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Molecular Systematic Investigations of Three Fin Fish Cichlid Species of *Oreochromis niloticus* (Nile Tilapia), *Oreochromis niloticus niloticus* (GIFT strain) and *Astronotus ocellatus* (Oscar Cichlid)

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ABS**TRACT**
Fisheries in India contribute significantly to the total GDP of agriculture and earn significant foreign exchange. Aquaculture is playing an important role in India and is bestowed with a huge biodiversity of aquatic organisms. India ranks second in total fish production in the world. Nile tilapia has become the world's second most popular farmed fish, after carps. Oscars is a popular aquarium fish around the world. In the present study, efforts were made to analyze the extent of divergence or similarity among three cichlid fin fishes, GIFT tilapia (*Oreochromis niloticus niloticus)*, Nile tilapia (*Oreochromis niloticus*) and Oscar cichlid (*Astronotus* ocellatus) using molecular biology techniques of Random Amplification of Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP). The Phylogenetic tree was constructed by using PhyElph software to study the evolutionary relationship between the three cichlid fin fish species. The Phylogenetic or evolutionary relationship was established for the three fishes Nile tilapia, GIFT and Oscar cichlid with the Phylogenetic tree. It was found that Nile tilapia and GIFT share a recent common ancestor while Oscar cichlid does not share any evolutionary relationship with Nile tilapia and GIFT.

KEYWORDS: Nile tilapia, Oscar, GIFT, RAPD, RFLP, Phylogenetic tree.

INTRODUCTION

More than 70 percent of the earth's surface is covered with water. The aquatic resources are rich in biodiversity and contribute greatly to the high protein dietary requirement of the ever-increasing global population. 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 (Crawford and March, 1989). In some countries, such as Bangladesh, Cambodia, Gambia, Sri Lanka and Small Island Developing States, fish makes up 50% or more of people's protein intake. On the global level, fisheries sector provides nearly 60 million people with direct employment. 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 the environment to address the well being and livelihood of people working in this sector. The fish and aquaculture production continues to expand increasing from 171 million tonnes to 201 million tonnes by 2030 (18% increase)(FAO, 2018). Nile tilapia is a freshwater cichlid native to the Nile River basin; the south-western Middle East; the Niger, Benue, Volta and Senegal rivers, and the lakes Chad, Tanganyika, Albert, Edward, and Kivu.

MATERIALS AND METHODS

Collection of Samples

GIFT tilapia (*Oreochromis niloticus niloticus)*, Nile tilapia (*Oreochromis niloticus*) and Oscar cichlid (*Astronotus* ocellatus) samples were collected from Fisheries Research and Information Centre (FRIC), Karnataka Verterinary Animal and Fisheries Sciences University (KVAFSU), Hesaraghatta, Bangalore, Karnataka, India (Fig. 1**)**.
The fish samples were anaesthetized using 3 drops of clove oil. 2 g of muscle sample was collected from the individual fish and transferred aseptically into labelled eppendorf tubes and preserved in absolute ethanol for further studies.

Fig. 1: Three species of Tilapia used in the study: 1-GIFT Tilapia, 2-Nile Tilapia, 3- Oscar fish

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 slits. The fishes were sacrificed and meristic characters were analysed for GIFT tilapia, Nile tilapia and Oscar cichlid. The meristic characters were counted under dissection microscope for better accuracy.

Extraction of genomic DNA

The preserved fish muscle tissue samples of three cichlid species were individually subjected to genomic extraction (Bardakci and Skibinski, 1994). The concentration of genomic DNA was estimated using UV-VIS Spectrophotometer at 260 nm. The isolated genomic DNA from fish tissue was checked with 0.8 % agarose gel.

RAPD Analysis

The RAPD analysis of genomic DNA of three fishes GIFT tilapia, Nile tilapia and Oscar cichlid were subjected to PCR amplification using primers OPA10, OPA08 and OPA04 (Table 1) for the amplification of gene (Ahmed *et. al.,* 2004). These primers were used to study variation in *Oreochromis* species (Bardakci and Skibinski, 1994).

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

Primer Name	Primer Sequence	GC %	T_m (°C)
OPA 10	GTGATCGCAG	60	25
OPA 08	GTGACGTAGG	60	25
OPA 04	AATCGGGCTG	60	25

Construction of phylogenetic tree

The DNA bands was generated from the RAPD analysis of three fishes GIFT tilapia, Nile tilapia and Oscar cichlid using three primers. The electrophoretic bands were analysed by PyElph gel image analysis program for construction of phylogenetic tree (Pavel and Vasile, 2012). A dendrogram was constructed based of genetic identity and genetic distance using the unweighted pair group method average 'UPGMA' clustering method.

