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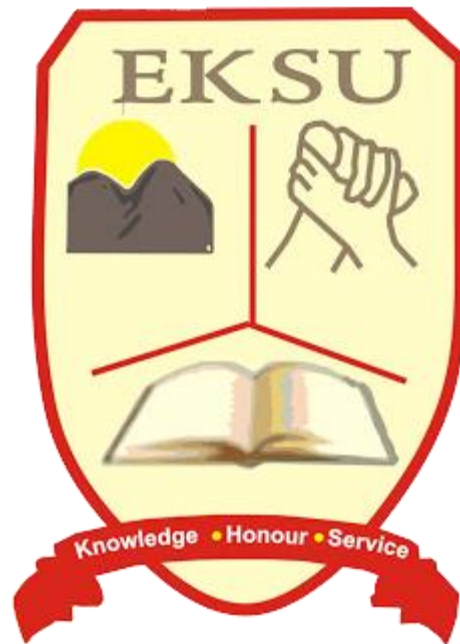
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Genetic Variability of some Tilapia Species from three different Reservoirs in Southwestern Nigeria

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Abstract

The technique of random amplified polymorphic DNA (RAPD) marker has been used for stock identification and discreteness and population analysis in fish but the potential for species and stock characterization has not yet been fully established for tilapias in Nigeria. A study on the genetic similarity within and between three species of tilapia (*Sarotherodon galilaeus*, *Tilapia guineensis*, and *Oreochromis niloticus*) was carried out to identify novel traits for the improvement of breeding techniques and production in aquaculture. One hundred and eighty-five tilapias were collected from three reservoirs: Owena, Asejire, and Aiba in Southwestern Nigeria. RAPD primers were used to estimate the genetic variation and their phylogenetic relationships. A total of thirty-five RAPD primers were screened for this study out of which, twelve RAPD markers gave reproducible bands and four primers yielded a clear and consistent DNA banding pattern. Seventy-seven detected alleles were generated by the twelve RAPD primers consisting of thirty-two alleles as monomorphic and forty-five as polymorphic. UPGMA cluster analysis of genetic data showed a high level of genetic similarity and produced two main clusters: CI and CII at 59.8% similarity level. Cluster I had twenty-four samples and Cluster II had eleven samples. The result of this study on the genetic similarity of fish and its genome is vital for improved fish breeding techniques.

Keywords: Random amplified polymorphic

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Introduction

Tilapia fish species represent the most important group of the family Cichlidae and common fishes' native to the fresh waters of Africa and found in the mouthbrooding genera *Oreochromis* and *Sarotherodon* and substrate spawning genus *Tilapia* (Trewavas, 1983). The species are characterized by delicious taste and cheap price which make them a valuable part of national income. Furthermore, many researchers use it as a fish model to investigate different items (El-Serafy *et al*, 2003). The world

aquaculture production of tilapia is second only to carp and exceeds 1.5 million tons per year (FAO, 2004) being increasingly important in tropical and subtropical countries: the Philippines, Taiwan, Israel, and several Sub-Saharan African countries- Cote d'Ivoire, Malawi, Benin Republic, etc (Liu and Cordes, 2004). Tilapia has been cultured intensively for more than four decades but its genetic resources have been poorly managed. Perdices *et al*. (2005) considered that the application of molecular

techniques would permit enhanced detection of evolutionary structure and taxonomy across the widespread species. Some of the modern approaches in genetic characterization of aquatic species to study the genetic variation include Restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) (Supungul *et al.*, 2000), Microsatellite DNA analysis (Sekino *et al.* 2002), Allozyme analysis (Klinbunga *et al.* 2000) and Random amplification of polymorphic DNA (RAPD) (Degani *et al.* 2000). Despite some progress made (Rutten *et al.* 2004; Shirak *et al.* 2009), the potential of using DNA markers for species and stock characterization has not yet been fully established for tilapias. The technique of the RAPD marker (Welsh and McClland, 1990; Williams *et al.*, 1990) has been successfully exploited for stock identification and population analysis in fish. Also, many RAPD fingerprinting studies on fish provide evidence of stock discreteness (Bartfai *et al.*, 2003; El-Zaeem *et al.*, 2001). The objective of this study is to determine the genetic variation within and between the tilapia populations in the three reservoirs at different locations in Southwestern Nigeria.

