GENETIC VARIATION AMONG TILAPIA SPECIES (*Tilapia zilli, Oreochromis niloticus* and *Hemichromis fasciatus)* POPULATIONS USING SIMPLE SEQUENCE REPEATS (SSR) MARKERS

BY

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Abstract

Tilapia belonging to the family Cichlidae is one of the most important cultured fish species all over the world due to their rapid growth, resistance to disease, high reproductive rate, and easy to culture in a variety of aquaculture systems. This study investigated morphological and genetic divergence of three (3) cichlids species (*Tilapia zilli, Oreochromis niloticus, and Hemichromis fasciatus*) collected from four (4) water bodies (Oyun dam, Moro river, Apodu dam, and Asa dam). A total of 70 specimens were collected from the various rivers. Morphometric data were then obtained from the fish. The morphometric data ranges from the body-related characters such as Body length (10.98-22.72); Body weight (15.95-32.21), Body width (6.15-9.38) to the head-related parameters like the Head length (3.48-5.91), Snout length (1.30-1.73), and Width of the head (3.59-6.05). Molecular analysis based on Simple Sequence Repeat (SSR) analysis was used to analyze variations within and among the Tilapia species. Genomic DNA from the crushed fish fin was extracted using the phenol-chloroform method. Five (5) oligonucleotide primers were screened, and four (4) primers were selected (OPA 01, OPB 02, OPC 03, and OPD 04) to amplify DNA from forty (40) samples of Tilapia species, and PCR was carried out. The Agarose gel electrophoresis results show SSR banding patterns with fragment sizes ranging from 100-1300bp and polymorphic DNA ranging from 84.61% (OPA-01 and OPC-03) to 100% (OPB-02 and OPD-04) confirming the presence of DNA. SSR is an efficient molecular marker for investigating genetic diversity in fish.

Keywords: *tilapia, genetic variation, PCR, Simple Sequence Repeats markers, DNA.*

Introduction

Tilapia fish is an indigenous African fish that is widely cultivated especially in Asia and the Middle East. It is much appreciated by consumers, being a good and affordable source of protein. Oreochromis niloticus (Nile Tilapia) and Tilapia zilli are two important fish species found in aquatic environments in Nigeria. Due to their great similarity, the taxonomic relationship between the two fish species is controversial. Although they can be discriminated physiologically, it is very difficult to identify them with meristic index for they have many overlapping characters especially at the juvenile stage. Nigeria is endowed with a lot of resources including fisheries resources that contribute immensely to the nutritional needs, economic growth and development of the nation. Fish provides essential amino acids like lysine and methionine which are limited in other animals. It is also rich in vitamins A and D as well as lipids which help to reduce thickness of blood thereby allowing it to flow easily in the body (Akande, 2020). The tilapiines have been divided into three major genera primarily on the basis of breeding habits: Oreochromis, the maternal mouth brooders (31 species); Sarotherodon, the biparental and paternal mouth brooders (9 species); and Tilapia, the substrate spawners (Trewavas [3]). Tilapias have become one of the most important groups of fish, with worldwide production of _ 2 million metrictons in 2002 (FAO, 2019). About 75% of this production is from aquaculture. The most important species are Oreochromis niloticus, Hemichromis fasciatus and Tilapia zilli (Bo-Young et al., 2005), because they grow fast, are easy to feed, are resistant to poor water quality and disease and are easily reproduced (Argue and Phelps 2019).

