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Variability of Genetically Improved Farmed Tilapia (Gift Tilapia) Crossed With Upm Red Tilapia Hybrids By Mitochondrial DNA Conserved Region Sequence

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ABSTRACT

Mitochondrial DNA conserved region (mtDNA CR) segment of a cross between GIFT tilapia and UPM red tilapia, their F1-hybrid (F1) and Backcross (BcF1) was used to define their genetic variability. DNA was extracted from 25 randomly selected specimens of each of the test strains and mtDNA CR primer ORMT-F ORMT-R was used. The MEGA software using Maximum Parsimony (MP) was conducted for the evolutionary analysis of the strains using *Tilapia rendalli* (AF484717.1 accession numbers from NCBI database). A total of 441 polymorphic regions of 99 sequencing sites were observed, the sites with 12 alignment gaps were missing while the invariable monomorphic sites were 360 (81.6%) with 69 (15.6%) variable polymorphic sites. Parsimony informative sites were 68 with 1 singleton variable site. The conserved regions were effective in discriminating between the sampled fish because they contained important elements that aid replication and expression of the mitochondria genome. The clustering of the strain in the sub-tree of the phylogeny verifies a monophyletic relationship although some of the strains showed more relatedness by their consistent overlap and clustering. Similarly with 0.1 genetic variability conserve region could be used for discrimination between the strains.

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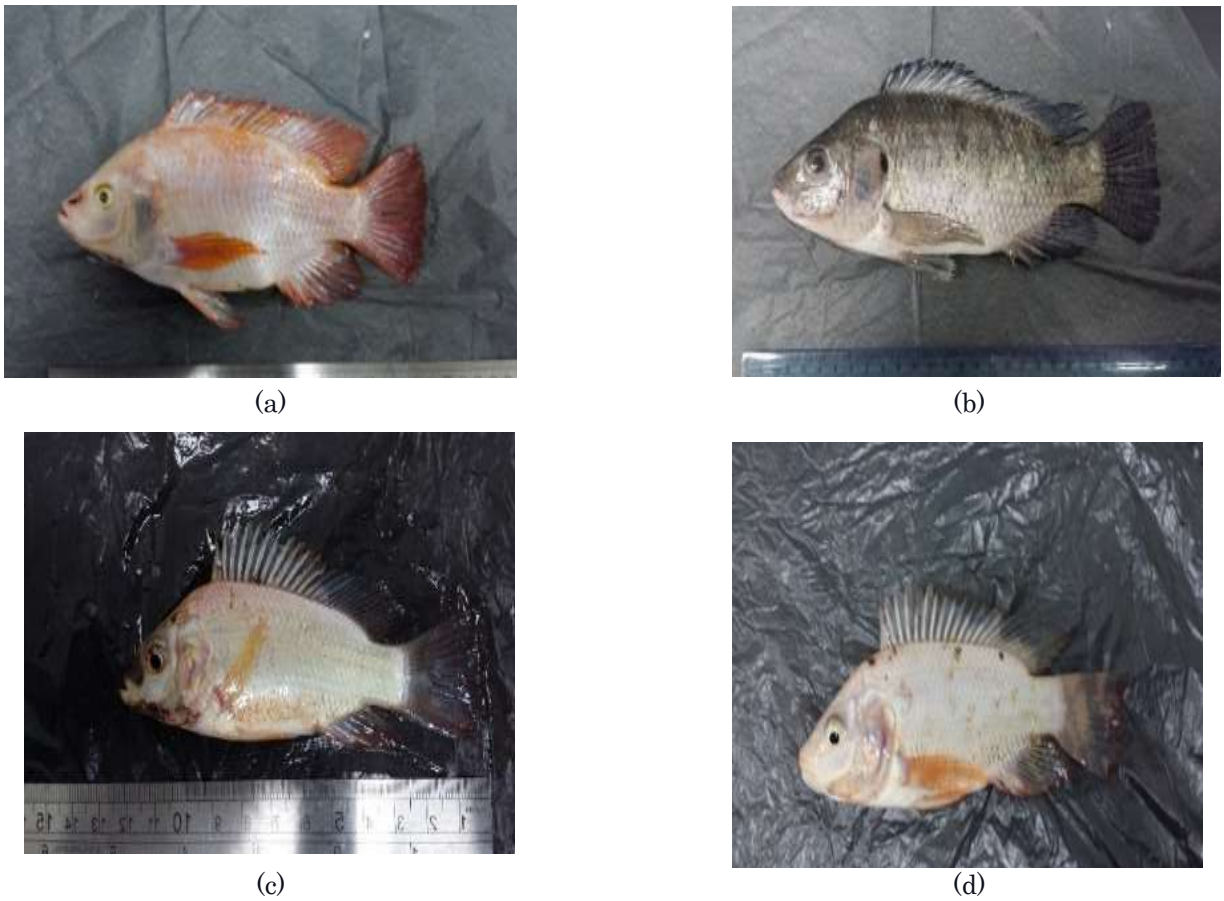
INTRODUCTION

