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Comparative Molecular Cytogenetic Analysis of Three Marine Fishes Species of Genus Epinephelus (Family: Serranidae) from Mediterranean Sea

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Abstract: ***The Metaphase chromosomes of three marine fishes species of genus Epinephelus (Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae (Family: Serranidae) and their karyological and molecular genetic relationships have been studied. All samples were collected from Egyptian Mediterranean Sea Coast. All three species have the same diploid chromosome number of 2n=48. Epinephelus aeneus and Epinephelus costae were identical in their karyotypes (all acrocentric), but the karyotype of Epinephelus marginatus was different. Nine of ten RAPD primers, showed polymorphic bands, were used for the construction of the dendrogram and a similarity matrix. A total of 51 bands were obtained; 35 of them were polymorphic. Similarity values among the studied samples ranged from 11% to 35%. High similarity values were obtained between Epinephelus aeneus and Epinephelus costae. (35%) and the low similarity values were obtained between Epinephelus aeneus and Epinephelus marginatus (11%). The cluster analysis clearly differentiated Epinephelus aeneus and Epinephelus costae from Epinephelus marginatus. RAPD analysis confirmed that the three Epinephelus species under study are genetically different from each other and a genetic variation was found between and within the three species tested in this study.***

Keywords: Zoology - Cytogenetics – karyotype - RAPD-PCR - DNA– Fishes – Serranidae Epinephelus - Mediterranean Sea.

1. Introduction

A rough estimation of more than 8500 species of macroscopic marine organisms should live in the Mediterranean Sea, corresponding to somewhat between 4% and 18% of the world marine species [1].

Cytogenetics refers to the study of heredity through the study of chromosomes (the bearers of the genes) and the cytological mechanisms of inheritance. Procedures involving preparations of mitotic chromosomes from actively dividing somatic tissues of live specimens or from embryos have been the most widely used among fish cytologists and have the dual advantages of being rapid and inexpensive. The soft organs (kidney, spleen, and liver) have proved to be fine sources of chromosomes. The discipline of “cytogenetics,” along with its practical application, has yet to be used extensively in fish breeding or fish culture [2]. Cytogenetic studies provide important basic knowledge which may have applications for many other studies, such as for the detection of ploidy in fishes [3,4].

In many vertebrate groups, the study of karyotypes and genome size has contributed along with analyses of mitochondrial and nuclear gene sequences to the resolution of challenges in biology systematics and evolution. However, in fishes, the most diverse of all vertebrate groups, higher taxa traditionally have been classified largely by morphology and paleontology, with a much smaller input of cytogenetic information. In part, karyotypes can be obtained only from living specimens, tissues, or cells, which makes it challenging to study the karyotypes of fishes that are difficult to collect alive (e.g., deep-sea fishes). Of course, even fresh material provides no guarantee that reliable chromosome figures can be obtained easily [5].

Karyotypes describe the number of chromosomes, and what they look like under a light microscope. Attention is paid to their length, the position of the centromeres, banding pattern, any differences between the sex chromosomes, and any other physical characteristics, the preparation and study of karyotypes is part of cytogenetic [6]. Karyotyping is the process of pairing and ordering all the chromosomes of an organism, thus providing a genome-wide snapshot of an individual's chromosomes. Karyotypes are prepared using standardized staining procedures that reveal characteristic structural features for each chromosome [7]. Most fishes studied have a diploid complement of 48 acro-centric chromosomes [8-10]. However, in some taxa, close species have been reported showing changes in chromosome numbers and formula [11-19]. Ozouf-Costaz, [20] reported that the diploid chromosome number in most fishes varies from 2n=22 to 2n = 260. A karyotype composed of 48 acrocentric chromosomes is considered to be ancestral for all teleosts [21]. Most fishes which belong to order Perciformes have the diploid chromosome numbers 2n = 48 [9,22-24]. In the Serranidae, most species have been chromosomally characterized and all show a diploid number of 48 and a majority of uniarmed chromosomes [23,25-29]. There are some variations of Epinephelus species, including E. malabalicus [30], E. fuscoguttatus [31,32], E. moara (E. bruneus) [33], and E. coioides [28], showing a chromosome number of 48 with varied karyotypes.

The technique of random amplified polymorphic DNA (RAPD) marker [34,35], has been successfully exploited for stock identification and population analysis in fish [36-41]; where Genetic information assists in solving problems of identity and defining con¬servation units for species [42]. Chromosomal studies in recent years gained a considerable importance, concerning species characterization, evolution

and systematic [43]. The aim of this study was to provide information about the chromosome numbers and karyotypes of the three species of genus Epinephelus, in addition to determine the molecular genetic variations and phylogenetic relationships among three species under this study. Three closely related species from the Egyptian Mediterranean Sea Coast fauna were studied in order to understand the types of chromosome changes that might have occurred during the differentiation of these species. To this aim, we used different cytogenetic techniques to characterize the molecular nature and patterns of distribution.

2. Materials and Methods

A total of 3 species of marine fishes were collected from the Mediterranean sea in Port-said, Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae of family Serranidae. Fishermen caught them then they were transported to the lab and kept alive until processed. Mitotic chromosomes were prepared from head kidney, spleen and gills as described by [44]. Each specimen was injected with 0.05% colchicines (1ml / 100g fish weight) the fish were maintained in a well aerated aquarium and after 2hr they were sacrificed. The kidneys, spleen and gills were removed and placed in a hypotonic solution of 0.56% kcl after nearly 30 min, the tissues immersed three times in a ethanol-acetic acid glacial mixture 3:1 every time was taken 20min, then the tissues squashed in 60% acetic acid , three droplets of the cellular suspension was dropped on a clean microscope slide, previously chilled in a freezer, from a height of 50 cm. the slides were briefly passed over a flame and then allowed to air-dry.for conventional karyotype the preparations were stained during 40 min with 5% Giemsa in phosphate buffer ph 6.8. The slides were examined under a research light microscope using ×10 or ×15 eyepieces, together with × 15 objectives for chromosomal analysis. Karyotypes were made from good spreads of chromosome. Classification of chromosomes in karyotype studies relating to centromeric index was done according to Levan [45].