Amplification and Isolation of 18s rRNA gene

The genomic DNA of three fishes GIFT tilapia, Nile tilapia and Oscar cichlid were subjected to PCR amplification using SSU I and SSU II primers (Table 2) for the amplification of 18s rRNA gene (Stothard and Rollinson, 1997). 5 ng of genomic DNA from each of the three fishes were used for the amplification of 18s rRNA. 25 µL PCR reaction mixture comprises of 2.5 µL of 10x PCR buffer 1.5 µL of dNTP mixtures, 2µL primer, 20 ng DNA template, 0.5 µl Taq DNA polymerase (1.25U), and 16 µL distilled water. PCR amplification of genomic DNA were performed in an Eppendrof Master cycle programmed for initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 56 °C for 30 sec, and extension at 72 °C for 2 min and a final extension step at 72 °C for 10 min. Reaction tubes were held at 4 °C. The visualization of PCR product was electrophoresed on a 1.5 % agarose gel stained with ethidium bromide.18s rRNA gene was isolated from PCR products by Glassmilk DNA purification method from the Electrophoresed agarose gel.

TABLE-2. Sequence of the primers used for the amplification of 18s rRNA gene

S.No	Primer Name	Primer Sequence	GC %	T_m (°C)
1	SSU I	CGACTGGTTGATCCTGCCAGTAG	56.5	68.9
2	SSU II	TCCTGATCCTTCTCAGGTTCAC	50	64.4

RAPD analysis of 18s rRNA gene

The extracted 18s rRNA gene from three fin fishes GIFT tilapia, Nile tilapia and Oscar cichlid was subjected to restriction digestion using the EcoRI, Ava I and Sma I (El-Serafy *et. al*., 2003). 25 µL of restriction digestion reaction mixture was comprises of 100ng 18s rRNA ,10x assay buffer and 0.5 units of EcoR I, Sma I and Ava I restriction enzymes respectively. The restriction digestion reaction for EcoR I and Ava I product was incubated at 37 for 2 hours and Sma I was incubated at 30 for 2 hours. The products of restriction digestion were subjected to gel electrophoresis using 2 % agarose gel with standard DNA markers (10,000 bp to 100 bp). The bands were using a UV transilluminator and the image was captured using a gel documentation unit.

RESULTS

Meristic characteristics

Nile tilapia contained a total of 16 - 17 dorsal spines and 11 - 15 dorsal rays. The number of anal spines was 3 and anal fin rays ranged from 10 - 11. In case of GIFT, the number of dorsal fin spines was 12 and dorsal fin rays were 13. The number of anal fin spines was 3 and anal fin rays were 9. In the Oscar Cichlid, the number of dorsal fin spines was 12 and rays were 21. The anal fin spines were 3 and anal fin rays were 19 (Fig.2, Table 3).

Fig. 2: Gill rakers : 1-GIFT Tilapia, 2-Nile Tilapia, 3- Oscar fish

TABLE-3. Meristic differences between the three fin fishes of cichlid species

Fish species	Dorsal fin		Anal fin		Gill Rakers
	Spines(No.s)	Rays(No.s)	Spines(No.s)	Rays(No.s)	(No.s)
Nile Tilapia	16-17	11-15	3	10-11	27 to 33
GIFT	16	13	3	9	24 to 25
Oscar	12	21	3	19	10 to 11

Extraction of genomic DNA

The genomic DNA was extracted from GIFT, *Oreochromis niloticus* (Nile tilapia), *Astronotus ocellatus* (Oscar).The concentration of genomic DNA was estimated using UV-VIS Spectrophotometer at 260 nm. The isolated genomic DNA from fish tissue was confirmed with 0.8 % agarose gel electrophoresis (Fig. 3).