Female Nile tilapia, in the presence of other females either visually or chemically, exhibit shortened interspawning intervals. Although parental investment by a female extends the interspawning period, female tilapia that abandon their young to the care of a male gain this advantage of increased interspawning periods. Studies have found that males have differential levels of gonadotropic hormones responsible for spermatogenesis, with dominant males having higher levels of the hormone. Thus, visual communication between Nile tilapia mates both stimulates and modulates reproductive behavior between partners such as courtship, spawning frequency, and nest building. Species belonging to the Oreochromis genus typically care for their young through mouth brooding, oral incubation of the eggs and larvae. Similar to other tilapia, Nile tilapia are maternal mouth brooders and extensive care is therefore provided almost exclusively by the female. After spawning in a nest made by a male, the young fry or eggs are carried in the mouth of the mother for a period of 12 days. Hemichromis fasciatus is found in rivers that have low speed of current, a strong height of water, a formed substrate of blocks and a few plant. According to Albaret (2012), the species is monogamist keeping and protecting eggs and alevins but not practising oral incubation. According to Daget (2015), the eggs are fixed on an immersed support, in a clean place, with the shelter of the current, a depth from 10 to 20 cm. From 50 mm, the young attacks only large preys; they devour all the young fish which pass to their carried. So H. fasciatus is used in pisciculture to limit the proliferation of the species to laying many and invading such as H. fasciatus. Used for tilapia control. Overfishing, loss of dry season refuges and rapids habitat due to development and dams all threaten populations of this species.

Redbelly tilapia zilli is a species of fish that has been introduced globally, mainly for aquaculture purposes or as a food fish. Native to Africa and southwest Asia, it is a highly successful species, capable of outcompeting both native and non-native species for food, habitat and spawning sites. The chest is pinkish and lips are bright green They prefer tropical environments with water temperatures of 25-30ºC, and optimal temperatures of 20-32ºC. However, it can tolerate temperatures between 11 and 36ºC, becoming lethargic and vulnerable to predators and disease below 16ºC (ISSG, 2014). Redbelly tilapia is a substrate spawner (Bailey, 2014) and larvae develop in close association with substrate but it is not a mouth brooding fish like some other congeneric species. Redbelly tilapia form monogamous pairs and exhibit biparental guarding behaviour. Both parents help in nest building, constructing nesting depressions 20-25 cm in width and 5-8 cm in depth, often in bottoms with sand or pebbles and ample vegetation. Identification of fish species mainly based on external morphological features including body shape, colors pattern, number and position of fines (Strauss and bond 1990). DNA marker technologies are not only the basis for genetic linkage mapping, but also for the analysis of genetic resources, strain differentiation, species differentiation, parentage identification, and preservation of genetic diversity and conservation of genetic integrity. DNA marker technologies are already used routinely for stock identification in some farmed fish species (Beacham et al., 2000, 2005; Duchesne and Bernatchez, 2007). Nowadays, molecular genetic markers are widely used to identify strain, define stock diversity, diagnosis of simply inherited traits and even to improve stocks (Rashed 2008, 2009). Some of these molecular markers are random amplified polymorphism (RAPD) and simple sequence repeat (SSR) or microsatellites.

SSR markers are the amplified products of less functional part of the genome that do not strongly respond to selection on the phenotypic level. Such DNA regions may accumulate more nucleotide mutations with potential to assess inter-population genetic differentiation. The amplification of genomic DNA by PCR with arbitrary nucleotide sequence primers, SSR can detect high levels of DNA polymorphisms. The technique detects coding as well as non-coding DNA sequences, and many of the most informative polymorphic sequences are those derived from repetitive (non-coding) DNA sequences in the genome.

Research Objective

This study attempts to identify and estimate polymorphisms and genetic diversity and variations between and within three Tilapia species; Oreochromis niloticus, Hemichromis fasciatus and Tilapia Zilli using SSR assay.

Literature Review

SSRs (Simple Sequence Repeats) have all the advantages of a PCR-based marker, with the added benefit that primers are commercially available and do not require prior knowledge of the target DNA sequence or genome organization (Haymer et al.,2014). Another useful methodology to assess genetic variations in fish populations is the RAPD (Random Amplified Polymorphic DNA) technique, which is used in fishery management and conservation genetics of wild populations. Based on the amplification of genomic DNA by PCR (Polymerase Chain Reaction) with arbitrary nucleotide sequence primers, SSR can detect high levels of DNA polymorphisms and can produce fine genetic markers (Williams et al., 2020; Welsh and McClelland, 2020). The potential use of SSR in genetic mapping and population genetics has been widely documented for a large variety of organisms, including fish (Postlethwait et al., 2014; Foo et al., 2015; Bielawski and Pumo, 2017; Caccone et al., 2017; Dergam et al., 2018; Nadig et al.,2019; Liu et al., 2019).Barriga-Sosa et al., 2004, Based on the current genetic variation, we can help develop informed management strategies. We have three objectives for this study: 1) identify which species are present; 2) determine the morphometric and genetic variation within and between populations; and 3) determine the population genetic structure and evaluate if the populations interbreed. Microsatellites consist of multiple copies of tandemly arranged simple sequence repeats (SSRs) that range in size from 1 to 6 base pairs. Abundant in all species studied to date, microsatellite motifs have been estimated to occur as often as once every 10 kb in fishes (Wright et al., 2013). Microsatellites recently have become an extremely popular marker type in a wide variety of genetic investigations.