DNA was extracted by using "Quick Genomic DNA Extraction Kit" Cat. No.1112. Ten primers (Alpha DNA, Montreal, Quebec, Canada) were used in RAPD – PCR analysis to study the difference between seven specimens of three families' Mugilidae, Serranidae and Sparidae, the code and sequences of these primers are shown in table (1).

Table 1: primers and primer sequences used for amplification and sequencing in this study

No.	Primer code No.	Nucleotide sequence(5` to 3`)	Annealing TmC/Sec	GC%
1	OPA-4	AATCGGGCTG	32	38.6
2	OPA-11	CAATCGCCGT	32	38.6
3	OPO-6	CCACGGGAAG	34	42.7
4	OPO-2	ACGTAGCGTC	32	38.6
5	OPO-4	AGGTCCGCTC	34	42.7
6	OPM-17	TCAGTCCGGG	34	42.7
7	OPM-2	ACAACGCCTC	32	38.6
8	OPE-5	TCAGGGAGGT	32	38.6
9	OPE-6	AAGACCCCTC	32	38.6
10	OPG-2	GGCACTGAGG	34	42.7

Each sample was analyzed in agarose gel prepared in 10 mM tris-HCL (PH 7.6), 10 mM EDTA, 0.005% bromophenol blue, 0.005% xylene cyanide and 10% glycerol. The gel was stained with ethidium bromide though adding 5 µl of this stain/100 ml buffer of agarose gel and photographed under ultraviolet light for visualizing the resulted bands. The banding patterns of DNA fragments were analyzed by Gene profiler computer software program showing the molecular weight and the intensity of each band. The marker is composed of 10 chromatography purified individual DNA fragments of molecular weight of 1000, 900, 800, 700, 600, 500, 400, 300, 200 and100 bp, respectively.

The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient [46]. Dice formula: GSij = 2a/ (2a+b+c),Where GSij is the measure of genetic similarity between individuals i and j, a is the number of bands shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i. The similarity matrix was used in the cluster analysis. At the first step, when each accession represents its own cluster, the distances between these accessions are defined by the chosen distance measure (Dice coefficient). However, once several accessions have been linked together, the distance between two clusters is calculated as the average distance between all pairs of accessions in the two different clusters. This method is called Unweighted Pair Group Method using Arithmetic Average (UPGMA) [46].

3. Results

Three species of Mediterranean Sea fishes, belonging to family Serranidae (Order: Perciformes), were cyto- and molecule-genetically studied, using air drying technique and RAPD-PCR analysis. The chromosomal numbers of all species under the study were the same, with 2n=48, but differ in the karyotype in some species. Ten single 10-mer primers (OPA-11, OPA-4, OPO-4, OPO-2, OPO-6, OPE-6, OPE-5, OPM-17, OPM-2 and OPG-2) - with G+C contents of 60% - were used in the present investigation to determine the genetic differences among three species of family Serranidae, Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae. The DNA fragments generated by the nine primers from the genomic DNA of the three species were separated using Agarose gel electrophoresis and illustrated in figs (4 to 12). The banding patterns of these DNA fragments were analyzed by Gene profiler computer software program and summarized in charts with each primer in table (5). Following are the kayotypes and amplification results of the three species obtained from this study.

3.1 Epinephelus aeneus

The photographs of cell spread and karyotypes of this species was found to have a diploid chromosome number of 2n=48, as shown in (Fig.1). These numbers of chromosomes are allocated into one group of acrocentric chromosomes with relative lengths varied from 2.16% to 6.35%, arm ratio of ∞ and centromeric index is zero. All these measurements are shown in table (2).

The RAPD PCR analysis indicated that all amplified primers produced fragments with this fish except OPE-6 and OPG-2 primers, 22 are all the bands varied from 1 by the primers OPE-5 to 4 by the primers OPA-11 and OPM-2, the size of these bands varies approximately from 150 bp to 1100bp by the primers OPA-11.

3.2 Epinephelus marginatus

The chromosomal analysis of the studied samples demonstrated that the diploid chromosomal number is 2n=48 (Fig. 2) arranged in three groups, groupA: consisted of four metacentric pairs with relative lengths ranged from 3.14% to 5.05%, arm ratios from 1 to 1.62 and centromeric indeces from 38.09% to 50%, group B: consisted of four submetacentric pairs with relative lengths ranged from 4.26% to 5.84%, arm ratios from 1.88 to 2.58 and centromeric indeces from 27.9% to 36.84%, group C: sixteen pairs of subtelocentric chromosomes with relative lengths varied from 2.47% to 5.39%, arm ratio of ∞ and centromeric index is zero. All these measurements are shown in table (3).

The RAPD-PCR analysis of Epinephelus costae, all primers reacted with this species except OPE-6 and OPM-17 generating 22 bands, the bands varied in number from 1 by the primer OPA-11 and OPO-6 to 5 bands by the primer OPO-2, the size of these bands ranged from 170bp to 900bp by the primer OPM-2.

3.3 Epinephelus costae

The karyotype of the studied samples of this apecies (fig. 3) also consisted of only one group of 48 acrocentric chromosomes (Fig.3). Relative lengths of these chromosomes varied from 2.56% to 6.30%, arm ratio of ∞ and centromeric index is zero. All these measurements are shown in table (4).

In the RAPD-PCR analysis of Epinephelus costae, all primers reacted with this species except OPE-6,OPO-6 and OPA-11 generating 24 bands, the bands varied in number from 1 by the primer OPE-5 and OPG-2 to 8 bands by the primer OPM-2, the size of these bands ranged from 140bp with OPG-2 primer to 1250 bp by the primer OPM-2.

A total of 51 DNA bands were generated by all primers in all specimen, out of these DNA bands 16 (31.4%) were conserved among all specimens while 35 bands were polymorphic with percentage (68.6%) of all the 9 tested primers produced polymorphism in all specimens (table 6).