Molecular studies of fish populations and examination of macromolecules are associated with the current development of starch gel electrophoresis with histochemical staining. This allows the detection of enzymes and a simple means of identification by the use of genetic markers. These markers provide genetic variants for population studies in fish. Genetic markers are of two types, protein and DNA. Protein markers are relatively cheaper, easy to analyze, and have a large database of different species while DNA requires a single tissue that could be preserved in ethanol for a scaly specimen, a small quantity for PCR analysis, and can detect changes in mutation that is not associated with protein electrophoretic mobility (Ferguson et al. 1995). Recent studies have shown the increasing rate at which mitochondria DNA (mtDNA) is used compared to nuclear DNA (nDNA) because of the formal high rate of mutation.

The use of mtDNA marker offers a better way for classifying related populations into strains because of its poor recombination ability and the inheritance through the maternal parent that allows placing into groups even after hybridization (Dasuki et al., 2023; Nwachi et al., 2020; Ogden 2008). Although much credit recently was given to the use of microsatellites for speciation, separate development per species due to the expensive and cumbersome transfer between species was needed when compared to the mitochondria gene that has traits that could be used to allocate strains to the parental population and could be transferred across species (Rastogi et al. 2007 & Esa et al. 2008). A report by (Brown et al. 2005 & Clayton 1982) infers that the conserved region is a parental template that is unique to the owner; it is used for H-strand synthesis and the monomeric mtDNA has a short H-strand with each conserved region attached to a molecule. This constraint made this region evolve faster than the mtDNA and a better target for any form of classification as described by Karamanlidis et al. (2016).



(a) (b)

(c) (d)

Plate 1 Strains of sampled fish (a) UPM red tilapia (b) GIFT tilapia (c) Backcross (BcF_1) and (d) Hybrid (F_1)

The sampled fish of UPM red tilapia, GIFT tilapia, F1, and BcF1 genomes (Plate 1) are the same but different at the level of DNA sequences and the protein-coding due to biological characteristics which largely depend on their amino acid content. The task of clarifying the strains based on their related taxa is connected to markers that are specific in action for intraspecific relationships. In the past, phenotypic markers (a direct means) were used to take measurements of dimension, size, and pigmentation as a means of discriminating between species. The genetic diversity method on the other hand is indirect and mostly based on markers with low inheritability even though they were genetically inherited, and the genetic basis of these markers measures genetic diversity.

Tilapia culture witnessed growth worldwide because of the increasing demand in local and `international markets and is the second most cultured fish after Carp (FAO 2009). Tilapia is also an important fish in aquaculture because of its useful model in population genetics (Chakrabarty 2006; Nagl et al. 2001; Nwachi & Esa 2016). The phylogenetic relationship among *Oreochromis niloticus*, O*reochromis mossambicus,* and *Oreochromis aureus* was studied by Esa et al (2008) using RAPD markers with the assertion that the highest genetic similarity occurred between *O. niloticus* and *O. mossambicus*. A report by Nagl et al (2001) on the mtDNA control region of *O. niloticus, O. mossambicus* and *O. aureus* was unable to explain the differences in their phylogenetic relationship although there is a relationship between the ND2 gene sequences of *O. Niloticus* and *O. mossambicus* (Klett & Meyer 2002). Studies by Betsen et al. (2016) and Ansah et al. (2014) show that Gift exhibited more potential of being productive than the normal tilapia. Hence, this study will examine the complete mtDNA conserve region of UPM red tilapia, GIFT tilapia, their hybrid (F1), and backcross (BcF1) assessing their phylogenetic and genetic relationship.

