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COMPARATIVE GENOMIC STUDIES ON SOME FRESH WATER AND MARINE FISHE USING F- AFLP AND SEQUENCING INTRON 5 OF GROWTH HORMONE GENE

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ABSTRACT

Four fish species; two fresh water fish (*Oreochromis niloticus* L. and *Tialpia zillii* L.), and two marine fish (*Liza ramada* L. and *Mugil cephalus* L.) were used in this study. These species were collected from Damietta and Port Said Governorate, respectively. The current Amplified Fragment Length Polymorphism (AFLP) analysis was successfully able to determine that phylogenetic and reflected the species type and the habitats type. The Nile tilapia and Green-belly were grouped in the same sub-cluster which belongs to fresh water fish, while Toubar and Bouri were found in the other sub-cluster which belongs to marine fish. In contrast to the Principal Coordinate Analysis (PCoA) that showed the habitat in accordance with the majority of life time in fresh water that Toubar spend more time during its life cycle in the fresh water than the Bouri. Structure analysis showed that the Toubar was more closely to the two fresh water species. The ability of these differences among and within species according to Analysis of Molecular Variance (AMOVA) analysis refered to using samples under study with wide environmental range. Our current study for sequence of intron 5 of growth hormone (GH) gene showed differences between *L. ramada*, *M. cephalus*, *O. niloticus* and *T. zillii* which were successfully able to determine mutations and Deletion and Insertion nucleotides in intron 5 of GH gene between *L. ramada*, *M. cephalus*, *O. niloticus* nad *T. zillii*. Polymophic single nucleotide and the numbers of A, C, G and T nucleotides for each fragment of intron 5 of GH gene also were determined.

Key words: AFLP, DNA sequencing, Intron, Growth hormone.

INTRODUCTION

Fish is the most important source of protein in tropical and subtropical countries especially those suffering chronically from lack of animal protein (Hickling, 1963). Fish caught from natural sources declined in many countries. The aquaculture is the most promising farming method to increase fish production. Freshwater fish are the most vertebrate groups exploited by human (Moralee *et al.*, 2000; Rashed *et al.,* 2002; Lee *et al*., 2011). Tilapias have been introduced and transferred around the world from their endemic origins in Africa and Middle East (Pullin 1983). According to Ladewig *et al.* (1984) and Bezrukov (1987) tilapia as a common name was divided into three main genera, *Tilapia*, *Sarotherodon and Oreochromis. Tilapias* (*Oreochromis niloticus, Oreochromis aureus* and *Tilapia zillii*) have become the most important fish species for freshwater aquaculture because of the relative ease of culture and breeding in variety of aquaculture systems and their favorable attributes as food fish. Marine fish are very important for many countries as economic source of food (Tidwell and Allan, 2001 ; Bell *et al*., 2009). *Mugil cephalus* L. (flathead mullet) is an important commercial marine fish and mainly inhabits tropical and subtropical coastal regions of the world (Nelson *et al*., 2004). The thinlip grey mullet *Liza ramada* L. (Mugilidae) is a migratory species classified as rare in the North

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Sea where it is sometimes caught along the Atlantic coast (Jonsson and Jonsson, 2008), it is also found in the Mediterranean Sea (Araújo *et al*., 2011). Amplified Fragment Length Polymorphism (AFLP) fingerprinting was used to detect genetic variability and comparative genomic in fish (*e.g. Liza ramada, Oreochromis niloticus* and *Oreochromis aureus*) (Bagley *et al*., 2001; Barreto and McCartney 2008). AFLP is a PCR-based technique that uses selective amplification of a subset of digested DNA fragments to generate and compare unique fingerprints for model and non model organisms (Vos *et al*., 1995). The power of this method relies mainly in that it does not require prior information regarding the targeted genome, as well as in its high reproducibility and sensitivity for detecting polymorphism at the level of DNA sequence. AFLP is employed for a variety of applications, such as: to assess genetic diversity within species or among closely related species (Maldini *et al*., 2006), to infer population-level phylogenies (Wang *et al*., 2009) and biogeographic patterns (Bagley *et al*., 2001). Variations of standard AFLP methodology have been also developed for targeting additional levels of diversity, like transcriptomic variation and DNA methylation polymorphism. Growth hormone (GH) gene plays a very important role in many regulatory, metabolic and developmental processes in various vertebrate tissues (Thompson *et al*., 2000). It is involved in the regulation of somatic growth and maintenance of protein, lipid, carbohydrate and mineral metabolism (Cruz *et al*., 2006; Khatab *et al*., 2014). This study aimed to study the genetic variation within and between freshwater and marine fish using both fingerprinting (*e.g.* fluorescence AFLP) and partial sequencing of growth hormone gene for each species, that isolate and sequence intron 5 of growth hormone (GH) gene to study inter specific variation among freshwater and marine fish and to test the genetic variation for being associated to the species habitats.

MATERIALS AND METHODS

Sampling

Four fish species; two freshwater fish (*Oreochromis niloticus* L. and *Tialpia zillii* L.), and two marine fish (*Liza ramada* L. and *Mugil cephalus* L.) were used in this study. These species were collected from Damietta and Port Said Governorate, respectively. This investigation was carried out in the laboratories of Molecular Genetics, Genetic Department, Faculty of Agriculture, Ain Shams and Zagazig Universities, Egypt, during the period from 2012 to 2015.