The number of fragments amplified per primer varied between 3 (OPE-5, OPM-17 and OPO-6) and 14 (OPM-2) (5.67 bands/ primer) and had a size range from 140 bp (OPG-2) to 1250 bp (OPM-2). Data of the presence / absence of DNA fragments of Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae, were used to calculate the genetic similarity, based on the calculated genetic similarity presented in table (7) and Dendrogram as in figure 13, an estimation of the relationship between the above species was concluded where the lowest genetic similarity 11 was observed between, Epinephelus aeneus and Epinephelus marginatus, while the highest value 35 was found between Epinephelus aeneus and Epinephelus costae.

The results of cytogenetic analysis (karyotyping) and of RAPD-PCR analysis were compared with those obtained from the classical methods in taxonomy using morphological and anatomical characters. This research is an initial study reporting the chromosome numbers, karyotypic characters and RAPD analysis of three species, Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae in Egypt.

4. Discussion

Considered as a monophyletic group, the family Serranidae is divided into three subfamilies, nearly 60 genera and 449 species, half of them belonging to the subfamily Epinephelinae [47]. From this total, less than 5% was karyotyped [48]. Grouper species of the Serranidae family are considered to be a highly desirable fish in Asia and around the world [49].

In the Serranidae, most species have been chromosomally characterized and all show a diploid number of 48 and a majority of uniarmed chromosomes [23,25-29]. Molina, [23] made cytogenetic studies on six individuals of Epinephelus adscensionis, five of Alphestes afer and one of Serranus flaviventris were collected along the coastline of Rio Grande do Norte and Bahia States, at Northeastern region of Brazil. Mitotic stimulation method [50] was performed prior to in vitro chromosomal preparations[51] and they reported that the cytogenetical surveys in A. afer, E. adscensionis and S. flaviventris showed that such Serranidae species share a similar karyotype, composed of 48 acrocentric chromosomes (2n=48), characterized by slight size differences amongst chromosomal pairs. There are some variations of Epinephelus species, including E. malabalicus [30], E. fuscoguttatus [31,32]. E. moara (E. bruneus) [33], and E. coioides [28], showing a chromosome number of 48 with varied karyotypes.

Cytogenetic studies made by [29] on Epinephelus bleekeri and E. coeruleopunctatus revealed that the chromosome number of both species was 48 with akaryotype of 48 acrocentric chromosomes and two submetacentric with 46 acrocentric chromosomes, respectively. Cytological analysis of early embryonic development in the white grouper, E.aeneus, was undertaken during the natural reproductive season of 1999, at the National Center for Mariculture (NCM) in Eilat. Cytological analyses showed in all normal embryos from different spawnings, parallel with normal diploid cells (2n=48) [26].

Cytogenetic analysis was carried out by [27] on 15 specimens of Epinephelus marginatus from three localities in the Mediterranean Sea and all specimens had 2n=48. [28] made a cytogenetic analysis of six individuals of the orange-spotted grouper (Epinephelus coioides) collected alive from coastal water of China and the karyotypic formula was 2n=2sm+46a.

The present study indicated that least genetic distance was observed in Epinephelus aeneus and Epinephelus costae,

these two species fell into a distinct cluster from other species Epinephelus marginatus . This points out that these two species are genetically closer and show divergence as compared to other species of Epinephelus marginatus. These two species are considered morphologically closer in contrast to the other species of Epinephelus. Although several amplified fragments were shared by all three Species, clearly distinguishable bands were observed only in a determined species (Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae) was evident from high number of polymorphic marker.

This study reports on the use of RAPD markers for studying genetic similarity among the three species (Family: Serranidae) of Mediterranean Sea fishes in Egypt. The RAPD assay has been used to construct phylogenetic trees for resolving taxonomic problems in many organisms [52-56]. RAPD bands in this study were always variant (i.e. strong, faint, fuzzy and sharp bands) generated with each primer because one or more copies of DNA may exist per genome or may be attributed to the varying of the annealing process between the primer and the DNA, this problem of mixed bands shows the well known sensitivity PCRs [57].

RAPD analysis occurred by [58] to investigate the genetic variation in two populations of yellow grouper (Epinephelus awoara) from the South China Sea. Muscle samples from fish were taken. Genomic DNA was extracted according to the DNA extraction method of [59]. 20 primers produced a total of 159 bands, among which 121 polymorphic bands (76.10%) were observed. The 38 monomorphic bands (23.90%) could be considered, on a preliminary basis, as population diagnostic bands that allow clear differentia¬tion among populations of Epinephelus awoara because they were present in all individuals analyzed.

Parenrengi, [60] made RAPD-PCR to determine the genetic variability and to establish the RAPD fingerprinting of groupers (Epinephelus spp) where a total of 100 individuals of E. tauvina from six populations and 36 individuals of E. merra and E. areolatus were studied using ten RAPD primers that generated a total of 403 fragments with 205 polymorphic fragments . Genetic distance among populations of E. tauvina varied from 0.20 to 0.41. Number of genotypes detected for each primer ranged from 3 to 5 for E. tauvina and E. areolatus; and 3 to 6 for E. merra. A total of 11 diagnostic markers were detected to be present in species, E. merra and E. areolatus, but not in E. tauvina. Five fragments were identified as the genus specific markers. Genetic distance among individuals of E. tauvina was 0.02-0.30, whilethat of E. merra and E. areolatus were 0.14-0.50 and 0.19-0.42, respectively. The genetic relatedness between E. merra and E. areolatus (0.52) was closer compared with E. merra and E. tauvina (0.67).

The RAPD method was successfully used to detect the variation between the different species of fishes. The results obtained in this study showed that RAPD could be used to generate useful fingerprints characteristic of fish species and for genotyping of individuals within the species. Thus, it provides an efficient and sensitive method which can be used to estimate genetic variability, relatedness, inbreeding levels, pedigree analyses, detection of economic traits and in other maker based studies in fishes [61].

In conclusion, the results of this study indicated that Epinephelus aeneus and Epinephelus costae were identical in their karyotypes, but the karyotype of Epinephelus marginatus was different. In addition, to the results indicated that each species has different molecular genetic characteristics. The cluster analysis clearly differentiated Epinephelus aeneus and Epinephelus costae from Epinephelus marginatus. The molecular genetic taxonomic relationship among three species of Serranidae fishes (Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae) were investigated using cytogenetic analysis and RAPD markers for first time in Egypt. A further molecular genetic study is a matter of interest, to study the gene sequence homology between species studied, in our lab.