MATERIAL and METHOD

Laboratory work

The procedures for this experiment were considered and approved by the Board of Studies; Department of Aquaculture, University Putra Malaysia.

Total DNA extraction.

A total of 104 fish consisting of 26 specimens each per strain of (UPM red tilapia, GIFT tilapia, F1, and BcF1) was

used. DNA samples were extracted from the muscular part of the caudal region, at a prior preservation workstation to avoid cross contamination. The samples were properly labeled, preserved in 95 % ethanol, and stored in a -20-degree freezer for further use. Total DNA extraction from the sampled tissue was obtained with the aid of ReliaPrepTM gDNA Tissue Miniprep System (Promega, USA) according to the manufacturer's instructions. The quality and concentration of the DNA were assessed using agarose gel electrophoresis, the resulting sample was diluted to 100ng while the resulting genomic DNA was stored in a -20℃ until further analysis.

Polymerase chain reaction (PCR) amplification of mitochondria conserve region (mtDNA)

Polymerase chain reaction (PCR) for mitochondria conserve region (mtDNA CR) was carried out using a set of primers in Table 1, the total PCR reaction volume for the mtDNA CR was 25µL cocktail which includes 2× power Taq PCR master mix from Bioteke technology (Beijing China), dH20, primer (forward and reverse) and DNA template (Table 2).

Table 1. The primer sequence of ORMT-F and ORMT-R

No	Region	Sequence
1	ORMT-F (forward primer)	5'-CTAACTCCCAAAGCTAGGAATTCT-3'
2	ORMT-R (reverse primer)	5'-CTTATGCAAGCGTCGATGAAA-3'

(Gu et al. 2016)

Table 2. Reagents final volume and Concentration for PCR reactions

Reagent	Final concentration	Final volume (µl)
Power Taq PCR master mix	2×	12.5
ddH20	Mm	9.5
Forward primer	25 pmol/µl	0.5
Reverse primer	25 pmol/µl	0.5
DNA template	-	2.0
Total	-	25

The PCR conditions were adopted and involved initial denaturation at 94 °C for 3 minutes in a Thermocycler followed by 35 cycles for 94 °C for 30 seconds, 54 °C for 40 seconds, and 72 °C for 40 seconds and a final extension step of 72 °C for 10min. The negative control was used in all the reactions for contamination detection during the reaction system. The PCR products were separated on 1 % agarose gels and ran at approximately 45min at 75 V at a current of 300 amps, the size was quantified using a digested (Promega, USA) DNA ladder (GeneRulerTM50-bp DNA Ladder) with a visualized product size of 413 - 450bp expected. The products were purified using DNA Wizard© SV Gel and PCR clean-up kit (Promega, USA) according to the manufacturer's instructions. The purified products were sequenced using the BigDye® Terminator v3.1 Cycle sequencing kit (ACGT) on an ABI 377 automated sequencer (PE Applied Biosystems) using only the forward primer ORMT-F.

The multiple sequence was aligned with ClustalW in BioEdit software (Hall 2011). Sequencing reaction using the reverse primer ORMT-R was subsequently carried out on some of the samples to verify the polymorphism in the DNA sequences detected using the forward primer. The Evolutionary analysis of the strains was conducted with MEGA software as described by Tamura et al. (2013) using neighbor-joining (NJ) tree, maximum parsimony, and maximum likelihood with conserve region sequence and using *Tilapia rendalli* as an out-group (AF328854.1; GenBank accession number) was constructed with the Kimura 2 parameter distance model (Saitou & Nei 1987 and Kimura 1980). The branching order of the tree was tested by bootstrapping at 550 replicate data.