Methodology

Tilapia zilli, Oreochromis niloticus and Hemichromis fasciatus species were obtained in Kwara state from Oyun river, Ilorin (08°28.006'N; 004°39.940'E), Moro river, Shao town, Shao (08°36.329'N; 004°33.790'E), Asa river, Asa-dam (08°45.151'N; 004°37.640'E) and Apodu river, Malete (08°28.006'N; 004°39.940'E) using fishing nets by commercial fishermen at the various locations. The species were identified using their various morphological features and characteristics like their body shape, colors pattern, number and position of fines.

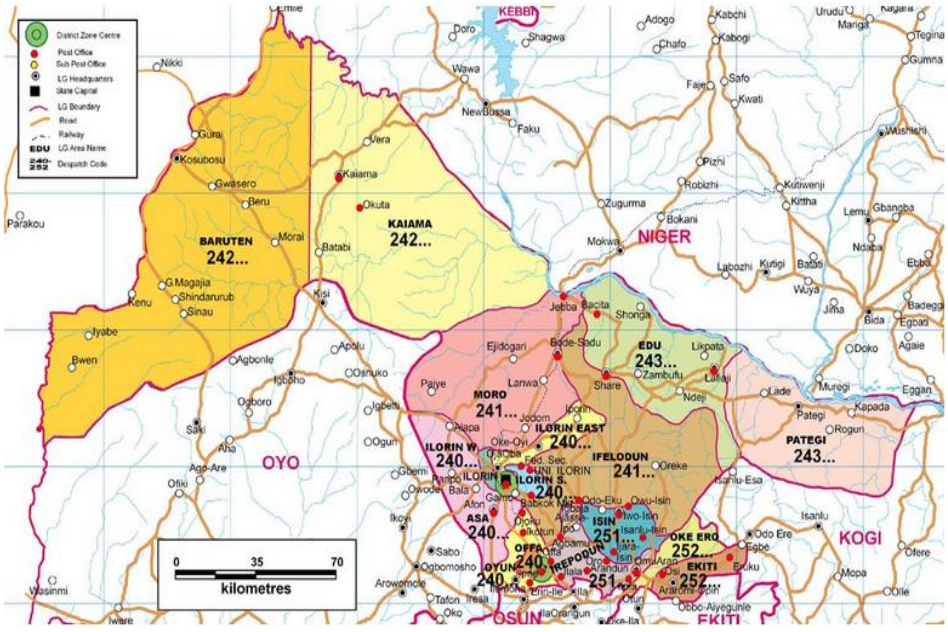

FIG 1: Map of Kwara State, Showing the Sampling Sites

Approximately 100 mg of fin tissue from individuals of each population were collected. The fins were then cut into small pieces and placed in a 5 ml-Plain tube and were preserved in 95% ethanol. Specimens were preserved in a refrigerator in the laboratory. Each collection site was considered *a priori* as a discrete group. Fish were first assessed as to whether it belongs to either the genus *Tilapia* or *Oreochromis and Hemichromis* following established criteria (Trewavas, 2013) and analysis of the morphology of the pharyngeal teeth (*Oreochromis* - monocuspid and bicuspid and *Tilapia* – tricuspid and *Hemichromis*- body lining). To identify fish samples at the species level, we assessed 13 morphometric variables (*M*) (Vreven *et al*., 1998; Barriga-Sosa *et al*., 2004).