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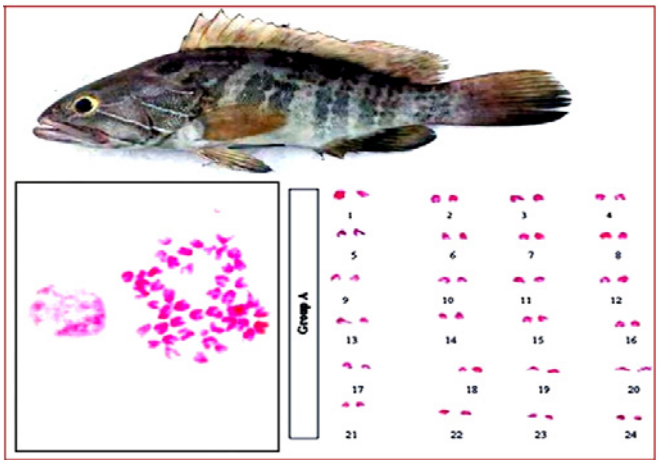


Figure 1 (a): A coloured photograph, chromosome spread and karyotype of *Epinephelus aeneus.*

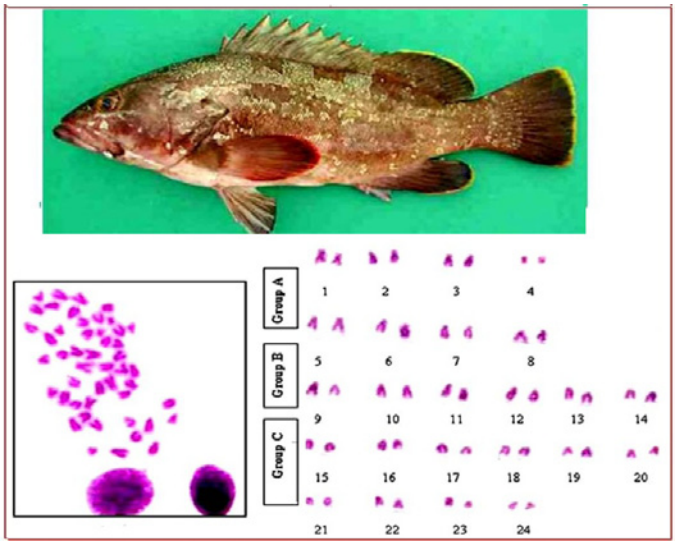


Figure 2 (a): A coloured photograph, chromosome spread and karyotype of Epinephelus marginatus

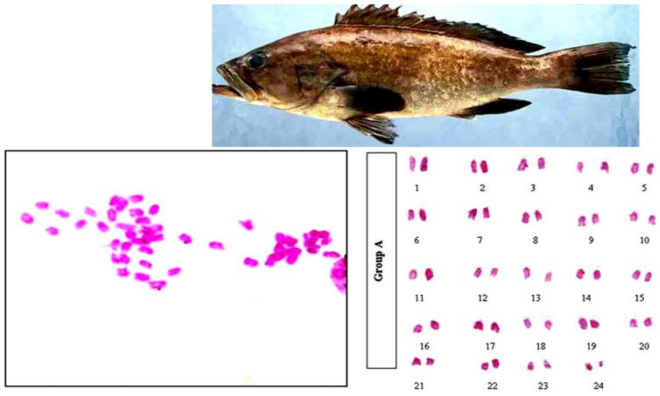


Figure 3 (a): A coloured photograph, chromosome spread and karyotype of EpinephelusCostae

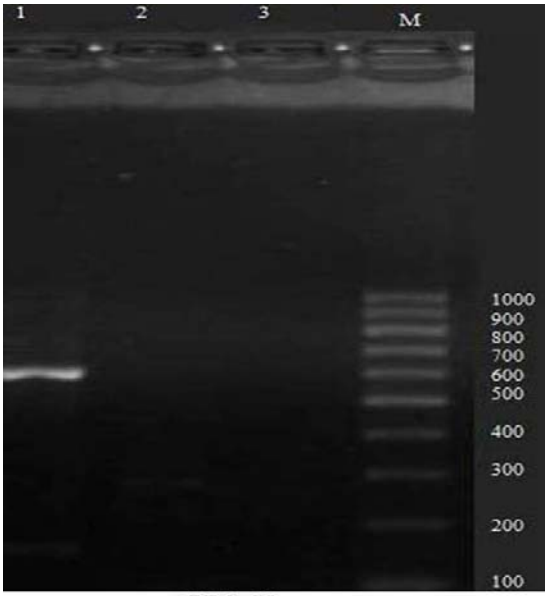


Figure 4: Agarose gel electrophoresis of RAPD products generated with OPA-11. *Where 1- Epinephelus aeneus. 2- Epinephelus marginatus 3- EpinephelusCostae*

