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Assessment of genetic variation and changes in protein subunits induced by petroleum oil in three species of Red Sea fishes using SDS-PAGE and ISSRs markers

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Abstract: *Genetic variability among three species of red sea fishes (Lethrinus borbonicus, Siganus rivulatus and Mulloidichthys flavolineatus) were determined to evaluate the effect of petroleum oil component pollutions on the fishes using Inter-simple sequence repeats (ISSRs) and sodium dodecyl sulfate-polyacrlamide gel electrophoresis (SDS-PAGE) markers. According to ISSR analysis of DNA, nineteen (19) ISSR primers generated a total of 465 bands with an average 24.5 bands per primer. Analyses of SDS-PAGE protein, 137 bands were identified variant bands from 44, 43 and 50 in sample Lethrinus borbonicus, Siganus rivulatus and Mulloidichthys flavolineatus respectively. Analysis of SDS-PAGE protein provided more precise information concerning of the effect of petroleum oil components on protein subunits and genetic variation in Siganus rivulatus species than ISSR-PCR of DNA. Whereas ISSR technique showed more polymorphism or relatively a close to the percentage of SDS-PAGE result among the tow species Lethrinus borbonicus and Mulloidichthys flavolineatus compared to there controls. A remarkable result from this study was identifying that petroleum oil components pollution have distinct effect in genetic structure of fishes and lead to disappear of some protein subunits or appear new some protein subunits in fish muscle tissues.*

Key words: *Lethrinus borbonicus, Siganus rivulatus, Mulloidichthys Flavolineatus, protein, Inter- simple sequence repeats.*

I. Introduction

Fishes an excellent protein source necessary for good health and also delivers various minerals and vitamins. Scientists reported that societies with high fish intake have considerably lower rates of acute myocardial infections, other ischemic heart diseases and atherosclerosis (Bang and Dyerberg, 1980 and Blanchet *et al*., 2000). However, the fish meal is a finite resource that has steadily increased in price in recent years and will continue to become increasingly expensive relative to other protein supplements in the ingredient market (Elhalfawy *et al*., 2013).

Fish also used as excellent indicator of certain aquatic pollution due to their high sensitivity to variety of benthic prey, such as crustaceans, polychaetes and small bivalves (Randall and Heemstra, 2009). The following techniques are those mostly used and are listed in chronological order, simple sequence repeats or just microsatellites (SSR) (Tautz, 1989) and randomly amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) or arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990), inter-simple sequence repeats (ISSR) (Zietkiewicz *et al*., 1994), amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995), single nucleotide polymorphisms (SNPs) (Chen and Sullivan, 2003) and more recently, diversity array technology (DarT) (Kilian *et al*., 2005). These different types of molecular markers are also different as to there potential to detect differences between individuals, their cost, facilities required, consistency and replication of results (Schlotterer, 2004 and Schulman, 2007).

This study aimed to investigate and to assess the selective effect of petroleum oil component pollution of red sea environment on genetic structure and protein subunits among three species of red sea fishes and its control using inter-simple sequence repeats (ISSR) of total genomic DNA and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of muscle proteins.

II. Material and method

Study area

The field work and sample collection were carried out during May, 2013 to Jun, 2014 on three species of red sea fishes from two sites, El-shalateen as control site and Ras-Gareb as pollutant site.

Sample collection and DNA extraction

Twenty four (24) samples of three species of male live fishes of size 15-20 cm. and 80-90g. *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* were collected, Muscles were stored at -20 ºC until DNA extraction, DNA was extracted Using the method of DNeasy Kit Qiagen.

Inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) amplification

A set of (19) primers ISSR (Table 1) was used in the detection of polymorphism. The amplification reaction was carried out in 25 µL of reaction volume containing 30 ng of genomic DNA, 1 U Taq DNA polymerase,0,2 µM dNTP, 1 µM primer, and 1X PCR buffer (containing 1.5 mM MgCl2). The condition for amplification was an initial denaturation temperature 94ºC for five min, followed by 40 cycles of 1 min at 94ºC, then an annealing step at 36ºC for 1 min followed by 1,5 min at 72 ºC, and then by a final extension step for 7 min at 72 ºC. The amplification products were resolved by electrophorsis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 95 volts. A 1kb DNA ladder was used as a molecular size standard.