Result

The morphometric parameters measured include: Body length, Body weight, Body width, Body depth/height, Head length, Head length excluding snout, Width of the head, Snout length, Length of the dorsal fin, Length of the anal fin, Length of the pelvic fin, Length of the pectoral fin, Length of the caudal fin. In Table 3, the three species sampled in Oyun (Unilorin) Dam shows reduced significant differences in most of the parameters studied except in Body length, body weight, body width, and body depth/height. *H. fasciatus* was observed to have higher values of morphometric parameters in Body length (20.43), body weight (30.38), body depth/height (2.25) and head length (5.7) measured compared to other species and *T. zilli* have more meristic count than any other species under study. *O. niloticus* however have higher significant values in five out of thirteen parameters under study among the species. In Table 4, *O. niloticus and H. fasciatus* were the only two species found during the course of sampling in Moro River, the morphometric variation among these two species shows reduced significant differences in Width of the head, Snout length, Length of the dorsal fin, Length of the anal fin, Length of the pelvic fin, Length of the pectoral fin, Length of the caudal fin but *O. niloticus* have higher values of morphometric parameters in body length (16.78), body weight (20.8), body width (6.23), body depth/height (2.35), head length (5.13) and head length excluding snout (4.53) and also meristic count compared to *H. fasciatus*. In most of the parameters, *O. niloticus* have higher significance values in ten out of thirteen parameters under study.

In Table 5, only *T. zilli* and *H. fasciatus* were found during the course of sampling in Apodu reservoir, Malete, there were reduced significant differences among the all the morphometric parameters under study, but *T. zilli* have higher value of morphometric parameters in body length (19.75) and *H. fasciatus* have higher value in body weight (30.12) and *T. zilli* meristic count than *H. fasciatus*. *T. zilli* have higher significant values in seven out of thirteen parameters under study. In Table 6, all the three were found during the cause of sampling in Asa Dam, Ilorin. There were little significant differences among all the morphometric parameters but *O. niloticus* have higher values in Body length (22.72) and Body weight (32.21). *O. niloticus* also high number of meristic count. *O. niloticus* also have significant values in twelve out of thirteen parameters under study.

Table 1: Morphometric measurements of *Tilapia* species collected from Moro River in SHAO district

s/n	Parameters	*Tilapia zilli*	*Oreochromis niloticus*	*Hemichromis fasciatus*
1	Body length	-	16.78 ±0.464	10.98 ±0.317
2	Body weight	-	20.8 ±1.518	15.95 ±0.811
3	Body width	-	6.23 ±0.236	3.13 ±0.149
4	Body depth/height	-	2.35 ±0.247	1.15 ±0.048
5	Head length	-	5.13 ±0.175	3.48 ±0.256

6	Head length excluding snout	-	4.53 ±0.229	3.08 ±0.256
7	Width of the head	-	4.28 ±0.200	3.08 ±0.256
8	Snout length	-	1.35 ±0.259	1.40 ±0.083
9	Length of the dorsal fin	-	9.57 ±0.371	9.32 ±0.340
10	Length of the anal fin	-	2.13 ±0.28	1.90 ±0.168
11	Length of the pelvic fin	-	1.33 ±0.269	1.90 ±0.077
12	Length of the pectoral fin	-	1.95 ±0.225	1.90 ±0.129
13	Length of the caudal fin	-	2.35 ±0.17	2.73 ±0.110

Table 2: Morphometric measurements of *Tilapia* species collected from Asa Dam in Asa river district