Materials and Methods

Study areas

Owena, Asejire, and Aiba reservoirs in Southwestern Nigeria were selected for the study. Owena reservoir is situated across River Owena in Ondo State (latitude 7.19°N and longitude 5.01°E). It is about 300 m long and 9 m in its deepest part with the capacity of approximately 600,000 m³ of water and with a catchment area of 790 km² (FAO, 2004). Aiba reservoir is located in Iwo, Osun State on latitude 7.63°N and longitude 4.19°E. It has an average altitude of 245 m above the sea level and a surface area of 28.75 km². It was built in the early 1950s primarily for the provision of potable water. The reservoir drains into River Osun via River Oba and attains its flood level peak between June and July yearly. Asejire reservoir is a man-made lake situated along with River Osun about 30 km east of Ibadan, Oyo State, and located on latitude 7.35°N

and longitude 4.13°E. It has an impounded area of 2342 hectares.

Collection of blood samples and storage

Blood sample (1 ml) was collected aseptically from each fish sample into EDTA bottles using 2 ml disposable syringe by gill vein puncture. The blood samples were stored on ice blocks and transported in a 10 L closed lid Plastic cooler to the Molecular Biology Laboratory, Biotechnology Centre, Federal University of Agriculture, Abeokuta for molecular analysis.

Extraction of genomic DNA

Total DNA was extracted from the whole blood samples using CTAB (hexadecyl trimethyl ammonium bromide) DNA extraction modified protocol of Doyle and Doyle (1987). 200 µl of the blood sample was transferred into an Eppendorf tube containing 600 µl of 2X CTAB extraction buffer (100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, 2% CTAB and 0.1 mg mL⁻¹ proteinase K, pH 8.0) for nuclear-lysis. The sample was incubated at 65°C for 1 h and allowed to cool to room temperature. Proteins and polysaccharides were removed by adding 500 µl of chloroform/isoamyl alcohol (24:1) solution, mixed for 5 min, and spun for 10 min at 14,000 rpm. 1 µL RNase was added into the tube to remove RNA, thereafter incubated at 37°C for 30 min. The sample was vortexed for 5 min and spun at 14,000 rpm for 15min. The supernatant was transferred into a new Eppendorf tube leaving the white interphase. DNA was then precipitated from the aqueous layer by adding 500 µl of cold isopropanol, kept in the freezer for 2 h. at -20°C and later spun at 14,000 rpm for 10 min. The DNA pellets were then washed with 500 µl 70% ethanol, air-dried for 30 min on the bench, and re-suspended in 100 µl of sterile water. The extracted DNA was kept at -20°C for further analysis. DNA quantification was performed and a dilution of 20-50 ng µl⁻¹ was used in the downstream application. The DNA concentration (µg µl⁻¹) was determined by absorbance at 260 nm and 280 nm in each sample

using Nanodrop®ND-1000 spectrophotometer. The ratio A₂₆₀/A₂₈₀ in each sample was used as an indicator of the genomic purity. The DNA integrity was checked by size fractionation on 1% agarose gel stained with ethidium bromide, visualized, and photographed on a gel documentation system (Gel Doc™, *BIO-RAD*). The quantified DNA was subjected to PCR amplification.

RAPD-PCR analysis

A total of thirty-five Operon random primers was tested for RAPD-PCR analysis. The reaction mix was carried out in 20 µl final volume containing 60 ng genomic DNA, 2 mM MgCl₂, 125 µM of dATP, dCTP dGTP and dTTP each, 0.1 µM of the primers and 1 unit of Taq DNA polymerase. Amplification was performed in a PCR thermocycler. (Biotop AGCT Thermal Cycler) The thermocycler profiles were an initial denaturation step for 3 min at 94°C followed by 45 cycles of denaturation step at 94°C for 20 seconds, annealing at 37°C for 40 seconds, and primer extension at 72°C for 40 seconds, and final extension temperature at 72°C for 5 min. The total volume of the PCR products was evaluated in 2% agarose gels and visualized by ethidium bromide staining. Bands obtained on the gel were measured by comparing the PCR product with the DNA ladder mix.

Molecular data analysis

The banding pattern was transformed into numerical values, where the presence of a band was scored 1 and the absence scored as 0. The binary value was transferred into NTSYS (Numerical Taxonomic Systems) software. A dendrogram was constructed from the matrix of similarity coefficients, using the unweighted pair group method of the arithmetic average (UPGMA) (Rolf, 2000). Genetic distances (GD %) were obtained by subtracting the similarity indices from 1 and multiplying the outcome by 100 [(1-S_{ij}) x 100] (Kim *et al.*, 1992).

