GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

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STUDIES ON THE MOLECULAR TAXONOMY OF THREE CICHLID SPECIES OF FIN FISH - *OREOCHROMIS MOSSAMBICUS* (PETERS 1852), *OREOCHROMIS NILOTICUS NILOTICUS* (LINNAEUS, 1758) AND *LABIDOCHROMIS CAERULEUS* (FRYER, 1956)

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ABSTRACT

In India, aquaculture plays an important role in the national economy and in the socioeconomic development of the country. Fish production prevents malnutrition in least developed countries. It helps in the war against global hunger and extreme poverty. Tilapia is farmed in at least 85 countries, with most production coming from the developing countries of Asia and Latin America. In this study, Random Amplification of Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) analyses were done for three cichlid species of fin fishes - Oreochromis mossambicus (Mozambique tilapia), *Oreochromis niloticus* niloticus (GIFT) and *Labidochromis caeruleus* (Banana cichlid). The characterization results were used for the construction of Phylogenetic tree using PyElph software to evaluate the evolutionary relationship between the species. This work briefly explains some evidences of molecular taxonomy of three cichlid species of fin fishes and also reveals the relationship among the tilapia species and evaluation of polymorphogenetic and polyphylogenetic relationship.

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KEYWORDS: Mozambique tilapia, GIFT tilapia, Banana cichlid, RAPD, RFLP, phylogenetic tree

INTRODUCTION

Fish is the primary source of protein and an important part of the diet worldwide. Globally, fish contributes to 16% of the total animal protein intake of humans and are rich in minerals and essential fatty acids. Fish is the primary source of omega-3 fatty acids in the human diet[3]. The fisheries and aquaculture sector also aim to tackle hunger, malnutrition, poverty and contributes to the economic growth in the world. It also focuses on the conservation of resources, biodiversity and protection of the environment to address the well beings, livelihood and other people working in this sector. The Mozambique tilapia is a deep bodied cichlid fish native to the eastward-flowing rivers of central and southern Africa. It is a dull coloured fish which lives up to a decade in its native habitat. It varies in its appearance due to its ability to inter breed with related species of cichlids. One of the popular fish varieties in farming is 'GIFT' (Genetically Improved Farmed Tilapia). It is known as the 'Aqua chicken' of the 21st century. Intensive research involving the genetic improvement of farmed tilapia for desirable traits of high food conversion efficiency, high growth rate, high percentage of meat yield, cold tolerance, saltwater tolerance and high disease resistance, led to the development of GIFT strain (Genetically Improved Farmed Tilapia) and GIFT derived strains are contributing to higher aquaculture production the world over. Banana Cichlid is a moderately aggressive freshwater fish native to Lake Malawi, Africa. The scientific name of this fish is *Labidochromis caeruleus*.

RAPD (Random Amplified Polymorphic DNA) is a commonly used technique; it can be accessed to identify genetic variation among the species. DNA fragments were obtained by PCR amplification of their random segments of genomic DNA of arbitrary nucleotide sequence by single primer. This study applies to identification of molecular taxonomy in fin fishes. RFLP (Restriction Fragment Length Polymorphism) is based upon variation in the DNA sequence recognized by restriction enzymes. Bacterial enzymes are used to cut DNA molecules at specified locations. RFLP are used as markers on genetic maps. According to the presence or absence of restriction enzyme sites and restriction enzymes recognize and cut at the particular site in genomic DNA were its differentiated. A variable domain of the nuclear small subunit (18S) rRNA gene was found to be useful for phylogenetic studies because of the consistent differences in this genomic DNA between fin fish species.

MATERIAL AND METHODS

Collection of samples

Oreochromis mossambicus (Mozambique Tilapia), *Oreochromis niloticus niloticus* (GIFT) and

Labidochromis caeruleus (Banana Cichlid) fish samples were collected from Fisheries Research and Information Centre (FRIC), Karnataka Veterinary, Animal and Fisheries Sciences University, Hesaraghatta, Bengaluru (Fig. 1). The fish samples were anaesthetized using 20 µl clove oil and 2 g of muscle sample was collected from the individual fish and transferred aseptically into labelled eppendorf tubes and preserved in 80 % ethanol for further studies.

Fig.1: The three species of Tilapia used in the study: MT- Mozambique Tilapia, GT- GIFT Tilapia, BC- Banana Cichlid

Meristic characteristics

Meristic characters in fishes are important to differentiation of taxonomic units and are able to spot differences between fish populations. Meristic characters are countable characters of a fish such as fin rays, fin spines and Gill rakers. The meristic characters analyses were performed in freshly sacrificed fish of *Oreochromis mossambicus* (Mozambique Tilapia), *Oreochromis niloticus niloticus* (GIFT) and *Labidochromis caeruleus* (Banana Cichlid)(Fig. 2).