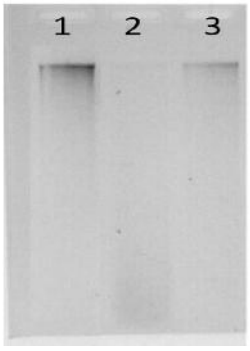

Fig. 3: Electrophoretogram of genomic DNA: Lane 1- GIFT Tilapia, Lane 2-Nile tilapia and Lane 3-Oscar fish

RAPD Analysis using three primers

The RAPD analysis of genomic DNA of three fishes GIFT, *Oreochromis niloticus* (Nile tilapia), *Astronotus ocellatus* (Oscar) were subjected to PCR amplification using primers OPA10, OPA08 and OPA04 (Table 2) for the amplification of gene. The primers were monitored for the capability to generate fingerprint banding pattern and to assess polymorphism among three Cichlid species (Table 4). All primers produced a total of 34 amplified bands with an average of 11.3 bands per primer from which 7 bands were common exhibiting low level of monomorphism of % 20.657, and 37 bands were polymorphic displaying high level of polymorphism of 79.33% with an average number of Polymorphic fragments per primer is 12.3. An instructive RAPD fingerprint profile was generated by the 16 primers with various band size lengths ranging from 100 to 4000 base pair comparing to a 100bp step up ladder and **5**00bp step up ladder. Three primers OPA10, OPA08 and OPA04 produced 8, 10 and 9 numbers of polymorphic bands respectively. The highest number of polymorphic bands (10) was generated with primer OPA08. The band frequency per species was 0.824, 0.794 and 0.735 for GIFT, *Oreochromis niloticus* (Nile tilapia), *Astronotus ocellatus* (Oscar) respectively while band frequency for each primer was from 0.294 to 0.353 (Fig. 4).

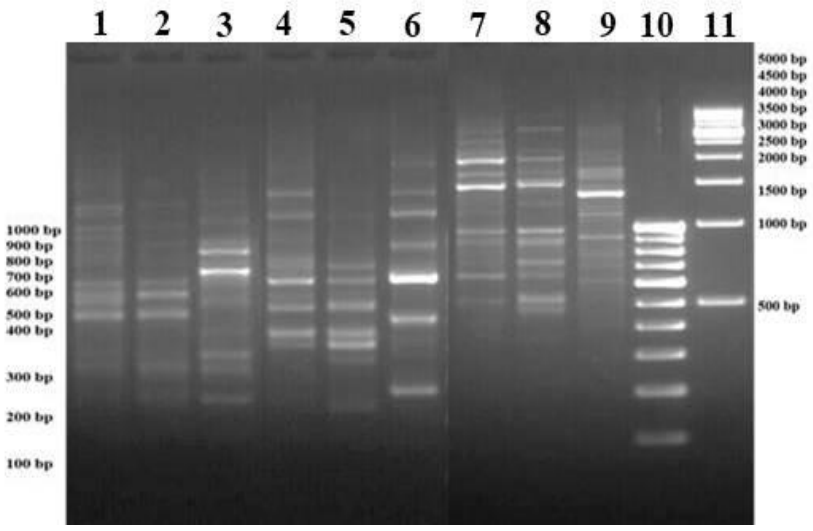

Fig. 4: Electrophoretogram of RAPD amplification products generated: Lanes 1, 4,7 - GIFT Tilapia; Lanes 2, 5,8- Nile tilapia; Lanes 3,6,9- Oscar fish; Lane 10- 100bp Stepup Ladder; Lane 11- 500bp Stepup Ladder; Lanes 1,2,3- primer OPA10; Lanes 4,5,6-primer OPA08; Lanes 7,8,9- primer OPA04

TABLE-4 RAPD band frequency for each primer, per species

Primer code	No of amplified band			TNA	NP	NM	Band freq/	%P	RA
	GIFT	Nile tilapia	Oscar	Bands	Bands	Bands	primer		Bands (bp)
OPA 10	9	8	9	10	8	2	0.294	80	200-1600
OPA 08	9	8	7	12	10	2	0.353	83.33	200-2500
OPA 04	10	11	9	12	9	3	0.353	75	300- 3100
Total	28	27	25	34	27	7		79.44	
Band freq/species	0.824	0.794	0.735						