S/N	Parameters	*Tilapia zillii*	*Oreochromis niloticus*	*Hemichromis fasciatus*
1	Body length	20.35 ±1.309	22.72 ±1.175	21.63 ±1.199
2	Body weight	29.75 ±5.833	32.21 ±6.350	30.98 ±5.981
3	Body width	9.38 ±0.349	9.35 ±0.514	9.29 ±0.393
4	Body depth/height	2.48 ±0.295	2.98 ±0.345	2.57 ±0.312
5	Head length	5.68 ±0.307	5.91 ±0.751	5.81 ±0.411
6	Head length excluding snout	4.30 ±0.682	4.51 ±0.714	4.48 ±0.592
7	Width of the head	3.60 ±0.692	3.82 ±0.531	3.59 ±0.323
8	Snout length	1.98 ±0.214	2.13 ±0.491	2.02 ±0.295
9	Length of the dorsal fin	10.18 ±1.128	10.21 ±0.563	9.58 ±0.827
10	Length of the anal fin	3.33 ±0.249	3.48 ±0.511	3.12 ±0.336
11	Length of the pelvic fin	1.43 ±0.165	1.56 ±0.292	1.49 ±0.195
12	Length of the pectoral fin	1.78 ±0.225	2.1 ±0.314	1.90 ±0.296
13	Length of the caudal fin	4.45 ±0.312	4.62 ±0.456	4.51 ±0.393

Table 3: Morphometric measurements of *Tilapia* species collected from Unilorin Dam in Oyun river district

1	Body length	18.78 ±0.647	19.93 ±0.539	20.43 ±1.057
2	Body weight	23.0 ±1.958	26.5 ±1.695	30.38 ±1.793
3	Body width	6.15 ±1.072	6.95 ±0.557	6.55 ±0.312
4	Body depth/height	1.65 ±0.301	1.63 ±0.175	2.25 ±0.184
5	Head length	4.28 ±0.388	5.38 ±0.345	5.7 ±0.234
6	Head length excluding snout	3.93 ±0.281	4.25 ±0.222	4.2 ±0.128
7	Width of the head	6.05 ±0.284	5.80 ±0.258	5.55 ±0.35
8	Snout length	1.30 ±0.219	1.73 ±0.149	1.35 ±0.155
9	Length of the dorsal fin	8.85 ±0.312	9.75 ±0.403	8.63 ±0.301
10	Length of the anal fin	3.05 ±0.316	2.85 ±0.171	2.93 ±0.175
11	Length of the pelvic fin	1.65 ±0.250	1.68 ±0.149	1.5 ±0.108
12	Length of the pectoral fin	1.86 ±0.128	1.60 ±0.208	1.55 ±0.090
13	Length of the caudal fin	2.78 ±0.163	2.65 ±0.194	2.53 ±0.268

Table 4: Morphometric measurements of *Tilapia* species collected from Apodu Dam in Malete river district

S/N	Parameters	*Tilapia zilli*	*Oreochromis niloticus*	*Hemichromis fasciatus*
1	Body length	19.75 ±0.445	-	18.69 ±0.614
2	Body weight	29.25 ±2.625	-	30.12 ±2.064
3	Body width	7.38 ±0.384	-	6.98 ±0.539
4	Body depth/height	1.83 ±0.214	-	2.03 ±0.179
5	Head length	4.15 ±0.175	-	4.39 ±0.289
6	Head length excluding snout	4.03 ±0.214	-	4.10 ±0.345
7	Width of the head	5.93 ±0.175	-	5.65 ±0.267
8	Snout length	1.63 ±0.175	-	1.22 ±0.137
9	Length of the dorsal fin	9.35 ±0.312	-	9.72 ±0.345
10	Length of the anal fin	2.68 ±0.175	-	2.29 ±0.145
11	Length of the pelvic fin	1.23 ±0.111	-	1.95 ±0.095
12	Length of the pectoral fin	1.58 ±0.119	-	1.23 ±0.265
13	Length of the caudal fin	2.38 ±0.149	-	2.32 ±0.189

Molecular Analysis Results

Extraction of DNA

Extraction DNA from fish samples is the first step for all molecular marker type. Genomic DNA can be extracted either from fresh, preserved or dried samples. As shown in Fig. 5, genomic DNA was successfully extracted from the fish samples and observed to have a very distinct band. Using this DNA agarose gel electrophoresis method, the clear band of DNA was obtained and photographed using gel documentation (Fig. 5).

Screening of SSR primers

For the forty samples analysed, Four SSR primers were used to analyse diversity. Chosen suitable primers are very important process for PCR-SSR techniques in order to obtain clear, highly informative and reproducible band. FOUR primers (5) of SSR were purchased from Eurofin (India).