Data analysis

To validate the similarity of the current study, sequences were compared with data from the National Centre for Biotechnology Information (NCBI) database with the aid of Basic Local Alignment Search Tools (BLAST). Sequences with higher ident values were considered and the sequences were corrected manually to reduce errors of mismatch with the actual sequencing report. Sequencing was aligned with clustalW of BioEdit version 7.2.5 (Chakrabarty, 2006). The genetic distance (D) of GIFT tilapia, UPM red tilapia, hybrid (F1), and backcross (BcF1) were carried out using distance-related statistical tools in MEGA version 6.06 software. The genetic diversity index was computed using DnaSP version 5.10 including nucleotide diversity (π), haplotype diversity (H), and the average number of nucleotide differences (K).

RESULTS

Phylogenetic analysis

A total of 104 tilapia tissue samples were collected from 4 populations of parent, hybrids, and backcross. Genomic DNA was extracted from 100 tissue samples and used for PCR amplification of mtDNA CR (conserve region) out of which 99 samples were sequenced. The fluorescent-based DNA sequencing results were displayed with DnaSp (version 5) referring to (Librado & Rozas 2009). A total of 441 polymorphic regions of 99 sequencing sites were observed, the sites with alignment gaps missing were 12 while the invariable monomorphic sites were 360 (81.6%) with 69(15.6%) variable polymorphic sites. Parsimony informative sites were 68 with 1 singleton variable site.

Table 3. Distribution of observed nucleotide diversity, haplotypes and diversity and segregation sites among the samples.

Haplotype	*UPM red tilapia*	*GIFT tilapia*	*F_1-Hybrid*	*BcF_1 backcross*
1	*1.00*	-	*0.96*	*1.00*
2	-	*0.84*	-	-
3	-	*0.08*	-	-
4	-	*0.08*	-	-
5	-	-	-	-
Parameters				
Nucleotide diversity	*0.0000*	*0.0302*	*0.0003*	*0.0000*
Number of haplotypes	*1*	*3*	*2*	*1*
Nucleotide (Pijc)	*0.0000*	*0.0000*	*0.0003*	*0.0326*
Haplotype diversity	*0.000*	*0.2933*	*0.0800*	*0.080*
Number of segregation sites	*0*	*66*	*2*	*0*

In Table 3 GIFT tilapia recorded the highest number of observed haplotypes at 3 with a haplotype diversity (H) of 0.2933, a segregation sites value of 66, and a nucleotide diversity (Ω) of 0.0302. F1 recorded nucleotide diversity (Ω) of 0.0003 with 1 haplotype. A total of 2 haplotypes and 0.0000 nucleotide diversity (Ω) was recorded by UPM red tilapia. UPM tilapia, F1, and BcF1 have one haplotype in common. In Table 5 GIFT tilapia recorded 4 conserved regions at conservation values of 0.942, 0.944, 0.938, and 0.955 while UPM red tilapia, F1, and BcF1 have only 1 respectively.

The Tajima's D of UPM red tilapia was -0.3543 but none significant while F1 has -1.9617 but significant. Both GIFT tilapia and BcF1 have -1.0851 and -1.1575. The Fu's fs were -0.289 for UPM red tilapia. GIFT tilapia recorded the highest mutation rate of 82 (Table 4).

Table 4. Hierarchical Analysis of Molecular Variance showing amount of population genetics

Parameters	UPM red tilapia	GIFT tilapia	F_1	BcF_1
Population	24	25	25	25
Mutation sites	2	82	12	1
Tajima's D	-0.3543	-1.0851	-1.9617	-1.1575
P-Value	p>0.10	p>0.10	P<0.05	p>0.10
Fu's fs statistic	-0.289	-0.148	-1.035	-1.061

The pairwise population frequencies in Figures 1a to 1d reveal that there are discrepancies between the expected and observed frequency of occurrence for the sampled fish.

The backcross (BcF1) and UPM red tilapia have frequencies that correlate to the observed pairwise values at 0 - 0.8 at 20 pairwise distances and 0 - 0.6 at 20 pairwise distances while the hybrid (F1) has a slight variation between the observed and expected value (0 - 0.05 at 20). GIFT tilapia recorded a variation of 0 - 0.2 at 60 pairwise distances between the observed and expected pairwise frequency.