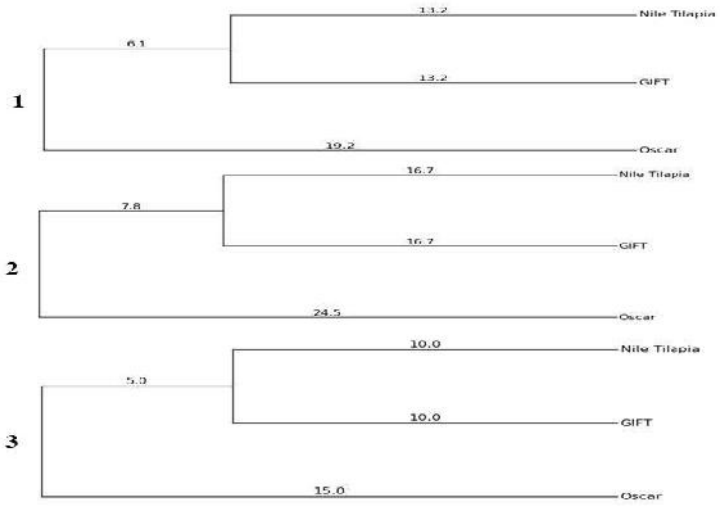

Fig. 5: Phylogenetic tree constructed for RAPD amplification products generated for GIFT Tilapia, Nile tilapia and Oscar fish: 1-primer OPA10, 2-primer OPA08 and 3-primer OPA04

Construction of phylogenetic tree

RAPD analysis was used to construct the phylogenetic tree to find evolutionary relationship among GIFT, *Oreochromis niloticus* (Nile tilapia), *Astronotus ocellatus* (Oscar). The primers gave polymorphic bonds among the three given species. The genetic difference in these fishes may be due to evolutionary relationship, habitat, temperature and phenotype of each genus. All the three primers OPA 10, OPA 08 and OPA 04 produced polymorphic bonds with Nile tilapia, GIFT and Oscar tilapia. The molecular weight of the bands showed that bands formed at different base pairs which showed the difference of the three fish species[1].The phylogenetic tree was constructed to study the evolutionary relationship between Nile tilapia, GIFT and Oscar cichlid (Fig.5). The phylogenetic tree constructed from the RAPD analysis of the three primers proved that Nile Tilapia and GIFT shared a recent common ancestor. The reason for that kind of conclusion was the formation of internodes by both Nile tilapia and GIFT in all the three phylogenetic trees. When two or more species form an internode in a phylogenetic tree, it shows that those species within these nodes share a common ancestor. Thus it can be concluded that Nile tilapia and GIFT share a very recent common ancestor but in case of Oscar cichlid, it did not form any kind of nodes with Nile tilapia or GIFT. This showed that Oscar cichlid does not share any type of evolutionary relationship between the other two species. It formed as a unique species in all the three phylogenetic trees thus giving us a clear cut view that Oscar does not have any kind of evolutionary relationship with the tilapia species.

PCR amplification of 18s rRNA gene

The visualization of PCR product was done on 1.5 % agarose gel, electrophoresed and stained with ethidium bromide (Fig. 6). In the present study, 1800bp of the 18s r RNA gene-PCR produce was amplified.

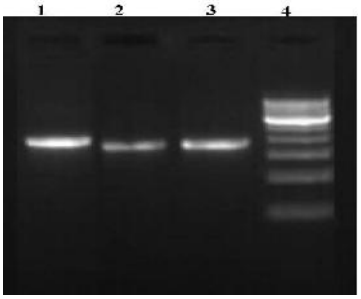

Fig. 6: 18s rRNA gene amplified products generated: Lane 1- GIFT Tilapia, Lane 2-Nile tilapia, Lane 3-Oscar fish, Lane 4- 100bp Stepup Ladder

RFLP analysis of 18s rRNA gene

RFLP analysis was done with the help of three restriction enzymes namely Ava I, Eco RI and Sma I for Nile tilapia, GIFT and Oscar cichlid in the 18s rRNA extracted from the samples. RFLP analysis was done to find the genetic variations and the phylogenetic relationship between the three species (Fig. 7). The restriction digestion of 18s rRNA gene of Nile Tilapia with Ava I restriction endonuclease resulted in three restriction fragments (1067 bp, 377 bp and 351 bp). It yielded the same result for GIFT Tilapia (1067 bp, 361 bp and 348 bp). Ava 1 generated similar bands for GIFT tilapia also. However, in case of Oscar Ava 1 generated a total of 5 bands with the molecular size of 3192 bp, 1516 bp, 1067 bp, 377 bp and 350 bp with the R_f values 0.20, 0.32, 0.36, 0.72 and 0.81 respectively. EcoR1 generated the similar bands in GIFT tilapia also as that of Nile tilapia and no polymorphic band was found between them. However in Oscar it generated a total of 3 bands with only one polymorphic band with molecular size of 1962 bp with the R_f value of 0.26.