Molecular Analysis

DNA extraction and purification

DNA was isolated from caudal fin tissue from 12 samples which represented four species; two freshwater fish (*Oreochromis niloticus* L. and *Tialpia zillii* L.), and two marine fish (*Liza ramada* L. and *Mugil cephalus* L.). Three different samples from each species were used as described by Bardakci and Skibinski (1994) with slight modifications**.**

AFLP fluorescence technique

The AFLP technique involves one preparatory step in addition to three major steps according to Vos *et al.* (1995), Preparation of adaptors and primers, Restriction "R" and ligation "L" of adaptors, Pre-selective PCR and Selective PCR. Then the product visualized using gene scan method. AFLP technique was carried out according to the original protocol fluorescently labeled as follows (Table 1).

AFLP Data Analysis

Fragment analysis

AFLP chromatogram data obtained from the fragment analysis method are very sensitive to factors like small bands, stutter bands, dimers, and noisy background. To minimize the influence of such factors, an automated AFLP scoring was performed using both programs, PEAKSCANNER for peak analysis and RAWGENO V2 for automated scoring (Arrigo *et al*., 2009).

Data analysis

The analysis of the AFLP data was performed in two known approaches. The first is based on the band-binary criterion (*i.e.* codifying the detected bands to 1 when presence and 0 when absent) and the other is based on the allele frequency (*i.e.* number of a band presence relatively to the number of all individuals). Each has its advantages and disadvantages, therefore, both forms were combined to obtain the maximum number of valuable indices according to the methodology of Bonin *et al*. (2007).

Table 1. Sequence 5´- 3´of primers and adaptors used to establish the AFLP technique

Primer/Adaptor	5´ - sequence - 3´
EcoRI - A1	CTCGTAGACTGCGTACC
EcoRI - A2	AATTGGTACGCAGTC
Mse I - A1	GACGATGAGTCCTGAG
Mse I - A2	TACTCAGGACTCAT
Eco + A	GACTGCGTACCAATTCA
Mse + C	GATGAGTCCTGAGTAAC
Eco + ACA	*FAM*-GACTGCGTACCAATTCACA
Eco + AGG	*HEX*-GACTGCGTACCAATTCAGG
Eco + ATA	*CY3*-GACTGCGTACCAATTCATA
Mse + CAA	GATGAGTCCTGAGTAACAA

Genetic polymorphism

Genetic variability was demonstrated by measuring valuable indices that were measured using FAMD (Fingerprint Analysis with Missing Data Software) (Schlüter and Harris 2006) and STRUCTURE (Pritchard *et al.,* 2000) in which number of different alleles (Na), number of effective alleles (Ne), Shannon information index (I), and genetic diversity (H) were estimated; where for Haploid Binary data, p = Band Freq. and q = 1 - p.

Ne = No. of Effective Alleles = $1 / (p^{\wedge 2} + q^{\wedge 2})$

I = Shannon's Information Index = $-1 \times (p \times Ln(p) + q \times Ln(q))$

h = Diversity = $1 - (p^{\wedge 2} + q^{\wedge 2})$

Population structure and phylogeny

FAMD was used to calculate gene flow (Nm) and F-statistics (FST) pairwise genetic distance matrix, population genetic distance and to test the population differentiation measurement by performing the analysis of molecular variance (AMOVA).

Isolation and sequencing of Growth hormone (GH) gene

Isolation of intron 5 for GH gene using PCR

Specific primer pair for intron 5 of growth hormone gene (forward primer: GHTILAPIA-F mod; 5` GAGAGCTCTCTGCAGAC SGAGGA G 3`, and reverse primer: GHTILAPIA-R mod; 5` ATYTCKGCYRSRTCCTGATTGGCC 3`) were newly designed and tested on the species under study, using the optimized PCR conditions.

DNA Sequencing of GH gene

PCR purification and elution

All PCR sample reactions, when successful, were prepared for the cleanup step (purification) in order to eliminate dimers, RNA, PCR residues and unamplified DNA by using Gene JETTM PCR purification kit (Thermo scientific, K0701).

PCR sequencing

After successful purification, the purified DNA was prepared to be sent to the sequence service to proceed with the sequencing process. To sequence all samples for each selected region, 96-well PCR-plates were prepared and both strands of each sample were sequenced. Each sample was prepared by adding 1 µl of primer (10 µM) to 10 µl of purified DNA product (approx. 50 µg).

Sequencing alignment and data analysis of intron 5 for GH gene

For each region, the sequence chromatograms were compiled using BIOEDIT V3 (Hall, 1999). MEGA V6. Tamura *et al*. (2013) a phylogenetic program was used for assembling data, alignment evolutionary analysis and visualizing

tree editor. All phylogenetic analysis based on Jukes-Cantor (JC) substitution model unless otherwise is mentioned.

RESULTS AND DISCUSSION

Amplified Fragment Length Polymorphism (AFLP) Fingerprint

Amplification, scoring and readability

PCR amplification was successful for three pairs of primers which represented four species under study. The total number of scored bands was 382 as 111, 147 and 124 bands for the three primer pairs, respectively, all bands ranged between 50 – 700 bp.