Results

DNA extraction and primer screening

The concentrations of the DNA ranged between 150 to 300 µg µl⁻¹ and the percentage genome purity of the extracted DNA was within the purity level of 1.8-2.0 for all the DNA samples and the fragment obtained was over 35 kb for all the samples (Fig. 1). Twelve decamer primers were used for this study (Table 1). The extracted genomic DNA from the different samples was screened using random primers (Operon Technologies, Alameda, 6 kits: OPAE, OPAD, OPI, OPC, OPB, and OPAF). A total of thirty-five RAPD primers were screened to test their ability to provide scorable DNA banding patterns. Among the primers screened, twelve RAPD markers gave informative bands and out of which five primers (OPAD-09, OPAF-09 OPB-15, OPC-09, and OPI-03) showed clear and distinct DNA banding patterns.

RAPD-PCR profiles

All the primers showed a different percentage of polymorphism. The number of bands and banding pattern were variable depending upon the primers and type of species tested and ranged from 3 to 15. The maximum polymorphism was produced by the primers OPC-09, OPAD-09, OPAF-09, OPB-15, and OPI-03 having 80, 92, 67, 75, and 75% respectively. The highest percentage of polymorphism loci was obtained for OPAD-09 (92%) while OPAE-04 showed the least polymorphism (20%). A total of 77 bands were amplified. The size of RAPD fragments ranged from 150 to 5000 bp (Table 1). Out of the total bands, 45 (58.4%) were polymorphic while 32 (41.6%) were monomorphic (Table 2).

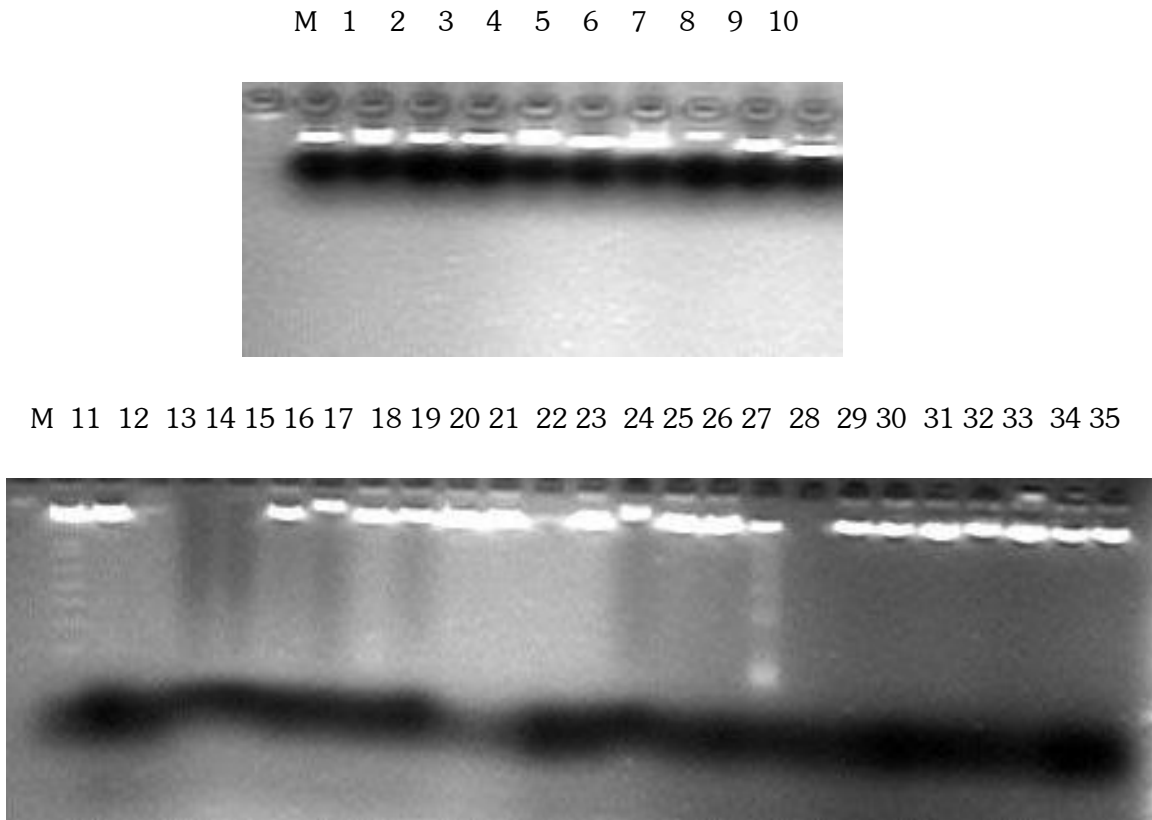


Fig.1: Electrophoresis gel for DNA extraction

Table: 1 Sequence and Operon codes of the random primers used to study variation in Tilapia species