Data analysis

The banding patterns generated by ISSR-PCR marker analysis were compared to determine the genetic variation of the samples under study. Clear and distinct amplification products were scored as '1' for presence and '0' for absence of bands.

Table1: Characteristics of selected ISSR primers

Primer	Primer sequence (5'-3')	
P1	(AG)8YC	AGAGAGAGAGAGAGAGYC
P2	(AG)8YG	AGAGAGAGAGAGAGAGYG
P3	(AC)8YT	ACACACACACACACACYT
P4	(AC)8YG	ACACACACACACACACYG
P5	(GT)8YG	GTGTGTGTGTGTGTGTYG
P6	CGC(GATA)4	CGCGATAGATAGATAGATA
P7	GAC(GATA)4	GACGATAGATAGATAGATA
P8	(AGAC)4GC	AGACAGACAGACAGACGC
P9	(GATA)4GC	GATAGATAGATAGATAGC
P10	(GACA)4AT	GACAGACAGACAGACAAT
P11	(TA)10G	TATATATATATATATATATAG
P12	(AC)9T	ACACACACACACACACACT
P13	(AC)9C	ACACACACACACACACACC
P14	(GA)9A	GAGAGAGAGAGAGAGAGAA
P15	(GA)9T	GAGAGAGAGAGAGAGAGAT
P16	(GA)9C	GAGAGAGAGAGAGAGAGAC
P17	(TA)5GT	TATATATATAGT
P18	(CA)6T	CACACACACACAT
P19	(CA)8A	CACACACACACACACAA

A: Adenine, T: Thymine, G: Guanine, C: Cytosine and Y= Pyrimidine

SDS-PAGE of muscle protein method

Total cellular proteins from muscles of thee species of red sea fishes were analyzed by SDS-PAGE. Pellets were collected by centrifugation at 12000 rpm at 4 ºC, washed once with distilled water and then with 1 ml of 1mM NaCl containing 5 mM EDTA. Then heated in the present of low molecular weight thiol (2-meracoptoethanol) and SDS denatured total cellular protein from fish's muscle cells. One volume of the cell suspension was mixed with one volume of 2X treated buffer 0.25M Tris-HCL PH 6.8, 4% SDS, 20% glycerol and 10% 2- meracoptoethanol) and boiled in a water bath for 90 second then quickly transferred to ice water and kept until loading the gel. The SDS-PAGE was performed to analyze protein profile in muscle of control and petroleum oil exposed tissues in the three species of the red sea fishes on the basis of molecular weight by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophorsis (SDS-PAGE) (Sharaf-Eldeen *et al.*, 2006).

The gels were prepared as separating gel containing 10% acrylamid monomer and staking gel 4% and sample extract was loaded in each lane of the gel. The electrophoresis was carried at 100 volts in 1X Tris⁄glycine-SDS-running buffer for 2hrs. After electrophoresis, the gel was stained in 50ml of staining solution (0.125% Coomassie blue R-250, 50% methanol and 10% acetic acid). The presence or absence of each band was treated as binary character in a data matrix that is, coded 1 and 0, respectively. Data were statistically analyzed by using gel Doc. 2000 Bio-Rad system.

III. Results

Inter-simple sequence repeats (ISSR) amplification

Photos of the produced banding patterns by application of ISSR techniques on three studied species of red sea fishes are shown in (Fig. 1,2 and 3). The number and types of the amplified DNA bands and percentage of the total polymorphism are given in (Tables 2, 3 and 4).

