001, *Oreochromis aureus* (n=100; accession number: MF385002) and *Oreochromis mossambicus* (n= 100; accession number: MF384326)] were collected from five locations. The five locations covered the five states in the Niger Delta region of Nigeria. In each location, 20 samples per species (*O. niloticus, O. aureus* and *O. mossambicus*) were collected giving a total of 60 tilapia per state. The fish included both males and females with an average weight of 0.734 kg, but sex was not considered a factor in the research. The locations of the samples were recorded with the global positioning system (GPS). These were Itu River in Akwa Ibom State at approximately 5°12′5″N and 7°58′39″E, Anangtigha River in Cross River State at 4°54′56″N and 8°20′39″E, The New Calabar River in Ikwerre, Rivers State at 4°59′92″N and 6°53′75″E, Kpansia River in Bayelsa State at 4°56′55″N and 6°19′52″E and Ethiope River in Delta State at 5°54′25″N and 5°40′58″E.

Ethical statement

This research was conducted under the ethical approval of the Faculty of Biological Sciences Ethical Committee, University of Calabar, Nigeria. The ARRIVE guidelines for experimenting with animals were fully adopted.

DNA extraction from blood samples

Extraction of mtDNA was carried out in the Biotechnology Laboratory Unit of the Animal Science Department, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. The DNA was extracted from the whole blood of the fish using Quick-DNA MiniPrep kit from Zymo Research, USA. Beta-mercaptoethanol was added to the lysis buffer to a final solution of 500 µl per 100 ml according to the manufacturer's instruction. Lysis buffer was added to 200 µl of the blood sample in Ependorff tube in a ratio of 4: 1 (800: 200 µl). The tube was vortexed for five seconds and then allowed to stand at room temperature for ten minutes. The mixture was transferred to a Zymo-Spin column in a collection tube and centrifuged for one minute at 10000 revolutions per minute (rpm) using a Centurion Scientific microcentrifuge (Model: C2015, USA). The collection tubes with the flow through were discarded, while the Zymo-Spin column was transferred to a new collection tube. 200 µl of DNA pre-wash buffer was added to the spin column and centrifuged for one minute at 10000 rpm. After that, 500 µl of g-DNA wash buffer was added to the spin column and centrifuged at 10000 rpm for one minute. The spin column was transferred to a clean microcentrifuge tube and ≥ 50 µl of DNA elution buffer was added to the spin column. This was incubated for five minutes at room temperature and then centrifuged at top speed (15000 rpm) for 30 seconds to elute the DNA. The eluted DNA was stored at -20 °C for further analysis.

Polymerase chain reaction (PCR) amplification

PCR amplification was performed in STABVIDA Laboratory, Quinta de Torre, Portugal. The primers Marinefish_Dloop_Thr_F (5'- AGCACCGGTCTTGTAAACCG- 3') and Marinefish_Dloop_Phe_R (3'- GGGCTCATCTTAACATCTTCA- 5') for D-loop region was used. PCR for each mtDNA region was carried out using 15 µl reaction volume containing 2 µl genomic DNA, 8.6 µl ddH2O, 0.5 µl $MgCl_2$, 1.5 µl of dNTPs, 1.5 µl of 10xPCR buffer, 0.37 µl of each forward and reverse primers and 0.15 µl of STABVIDA proprietary Taq polymerase. This was performed using the GeneAmp® PCR system (9700 thermal cycler, USA) with the cycling condition of the initial denaturation step at 95 °C for five minutes, followed by 25 cycles of denaturation at 94 °C for 40 seconds, annealing at 54 °C for 45 seconds, extension at 72 °C for one minute and final extension at 72 °C for seven minutes. PCR products were purified using exofast protocol according to the manufacturer.

Sequencing of D-loop

D-loop of the mtDNA was sequenced for all tissue-DNA extracts using the primers Forward Marinefish_Dloop_Thr_F (5'- AGCACCGGTCTTGTAAACCG- 3') and Marinefish_Dloop_Phe_R (3'- GGGCTCATCTTAACATCTTCA- 5'). The sequencing reaction was performed in STABVIDA Laboratory,

Quinta de Torre, Portugal with AB13730×L sequencer using 20 µl reaction comprising approximately 20 ng of purified PCR product as template DNA, 8 µl of Big Dye Terminator Reaction Mix (dNTPs, ddNTPs, buffer, enzyme and $MgCl_2$), 8 µl of deionized water, 2 µl of primer programmed as 25 cycles at 96°C for 10 seconds, 60 °C for five seconds and 60°C for four minutes.