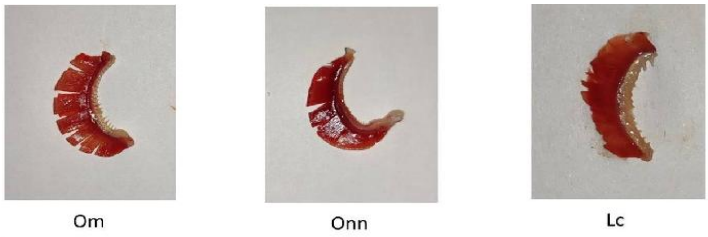

Fig.2 : Gill rakers of Om-*Oreochromis mossambicus*, Onn-*Oreochromis niloticus niloticus* and Lc-*Labidochromis caeruleus*

Extraction of genomic DNA

The preserved fish tissue samples of all the three cichlid species of fin fish were individually subjected to whole genomic DNA extraction. DNA was extracted from the muscle tissue (Bardakci and Skibinski, 1994). The concentration of genomic DNA was estimated using UV-VIS Spectrophotometer at 260 nm and the isolated genomic DNA from fish tissue was checked on 0.8% agarose gel electrophoresis for its presence.

RAPD analysis

The RAPD analysis of the genomic DNA was done with the help of three set of primers OPA 10, OPA 08 and OPA 04 also named as primer 1, 2 and 3 (Table 1). The term OPA refers to the operon codes of the three primers. These primers were used to study variation in *Oreochromis* species (Bardakci and Skibinski, 1994). RAPD analysis was done using these three primers (Ahmed *et. al.,* 2004).

TABLE-1. Details of the random primers used during RAPD-PCR

S.No	Primer Name	Primer Sequence	GC content (%)	Tm (°C)
1	Primer 1	GTGATCGCAG	60	25
2	Primer 2	GTGACGTAGG	60	25
3	Primer 3	AATCGGGCTG	60	25

Phylogenetic tree

The Phylogenetic tree was constructed by the polymorphic DNA bands with the help of PyElph software and the original measure of genetic distance using the unweighted pair group method average UPGMA clustering method each tree was constructed by using different RAPD primers, for each primers polymorphic bands were recorded.

Amplification and Purification of 18s rRNA Gene

5ng of genomic DNA from each of the 3 fish species, *O. mossambicus, O. niloticus niloticus* and *L. caeruleus* were used for the amplification of 18s rRNA gene (El-Serafy *et al.,* 2003). The genomic DNA of these fishes was subjected to PCR amplification using the specific primers (SSU1 and SSU2) named as primer-4 and primer -5 (Stothard and Rollinson, 1997). The entire nuclear 18s rRNA was amplified by using certain PCR conditions they are subjected to agarose gel electrophoresis (0.8g agarose) the gel was visualized under UV light and specific fragments from the gel was cut and isolate the specific gene from the agarose gel by Glass milk DNA purification.

RFLP analysis of 18s rRNA Gene

The extracted, amplified 18s rRNA gene from each of the 3 fin fish species were subjected to restriction digestion using the restriction endonuclease, EcoR I, Ava I and Sma I (El-Serafy *et. al*., 2003). EcoR I and Ava I was incubated at 37 with 18s rRNA gene for 2 hours and Sma I was incubated at 30 with 18s rRNA gene for 2 hours The products from the restriction digestion reaction for each

fin fish and with each of the restriction endonuclease were subjected to gel electrophoresis (2% agarose) with standard DNA markers (10,000bp to 100 bp). The bands were imaged and documented using a UV transilluminator and the gel documentation unit.

RESULTS

Meristic characteristics: The morphometric characters including the total number of dorsal fins, anal fins and gill rakers of three cichlid fish samples under study were found out as shown in table 4.1. The Mozambique tilapia was found to have a total of 16 dorsal spines and 12 dorsal rays, 3 anal spines and 11 anal rays together with 18 to 19 gills. In GIFT tilapia, there were about 16 dorsal spins and 13 dorsal rays, 3 anal spines and 9 anal rays along with 24 to 25 gills. In case of Banana cichlid, it was found to have 16 dorsal spines and 9 dorsal rays, 3 anal spines and 8 anal rays together with 16 to 17 gill rakers (Table 2, Fig.2).