Results of SSR profiles

SSR technique has been used in genetic study of Tilapia fish (Fawzia *et al.*, 2017).
Four SSR primers were applied to forty individuals containing three different Tilapia species for DNA amplification by PCR. The results showed different primers generated different fragment numbers and length of DNA amplification products as shown in Table 7. Different primers produced different fragment patterns. The banding patterns which were clear and reproducible bands selected. The selected primers were OPA 01, OPB 02, OPC 03 and OPD 04. These primers were selected to generate SSR pattern for all individuals of Tilapia species samples.

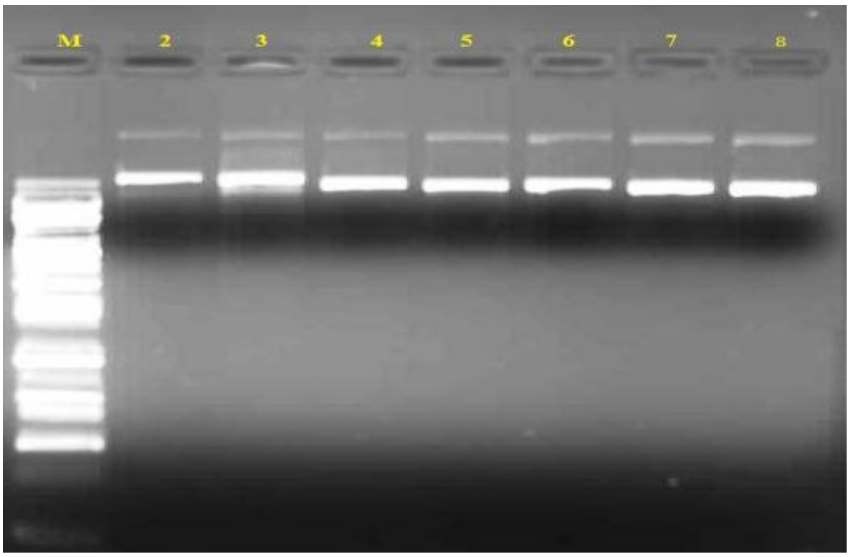

Fig 2. Agarose gel electrophoresis of genomic DNA isolated from Tilapia fins. Lane 1: DNA ladder (M); Lane 2-8. Genomic DNA of Tilapia fins isolated from adult Tilapia spp