Statistical analysis of mitochondrial D-loop

ChromasPro version 2.6.6 and Bioedit were used to view and edit the sequences. MEGA 6.06 was used for multiple sequence alignment of all the samples.[33] Estimation of polymorphism in the aligned regions including nucleotide diversity (π) and haplotype diversity (Hd) values were carried out using DnaSP 5.1 software [34]. The genetic distance within and between species was performed using MEGA 6.06.[33] Assessment of natural selection on each codon in the different species of tilapia fish was determined using the HyPhy method on MEGA 6.06 [33]. CodonCode Aligner version 6.06 was used to analyze the mutation of SNPs in the aligned sequences.

Results

Genetic variation

The mitochondrial DNA polymorphism of the three species of tilapia used in the study is presented in Table 1. Comparative assessment of the three species showed that the highest polymorphism was recorded in *O. aureus* with 225 polymorphic sites followed by *O. niloticus* and *O. mossambicus* with 129 and 84 polymorphic sites, respectively. The number of haplotypes were five in *O. niloticus,* three each in *O. aureus* and *O. mossambicus*. Haplotype diversity was highest in *O. niloticus* (0.796 ± 0.001), followed by *O. mossambicus* (0.703 ± 0.004) and *O. aureus* (0.692 ± 0.002), while nucleotide diversity was highest in *O. aureus* (0.139 ± 0.001) followed by *O. niloticus* (0.058 ± 0.00012) and *O. mossambicus* (0.052 ± 0.0002). Sequence conservation was highest among *O. mossambicus* with 87% and lowest among *O. aureus* with 72.6%. Table 2 is the result of the mt polymorphism between the three tilapia species within each location. The polymorphic site was highest

Table 1. *Variation in mitochondrial DNA among three species of tilapia*

Polymorphism indices	*O. niloticus*	*O. aureus*	*O. mossambicus*
Number of sequences (NSQ)	100	100	100
Number of sites (NS)	813	807	776
Monomorphic sites (MNS)	684	582	692
Polymorphic sites (PS)	129	225	84
Singleton variable sites (SVS)	08	14	10
Parsimony information sites (PIS)	121	211	74
Number of haplotype (NH)	5	3	3
Haplotype (gene) diversity (Hd)	0.796 ± 0.001	0.692 ± 0.002	0.703 ± 0.004
Nucleotide diversity (Nu)	0.058 ± 0.00012	0.139 ± 0.001	0.052 ± 0.0002
Average number of nucleotide difference (ANND)	42.35	112.830	40.527
Sequence conservation (SC)	0.839 (83.9%)	0.726 (72.6%)	0.870 (87.0%)
Minimum number of recombination (MNR)	6	0	0

Table 2. *Variation in mitochondrial DNA between species of tilapia from five rivers of South South, Nigeria*

		Polymorphism Indices											
Locations	**Species**	**NSQ**	**NS**	**MNS**	**PS**	**SVS**	**PIS**	**NH**	**Hd**	**Nu**	**ANND**	**SC**	**MNR**
ITU-RV	O.n	20	825	759	66	66	0	2	0.333 ± 0.046	0.027 ± 0.0003	22	0.920 (92.0%)	0
	O.a	20	807	582	225	215	10	3	0.700 ± 0.048	0.119 ± 0.004	96.200	0.727 (72.7%)	0
	O.m	20	823	776	47	47	0	2	0.500 ± 0.270	0.029 ± 0.0003	23.500	0.943 (94.3%)	0
ANT-RV	O.n	20	818	766	52	52	0	2	0.167 ± 0.0018	0.0011 ± 0.00008	8.667	0.937 (93.7%)	0
	O.a	20	807	582	225	200	25	3	0.833 ± 0.049	0.150 ± 0.007	121.333	0.726 (72.6%)	0
	O.m	20	816	811	5	5	0	2	1.00 ± 0.250	0.006 ± 0.00002	5.00	0.994 (99.4%)	0
IKW-RV	O.n	20	814	723	91	91	0	2	0.222 ± 0.028	0.0248 ± 0.00031	20.222	0.889 (88.9%)	0
	O.a	20	815	715	100	100	0	2	0.400 ± 10.056	0.049 ± 0.0001	40.00	0.878 (87.8%)	0
	O.m	20	778	749	29	09	0	2	0.500 ± 0.070	0.019 ± 0.0001	14.50	0.998 (99.8%)	0
KPN-RV	O.n	20	882	805	17	17	0	2	0.167 ± 0.018	0.0035 ± 0.00001	2.833	0.979 (97.9%)	0
	O.a	20	808	605	200	200	0	2	0.50 ± 0.070	0.124 ± 0.005	100	0.757 (75.7%)	0
	O.m	20	776	692	84	84	0	3	1.00 ± 0.074	0.076 ± 0.002	59.333	0.870 (87.0%)	0
ETH-RV	O.n	20	820	769	51	51	0	2	0.400 ± 0.056	0.025 ± 0.0002	20.40	0.938 (93.8%)	0
	O.a	20	808	592	216	216	0	2	0.400 ±0.056	0.107 ± 0.004	86.400	0.738 (73.8%)	0
	O.m	20	778	745	33	33	0	2	0.500 ± 0.070	0.021 ± 0.0002	16.500	0.958 (95.8%)	0