DNA polymorphism

Genetic diversity and polymorphism

A total of 382 bands were scored from three primer pairs for all 12 samples which represented 4 species. All bands were polymorphic. Percentage of polymorphic loci was 25.13% in Bouri, 51.31% in Toubar, 45.55% in Green-belly tilapia and 45.03% in Nile tilapia (Table 2).

Similar results were obtained by Maldini *et al*. (2006) who measured the percentage of polymorphic loci between 20 species, ranged between 2.75 - 37.27%, while the most relevant to our work, the highest percentage was found between two *Liza* species (31.19 - 37.27%). Wang *et al*. (2000) obtained 299 selectively amplified DNA fragments of which 66.2% were polymorphic using three primer set combinations in Common carp (*Cyprinus carpio* L.).

The highest mean number of different allele (Na) was 1.22 ± 0.05 in *L. ramada* (Toubar) and the lowest was 0.85 ± 0.04 in *M. cephalus* (Bouri). The highest mean number of effective allele (Ne) was 1.41 ± 0.02 in *L. ramada* (Toubar) and the lowest was 1.20 ± 0.02 in *M. cephalus* (Bouri). The highest mean of Shannon`s information index (I) was 0.33 ± 0.02 in *L. ramada* (Toubar) and the lowest was 0.16 ± 0.01 in *M. cephalus* (Bouri). The highest mean of diversity (H) was 0.23 ± 0.01 in *L. ramada* (Toubar) and the lowest was 0.11 ± 0.01 in *M. cephalus* (Bouri) (Table 2).

Shannon's diversity index was calculated to provide a relative estimate of the degree of genetic variation within each population, Kader *et al*. (2013) measured Shannon's Information index (I= 0.36 ± 0.30) for *T. zillii* and (0.319, 0.35) for *O. niloticus* and *O. aureus,* respectively, while considering the Nei's gene diversity (H), higher diversity was found in *T. zillii* (0.25) than in *O. niloticus* (0.22) and *O. aureus* (0.24) populations, this means that *T. zillii* population has a higher proportion of heterozygous genotypes than *O. niloticus* and *O. aureus* populations.

Comparative analysis of AFLP product

Similarity and dissimilarity

The calculated Jaccard similarity coefficients based on AFLP data ranged from 0.28 between Green-belly and Nile tilapia which displayed low genetic similarity of 0.58 between Bouri and Nile tilapia which displayed a higher genetic similarity. Diagonal pairwise Fst values were written according to standard Jaccard distance transformation (1-similarity), (Table 3).

Inter species analysis

Principal coordinate analysis

A PCA variable showed clustering in four quadrants variables (Fig. 1). PCA analysis showed that Nile tilapia and Green-belly (*zillii*) set in quadrant II. Toubar set in quadrant III, while Bouri set in quadrant I on a distance from the other studied species, it worth mentioning, the two marine species are obviously apart while Toubar species are located near the freshwater species than to its mate.

Sønstebø *et al*. (2007) mentioned that the results of the PCO of the pairwise individual genetic distances in brown trout (*Salmo trutta* L.) presented that in the two first Principal Coordinates (PCoA) axes of both the AFLP and the microsatellite plot describe almost 50% of the variation. Lucek *et al*. (2010) mentioned that the principal coordinate analysis based on the statistical principal coordinate analysis (PCA) used to show the genetic variation on the scale between two axes, the first (17.4%) and the second axis (10.8%) of the variation in stickleback fresh water fish. Applying the same method revealed successfully the ability of the

Table 2. Genetic parameters: number of different alleles (Na), number of effective alleles (Ne), Shannon information index (I), genetic diversity (H) and (%) of polymorphic

Common name			Na	Ne	I	H	(%) of polymorphic
Marine fish	Bouri	Mean	0.85	1.20	0.16	0.11	
		SE	0.04	0.02	0.01	0.01	25.13
	Toubar	Mean	1.22	1.41	0.33	0.23	
		SE	0.05	0.02	0.02	0.01	51.31
Freshwater Fish	Green-belly tilapia	Mean	1.05	1.36	0.29	0.20	
		SE	0.05	0.02	0.02	0.01	45.55
	Nile tilapia	Mean	1.11	1.36	0.29	0.20	
		SE	0.05	0.02	0.02	0.01	45.03

Table 3. Genetic similarity matrix among four species genotypes, as computed according to Jaccard and Bayesian similarity coefficient from AFLP generated data

	Bouri	Toubar	Green-belly	Nile tilapia
Bouri	1.00			
Toubar	0.56	1.00		
Green-belly	0.52	0.32	1.00	
Nile tilapia	0.58	0.39	0.28	1.00