Phylogenetic tree

A phylogenetic tree based on mtDNA CR conserved region was constructed. The sequences from the sample fish haplotype and fish of the same family *Tilapia rendalli* (accession number AF484717.1 as obtained from NCBI database) were used as our group and a root for the tree. The evolutionary history that was inferred by the maximum parsimony (MP) method in Figure 2 reveals that 1 out of every 3 trees was parsimonious. The length observed was 86, with a consistency index of 0.870968, and a retention index of 0.870968 while the composite index and parsimony-informative sites are 0.830458 and 0.758585 respectively.

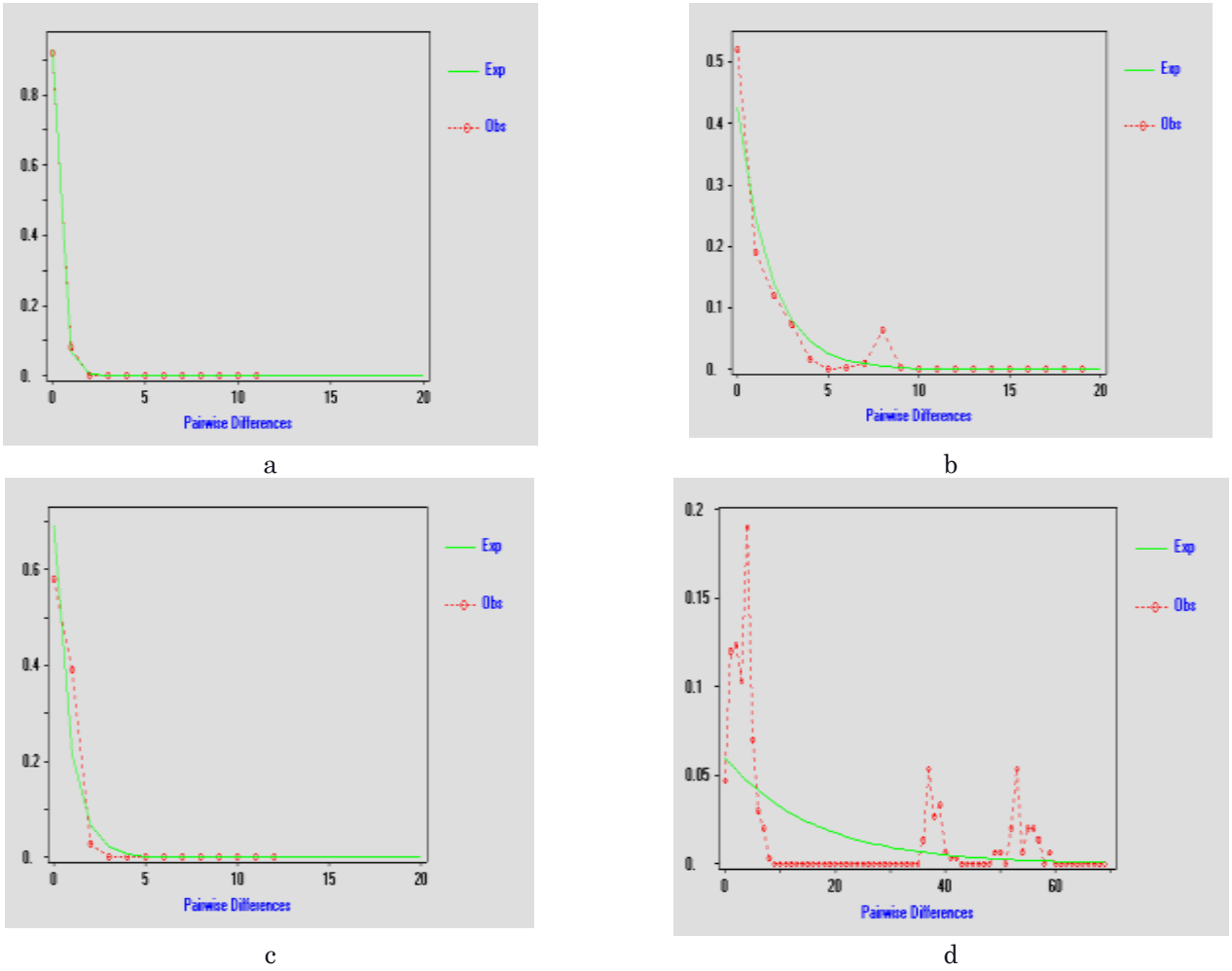


Figure 1 Pairwise distribution of sampled fish (a) (BcF_1) (b) F_1 (c) GIFT tilapia and (d) UPM red tilapia

Table 5. Conserve region of mtDNA analysis

Parameters	Region	UPM red tilapia	GIFT tilapia	F1	BcF1
Start to end	1	13-448	28-96	38-447	2-436