Table 3. *Genetic distance between three species of tilapia*

Species	*O. niloticus*	*O. aureus*	*O. mossambicus*
O. niloticus	0	0.294	0.217
O. aureus	0.294	0	0.388
O. mossambicus	0.217	0.388	0

in *O. aureus* across the five locations followed by *O. niloticus* and *O. mossambicus* except in Kpansia River where mtDNA polymorphism was in the order *O. aureus* > *O. mossambicus* > *O. niloticus*.

Genetic distance

The genetic distance between the three species of tilapia fish is presented in Table 3. The highest genetic distance was observed between *O. aureus* and *O. mossambicus* (0.388), while the species with the closest genetic distance were *O. niloticus* and *O. mossambicus* (0.217). Table 4 shows the genetic distance among the three tilapia species within and between the different study locations. The highest genetic distance was observed between *O. niloticus* from Kpansia River and *O. aureus* from New Calabar River (0.509). Generally, higher genetic distance values were observed between *O. aureus* and all species in the different locations. The lowest genetic distance values were recorded within species between locations. For instance, *O. aureus* between Itu and Ethiope River was 0.000. Similar results were obtained between *O. aureus* from Anangtigha, Itu and Ethiope rivers. The within-species genetic distance between *O. mossambicus* from Ikwere and Ethiope Rivers; Anangtigha and Kpansia rivers was also 0.000. Thus, the genetic distance was higher between species than within species, irrespective of location.

Table 4. *Genetic distance among three species of tilapia within and between five rivers of South-South, Nigeria*

Species and location	O. n ETH-RV	O. n KPN-RV	O. n ITU-RV	O. n IKW-RV	O. n ANT-RV	O. a ETH-RV	O. a KPN-RV	O. a ITU-RV	O. a IKW-RV	O. a ANT-RV	O. m ETH-RV	O. m KPN-RV	O. m ITU-RV	O. m IKW-RV	O. m ANT-RV
O. n KPN-RV	0.105	0													
O. n ITU-RV	0.079	0.153	0												
O. n IKW-RV	0.023	0.107	0.070	0											
O. n ANT-RV	0.018	0.101	0.068	0.021	0										
O. a ETH-RV	0.176	0.242	0.204	0.177	0.169	0									
O. a KPN-RV	0.133	0.204	0.182	0.124	0.131	0.064	0								
O. a ITU-RV	0.176	0.242	0.204	0.177	0.169	0.000	0.064	0							
O. a IKW-RV	0.452	0.509	0.464	0.453	0.453	0.356	0.328	0.356	0						
O. a ANT-RV	0.176	0.242	0.204	0.177	0.169	0.000	0.064	0.000	0.356	0					
O. m ETH-RV	0.156	0.240	0.212	0.157	0.151	0.208	0.171	0.208	0.482	0.208	0				
O. m KPN-RV	0.177	0.248	0.238	0.176	0.166	0.223	0.180	0.223	0.491	0.223	0.043	0			
O. m ITU-RV	0.226	0.299	0.279	0.224	0.219	0.282	0.231	0.282	0.553	0.282	0.098	0.096	0		
O. m IKW-RV	0.156	0.240	0.212	0.157	0.151	0.208	0.171	0.208	0.482	0.208	0.000	0.043	0.098	0	
O. m ANT-RV	0.177	0.248	0.238	0.176	0.166	0.223	0.180	0.223	0.491	0.223	0.043	0.000	0.096	0.043	0