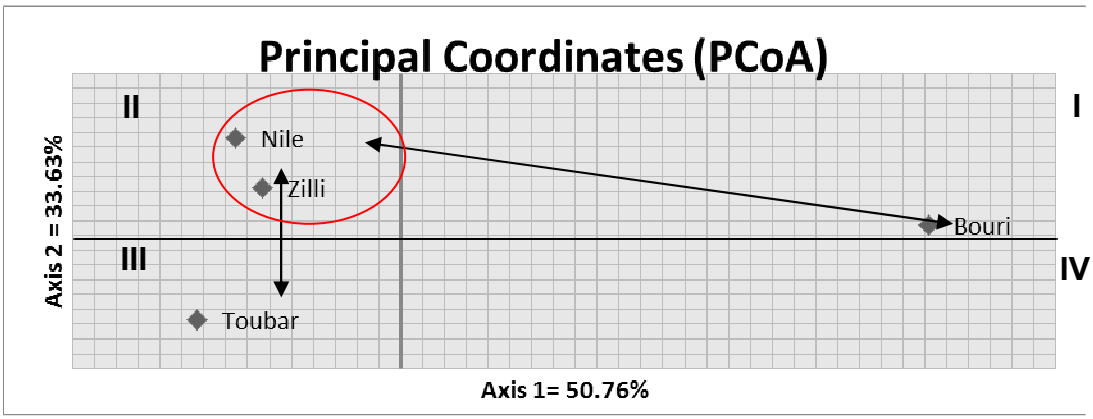

Fig. 1. PCA plot for explained by two axes. Axis 1 = 50.76 % and axis 2 = 33.63%. Four quadrants were defined

PCoA to reveal the genetic variation among the studied species. Where the two fresh water species where closely related while the two marine were apart. It was observed that Toubar was more closely to the two fresh water species than to its relative of the same family (Miguliadea). Such observation would reflect the success of the amplified AFLP markers to reflect the fact that Toubar spend more time during its life cycle in the fresh water than Bouri.

Structure analysis (Clustering)

Structure analysis between marine and fresh water fish under study showed that Toubar was definitely related to the two fresh water species Green-belly and Nile tilapia than to its relative species (Bouri). Based on STRUCTURE results (fingerprinting technique) (Fig. 2), samples that are genetically related are from different geographical water type, which reflects that the detected genetic variation is more related to the water type than to the species.

Sønstebø *et al.* (2007) used structure analysis in *Salmo trout* L. that places individuals population information into subgroups that had distinctive allele frequencies.

Analysis of molecular variance (AMOVA)

An AMOVA test was used to measure the changes in the pairwise differentiation of the F-statistics analog for the AFLP, FST value was 0.369 (P-value=0.00), the variance was partitioned into variation among water type (3%), among species 63% and 34% within species (Table 4 and Fig. 3). The genetic variation mainly found to be affected by the differences between the samples of the same species, and less between the species, while very low among the differences between its habitats. High genetic differentiation was significantly found, whereas in Lin *et al*. (2009), they found that the overall genetic differentiation among small yellow croaker fish from the six populations was small but significant (FST = 0.030, P- value < 0.001), suggesting significant genetic differentiation among localities, and a significant genetic structure (FST =0.058, P < 0.05) was detected among the three groups based on the breeding migration routes and over-wintering grounds that according to AMOVA analysis,.

Phylogenetic relationships

Dendrogram constructed from the AFLPs generated data separated 12 samples of four species of marine and fresh water fish from Neighbor joining cluster analysis into two major clusters (Fig. 4).

Cluster one included Nile tilapia 1, Nile tilapia 2, Nile tilapia 3, Green-belly (Zillii) 1, Green-belly (Zillii) 2 and Green-belly (Zillii) 3. Cluster two included Toubar 1, Toubar 2, Toubar 3, Bouri 1, Bouri 2 and Bouri 3.

Within cluster one; one sub-cluster contained Nile tilapia 3 and Nile tilapia 2,this sub-cluster joined with Nile tilapia 1. The other sub-cluster contained Green-belly (Zillii) 2 and Green-belly (Zillii) 1, which joined with Green-belly 3. Cluster two was divided into two sub-clusters; Toubar 2 and Toubar 1, which joined with Toubar 3 in first sub-cluster, however the second sub-cluster contained Bouri 2 and Bouri 1, which joined with Bouri 3. The phylogenetic analysis perfectly reflected the species type and the habitats type.

Kader *et al*. (2013) mentioned that the genetic identity and distance between three Tilapia species showed that *O. niloticus* was closest to *O. aureus* with a genetic identity of (0.736), whereas *T. zillii* was farthest to *O. aureus* and *O. niloticus* with a genetic distance of (0.377, 0.405, respectively, the UPGMA-tree dendogram indicated the relationship among the three Tilapia species which are of family Cichlidae, taking into consideration the close relationship between *O. niloticus* and *O. aureus*.

Nucleotide Polymorphism (DNA Sequencing)

Four samples (each represent a single species) were used for DNA amplification, purification and sequencing of intron 5 of Growth Hormone gene. When successful, sequences were aligned to a reference sequence to detect the overall variation ratio (variation test)