TABLE -2. Meristic differences between the three fin fishes of cichlid species

Fish species	Dorsal fin (No.s)		Anal fin (No.s)		Gill
	Spines	Rays	Spines	Rays	Rakers (No.s)
Mozambique tilapia	16	12	3	11	18 to 19
GIFT	16	13	3	9	24 to 25
Banana cichlid	16	9	3	8	16 to 17

Fig.2 : Gill rakers of Om-*Oreochromis mossambicus*, Onn-*Oreochromis niloticus niloticus* and Lc-*Labidochromis caeruleus*

Extraction of Genomic DNA: The genomic DNA was extracted from muscle tissue the figure show the movement of genomic DNA isolated thus, indicates the presence of genomic DNA in the three fin fish samples (Fig.3).

Fig. 3 : Gel showing genomic DNA bands of 1- Mozambique Tilapia, 2- GIFT Tilapia, 3- GIFT Tilapia

RAPD analysis using three Random Primers: RAPD analysis of genomic DNA by using three different primers of OPA10, OPA08, OPA04 on three different fin fishes fishes *Oreochromis mossambicus* (Mozambique Tilapia), *Oreochromis niloticus niloticus* (GIFT) and *Labidochromis caeruleus* (Banana Cichlid) shows the monomorphic and the polymorphic bands (Table 3). RAPD-PCR with different primers shows the variation in their molecular weight with both the monomorphic and the polymorphic bands for different fish samples shows different (TNA) total number of amplified bands and the three fish samples with different primers shows same number of polymorphic bands and percentage of polymorphism (Table 3, Fig.4)

TABLE- 3. R_f Values and Molecular weight of the DNA bands generated by the three random primers in the three fin fish species

Sample	Primer -1		Primer-2		Primer-3	
	R_f Values	Mol. Wt. (bp)	R_f Values	Mol. Wt. (bp)	R_f Values	Mol. Wt. (bp)
Mozambique	0.33	2500	0.40	1700	0.25	1750
	0.38	2300	0.43	1400	0.30	1800
	0.46	2000	0.51	1250	0.34	1400
	0.53	1750	0.53	1150	0.36	1250
	0.56	1200	0.60	800	0.40	1100
	0.64	1000	0.66	750	0.43	600
	0.66	600	0.70	700	0.59	550
	0.69	575	0.75	600	-	-
	0.75	500	0.81	475	-	-
	0.91	350	0.85	450	-	-
	1.04	300	0.88	400	-	-
	-	-	1.02	300	-	-
GIFT	0.46	1400	0.40	1500	0.29	3200
	0.48	1300	0.47	1250	0.33	3000
	0.51	950	0.60	950	0.39	2800
	0.54	800	0.62	800	0.42	2000
	0.60	650	0.66	780	0.47	1750
	0.67	650	0.70	700	0.54	1250
	0.70	550	0.75	600	0.60	1000
	0.77	500	0.81	550	0.64	900
	0.89	350	0.84	450	0.75	700
	0.93	310	-	-	0.83	550
Banana Cichlid	0.39	2000	0.35	2000	0.24	1750
	0.46	1200	0.38	1750	0.33	1400
	0.51	1000	0.40	1500	0.37	1250
	0.53	800	0.43	1250	0.40	1100
	0.59	700	0.43	1000	0.45	900
	0.62	650	0.5	800	0.51	800
	0.65	500	0.56	750	0.55	750
	0.73	550	0.73	700	0.61	550
	0.77	400	0.77	600	0.73	450
	-	-	0.91	390	-	-
	-	-	0.97	380	-	-
	-	-	1.05	300	-	-