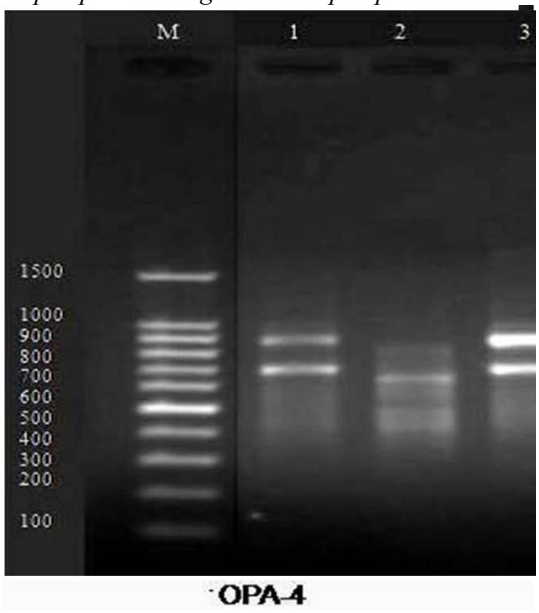


Figure 5: Agarose gel electrophoresis of RAPD products generated with OPA-4

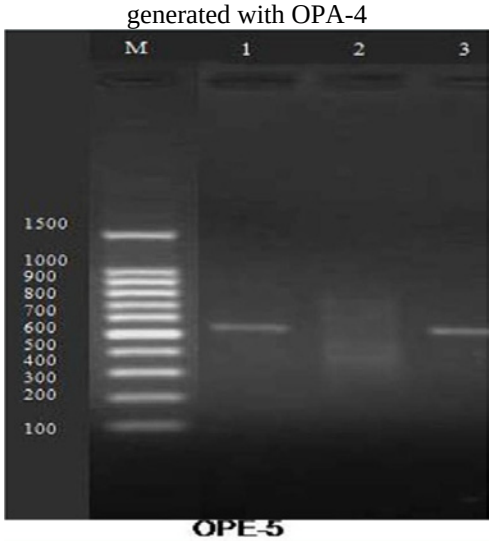


Figure 6: Agarose gel electrophoresis of RAPD products generated with OPE-5

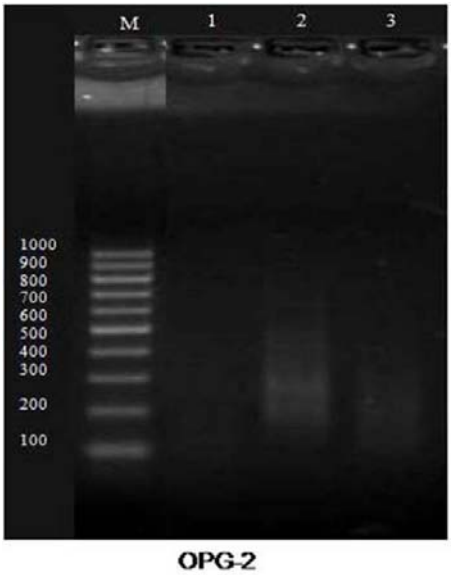


Figure 7: Agarose gel electrophoresis of RAPD products generated with OPG-2

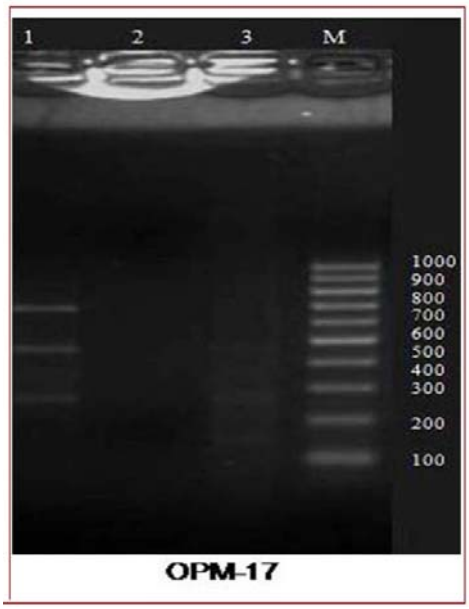


Figure 8: Agarose gel electrophoresis of RAPD products generated with OPM-17

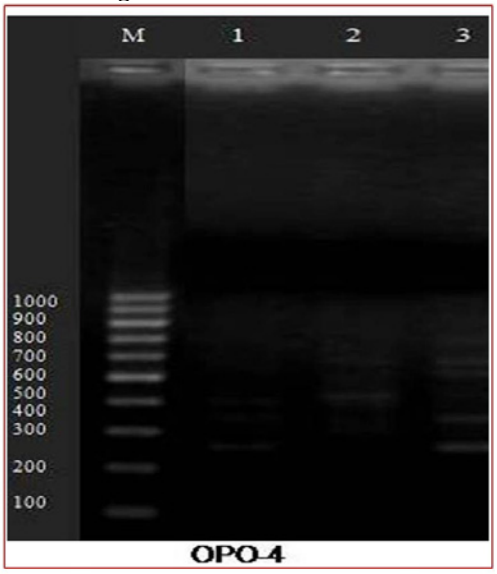


Figure 9: Agarose gel electrophoresis of RAPD products generated with OPO-4

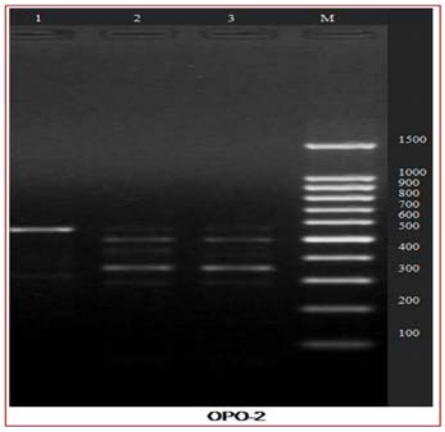


Figure 10: Agarose gel electrophoresis of RAPD products generated with OPO-2

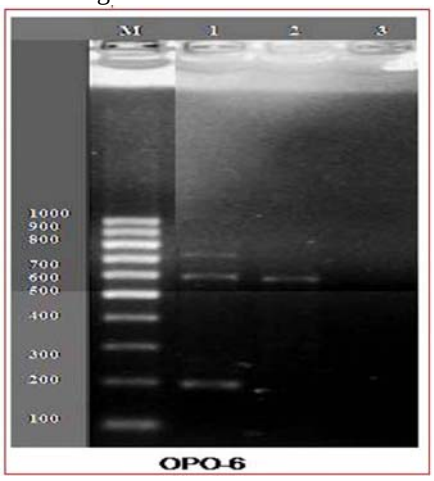


Figure 11: Agarose gel electrophoresis of RAPD products generated with OPO-6

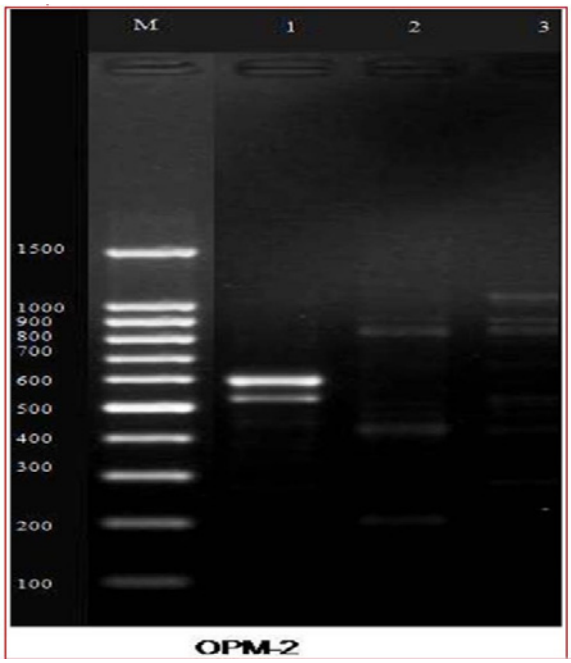


Figure 12: Agarose gel electrophoresis of RAPD products generated with OPM-2

Table 2: Averages of chromosomes measurements and classification, obtained from observations on ten cell spreads of Epinephelus aeneus