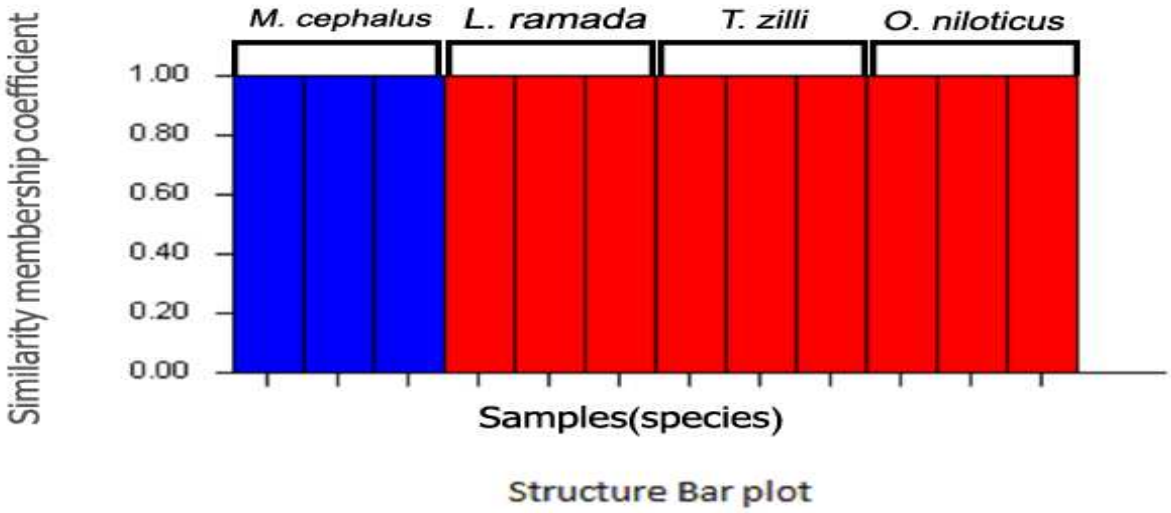

Fig. 2. Graphical plotting of STRUCTURE output data based on AFLP loci. which1, 2, 3 represent (1= *M. cephalus*); 4, 5, 6 represent (2= *Liza ramada*); 7, 8, 9 represent (3= *T. zilli*) and 10, 11, 12represent (4= *O. niloticus*)

Table 4. Genetic differentiation through AMOVA of marine and freshwater fish based on the AFLP. The source of variance (among and within locations), the degree of freedom (df),the sum of squares (SS), the mean of squares (MS), the estimated variance and the percentage of variation, are shown

Source	Df	SS	MS	Est. Var.	(%)
Among water type	1	153.750	153.750	2.472	3%
Among species	2	277.833	138.917	28.583	63%
Within species	8	425.333	53.167	53.167	34%
Total	11	856.917		84.222	

Fst=0.369 (P- value=0.00)

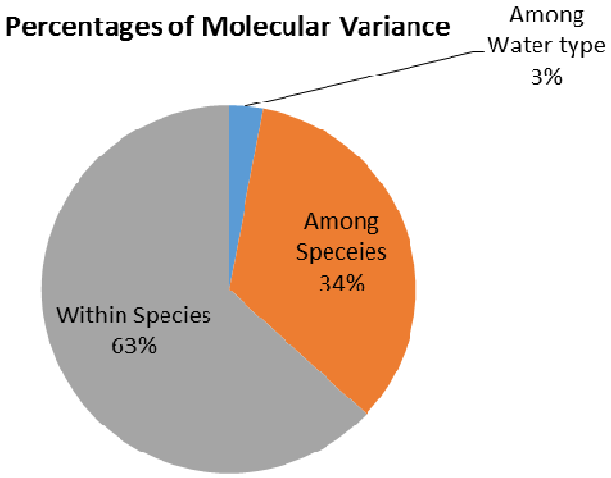

Fig. 3. Fst partitions based on AMOVA test of the AFLP *loci*.

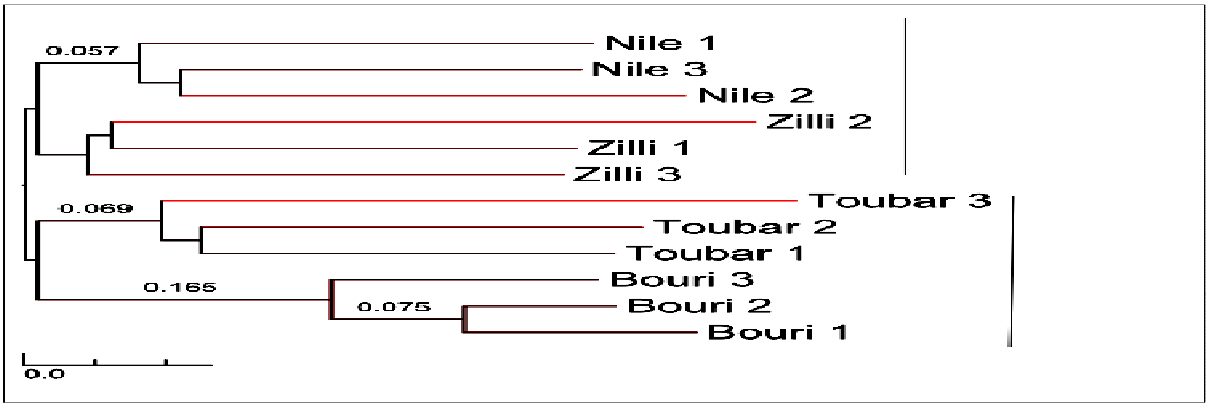

Fig. 4. Dendogram for the genetic relationships among species using Neighbor joining cluster analysis

by dividing the total number of variations in nucleotide sequence by the total length of the sites. Successful reasonably variable candidate regions were used for sequencing all samples. Growth hormone (GH) has many important physiological roles in the control of growth, metabolism and reproduction, which is mediated by growth hormone receptor (GHR) (Ma *et al.,* 2007). Genotypic data were analyzed to estimate genetic variability, DNA polymorphism and to generate phylogenetic trees.

Genotyping of intron 5 of growth hormone (GH) gene

PCR and sequencing product

One sample from each species was chosen to be sequenced which PCR products resulting from DNA extraction from each species under study. Sequence data generated 116 nucleotides which represent intron 5 of GH gene from all species under study (Fig. 5).