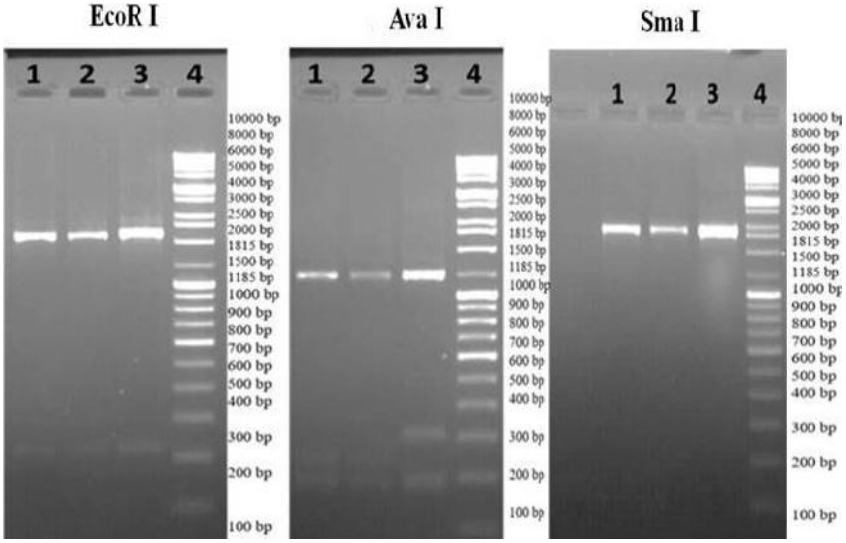

Fig. 7: RFLP analysis of 18s rRNA gene using EcoRI, Ava I and Sma I:Lanes 1- GIFT Tilapia, Lanes 2-Nile tilapia, Lanes 3-Oscar fish, Lanes 4- 100bp Stepup Ladder

DISCUSSION

The RAPD analysis of genomic DNA of three fishes GIFT tilapia, Nile tilapia and Oscar cichlid were subjected to PCR amplification using primers OPA10, OPA08 and OPA04 (Table 2) for the amplification of gene. The primers were monitored for the capability to generate fingerprint banding pattern and to assess polymorphism among three Cichlid species (Table 4). All primers produced a total of 34 amplified bands with an average of 11.3 bands per primer from which 7 bands were common exhibiting low level of monomorphism of % 20.657, and 37 bands were polymorphic displaying high level of polymorphism of 79.33 % with an average number of Polymorphic fragments per primer is 12.3. An instructive RAPD fingerprint profile was generated by the 16 primers with various band size lengths ranging from 100 to 4000 base pair comparing to a 100bp step up ladder and **5**00bp step up ladder.

Three primers OPA10, OPA08 and OPA04 produced 8, 10 and 9 numbers of polymorphic bands respectively. The highest number of polymorphic bands (10) was generated with primer OPA08. The band frequency per species was 0.824, 0.794 and 0.735 for GIFT tilapia, Nile tilapia and Oscar cichlid respectively while band frequency for each primer was from 0.294 to 0.353 (Fig. 4).

Construction of phylogenetic tree

RAPD analysis was used to construct the phylogenetic tree to find evolutionary relationship among GIFT tilapia, Nile tilapia and Oscar cichlid. The primers gave polymorphic bonds among the three given species. The genetic difference in these fishes may be due to evolutionary relationship, habitat, temperature and phenotype of each genus. All the three primers OPA 10, OPA 08 and OPA 04 produced polymorphic bonds with Nile tilapia, GIFT and Oscar tilapia. The molecular weight of the bands showed that bands formed at different base pairs which showed the difference of the three fish species[1].The phylogenetic tree was constructed to study the evolutionary relationship between Nile tilapia, GIFT and Oscar cichlid (Fig.5). The phylogenetic tree constructed from the RAPD analysis of the three primers proved that Nile Tilapia and GIFT tilapia shared a recent common ancestor. The reason for that kind of conclusion was the formation of internodes by both Nile tilapia and GIFT tilapia in all the three phylogenetic trees. When two or more species form an internode in a phylogenetic tree, it shows that those species within these nodes share a common ancestor. Thus it can be concluded that Nile tilapia and GIFT tilapia share a very recent common ancestor but in case of Oscar cichlid, it did not form any kind of nodes with Nile tilapia or GIFT tilapia. This showed that Oscar cichlid does not share any type of evolutionary relationship between the other two species. It formed as a unique species in all the three phylogenetic trees thus giving us a clear cut view that Oscar does not have any kind of evolutionary relationship with the tilapia species.