Chromosome Number	Chromosome Length			Relative Length %			Arm Ratio Mean ± S.D.	Centromeric Index Mean± S.D.	Classification
	Long Arm Mean ± S.D.	Short Arm Mean±S.D.	Total Mean ± S.D.	Long Arm Mean± S.D.	Short Arm Mean ± S.D.	Total Mean ± S.D.			
1	0.47±0.03	0	0.47±0.03	6.35±0.04	0	6.35±0.04	∞	0	Acro.
2	0.44±0.03	0	0.44±0.03	5.94±0.04	0	5.94±0.04	∞	0	Acro.
3	0.43±0.05	0	0.43±0.05	5.81±0.04	0	5.81±0.04	∞	0	Acro.
4	0.41±0.04	0	0.41±0.04	5.54±0.03	0	5.54±0.03	∞	0	Acro.
5	0.40±0.05	0	0.40±0.05	5.40±0.03	0	5.40±0.03	∞	0	Acro.
6	0.39±0.03	0	0.39±0.03	5.27±0.03	0	5.27±0.03	∞	0	Acro.
7	0.38±0.03	0	0.38±0.03	5.13±0.03	0	5.13±0.03	∞	0	Acro.
8	0.36±0.04	0	0.36±0.04	4.86±0.04	0	4.86±0.04	∞	0	Acro.
9	0.35±0.04	0	0.35±0.04	4.72±0.04	0	4.72±0.04	∞	0	Acro.
10	0.34±0.04	0	0.34±0.04	4.59±0.04	0	4.59±0.04	∞	0	Acro.
11	0.33±0.03	0	0.33±0.03	4.45±0.04	0	4.45±0.04	∞	0	Acro.
12	0.31±0.03	0	0.31±0.03	4.18±0.03	0	4.18±0.03	∞	0	Acro.
13	0.30±0.03	0	0.30±0.03	4.05±0.05	0	4.05±0.05	∞	0	Acro.
14	0.29±0.03	0	0.29±0.03	3.91±0.04	0	3.91±0.04	∞	0	Acro.
15	0.28±0.04	0	0.28±0.04	3.78±0.05	0	3.78±0.05	∞	0	Acro.
16	0.26±0.04	0	0.26±0.04	3.51±0.03	0	3.51±0.03	∞	0	Acro.
17	0.25±0.04	0	0.25±0.04	3.37±0.03	0	3.37±0.03	∞	0	Acro.
18	0.24±0.04	0	0.24±0.04	3.24±0.04	0	3.24±0.04	∞	0	Acro.
19	0.23±0.04	0	0.23±0.04	3.10±0.04	0	3.10±0.04	∞	0	Acro.
20	0.21±0.03	0	0.21±0.03	2.83±0.04	0	2.83±0.04	∞	0	Acro.
21	0.20±0.03	0	0.20±0.03	2.70±0.03	0	2.70±0.03	∞	0	Acro.
22	0.19±0.03	0	0.19±0.03	2.56±0.04	0	2.56±0.04	∞	0	Acro.
23	0.18±0.03	0	0.18±0.03	2.43±0.03	0	2.43±0.03	∞	0	Acro.
24	0.16±0.03	0	0.16±0.03	2.16±0.03	0	2.16±0.03	∞	0	Acro.
Sum.			7.4±0.03						

Table 3: Averages of chromosomes measurements and classification, obtained from observations on ten cell spreads of Epinephelus marginatus

Chromosome Number	Chromosome Length			Relative Length %			Arm Ratio Mean ± S.D.	Centromeric Index	Classification
	Long Arm Mean ± S.D.	Short Arm Mean ± S.D.	Total Mean ± S.D.	Long Arm Mean± S.D	Short Arm Mean ± S.D.	Total Mean ± S.D.			
1	0.24±0.04	0.21±0.03	0.45±0.05	2.69±0.04	2.36±0.04	5.05±0.05	1.14±0.03	46.66±0.08	M.
2	0.26±0.03	0.16±0.03	0.42±0.04	2.92±0.04	1.79±0.03	4.71±0.04	1.62±0.05	38.09±0.04	M.
3	0.23±0.05	0.16±0.06	0.39±0.05	2.58±0.03	1.79±0.03	4.37±0.03	1.44±0.04	41.02±0.04	M.
4	0.14±0.04	0.14±0.06	0.28±0.04	1.57±0.05	1.57±0.03	3.14±0.04	1.00±0.05	50.00±0.04	M.
5	0.34±0.05	0.18±0.04	0.52±0.03	3.82±0.04	2.02±0.03	5.84±0.04	1.88±0.03	34.61±0.05	S.M.
6	0.30±0.03	0.16±0.03	0.46±0.04	3.37±0.05	1.79±0.05	5.16±0.04	1.88±0.03	34.78±0.05	S.M.
7	0.31±0.03	0.12±0.04	0.43±0.04	3.48±0.03	1.34±0.04	4.82±0.05	2.58±0.04	27.90±0.03	S.M.
8	0.28±0.04	0.14±0.04	0.38±0.04	2.69±0.03	1.57±0.05	4.26±0.03	2.00±0.04	36.84±0.03	S.M.
9	0.48±0.03	0	0.48±0.03	5.39±0.05	0	5.39±0.05	∞	0	Acro.
10	0.47±0.04	0	0.47±0.04	5.28±0.04	0	5.28±0.04	∞	0	Acro.
11	0.45±0.04	0	0.45±0.04	5.06±0.03	0	5.06±0.03	∞	0	Acro.
12	0.44±0.04	0	0.44±0.04	4.94±0.03	0	4.94±0.03	∞	0	Acro.
13	0.42±0.03	0	0.42±0.03	4.72±0.03	0	4.72±0.03	∞	0	Acro.
14	0.39±0.03	0	0.39±0.03	4.38±0.08	0	4.38±0.08	∞	0	Acro.
15	0.37±0.04	0	0.37±0.04	4.16±0.04	0	4.16±0.04	∞	0	Acro.
16	0.35±0.04	0	0.35±0.04	3.93±0.05	0	3.93±0.05	∞	0	Acro.
17	0.33±0.04	0	0.33±0.04	3.71±0.04	0	3.71±0.04	∞	0	Acro.
18	0.31±0.03	0	0.31±0.03	3.48±0.04	0	3.48±0.04	∞	0	Acro.
19	0.29±0.03	0	0.29±0.03	3.26±0.05	0	3.26±0.05	∞	0	Acro.
20	0.28±0.03	0	0.28±0.03	3.14±0.04	0	3.14±0.04	∞	0	Acro.
21	0.27±0.03	0	0.27±0.03	3.04±0.05	0	3.04±0.05	∞	0	Acro.
22	0.26±0.05	0	0.26±0.05	2.92±0.04	0	2.92±0.04	∞	0	Acro.
23	0.23±0.04	0	0.23±0.04	2.58±0.03	0	2.58±0.03	∞	0	Acro.
24	0.22±0.05	0	0.22±0.05	2.47±0.03	0	2.47±0.03	∞	0	Acro.
Sum.			8.89±0.06						