Genetic inter-specific polymorphism in intron 5 of growth hormone (GH) gene

The number of each nucleotide in each sequenced fragment of the four species under study as shown in Table 5. Where the number of Adenine (A) and Thymine (T) bases were 31 and 25 bp in *L. ramada* and *M. cephalus*, respectively. While the cytosine (C) bases were 11 bp in *M. cephalus* and *O. niloticus*. Moreover, the number of guanine + cytosine (G+C) was 48 bp for *L. ramada* and *M. cephalus* and 45 bp for *O. niloticus* and *T. zillii*. While the number of A+T was 56 bp for *L. ramada* and *M. cephalus* and 59 bp for *O. niloticus* and *T. zillii*.

Freshwater fish

More than a mutation was detected between *O. niloticus* and *T. zillii.* Nucleotide substitutions were seen in a form of three transversions (T □ C and G □ A) were sited in base number 20, 45 and 101, of the aligned sequences respectively. Nucleotide T mutated to nucleotide C at the base number 20. However, nucleotide G mutated to nucleotide A at base number 45 and 101, respectively. All these mutations represent transversion mutations, as shown in Table 6.

Ber and Daniel (1992) studied the nucleotide sequence of the growth hormone (GH)-encoding gene (GH) of the tilapia fish (*Tilapia nilotica*) comparing with salmonidae fish and reported that the *T. nilotica* GH gene, similar to that of the salmonidae fish. Palaiokostas *et al*. (2013) used Restriction Associated DNA (RAD) sequencing in *O. niloticus* (Nile tilapia) in two families derived from crossing XY males with females from an isogenic clonal line, in order to identify Single Nucleotide Polymorphisms (SNPs).

Marine fish

Several mutations were detected between *L. ramada* and *M. cephalus.* Nucleotide substitution were seen in a form of four transitions (C □ G and G □ C) were sited at the base number 37, 48, 64 and 98, of the aligned sequences, respectively, C mutated to nucleotide G at the base number 37, 48 and 98, respectively. However, nucleotide G mutated to nucleotide C at base number 64. All these mutations were transition mutations, as shown in Table 7.

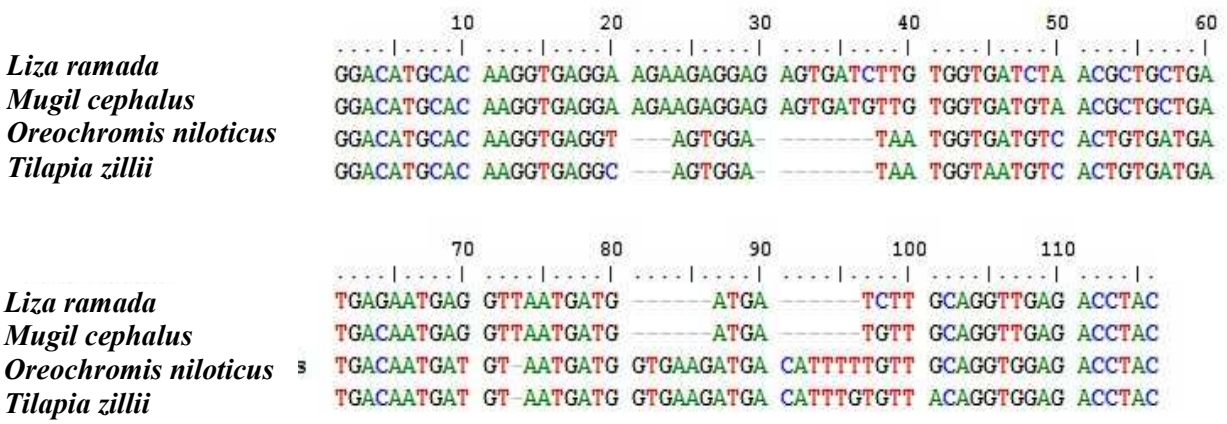

Fig. 5. Sequences in intron 5 of GH gene for species under study

Table 5. The numbers of A, C, G and T nucleotides for each fragment of intron 5 of GH gene

	A	C	G	T	G+C	A+T
L. ramada	31	13	35	25	48	56
M. cephalus	31	11	37	25	48	56
O. niloticus	30	11	34	29	45	59
T. zillii	32	12	33	27	45	59

Table 6. Polymorphic single nucleotide sites in intron 5 of GH gene between *O. niloticus* and *T. zillii*

	Code	*O. niloticus*	*T. zillii*
Transversion	g.20T □ C	T	C
Transversion	g.45G □ A	G	A
Transversion	g.101G □ A	G	A

Table 7. Polymorphic single nucleotide sites in intron 5 of GH gene between *L. ramada* and *M. cephalus*

	Code	*L. ramada*	*M. cephalus*
Transition	g.37C □ G	C	G
Transition	g.48C □ G	C	G
Transition	g.64G □ C	G	C
Transition	g.98C □ G	C	G

Livi *et al.* (2011) sequenced partially cytochrome *b* for *M. cephalus* in order to investigate the genetic divergence and the phylogeographic relationship among populations, while Raingeard *et al.* (2006) had approached the phylogenetic and evolutionary relationships of seven species of Mugilidaeon. The DNA sequences of the two species of the genus *Mugil* (*M. cephalus* and *M. curema*) showed a remarkable genetic divergence compared to all the other members of the family. The relative rate test revealed a significantly higher rate of evolution along the *Mugil* lineage.