ITU-RV (Itu River); ANT-RV (Anangtigha River); IKW-RV (New Calabar River); KPN-RV (Kpansia River); ETH-RV (Ethiope River)
O.n (Oreochromis niloticus); O.a (Oreochromis aureus); O.m (Oreochromis mossambicus)

Table 5. *Selection pressure in three species of tilapia*

Species	Selective types	dN	dS	dN-dS	Site index	p-value
O. niloticus	Positive	25.985	0	25.985	48	0.04
	Negative	6.829	33.871	-27.042	33	0.02
	Neutral	0	0	0	149	N.A
O. aureus	Positive	46.166	4.666	41.5	83	0.05
	Negative	14.626	58.054	-43.428	56	0.05
	Neutral	0	0	0	108	N.A
O. mossambicus	Positive	13.598	0	13.598	45	0.02
	Negative	4.129	44.028	-39.899	22	0.05
	Neutral	0	0	0	174	N.A

NA (Not available); dN (Non-synonymous); dS (Synonymous)

Table 6. *Selection pressure of tilapia from five rivers of South-South, Nigeria*

Locations	Selection types	dN	dS	dN-dS	Site index	P-value
ITU –RV	Positive	73.122	6.383	66.739	100	0.0101
	Negative	29.852	96.214	-66.362	81	0.0101
	Neutral	0	0	0	50	N.A
ANT-RV	Positive	61.119	7.181	53.938	95	0.01
	Negative	26.357	107.179	80.822	84	0.002
	Neutral	0	0	0	57	N/A
IKW-RV	Positive	57.999	7.993	50.006	98	0.606
	Negative	17.791	77.722	59.931	70	0.007
	Neutral	0	0	0	56	N.A
KPN-RV	Positive	68.768	12.859	55.909	90	0.002
	Negative	32.202	114.598	82.396	88	0.002
	Neutral	0	0	0	43	N.A
ETH-RV	Positive	63.139	3.617	59.522	86	0.005
	Negative	33.609	112.603	78.994	92	0.005
	Neutral	0	0	0	43	N.A

NA (Not available); dN (Non-synonymous); dS (Synonymous

Table 7. *Single nucleotide polymorphisms (SNPs) variation in three species of tilapia fish from five rivers of South-South, Nigeria*

SNP	*O. niloticus* Amino Acid change	ds/ dN	Transver-sion/ Transition	SNP	O. aureus Amino Acid change	ds/ dN	Transver-sion/ Transition	SNP	O. mos-sambicus Amino Acid change	ds/ dN	Transversion/ Transition
1T>C	STP1Gln	dN	Transition	1T>C	STP1Gln	dN	Transition	1T>A	STP1Lys	dN	Transversion
7A>T	Lys3STP	dN	Transversion	3A>G	STP1del	dN	Transition	7A>C	Arg3Arg	dS	Transversion
13T>C	Phe5Leu	dN	Transition	4T>A	STP2Lys	dN	Transversion	18A>T	STP6Tyr	dN	Transversion
21C>G	Pro7Pro	dS	Transversion	7T>G	Leu3Glu	dN	Transversion	131A>T	Lys44Ile	dN	Transversion
23T>G	Leu8Arg	dN	Transversion	8T>A	Leu3Glu	dN	Transversion	153A>C	Thr51Thr	dS	Transversion
24A>G	Leu8Leu	dS	Transition	11G>A	Gly4Glu	dN	Transition	216G>A	Lys72Lys	dS	Transition
26C>T	Pro9Leu	dN	Transition	14G>C	Ser5Thr	dN	Transversion	266C>G	Thr89Arg	dN	Transversion
37A>T	Lys13Trp	dN	Transversion	17T>G	Leu6STP	dN	Transversion	283G>A	Ala95Thr	dN	Transition
38A>G	Lys13Trp	dN	Transition	19A>C	Asn7Leu	dN	Transversion	289T>C	Leu97Leu	dS	Transition
39A>G	Lys13Trp	dN	Transition	20A>C	Asn7Thr	dN	Transversion	315T>C	Ser105Ser	dS	Transition
40G>A	Ala14Thr	dN	Transition	21T>C	Asn7Thr	dN	Transition	352G>A	Val118Ile	dN	Transition
44G>C	Arg15Thr	dN	Transversion	23C>T	Pro8Leu	dN	Transition	369G>C	Gln123His	dN	Transversion
49C>G	Leu17Val	dN	Transversion	24T>G	Pro8Leu	dN	Transversion	465G>A	Gln155Gln	dS	Transition
54T>G	Thr18Thr	dS	Transversion	25G>A	Gly9Ser	dN	Transition	472T>A	STP158Lys	dN	Transversion
83C>G	Ser28STP	dN	Transversion	26G>C	Gly9Ala	dN	Transversion	485T>C	Phe162Ser	dN	Transition
97T>G	Tyr33Asp	dN	Transversion	27C>T	Gly9Gly	dS	Transition	556A>G	Ser186Gly	dN	Transition
103T>A	Tyr35Ser	dN	Transversion	28T>C	Cys10Pro	dN	Transition	597T>G	Phe199Leu	dN	Transversion
104A>G	Tyr35Ser	dN	Transition	29G>A	Cys10Asp	dN	Transition	602T>C	Phe201Ser	dN	Transition
114A>G	Ser38Ser	dS	Transition	30T>A	Cys10STP	dN	Transversion	603C>A	Phe201Leu	dN	Transversion
118T>C	Leu40Leu	dS	Transition	32A>C	Asn11Thr	dN	Transversion	604A>C	Ile202Leu	dN	Transversion