Table (4): Averages of chromosomes measurements and classification, obtained from observations on ten cell spreads of Epinephelus costae

Chromosome Number	Chromosome Length			Relative Length %			Arm Ratio Mean ± S.D.	Centromeric Index Mean±	Classification
	Long Arm Mean ± S.D.	Short Arm Mean± S.D.	Total Mean ± S.D.	Long Arm Mean ± S.D.	Short Arm Mean ± S.D.	Total Mean ± S.D.			
1	0.64±0.05	0	0.64±0.05	6.30±0.05	0	6.30±0.05	∞	0	Acro.
2	0.59±0.03	0	0.59±0.03	5.81±0.04	0	5.81±0.04	∞	0	Acro.
3	0.55±0.03	0	0.55±0.03	5.41±0.03	0	5.41±0.03	∞	0	Acro.
4	0.52±0.06	0	0.52±0.06	5.12±0.04	0	5.12±0.04	∞	0	Acro.
5	0.51±0.06	0	0.51±0.06	5.02±0.04	0	5.02±0.04	∞	0	Acro.
6	0.50±0.04	0	0.50±0.04	4.92±0.04	0	4.92±0.04	∞	0	Acro.
7	0.49±0.03	0	0.49±0.03	4.82±0.05	0	4.82±0.05	∞	0	Acro.
8	0.48±0.04	0	0.48±0.04	4.72±0.03	0	4.72±0.03	∞	0	Acro.
9	0.46±0.04	0	0.46±0.04	4.53±0.06	0	4.53±0.06	∞	0	Acro.
10	0.45±0.04	0	0.45±0.04	4.43±0.04	0	4.43±0.04	∞	0	Acro.
11	0.44±0.03	0	0.44±0.03	4.33±0.03	0	4.33±0.03	∞	0	Acro.
12	0.43±0.03	0	0.43±0.03	4.23±0.04	0	4.23±0.04	∞	0	Acro.
13	0.42±0.03	0	0.42±0.03	4.13±0.03	0	4.13±0.03	∞	0	Acro.
14	0.41±0.03	0	0.41±0.03	4.03±0.03	0	4.03±0.03	∞	0	Acro.
15	0.40±0.04	0	0.40±0.04	3.94±0.03	0	3.94±0.03	∞	0	Acro.
16	0.38±0.04	0	0.38±0.04	3.74±0.04	0	3.74±0.04	∞	0	Acro.
17	0.36±0.04	0	0.36±0.04	3.54±0.03	0	3.54±0.03	∞	0	Acro.
18	0.35±0.04	0	0.35±0.04	3.44±0.03	0	3.44±0.03	∞	0	Acro.
19	0.33±0.04	0	0.33±0.04	3.25±0.03	0	3.25±0.03	∞	0	Acro.
20	0.32±0.03	0	0.32±0.03	3.15±0.04	0	3.15±0.04	∞	0	Acro.
21	0.30±0.03	0	0.30±0.03	2.95±0.04	0	2.95±0.04	∞	0	Acro.
22	0.29±0.03	0	0.29±0.03	2.85±0.05	0	2.85±0.05	∞	0	Acro.
23	0.27±0.03	0	0.27±0.03	2.66±0.04	0	2.66±0.04	∞	0	Acro.
24	0.26±0.03	0	0.26±0.03	2.56±0.04	0	2.56±0.04	∞	0	Acro.
Sum.			10.15±0.04						

Table 5: Survey of RAPD Markers using nine primers. (1- Epinephelus aeneus, 2- Epinephelus marginatus 3- Epinephelus costae), where (1) means present and (0) means absence.