	2		45-98		
	3		284-380		
	4		382-448		
Conservation	1	1.00	0.942	1.00	1.00
	2		0.944		
	3		0.938		
	4		0.955		
Homozygosity	1	1.00	0.987	1.00	1.00
	2		0.989		
	3		0.985		
	4		0.993		
P-value	1	0.000	0.0051	0.000	0.000
	2		0.0127		
	3		0.0010		
	4		0.0018		

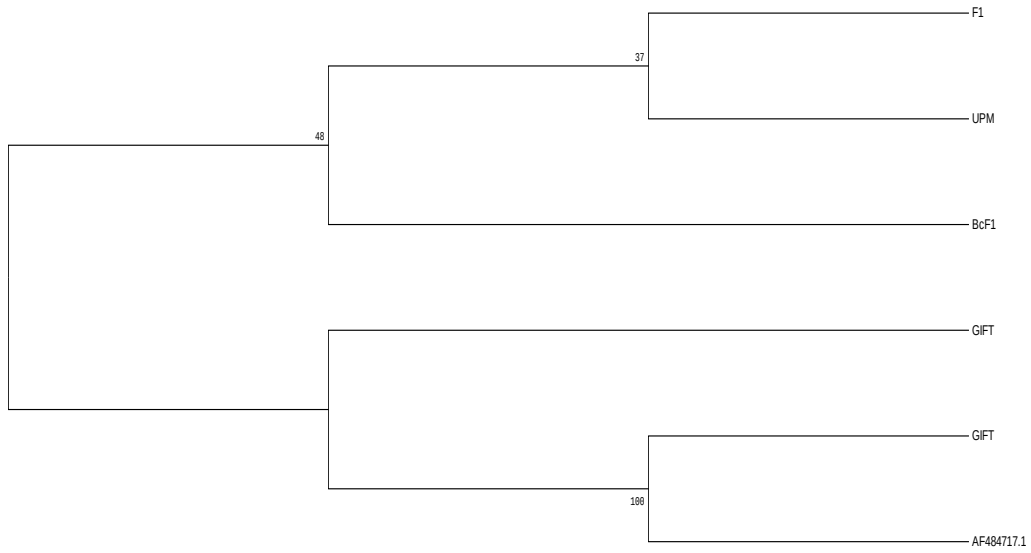


Figure 2: Maximum Parsimony (MP) tree showing relationships among Mitochondria conserve region (mtDNA) of UPM red tilapia, GIFT tilapia, F_1, and BcF_1 with Tilapia rendalli (AF484717.1) out-group. The bootstrap value (%) based on 50 pseudo replications for the MP analysis

The clustered percentage for the bootstrap test is at 1050 replicates while the MP tree was obtained with the aid of Subtree-Pruning-Regrafting (SPR) with 1 search level which the initial tree was obtained by the addition of 10 replicates sequences. A total of 55 nucleotide sequences were analysed at 1st+2nd + 3rd+ non-coding at 420 positions.