A total of 465 clear and distinguishable ISSR bands (147 bands for *Lethrinus borbonicus* species, 152 for *Siganus rivulatus* and 166 for *Mulloidichthys flavolineatus*) were generated by the nineteen used ISSR primers of this study (Table 1), where sizes ranged from 0.14 to 1.7 Kb (Tables 2, 3 and 4). The number of bands per primer showed variant range among the three species, in *Lethrinus borbonicus* species ranged from 5 to 22 with a mean of 13.3 per primer, in *Siganus rivulatus* species ranged from 7 to 27 bands with a mean of 15.2 per primer and in *Mulloidichthys flavolineatus* species ranged from 8 to 28 with a mean of 16.6 per primer. Primers 2, 6, 10, 11,14,15 and 18 amplified polymorphic markers for *Lethrinus borbonicus* species, primers 1, 2, 6, 8, 9, 10, 11 and 12 revealed polymorphic markers for *Siganus rivulatus* species and primers 2, 4, 5, 6, 7, 10 and 11 amplified polymorphic markers for *Mulloidichthys flavolineatus* (Tables 2, 3 and 4).

The highest number of bands was generated from the primers P14, P11 and P8 for the species *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* respectively (Figures 1, 2 and 3). The highest percentage of polymorphism (44.4%, 25% and 27.3%) for the species *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* respectively was generated from the primers P15, P8 and P6 respectively. The number of polymorphic bands produced per primer for the species *Lethrinus borbonicus* ranged between 1 (P2, P10 and P11) to 8 (P15), for the species *Siganus rivulatus* ranged from 1 (P1, P2, P9, P10, P11, P12) to 2 (P6 and P8) and for the species *Mulloidichthys flavolineatus* ranged from 1 (P5) to 3 (P2, P6 and P11) out of 19 polymorphic bands for the species *Lethrinus borbonicus*, 10 for the species *Siganus rivulatus* and 16 for the species *Mulloidichthys flavolineatus*. The ISSR banding patterns were amplified by nineteen primers (Figures 1, 2 and 3). The profiles generated by ISSR primers showed polymorphism among the control and the investigated species from polluted environment with petroleum oil of the three species of fishes. The extent of the polymorphism varied. The percentage polymorphic loci were calculated to be 12.9, 6.6 and 9.6 for *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* respectively.

SDS-PAGE analysis

The product SDS-protein profile of the three test species is shown in (Fig. 4). A maximum number of 137 bands were detected at approximately molecular weights ranging between 11 KDa and 250 KDa. The protein profile was showed distinct polymorphism among the three species compared to the control.

The electrophoretogram (Fig. 4) represents the decrease in the intensity of muscle protein subunits in *Mulloidichthys flavolineatus* species exposure tissue samples compared to control, while *Lethrinus borbonicus* and *Siganus rivulatus* species exposed muscles protein showed increase intensity in banding pattern compared to control tissue sample, *Siganus rivulatus* showed more increase proportion in muscle protein subunits than *Lethrinus borbonicus* species, after electrophoresis and Coomassie blue.

Staining of cellular muscle proteins, 43 bands were observed in *Siganus rivulatus* species, by comparison of molecular weight to control group, 13 bands were appeared in exposure petroleum oil tissue. 9 of higher molecular weight bands were observed at (350, 265, 240, 210, 190, 170, 120, 90 and 68 KDa), four other lower molecular weight bands (30, 21, 19 and 12 KDa) were appeared, and two bands at (140 and 18 KDa) component were lost in exposure sample as indicated by the increase of band intensity. Whereas In *Lethrinus borbonicus* species, 44 bands were observed with six polymorphic bands were appeared in petroleum oil exposure sample at molecular weight (400, 125, 90, 68, 19 and 17 KDa) and the other protein bands remained unchanged in exposure sample as indicated by the increase of band intensity. In *Mulloidichthys flavolineatus* 50 protein subunit bands were produced through the Coomassie blue stained gel with six polymorphic bands compared to the control, four bands were disappear in exposure sample at molecular weight (400, 120, 17 and 14 KDa) and two new bands were appeared at (130 and 16 KDa) molecular weight as indicated by the decrease of band intensity. This indicates that petroleum oil may be not toxic or more toxic. The variations in protein subunit band patterns effect may be due to change in the turn over (Synthesis/degradation) of various proteins. This result showed that pollutant environment with petroleum oil may lead to disappear of some protein subunits in some fishes or appear new some protein subunits in other fishes.