Freshwater and marine fish

There are many point mutations occurred in intron 5 of GH gene between *L. ramada*, *M. cephalus*, *O. niloticus* and *T. zillii* in different sites. There are specific nucleotide sites distinguish between marine and freshwater fish, A→T, T→A, G→A, A→C, G→T, C→G, C→A, G→T and T→G at sites 26, 39, 40, 50, 53, 54, 57, 70 and 107, respectively. There are specific nucleotide sites distinguish species of freshwater fish, at the base number 20 (T/C), 45 (G/A) and 101 (G/A), of the aligned sequences respectively, while there are other sites distinguish species of marine fish, at base number 48 (C/G), 64 (G/C) and 98 (C/G), of the aligned sequences respectively. However site 37 doesn`t discriminate any of the species, as shown in Table 8.

Krück *et al.* (2013) showed that sustainable management of sea mullet (*M. cephalus*) fisheries needs to account for recent observations of regional-scale differentiation. Population genetic analysis is sought to assess the situation of this ecologically and economically important fish species in eastern Australian waters.

Deletion and Insertion nucleotides in intron 5 of GH gene

There are many oligo-nucleotides inserted in certain sites and deleted in other sites. AGA and GAGTGATG nucleotides appeared in *L. ramada* and *M. cephalus* at sites 21- 33 and 30 – 37 sequence, respectively, while disappeared in *O. niloticus* and *T. zilli*. However, GTGAAG nucleotides appeared in *O. niloticus* and *T. zillii* at sites 81- 86, as shown in Table 9.

Genetic diversity

The evolutionary divergence among species was studied based on intron 5 of GH gene sequence. The largest genetic divergence was between *T. zillii* and *L. ramada* (0.15), on the contrary, the smallest genetic divergence between *T. zillii* and *O. Niloticus* (0.03), as shown in Table 10.

Romana-Eguia *et al*. (2005) studied two control and three selected generation of Chitralada Nile tilapia to determine the effect of size-specific mass selection on genetic variability, Although estimated inbreeding levels were not significantly different among selected and control, the increase in the degree of inbreeding within the selected line was higher than the control line after four generations.

Phylogenetic relationship

The phylogenetic tree based on the intron 5 of GH gene sequence between four species of marine and fresh water fish was generated using neighbor joining (NJ) and confirmed by both maximum likelihood (ML) and minimum evaluation (ME) method (Fig. 6). The tree was divided into two clusters, the first cluster included *L. ramada* and *M. cephalus*, the bootstrap value of NJ, ML and ME was 100. The second cluster included *O. niloticus* and *T. ziili*, the bootstrap value of NJ, ML and ME was 100.

Imsiridou *et al.* (2007) studied that 5S rDNA sequences of six Mugilidae species found in the Mediterranean, namely *M. cephalus, M. soiuy, C. labrosus, L. aurata, L. ramada* and *L. saliens* were aligned and used for the examination of the phylogenetic relationships among them. He *et al*. (2011) studied nucleotide sequence of the genome for Nile tilapia (*O. niloticus*), blue tilapia (*O. aureus*) and Mozambique tilapia (*O. mossambicus*), phylogenetic analyses using three different computational algorithms (maximum parsimony, maximum likelihood and Bayesian method) show *O. niloticus* and *O. mossambicus* are closely related, and *O. aureus* has remotely phylogenetic relationship from them.

Table 8. Polymorphic single nucleotide sites in intron 5 of GH gene between *L. ramada*, *M. cephalus*, *O. niloticus* and *T. zillii*

Site	*L. ramada*	*M. cephalus*	*O. niloticus*	*T. zillii*
20	A	A	T	C
26	A	A	T	T
37	C	G	-	-
39	T	T	A	A
40	G	G	A	A
45	G	G	G	A
48	C	G	G	G
50	A	A	C	C
53	G	G	T	T
54	C	C	G	G
57	C	C	A	A
64	G	C	C	C
70	G	G	T	T
98	C	G	G	G
101	G	G	G	A
107	T	T	G	G

Table 9. Deletion and Insertion nucleotides in intron 5 of GH gene between *L. ramada*, *M. cephalus*, *O. niloticus* and *T. zillii*

Site	*L. ramada*	*M. cephalus*	*O. niloticus*	*T. zillii*
21-23	AGA	AGA	-	-
30-37	GAGTGATG	GAGTGATG	-	-
81-86	-	-	GTGAAG	GTGAAG
91-96	-	-	CATTTT	CATTTT

Table 10. Estimated of genetic distances (substitution rate) between *L. ramada*, *M. cephalus*, *O. niloticus* and *T. zillii* of intron 5 of GH sequences

	L. ramada	*M. cephalus*	*O. niloticus*	*T. zillii*
L. ramada	0.00			
M. cephalus	0.04	0.00		
O. niloticus	0.13	0.10	0.00	
T. zillii	0.15	0.12	0.03	0.00