DISCUSSION

Tilapia is an important food fish can be identified using molecular techniques like; microsatellites DNA restriction enzymes, barcoding, Snp, analysis of the nuclear fragment of rDNA, and the first internal transcribed spacer. Morphological techniques; Length, weight relationship, morphometric, and meristic traits analysis following (Oladimeji et al. 2015; Hsieh et al. 2010; Ward et al. 2005). Over time, the use of molecular techniques proved to be more accurate and practical compared to the use of morphological methods (Lim et al. 2015 and McManus & Katz 2009). The use of biomarkers as opined by (Huang et al. 2004) in both evolutionary and gene regulatory activities improves the accuracy level in species identification and evolutionary trend of a strain.

Phylogenetic analysis of the main coding region (Conserve Region) of the mitochondria (mt) DNA in the parents, hybrid (F1), and backcross (BcF1) of UPM red tilapia and GIFT tilapia were examined. The conserved regions were found to be effective in discriminating between the sampled fish because they contained important elements that aid replication and expression of the mitochondria genome (Zou et al., 2015; Kinaro et al., 2015 Xiao et al., 2016). In the present study, 4 conserved regions were identified in GIFT tilapia while UPM red tilapia, F1, and BcF1 have one conserved region each. The identified areas were able to serve as templates for differentiation. The mtDNA is maternally inherited thereby, making possible speciation through the identification of sib and half sib in general hence, parent stock from F1 and BcF1 respectively. The conserved region is more species-specific because it occurs as a template inherited as part of mtDNA with special features that enhance speciation (Jiang et al., 2004).

The sequenced conserved region of 99 sampled fish produced 5 haplotypes three populations (UPM red tilapia, F_1, and BcF_1) have one haplotype in common which agrees with the opinion of (Zou et al., 2015) with assertions that the sampled fish shared common ancestors of *O. niloticus*. Similar pairwise distance population frequencies were observed in the pair of BcF1 and UPM red tilapia compared to F1 with slight variations to the large fluctuation that is observed in GIFT tilapia. This could be explained by the fact that the GIFT tilapia projects involved a large population of stock with varying transfer of maternal DNA and multiple control regions compared to the other sampled fish.

Examining the genetic diversity of the population in Table 3 with the aid of their haplotype diversity (H) and nucleotide diversity (π) reveals that Gift tilapia has the highest value at 0.2933 haplotype diversity (H) in the

population that was examined. It also recorded the highest nucleotide diversity (π) at 0.0302. He et al (2015) opined that haplotype diversity (H) of a strain depends on the number of populations that hybridized, indicating that the higher (H and π) value recorded by GIFT tilapia is a result of the high number of introgressions. The genetic distance (D) average values as inferred by Zou et al (2015) were 0.90, 0.30, and 0.05 for individuals. In this study (0.1) was recorded for all the population, which is in-between the recommended score, this is an indication that the strains examined were very near to each other. We can infer that; all the sampled fish could be differentiated at their conserved region and the genetic sustainability of the BcF1 is possible because of the hybrid vigor of the F1 male parent. However, the test fish despite coming from different strains were genetically linked together.

Researchers like Wu & Yang (2012), Kinaro et al. (2015), and He et al. (2015) analyzed the differences between strains of interest. While this study uses a Maximum parsimony (MP) phylogenetic tree to examine the relationship between sampled fish, a monophyletic relationship was observed between the strains due to the relatedness of the haplotype; it can be seen that 2 of the haplotypes are shared. The phylogenetic tree produced was validated based on the value of consistency index (CI) and retention index (RI); a high value of CI tends to decrease the homoplasy index (HI) and an RI that is close to 1 is an indication that the characters that form the tree fit (Hidayat et al., 2005; Zou et al., 2015). The CI and RI values were 0.870968 and 0.870968 respectively in this study. The phylogenetic analysis verifies the monophyletic relationship although some of the strains showed more relatedness by their consistence overlap and clustering. It is of note that despite their positions on the Sub-tree each strain maintains a level of uniqueness that could be used for proper identification.

CONCLUSION

The genetic variability of parents (Gift Tilapia and UPM red Tilapia), hybrids (F1), and backcross (BcF1) were examined, their distances were (0.1), an indication that they were closely related. Some of the populations shared one haplotype on close examination. The phylogenetic tree shows a close relationship to each other and to an out-group signifying similarity in their genetic makeup which can be related to the fish coming from the same genus.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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