S/N	Primer Name	Nucleotide sequence (5' to 3')	Fragment size (bp)
1	OPAD 09	TCGCTTCTCC	200-2500
2	OPAE 04	CCAGCACTTC	200-2500
3	OPAE 05	CCTGTCAGTG	250-2500
4	OPAE 09	TGCCACGAGG	250-3000
5	OPAF 07	GGAAAGCGTC	250-3500
6	OPAF 08	CTCTGCCTGA	250-3500
7	OPAF 09	CCCCTCAGAA	200-4500
8	OPAF 11	ACTGGGCCTC	200-3500
9	OPAF 12	GACGCAGCTT	200-3000
10	OPB 15	GGAGGGTGTT	250-5000
11	OPC 09	CTCACCGTCC	200-3500
12	OPI 03	CAGAAGCCCA	150-4000

Table 2: The percentage polymorphism of the RAPD markers used

S/N	Primer name	No of polymorphic markers	No of monomorphic markers	Total no of markers	% polymorphism
1	OPAD 09	11	1	12	92
2	OPAE 04	1	4	5	20
3	OPAE 05	1	3	4	25
4	OPAE 09	1	3	4	25
5	OPAF 07	2	3	5	40
6	OPAF 08	1	3	4	25
7	OPAF 09	10	5	15	67
8	OPAF 11	1	3	4	25
9	OPAF 12	1	2	3	33
10	OPB 15	6	2	8	75
11	OPC 09	4	1	5	80
12	OPI 03	6	2	8	75
Total no of markers		45	32	77	

UPGMA cluster analysis of all primers

The dendrogram based on the similarity matrix differentiated the species into two distinct clusters: Cluster I (CI) and Cluster II (CII) at 59.8% similarity level. Cluster I had 24 samples of different species out of which seven (7) were *Oreochromis niloticus*, eleven (11) were *Sarotherodon galilaeus* and six (6) belonged to *Tilapia guineensis* from all the three reservoirs showing different levels of genetic relationships. Cluster II had eleven (11) samples out of which six (6) belonged to *Tilapia guineensis*, four (4) were *Sarotherodon galilaeus* and one (1) sample was *Oreochromis niloticus*. Cluster II consisted of samples from Aiba and Asejire reservoirs at different similarity coefficients.

Cluster I was further divided into two sub-clusters and had shown a high level of genetic variation at 69.4%. Sub-cluster I had eighteen (18) samples and consisted of samples from Asejire and Aiba reservoirs collectively showing a 74.4% similarity level. Sub-cluster II had six (6) samples which comprised samples from Owena reservoir only at 96.7% similarity level. Cluster II was also observed to consist of two sub-clusters: Sub-cluster I had three (3) samples from Asejire reservoir only showing 76.7% similarity level while Sub-cluster II had eight (8) samples which consisted of samples from Asejire and Aiba reservoirs together at 71.4% similarity level.