		Primer OPA-11					Primer OPA-4		
N	MW	1	2	3	N	MW	1	2	3
1	110	1	0	0	1	960	1	0	1
2	980	1	0	0	2	830	0	1	0
3	570	1	0	0	3	690	1	0	1
4	260	0	1	0	4	610	0	1	0
5	150	1	0	0	5	410	0	1	0
		Primer OPE-5			6	360	0	1	0
N	MW	1	2	3			Primer OPG-2		
1	720	0	1	0	N	MW	1	2	3
2	510	1	0	1	1	440	0	1	0
3	300	0	1	0	2	250	0	1	0
		Primer OPM-17			3	190	0	1	0
N	MW	1	2	3	4	140	0	0	1
1	670	1	0	0			Primer OPO-4		
2	430	1	0	1	N	MW	1	2	3
3	230	1	0	1	1	730	0	0	1
		Primer OPO-2			2	580	0	1	1
N	MW	1	2	3	3	500	0	0	1
1	580	1	1	1	4	390	1	1	0
2	490	0	1	1	5	340	1	0	1
3	410	0	1	0	6	260	1	0	1
4	400	0	0	1			Primer OPO-6		
5	330	0	1	1	N	MW	1	2	3
6	280	1	0	0	1	750	1	0	0
7	260	0	1	1	2	560	1	1	0
		Primer OPM-2			3	180	1	0	0
N	MW	1	2	3					
1	125	0	0	1					
2	100	0	0	1					
3	900	0	1	1					
4	700	0	0	1					
5	620	1	0	0					
6	540	1	0	0					
7	530	0	0	1					
8	470	0	0	1					
9	460	0	1	0					
10	410	0	1	1					
11	370	1	0	0					
12	260	0	0	1					
13	250	1	0	0					
14	170	0	1	0					

Table 6: Number of amplified and polymorphic DNA-fragments in the three species

No. of Primer	Primer code	No. of amplified bands			Total amplified bands	No. of polymerphic bands	Polymorphism %
		-1	-2	-3			
		Epinephelus aeneus	Epinephelus marginatus	Epinephelus costae			
1	OPA-11	4	1	0	5	5	100
2	OPA-4	2	4	2	6	4	66.7
3	OPE-5	1	2	1	3	2	66.7
4	OPG-2	0	3	1	4	4	100
5	OPM-17	3	0	2	3	1	33.3
6	OPO-4	3	2	5	6	2	33.3
7	OPO-2	2	5	5	7	3	42.9
8	OPO-6	3	1	0	3	2	66.7
9	OPM-2	4	4	8	14	12	85.7
total		22	22	24	51	35	68.6

Table 7: Genetic similarity values calculated from the DNA fragments amplified from ***Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae***, using nine OPERON primers

Epinephelus costae	*Epinephelus marginatus*	*Epinephelus aeneus*	#
		100	***Epinephelus aeneus***
	100	11	***Epinephelus marginatus***
100	17	35	***Epinephelus costae***

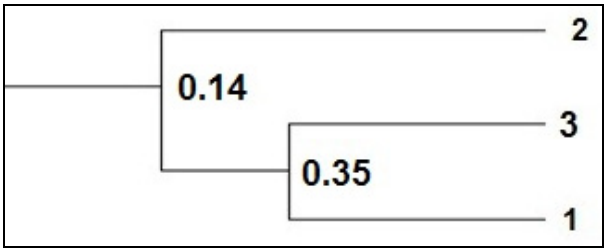
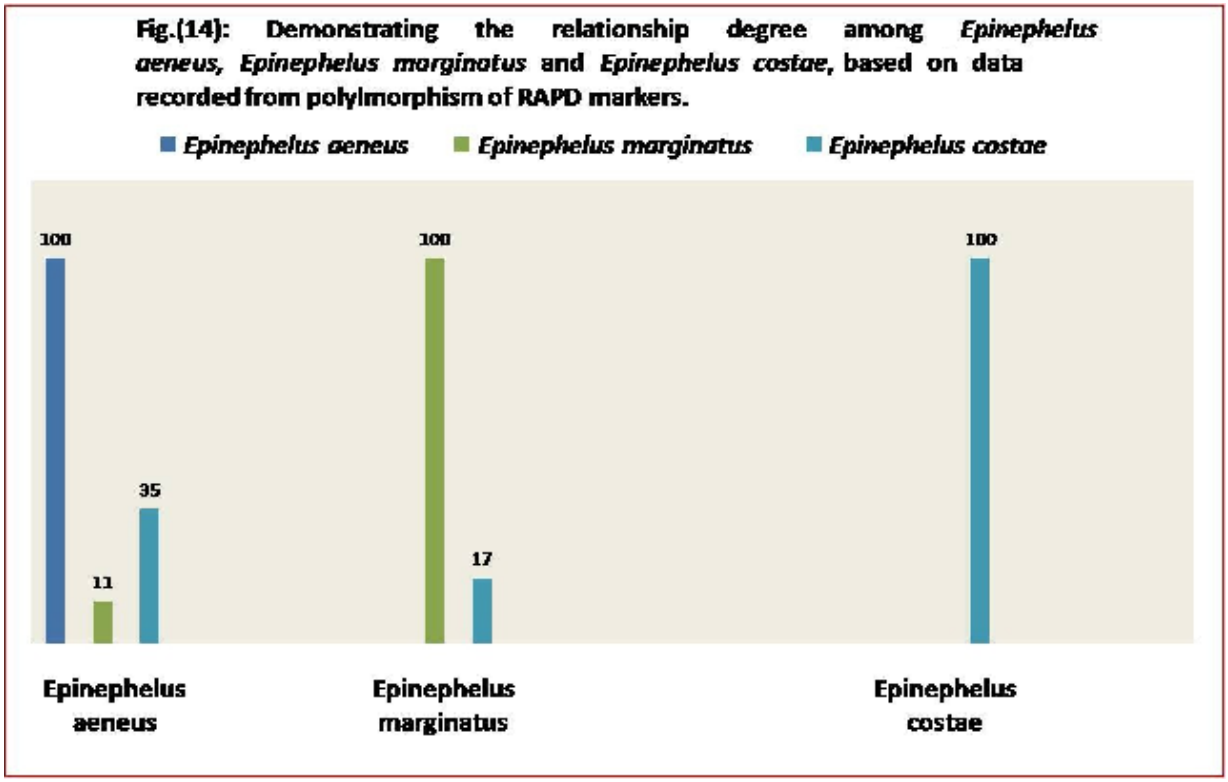


Figure 13: Dendrogram demonstrating the relationship among Epinephelus aeneus, Epinephelus marginatus and Epinephelus costae, the based on data recorded from polylmorphism of RAPD markers.

1- ***Epinephelus aeneus***, **2-** ***Epinephelus marginatus*** and **3-** ***Epinephelus costae.***

2-

Dendrogram consisted of two cluster**; (**A) contained ***Epinephelus marginatus*** and (B) contained ***Epinephelus aeneus*** and ***Epinephelus costae***.



Author Profile

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