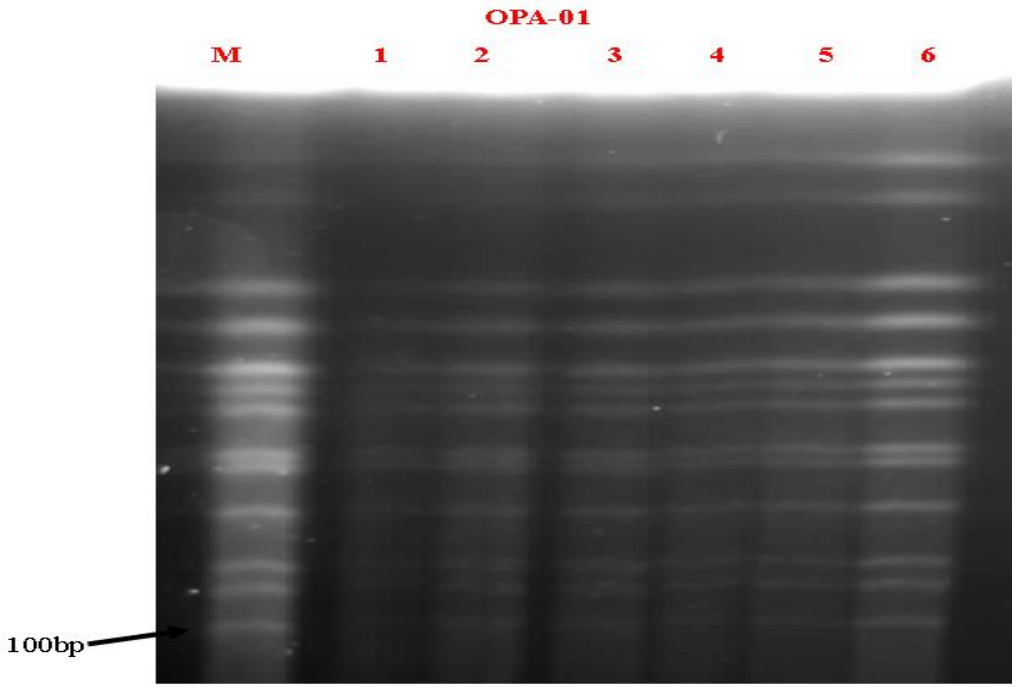

Fig 3: SSR-PCR patterns generated by OPA-01 primer. The fragment patterns are identified as follows: T. zillii(Lanes 1-2), O. niloticus(Lanes 3-4), H. fasciatus(5-6) and DNA marker (100 bp ladder was loaded in Lane M).

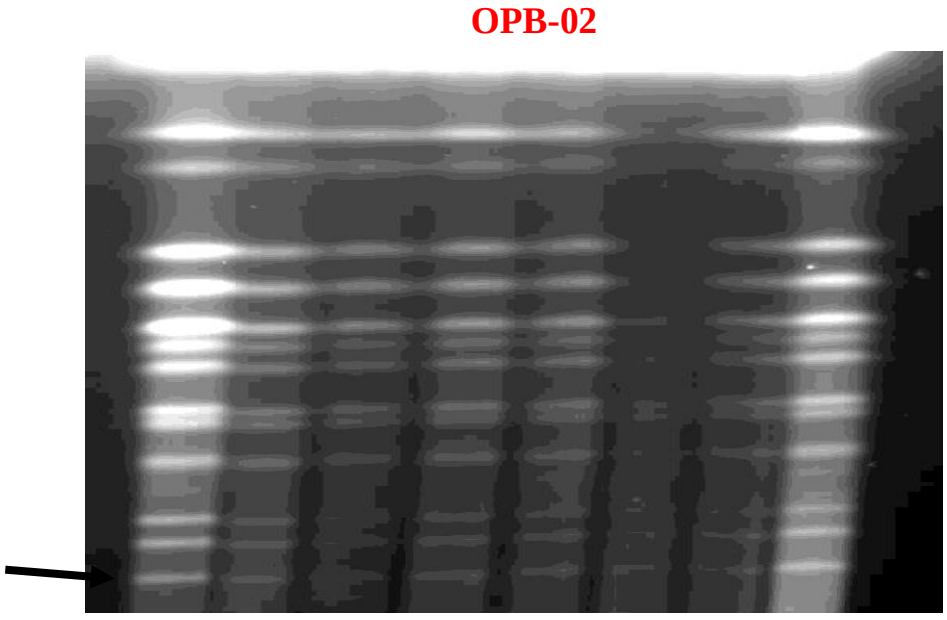

Fig 4: SSR-PCR patterns generated by OPD-04 primer. The fragment patterns are identified as follows: T. zillii(Lanes 1-2), O. niloticus(Lanes 3-4), H. fasciatus(5-6) and DNA marker (100 bp ladder was loaded in Lane M).

Table 5: Total number of bands, number and percentage of polymorphic bands generated by four primers in the three studied Tilapia spp.

Primer	Number of fragments	Size of fragments	Total number of fragments	Number of polymorphic DNA	Percentage of polymorphic (%)
OPA-01	0-6	100-1,300	13	11	84.61%
OPB-02	0-7	100-1,300	13	13	100%
OPC-03	0-7	100-1,300	13	11	84.61%
OPD-04	0-6	100-1,300	13	13	100%