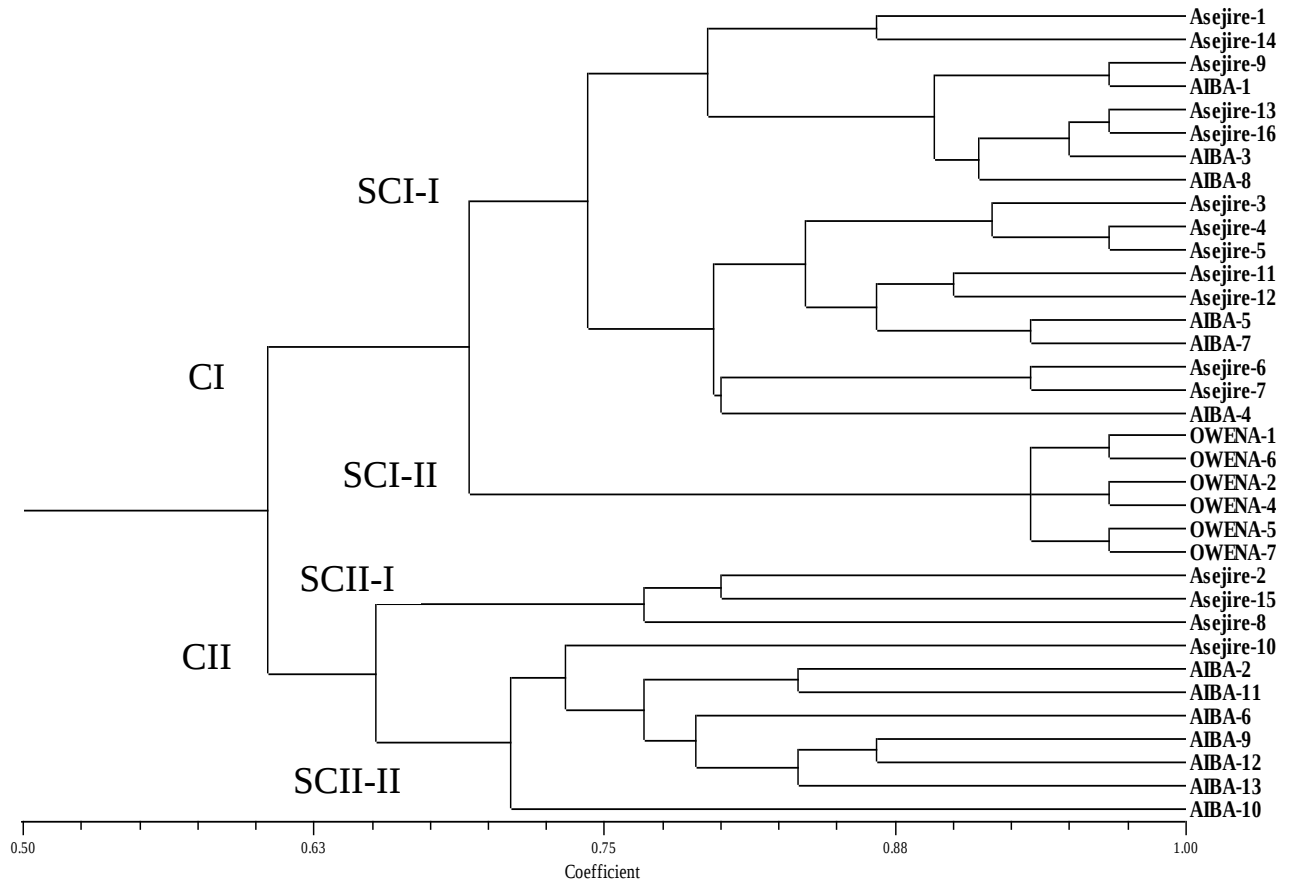


Fig. 2: Cluster analysis-based dendrogram depicting the genetic relationships among different Cichlid species (based on Nei's and Li's (1972) coefficients of similarity). CI = Cluster I; CII= Cluster II; SC = Sub clusters I and II:

Discussion

The presence of variability within species (among populations and also between individuals within a population) is essential to their ability to survive and successfully respond to environmental changes (Rehbein *et al.*, 1995). Out of the thirty-five primers screened, twelve primers (OPAD-09, OPAE-04, OPAE-05, OPAE-09, OPAF-07, OPAF-08, OPAF-09, OPAF-11, OPAF-12, OPB-15, OPC-09, and OPI-03) generated reproducible bands and gave satisfactory results whereas twenty-three primers produced highly inconsistent amplification products. The RAPD-PCR results of all genomic DNA isolated from the different samples were compared with each other. The primer OPAD-09 had the greatest degree of DNA polymorphism over the other four primers. Primer OPC-09 showed a greater percentage over primers OPI-03 and OPB-15 while

primers OPI-03 and OPB-15 are of the same percentages (75%).

The twelve random decamer primers generated a total of 77 unambiguous, readable, and reproducible amplified products. The number of amplified bands varied from three to fifteen and the size of the amplified products varied from 150 to 5000 bp. This agrees with Welsh *et al.* (1991) that the number and size of the bands generated strictly depend upon the nucleotide sequence of the primer used and the source of the template DNA, resulting in the genome-specific fingerprints of random DNA bands. The results showed that all the samples had different banding composition. OPI-03 generated a total of 8 bands, OPC-09 had 5 bands, OPB-15 generated 8 bands, OPAF-09 generated a total of 15 bands, and OPAD-09 had a total of 12 bands. The highest

number of bands was generated by OPAF-09 and revealed 67% polymorphism, while OPAD-09 showed the highest polymorphism (92%) across the samples. The variations in the number of bands amplified by different primers were influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome (Davis and Hetzel, 2000). Of the 77 bands, 45 (58.44%) were polymorphic while 32 (41.56%) were monomorphic in all the samples. The percentages of polymorphism RAPD bands ranged from 20 to 92% in all studied *Tilapia* species.