PCR amplification of 18s rRNA gene

The amplified 18s rRNA gene from GIFT, *Oreochromis niloticus* (Nile tilapia), *Astronotus ocellatus* (Oscar) was done using the primers SSU I and SSU II (Table 3).The 18s rRNA gene was selected because it reveals even genetic variation between the species (Stothard and Rollinson,1997). The visualization of PCR product was done on 1.5 % agarose gel, electrophoresed and stained with ethidium bromide (Fig. 6). However, many workers have used mitochondrial DNA as a marker in fish species identification (Unseld *et. al.,* 1995; Hisar *et. al.*, 2006)). The primers, 18s forward primer (5'CCG CTT TGG TGA CTC TTG AT) and 18s reverse primer (5'CCG AGG ACC TCA CTA AAC CA) were used to amplify 18s rRNA gene based on the sequence information of channel catfish (Nakajima *et. al*., 2012). In the present study, 1800bp of the 18s r RNA gene-PCR produce was amplified. However, a PCR product of 1400bp molecular size of the 18 s r RNA gene was reported using different primers (Nakajima *et. al*., 2012).

RFLP analysis of 18s rRNA gene

RFLP analysis was done with the help of three restriction enzymes namely Ava I, Eco RI and Sma I for Nile tilapia, GIFT and Oscar cichlid in the 18s rRNA extracted from the samples. RFLP analysis was done to find the genetic variations and the phylogenetic relationship between the three species (Fig. 7).

The restriction digestion of 18s rRNA gene of Nile Tilapia with Ava I restriction endonuclease resulted in three restriction fragments (1067 bp, 377 bp and 351 bp). It yielded the same result for GIFT Tilapia (1067 bp, 361 bp and 348 bp).Contradictory to the findings of the present study, Ava I generated 6 restriction fragments after digestion with 18s rRNA gene of Nile Tilapia (650 bp, 500 bp, 350 bp, 250 bp, 150 bp, 100 bp) (El-Serafy *et al*., 2003). In the present study, Ava 1 generated similar bands for GIFT tilapia also. However, in case of Oscar Ava 1 generated a total of 5 bands with the molecular size of 3192 bp, 1516 bp, 1067 bp, 377 bp and 350 bp with the R_f values 0.20, 0.32, 0.36, 0.72 and 0.81 respectively. The restriction digestion of 18s rRNA gene of the three cichlid fin fishes, Nile tilapia, GIFT and Oscar did not results in any polymorphic band in Nile and GIFT, while in Oscar, it generated 3 polymorphic bands. In the present study, the restriction digestion of this amplified 18s rRNA gene with EcoR1 resulted in 3 DNA bands of 1921 bp, 1613 bp and 323 bp with the R_f values 0.26, 0.29 and 0.76 respectively in Nile tilapia as well as GIFT tilapia resulted in 3 DNA bands of 1882 bp, 1613 bp and 320 bp with a values of 0.27, 0.29 and 0.77. While it generated three bands in Oscar cichlids of molecular size 1962 bp.169 bp and 322 bp with R_f values 0.26, 0.29 and 0.76. Some researchers observed that EcoRI digestion of 18 s rRNA gene generated only two bands in the four species of tilapia including Nile tilapia and their results are in agreement with those of the present study. 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).

EcoR1 generated the similar bands in GIFT tilapia also as that of Nile tilapia and no polymorphic band was found between them. However in Oscar it generated a total of 3 bands with only one polymorphic band with molecular size of 1962 bp with the R_f value of 0.26.Similar results were reported by El-Serafy *et al*. (2003) who observed that Sma 1 did not digest the 18s rRNA gene of the three species of tilapia, *O.niloticus, O.aureus* and *S.galilaeus.*

CONCLUSION

The present study was done to show the evolutionary relationship between three different cichlid fin fishes (Nile Tilapia, GIFT and Oscar Cichlid). The genomic DNA was extracted from the muscle sample of these three fishes (Nile Tilapia, GIFT and Oscar Cichlid). These relationships were studied with the help of RFLP and RAPD techniques. The RFLP analysis was done with the help of three enzymes and 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 value and molecular weight of the samples were calculated for both RAPD and RFLP. Phylogenetic tree was constructed by using software called PyElph and the phylogenetic relationships were studied. 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 (Nile Tilapia, GIFT tilapia and Oscar Cichlid). There have been various genetic variations among the fishes from one generation to another in the given analysis. The phylogenetic tree proved that Nile Tilapia and GIFT tilapia shared recent common ancestors.

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