SNP (Single nucleotide polymorphism); dN (Non-synonymous); dS (Synonymous)

Selection pressure

The result of the selection pressure among the three species of tilapia measured as positive, negative and neutral is presented in Table 5. It was revealed that the majority of the sites on mtDNA sequence of the three species were under neutral selection pressure. *O. niloticus, O. aureus* and *O. mossambicus* experienced more positive selection pressure with 48, 83 and 45 site indexes than negative selection pressure with 33, 56 and 22 site indexes. Table 6 shows selection pressure on the species based on location. It was revealed that the species were also experiencing more positive selection pressure in each location than negative pressure except in Ethiope River where a higher negative site index of 92 was obtained over a positive site index of 86.

Single nucleotide polymorphism (SNPs)

Table 7 shows single nucleotide polymorphisms (SNPs) detected on the mitochondrial sequences of the three species of tilapia. There were 129, 225 and 84 SNPs detected in *O. niloticus*, *O. aureus* and *O. mossambicus*, respectively. Out of the 129 SNPs in *O. niloticus,* 98 (76%) were non-synonymous mutations, while 31 (24%) were synonymous mutations. The 129 SNPs also resulted in 72 (55.8%) and 57 (44.2%) transversion and transition mutations, respectively. In *O. aureus,* there were 201 (89.3%) non-synonymous and 24 (10.7%) synonymous mutations from the 225 SNPs detected, which also resulted in 137 (60.9%) transversion and 88 (39.1%) transition mutations. *Oreochromis mossambicus* also had higher non-synonymous mutation than synonymous mutation [70 (83.3% and 14 (16.7%), respectively]. Transversion mutation (52= 61.9%) was also higher than transition mutation (32= 38.1%) in *O. mossambiscus.* In the three species of tilapia, it was generally observed that SNPs resulted in more non-synonymous mutations than synonymous mutations. There were also more transversion mutations than transition mutations.

Discussion

Mitochondrial DNA is one of the most utilized markers for distinguishing and characterizing organisms both at species and population levels. The major advantage of using mitochondrial DNA for genetic diversity studies over nuclear DNA is the high rate of mutation events in the region [35]. Out of the 37 genes of mtDNA, the D-loop also called the control region has the highest rate of mutation and by implication, the highest level of variation [36-37]. Due to this unique property of the mtDNA D-loop, there has been an upsurge of interest by researchers in evolutionary biology, population biology, genetics and related fields in utilizing this marker to answer important research questions relating to species and population discrimination. In fish, many previous researches indicated mtDNA D-loop to be a very reliable genetic marker for distinguishing populations as well as species [29-30,38-40]