Discussion

Morphometric Variation

The morphometric characteristics in this study shows reduced significant differences except in body length, body weight, body width and body depth/height which varies greatly among the three fish species from the four rivers. In table 1, O. niloticus was observed to have higher values of morphometric parameters (Body width (6.95), head length excluding snout (4.25), snout length (1.73), length of dorsal fin (9.75) and length of the pelvic fin (1.68)) measured compared to T. zilli and H. fasciatus, but T. zilli had more meristic count than the other species which is similar to work done by Olufeagba et al., (2015) where morphological variations of Cichlids showed significant differences in most morphometric parameters except in pectoral fin length, pelvic fin length, pre-dorsal distance, vomerine width and snout length, all meristic count however were statistically different among the species, Sarotherodon galilaeus was observed to have higher values of morphometric parameters measured compared to other species, but T. zilli had more meristic count in the study. This may be due to the uncertainty in many occasions because of geographical and ecological variability in form (Garg et al., 2009). Samaradivakara et al., (2012) reported the morphological variation among three populations of introduced Tilapia fish collected from Kurunegala, Anuradhapura and Polonnaruwa and the brood stock of Nile Tilapia (Oreochromis niloticus) collected from the Udawalawe tilapia breeding centre. Using multivariate analysis of twenty morphometric and fourteen meristic characters. Discriminant analysis and cluster analysis of conventional morphometric measures showed a high divergence among the populations while the conventional meristic measures did not show a divergence among the populations. This is in contrast with this study where O. niloticus found in three rivers (Oyun, Asa and Moro) shows high significant differences in both morphometric measures and meristic measures. The differences between the populations may have appeared due to either genetic differences or environmental factors (Nyingi et al., 2009).

The body weight of T. zilli (29.75), O. niloticus (32.21) and H. fasciatus (30.98) collected from Asa Dam were higher which may due to geographical and habitat variation or genetic component and Wimberger (1992) had highlighted environmental conditions such as food abundance and temperature as causes of fish high morphological plasticity. In table 4, there were no correlations in body length of T. zilli (20.35) and O. niloticus (22.72) and no correlation in their body weigth (T. zilli (29.75) and O. niloticus (32.21)) which is in contrast with Fagbuaro, et al., (2016) were the mean of their total body lengths were 20.10, 21.15 and 20.17, 20.42 while the mean of the body weights were 164.70, 167.80 and 170.20, 162.20 for females and males of Oreochromis niloticus and Tilapia zilli

respectively. And this result may be due to their inheritance, competition for food and other physical materials in the water body, size variations, weight or other unseen environmentally induced factors (Kumar et al 2014).

Molecular Analysis

Molecular analyses have solved problems encountered during morphological methods and give better accuracy to the confusion caused during morphological methods. Microsatellites or Simple Sequence Repeat (SSR) represent an abundant and dispersed evenly throughout genomes. They have become the markers of choice for a wide range of applications in population genetic, conservation and evolutionary biology (Mojekwu and Anumudu, 2013). Four SSR primers were used in this study to characterize the three Tilapia species which is similar to work done by Fawzia et al., (2017) where five SSR primer pairs were used to characterize three Nile tilapia populations to assess the genetic variation between them. The results in this study shows different primers generated different fragment numbers and length of DNA amplification products as shown in Table 5. Different primers produced different fragment patterns ranging from 100-1300bp as compared to the results which were reported by Liu et al., (1999) who generated RAPD for gene mapping and analysis of genetic variation of catfish. The study of the genetic variation is a key step in any selection program, for the reason that, the genetic improvement depends mainly on maintaining the genetic variation between the mixed populations (Ponzoniet al., 2005). This genetic marker can improve assessment and characterization of diversity in stocks, which increases the chances that re-stocked tilapia fisheries are being managed effectively for long-term survival.

Conclusion

Description of species has commonly relied on description of unique sets of morphological characters such as body shape, colors pattern, number and position of fines which are phylogenetically informative to distinguish the three species of Tilapia. Based on information available from the present study, the possible inference can be drawn that good genetic divergence existed in Tilapia species in the waterbodies (Oyun dam, Moro river Apodu dam and Asa dam). Microsatallites (SSR) have become the marker of choice for application in fish population genetic studies because it is simple and reliable tool for assessing the molecular genetic variability within and among the cichlid species. SSR analysis was successfully used to examine the genetic polymorphism, to detect species-specific SSR markers and to determine the variability among the three applied Tilapia species. An elaborated work with some more sensitive techniques like restriction fragment length polymorphism may be employed for monitoring of genetic status and breeding programmes of this valuable Tilapia fishes.

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