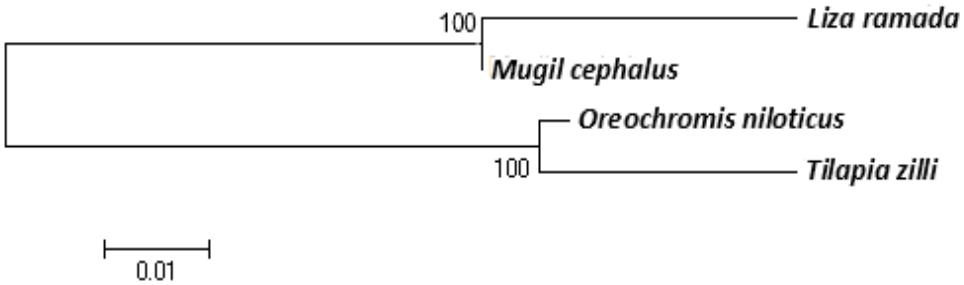

Fig. 6. The phylogenetic tree for the genetic relationships based on the intron 5 of GH gene sequence among the species under study

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دراسات جينومية مقارنه لبعض اسماك المياة العذبة والبحرية باستخدام AFLP الفلوروسنتي ودراسة تتابعات الأنترون ٥ الخاص بجين هرمون النمو

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تم استخدام ٤ سلالات من الاسماك، سلالتان من اسماك المياة المالحة (البورى– الطوبارة) وسلالتان من اسماك المياة العذبة (البلطى النيلى – البلطى الأخضر)، وكل سلالة ممثلة بثلاث عينات، تم الحصول على السلالات المستخدمة في هذه الدراسة من محافظتى دمياط وبورسعيد، تم دراسة التباين الوراثى داخل وبين اسماك المياة العذبة واسماك المياة المالحة بإستخدام تكنيك البصمة الوراثية (fluorescence AFLP) وتحديد التتابع الجزيئى لهرمون النمو الخاص بالأنواع محل الدراسة. تم عزل DNA من الذعنفة الذيلية من ١٢ عينة تمثل ٤ سلالات محل الدراسة، تم استخدام انزيمى القطع EcoR1 - Mse1 مع محولات متخصصة (adaptors) وثلاث بادئات انتخابية (ثلاث قواعد)، أظهر تحليل مكون التباينات الاساسية على مستوى الانواع (PCA) وتحليل الاستنتاج البيسيانى غير الوصفى (non-descriptive) Bayesian inference من خلال برنامج STRUCTURE أن هناك اختلافا وراثيا بين الأنواع حيث وجد أن اسماك المياة العذبة متقاربين بينما اسماك المياة البحرية وجد بينهم تباعد بالرغم من انتمائهم لنفس العائلة (العائلة البورية) وهذا يعكس نجاح استخدام تكنيك AFLP والذى يعكس حقيقة أن الطوبارة تقضى وقت أطول أثناء دورة حياتها فى المياة العذبة يزيد عن الوقت الذى يتواجد فية البورى فى المياة العذبة، تم استخدام اختبار AMOVA لقياس التغيرات عن طريق البرنامج الاحصائى F ، واتضح أن الاختلاف الوراثى أكثر تأثرا بين الأنواع واقل تأثرا بالاختلافات الراجعة للبيئة مما اظهر اختلافات معنوية، وأظهر تحليل القرابة بين الأنواع منشأها وتطورها، حيث أظهرت الدراسة نجاح استخدام تكنيك AFLP في تحديد الاختلافات الوراثية بين الأنواع محل الدراسة وتحديد درجة قرابتها، تم دراسة تتابعات الانترون ٥ الخاص بهرمون النمو لنوعين من أسماك المياة البحرية (الطوبارة – البورى) ونوعين من اسماك المياة العذبة (البلطى النيلى – البلطى الأخضر)، تم محازاة التتابعات وذلك لتحديد نسبة الاختلافات بين الأنواع، تم تحليل البيانات الوراثية لتقدير تعدد اشكال DNA المظهرية لتحديد شجرة التطور والقرابة، تم اختيار عينة واحدة لكل نوع لدراسة التتابعات الخاصة بكل عينة وتم استخلاص DNA منها، أظهرت نتائج تحليل التتابعات ١١٦ نيكلوتيدة تمثل الانترون ٥ لهرومون النمو، ظهرت اكثر من طفرة بين أسماك المياة العذبة محل الدراسه والتى تمثل طفرات استبدال (transversion mutations)، وظهور طفرات عديدة بين أسماك المياة البحرية محل الدراسة والتى تمثل طفرات انتقال (transition mutations)، هناك عديد من الطفرات حدثت فى المواقع المختلفة حيث وجد عديد من النيكلوتيدات داخل مواقع معينة ومحذوفة من مواقع أخرى، وجد من خلال دراسة الاختلافات التطورية هناك اختلافات كبيرة بين البلطى الأخضر والطوبارة (٠.١٥) بينما توجد اختلافات بسيطة بين البلطى النيلى والبلطى الأخضر (٠.٠٣)، ومن خلال الدراسة تم التعرف على شجرة القرابة بين أنواع اسماك المياة العذبة والبحرية الخاصة بالانترون ٥ الخاص بهرمون النمو- أظهرت دراسة الانترون ٥ الخاص بهرمون النمو للأنواع محل الدراسة نجاحا فى تحديد الطفرات ومواقع الحذف والإضافة وتحديد مواقع النيكلوتيدات المختلفة.

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