In this study, mtDNA D-loop was utilized to evaluate variation among three species of tilapia fish. Polymorphic sites were higher in *O. aureus* indicating the possibility of higher genetic variation within the species over *O. niloticus* and *O. mossambicus*. One of the ways to measure genetic variation within and between species is through the evaluation of gene diversity also called haplotype diversity. Haplotypes are genes conserved as sequences that survive many generations of reproduction. *Oreochromis niloticus* had the highest number of haplotypes, while *O. aureus* and *O. mossambicus* had similar haplotype numbers. The implication here is that *O. niloticus* shares more conserved genes and therefore have more relatedness within the species as compared to *O. aureus* and *O. mossambiscus.* Earlier researchers reported five haplotypes in *O. niloticus* and *O. aureus* [29]. In a similar study, six haplotypes were identified in the population of *O. niloticus* in South West Nigeria, which was closely similar to the number of haplotypes identified in the present study [30]. Authors [41] also identified 13, 11, 11 and 7 haplotypes in four populations of *O. niloticus*, while *O. esculentus* was identified with five and 11 haplotypes in two populations. The findings of the present study suggest that haplotype numbers may be specific to species and the population they inhabit. It is therefore possible that variation in haplotype number between the three species of tilapia used in this study is due to differences in their mitochondrial genome with preference to mtDNA D-loop.

Haplotype and nucleotide diversity are key parameters in measuring variation in the DNA sequence of organisms. Haplotype diversity is a measure of the likelihood that two or more random sequences are different [42], while nucleotide diversity is the measure of genetic variation that is influenced by mutation rate and effective population size [43]. They are usually measured as coefficients and the more the value approaches 1, the higher the diversity estimates. Of the three species of tilapia fishes, *O. aureus* had the highest nucleotide diversity, while *O. niloticus* and *O. mossambicus* had similar nucleotide diversity. Similar observations were made among the species within each location. Thus, *O. aureus* had higher within-species variation and was more genetically distant from *O. niloticus* and *O. mossambicus.* This was in line with the results obtained from genetic distance where *O. aureus* showed a higher genetic distance from *O. niloticus* and *O. mossambicus*. There was an earlier report of nucleotide diversity of 0.237 in 20 samples of *O. niloticus* and 0.276 in 26 samples of *O. aureus* [29]. Similarly, a range of 0.001 to 0.006 diversity in nucleotide of a fragmented population of tilapia fish was reported [44]. The differences in nucleotide diversity earlier reported by researchers in similar studies and the present study are likely due to environmental variations. Evaluation of sequence conservation between the three tilapia species showed the lowest conservation in *O. aureus* sequences, indicating the major reason why *O. aureus* had higher diversity within species and was more distantly placed from *O. niloticus* and *O. mossambicus*

The degree of genetic differences between and within species in a population defined through genetic distance [45] has been used very reliably to distinguish closely related species. When species have similar alleles, they will equally have a low degree of genetic distance, while species with dissimilar alleles will have high degree of genetic distance. From the results obtained on the genetic distance estimate of the three species of tilapia, *O. aureus* was different from *O. niloticus* and *O. mossambicus*, while *O. niloticus* and *O. mossambicus* had a lower genetic distance. The implication here is that *O. aureus* from the different locations were more dissimilar to *O. nilotius* and *O. mossambicus.* This can be attributed to the higher degree of variable alleles in *O. aureus* which were reflected in the higher polymorphic sites, nucleotide diversity and low sequence conservation in their mtDNA D-loop sequences.

According to Kavembe GD, et al. [45], a good estimate of genetic distance is critical for measuring genetic differences between populations. In terms of the different locations, *O. aureus* had higher allelic differences that resulted in a higher degree of genetic distance from other locations. This variation can be utilized by fish breeders as an indicator for selection and

breeding improvement. Therefore, it may be more profitable to select *O. niloticus* to perform a cross with *O. aureus* to combine their genetic heterozygosity since the two species had more variation in mt D-loop. Following the nucleotide diversity of species within each location, *O. niloticus* from Itu River and Ethiope River had the highest diversity compared to *O. niloticus* from other locations Therefore, since genetic improvement of farm animals involves selection and breeding of heterogeneous individuals with good quality traits of interest[45] for genetic gain (improved breeding value) in subsequent generations, our findings here suggest that selection of *O. niloticus* from Ethiope River and Itu River for breeding improvement may be a more profitable venture over other locations where *O. niloticus* were sampled. However, for inter-species breeding and genetic improvement, *O. niloticus* from Itu and Ethiope Rivers and *O. aureus* from New Calabar River may present better choices considering that *O. aureus* from New Calabar River had a wider degree of genetic distance from *O. niloticus* from Itu River and Ethiope River.