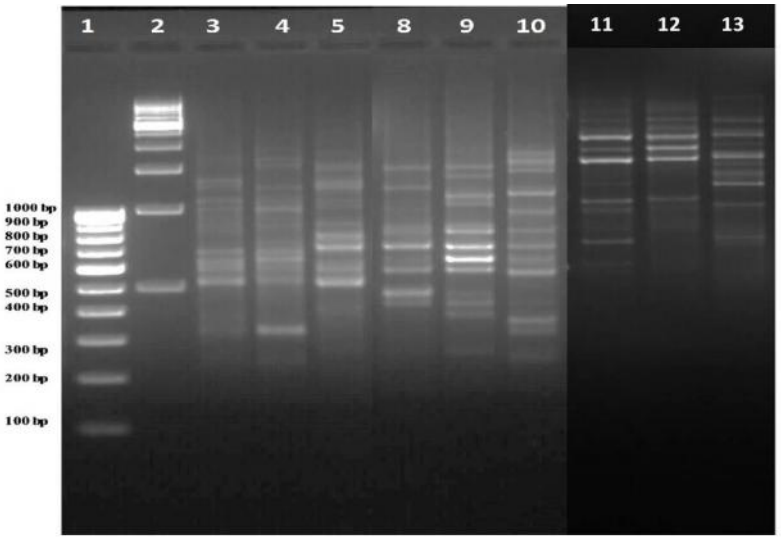

Fig. 4. RAPD-PCR pattern of three cichlid fish species showing DNA polymorphic bands using Random Primers: Lane -1: 100 bp step up ladder, Lane- 2: 500 bp step up ladder, Lanes: 3,4,5-Primer-1; Lanes: 8,9,10-Primer-2; Lanes: 3,4,5 Primer-3; Lanes:3,8, 11- *Oreochromis niloticus niloticus*, Lanes : 4, 9, 12- *O. mossambicus* and Lanes: 5, 10, 13-*Labidochromis caeruleus*

Phylogenetic tree construction from RAPD Analysis

The phylogenetic tree has been constructed for three primers using PyElph software. The phylogenetic tree contains taxa which represent the species name and bootstrap values which represent the reliability of the phylogenetic tree. Phylogenetic tree constructed which represents that Mozambique Tilapia and GIFT from the single node and the bootstrap values from all three primers for Mozambique and GIFT are same which share a recent common ancestor. The Banana cichlid does not have any common ancestry with either Mozambique or GIFT. The bootstrap values for the node is lowest in primer 1 and highest in primer 3 the bootstrap value for the Banana is highest in primer 3, Since Mozambique and GIFT share a recent common ancestor, they have the same bootstrap values and the banana is 100% distantly from the other two species (Fig. 5).

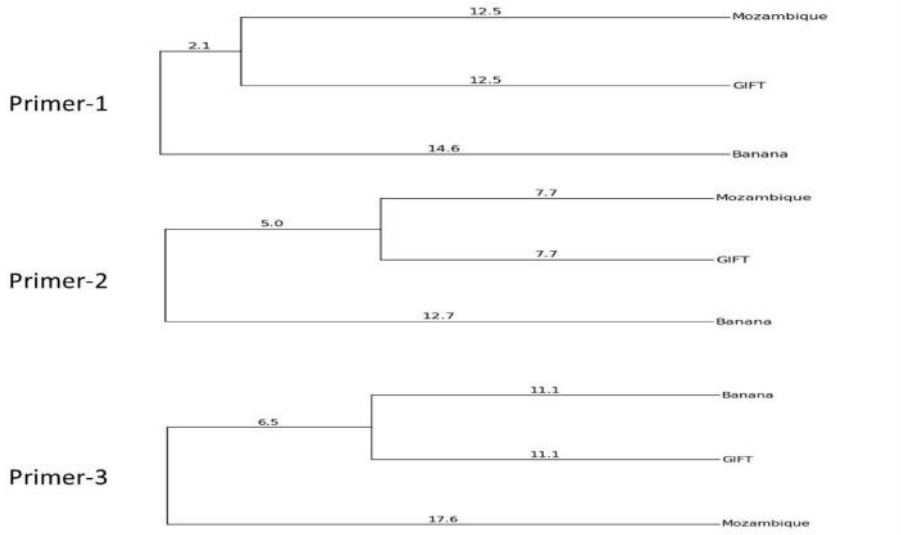

Fig. 5. Dendogram demonstrating the phylogenetic relationships among three cichlid fish species from the Rf values obtained by RAPD-PCR using three random primers

PCR amplification of 18s rRNA gene

The amplification of 18s rRNA gene from genomic DNA in three fin cichlid fishes was done using the primers, 18sf (5'CGA CTG GTT GAT CCT GCC AGT AG 3') and 18sr (5'TCC TGA TCC TTC TCA GGT TCA C 3').

The restriction digestion of 18s rRNA gene of Mozambique Tilapia with Ava I restriction endonuclease resulted in one restriction fragment (1185 bp). It yielded the same result for GIFT Tilapia (1185 bp, 200 bp and 250 bp) (Fig 6). Ava I generated similar bands for GIFT tilapia also. However, in case of Banana cichlid, Ava I generated a total of 3 bands with the molecular size of 1185 bp, 200 bp and 250 bp with R_f values of 0.82, 1.63 and 1.73 respectively.