This study has demonstrated that RAPD markers can differentiate between and determine genetic relatedness within some populations of cichlids species. The RAPD fragments observed in the populations showed a high degree of polymorphism within and between the populations. Unweighted pair-group method using arithmetic average (UPGMA) analysis of the RAPD markers and pair-wise genetic similarities among the populations from the three studied reservoirs was estimated to range from 0.292 to 0.967 indicating a high genetic distance among the sampled populations. The result of the cluster analysis among the cichlids showed that the species from Asejire and Aiba reservoirs interbreed thereby producing mixed breeds. This might be as a result of natural selection based on breeding partners and as a result of seasonal migration. The similarity coefficients recorded in the populations among the sampling reservoirs varied greatly being 0.900-0.967, 0.333-0.958, and 0.292-0.875 for the samples from Owena, Asejire, and Aiba reservoirs respectively.

The phylogenetic tree indicated that all the populations are polyphylogenetic. The results of the similarity coefficient also indicated the polyphylogenetic relationships of the different species. The populations from Asejire and Aiba reservoirs were closely related even though they are different species of cichlids as seen from the dendrogram. The populations from Owena reservoir were genetically related sharing some characteristics but not purely of the same strain. In

contrast, those with similar strain are paired together showing a high similarity coefficient of 0.967. This might be a result of the short period of reproduction (Barman *et al.*, 2003) and that the samples originated from a common ancestor, hence the results give solutions to cichlids diversities in their natural environments. It also means that the species *O. niloticus* collected from Owena reservoir are mono-phylogenetic (common ancestor) while the other populations display another origin. This supports Farias *et al.* (1999) and El-Serafy *et al.* (2003) who used restriction fragment length polymorphisms of nuclear and mitochondrial DNA-PCR products (RELPS/PCR) as a basis for examining the relationships among *Tilapia* species and found out that *Tilapia* species are poly-phylogenetic species and some are mono-phylogenetic. Similar results were obtained for *Tilapia* by Yapi-Gnaore (2001) who found that the molecular techniques provide good markers and significant genetic characterization for the studied species. The monophyletic relationship of *Tilapia* fish has been confirmed by Oberst *et al.* (1996), Zowail and Baker (1998), Yapi-Gnaore (2001) and Rognon and Guyomard (2003) by using several electrophoretic techniques including polyacrylamide gel electrophoresis, isoelectric focusing, immune electrophoresis, and allozyme electrophoresis. The present study also showed a significant correlation between genetic identity and geographical distance. Fernandez, (2001) reported that the population structure of freshwater organisms is dependent on the distributions of river systems. Among the three reservoirs studied, Asejire and Aiba are closer than the Owena reservoir.

The genetic similarity values obtained in the sampled populations from the three reservoirs were in line with the expected values for individuals of the same population. This is supported by the findings of Haroun, (1999) that 98% of populations of the same species have a genetic similarity of over 0.85. This could have enhanced interbreeding between the populations from Asejire and Aiba reservoirs, which resulted in the improvement of genetic variation

within the populations. The genetic distance values for the populations from the three reservoirs were 11.10-59.30 (Asejire), 3.30-10.00 (Owena), and 8.30-66.70 (Aiba) which might be due to isolation by distance. The similarity coefficient between *Sarotherodon galileus* in Asejire and Aiba reservoirs was 0.533-0.933; *O. niloticus* in Asejire and Owena

Conclusion

The knowledge of the genetic structure and variability of stocks should be a prerequisite for any fish breeding program as this would provide a greater understanding of the genetic resources and for proper management of genetic data using genetic

was 0.600-0.800 and *T. guineensis* was 0.400-0.767 in Asejire and Aiba reservoirs. The result showed that the populations from Owena reservoir were genetically related to the highest value of the coefficient of similarity such that the probability of having a pure breed is higher in the Owena reservoir.

tools to avoid inbreeding and to improve the quality of fish produced. The results of this study indicate that RAPDs markers are appropriate for the characterization of germplasm and estimation of genetic diversity in fish populations.

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