There is a growing interest in research involving the quantification of selection pressure and their contribution to genetic variation in populations. In this perspective, several methods have been proposed to determine new mutations that may have a selective advantage over others in a population [46]. Positive selection also called directional selection occurs when an extreme phenotype is favoured over other phenotypes in a population [47], while negative selection or purifying selection occurs when the effects of deleterious genes are gradually removed or selected against in a population until all the associated alleles are removed [48]. Negative selection leads to a stabilizing effect in the phenotypes of a population involved. Neutral selection (balancing selection) on the other hand does not affect an organism's ability to survive and reproduce [49]. In this study, most of the site indexes of the mitochondrial sequences were under balancing or neutral selection, which may have no affiliated influence over the fitness of the tilapia species. On the contrary, the positive site index associated with positive selection pressure and influence on fitness was higher than the negative site index. This was similar to the submission of Ikpeme EV, et al. [50] who also reported higher positive selection sites in frizzled feather chicken genotype. According to Rieseberg LH, et al. [51], positive selection takes place when populations experience new environmental pressures as a result of migration from one environment to another. These pressures lead to rapid changes in allelic frequency and speciation. Thus, the higher positive selection pressure identified among the tilapia species may not be unconnected with the high level of d_N-d_S substitution rate for the positive site index. This therefore is an indication that many alleles of mtDNA in the three tilapia species are under the positive selection advantage of perpetuity, which may eventually lead to population structuring and speciation over time. It may also be an indication that certain haplotypes in the population are under selection advantage that may positively influence the adaptation of these tilapia species in the advent of environmental hazards. Negative selection pressure was also recorded among the three tilapia species but in a lower d_N-d_S substitution rate and negative site index. This was an indication that the rate of negative/purifying selection among the species may be low. As earlier reported, negative selection reduces the rate of deleterious genes in a population.[52] Thus, it is possible that the selection pressure arising from negative selection in the tilapia may act to remove the effect of deleterious mutations in their habitat. This may have a positive impact on the survivability of these species in their habitats. The negative selection pressure may be effective in mitigating the effect of deleterious homozygosity arising from genetic drift and inbreeding activities within and between the tilapia species [53]

It has been reported that the selection on mitochondrial genes may be influenced by environmental factors affecting metabolic processes which may be specific to a taxon or population [54]. A combination of sequences of the three species in each study location also showed a higher positive selection index over negative selection, indicating that the environment plays a key role in selecting alleles to maintain through positive selection pressure or alleles to remove from the population through negative selection pressure. Single nucleotide polymorphisms among the three tilapia species indicated higher non-synonymous mutations than synonymous mutations and nucleotide changes resulting in higher transversion mutations over transition mutations. Non-synonymous mutation occurs when nucleotide substitution results in the production of an entirely new amino acid. Transversion mutation occurs when a purine is substituted by a pyrimidine and vice versa. These types of mutations result in the creation of variation in populations. Synonymous mutation does not result in any amino acid change following nucleotide substitution. Transition mutation on the other hand involves the substitution of a purine by purine or a pyrimidine by pyrimidine. It is therefore apparent that the variations that were observed among the tilapia species were majorly from non-synonymous and transversion mutations.

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Conclusion

The genetic analyses conducted on the three tilapia species revealed distinct genetic variations among them, as evidenced by mt D-loop sequences. These findings support the significance of genetic diversity in informing breeding strategies aimed at enhancing tilapia populations for sustainable aquaculture and food security initiatives. We recommend the crossbreeding of *O. niloticus* from the Itu and Ethiope Rivers with heterogeneous *O. aureus* from the New Calabar River in tilapia breeding progammes for possible genetic gains in hybrids. This can potentially contribute significantly to the genetic improvement of tilapia stocks, thereby advancing the prospects of robust and resilient aquaculture systems capable of meeting the escalating demands of burgeoning human populations as well as optimization of tilapia production for the betterment of global food security agendas.

Competing interest

We have no competing interest to declare.

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Data availability

All the data supporting the results of this study are included in the article itself.

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