The restriction digestion of this amplified 18s rRNA gene with EcoR I resulted in two DNA bands of 2000 bp and 1900 bp with R_f values 0.65 and 1.59 respectively in Mozambique tilapia as well as GIFT tilapia. While it generated three bands in Banana cichlids of molecular size 2000 bp, 1900 bp and 230 bp with R_f values 0.57, 0.65 and 1.59 respectively (Table 4). EcoR I generated similar bands in GIFT tilapia also as that of Mozambique tilapia and no polymorphic band was found between them. However, in Banana cichlid it generated a total of 3 bands with only one polymorphic band with molecular size of 230 bp with R_f value of 1.59. The 18s rRNA gene was not digested by Sma I.

TABLE-4. R_f Values and Molecular weight of the DNA bands generated by the three Restriction Endonucleases of the 18s rRNA gene in the three fin fish species

Sample	EcoRI		AvaI		SmaI	
	R_f Values	Mol. Wt. (bp)	R_f Values	Mol. Wt. (bp)	R_f Values	Mol. Wt. (bp)
Mozambique	0.65	2000	0.82	1185	0.35	1815
	1.59	1900	-	-	-	-
GIFT	0.69	2000	0.82	1185	0.35	1815
	1.67	1900	1.63	200	-	-
	-	-	1.71	250	-	-
	0.57	2000	0.82	1185	0.35	1815
	0.65	1900	1.63	200	-	-

Banana Cichlid	1.59	230	1.73	250	-	-

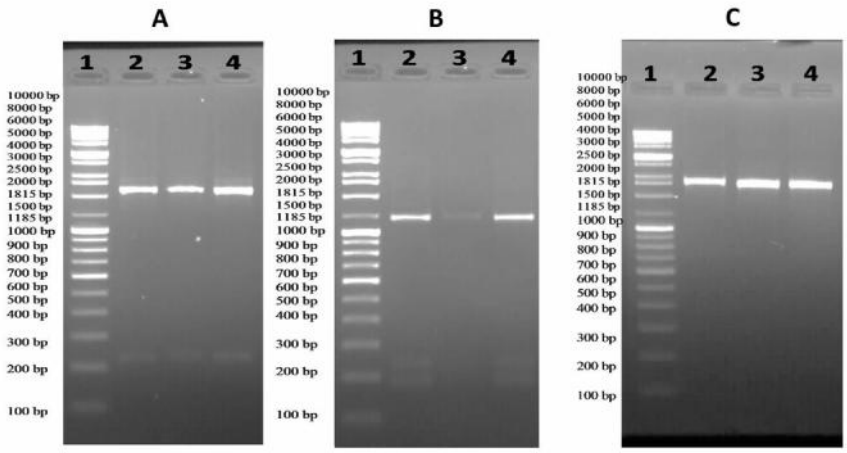

Fig. 6 : RFLP pattern of 18s r RNA of three cichlid species following digestion with Restriction Endonuclease **A**- *Eco*R I , **B** – *Ava*I, **C**- *Sma*I; Lane-1: DNA ladder, Lane 2: *Oreochromis niloticus niloticus*, Lane -3: *O. mossambicus* and Lane-4: *Labidochromis caeruleus*.

DISCUSSION

RAPD analysis of genomic DNA by using three different primers of OPA10, OPA08, OPA04 on three different fin fishes fishes *Oreochromis mossambicus* (Mozambique Tilapia), *Oreochromis niloticus niloticus* (GIFT) and *Labidochromis caeruleus* (Banana Cichlid) shows the monomorphic and the polymorphic bands (Table 3). RAPD-PCR with different primers shows the variation in their molecular weight with both the monomorphic and the polymorphic bands for different fish samples shows different (TNA) total number of amplified bands and the three fish samples with different primers shows same number of polymorphic bands and percentage of polymorphism (Table- 3, Fig. 4).

The phylogenetic tree has been constructed for three primers using PyElph software. The phylogenetic tree contains taxa which represent the species name and bootstrap values which represent the reliability of the phylogenetic tree. Phylogenetic tree constructed which represents that Mozambique Tilapia and GIFT from the single node and the bootstrap values from all three primers for Mozambique and GIFT are same which share a recent common ancestor. The Banana cichlid does not have any common ancestry with either Mozambique or GIFT. The bootstrap values for the node is lowest in primer 1 and highest in primer 3 the bootstrap value for the Banana is highest in primer 3, Since Mozambique and GIFT share a recent common ancestor, they have the same bootstrap values and the banana is 100% distantly from the other two species (Fig. 5).

The amplification of 18s rRNA gene from genomic DNA in three fin cichlid fishes was done using the primers, 18sf (5'CGA CTG GTT GAT CCT GCC AGT AG 3') and 18sr (5'TCC TGA TCC TTC TCA GGT TCA C 3'). The 18s rRNA gene was selected because it reveals even small genetic variation between the species (Stothard and Rollinson, 1997). However, many workers have used mitochondrial DNA as a marker in fish species identification (Unseld *et. al*., 1995; Hisar, *et al*., 2006). However, primers, 18sf (5'CCG CTT TGG TGA CTC TTG AT 3') and 18sr (5'CCG AGG ACC TCA CTA AAC CA 3') were used to amplify 18s rRNA gene based on the sequence information of channel catfish (Nakajima *et. al*., 2012). In the present study, 1800 bp of the 18s rRNA gene-PCR produce was amplified. However, a PCR product of 1400 bp molecular size of the 18s rRNA gene was reported using different primers (Nakajima *et al*., 2012).

In the present study, the restriction digestion of 18s rRNA gene of Mozambique Tilapia with Ava I restriction endonuclease resulted in one restriction fragment (1185 bp). It yielded the same result for GIFT Tilapia (1185 bp, 200 bp and 250 bp) (Fig 6). Contradictory to the findings of the present study, Ava I generated 6 restriction fragments after digestion with 18s rRNA gene of Mozambique Tilapia (650 bp, 500 bp, 350 bp, 250 bp, 150 bp, 100 bp) (El-Serafy *et. al.,* 2003). In the present study, Ava I generated similar bands for GIFT tilapia also. However, in case of Banana cichlid, Ava I generated a total of 3 bands with the molecular size of 1185 bp, 200 bp and 250bp with R_f values of 0.82, 1.63 and 1.73 respectively. The restriction digestion of 18s rRNA gene of the three cichlid fin fishes, Mozambique tilapia, GIFT and Banana cichlid did not result in any polymorphic band in Mozambique and GIFT while in Banana cichlid, it generated 3 polymorphic bands (Fig.6).

In the present study, the restriction digestion of this amplified 18s rRNA gene with EcoR I resulted in two DNA bands of 2000 bp and 1900 bp with R_f values 0.65 and 1.59 respectively in Mozambique tilapia as well as GIFT tilapia. While it generated three bands in Banana cichlids of molecular size 2000bp, 1900 bp and 230 bp with R_f values 0.57, 0.65 and 1.59 respectively (Table 4). The results of the present study are in agreement with those of some workers who observed that EcoR I digestion of 18s rRNA gene generated only two bands in the four species of tilapia including Mozambique tilapia. However, in their study they observed band with different molecular size (1650bp and 350bp) when compared with the results of the present study (El-Serafy *et al*., 2003). EcoR I

generated similar bands in GIFT tilapia also as that of Mozambique tilapia and no polymorphic band was found between them. However, in Banana cichlid it generated a total of 3 bands with only one polymorphic band with molecular size of 230 bp with R_f value of 1.59.

The 18s rRNA gene was not digested by Sma I in the present study. Similar results were reported that that Sma I did not digest the 18s rRNA gene of the three species of tilapia, *O. niloticus, O. aureus* and *S. galilaeus* (El-Serafy *et al*., 2003).

CONCLUSION

The present study was carried out to show the evolutionary relationship between three different cichlid species of fin fishes (Mozambique Tilapia, GIFT and Banana Cichlid). The genomic DNA was extracted from the muscle sample of these three fishes (Mozambique Tilapia, GIFT and Banana Cichlid). These relationships among species were studied with the help of RFLP and RAPD techniques. The RFLP analysis was done with the help of three enzymes and the bands were visualized. RFLP analysis showed that there were genetic variations after a certain point. The movement of the DNA was visualized under UV transilluminator. The R_f values and molecular weight of the samples were calculated for both RAPD and RFLP. A phylogenetic tree was constructed by using software called PyElph and the phylogenetic relationships were studied. Thus the study proved that there is an evolutionary relationship among the three cichlid fin fishes and it also showed the genetic variations occurring among these fishes (Mozambique Tilapia, GIFT and Banana Cichlid). There have been various genetic variations among the fishes from one generation to another in the given analysis.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the permission given to carry out the present study by the Principal, Adhiyamaan College of Engineering, Hosur, Tamil Nadu and FRIC, KVAFSU, Bengaluru, Karnataka.

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