IV. Discussion

ISSR techniques have been widely applied genetic diversity in several economically important animals and are useful for biological conservation. Amplification of polymorphic percentage ISSR bands in the three species of fishes (12.9%, 6.6% and 9.6%) in *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* respectively indicates the existence of genetic diversity in the three species compared to control species. Out of nineteen selected primers used in this study 10 primers showed distinguish among the three

species compared to its control. The ISSR markers seem to be quite valuable for identifying red sea fishes and for assess evaluating their genetic diversity for the species exposure to chemically structures in their environment.

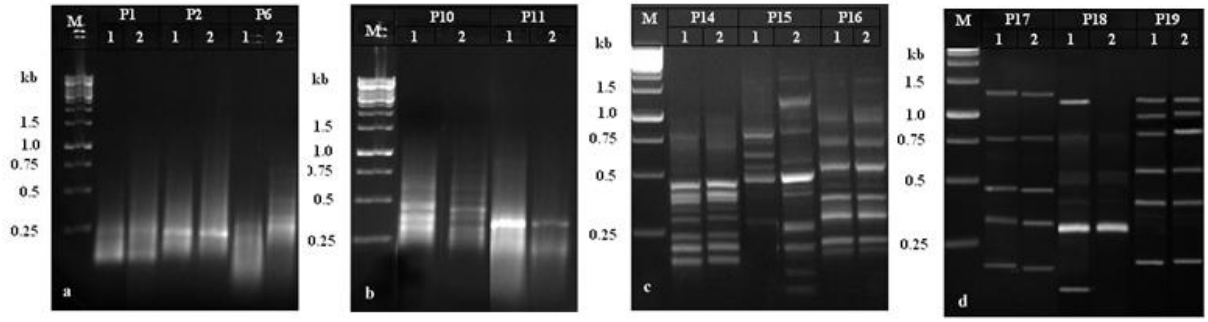

Fig. 1: ISSR amplification profiles using primers P1, P2, P6, P10, P11, P14, P15, P16, P17, P18 and P19 respectively. *Lethrinus borbonicus* sample codes are in lanes 1 is the control and 2 the exposure sample, DNA Marker (range 0.15 to 1.7 kb).

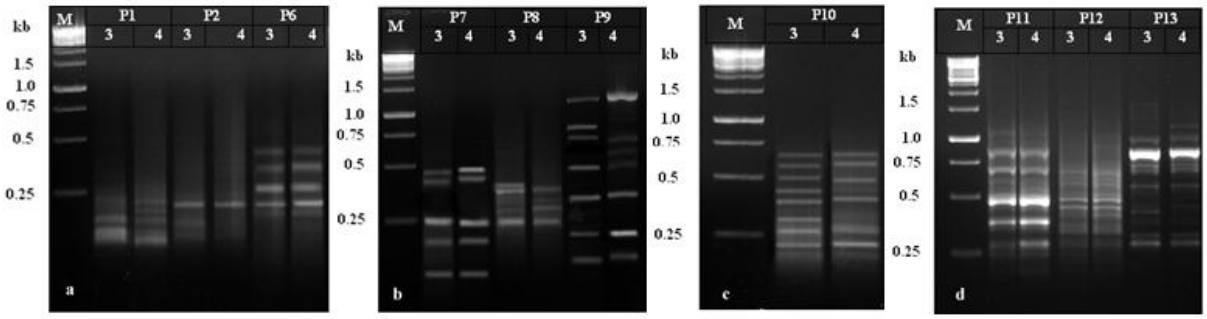

Fig. 2: ISSR amplification profiles using primers P1, P2, P6, P7, P8, P9, P10, P11, P12 and P13 respectively. *Siganus rivulatus* sample codes are in lanes 3 is the control and 4 the exposure sample, DNA Marker (range 0.2 to 1.5 kb)

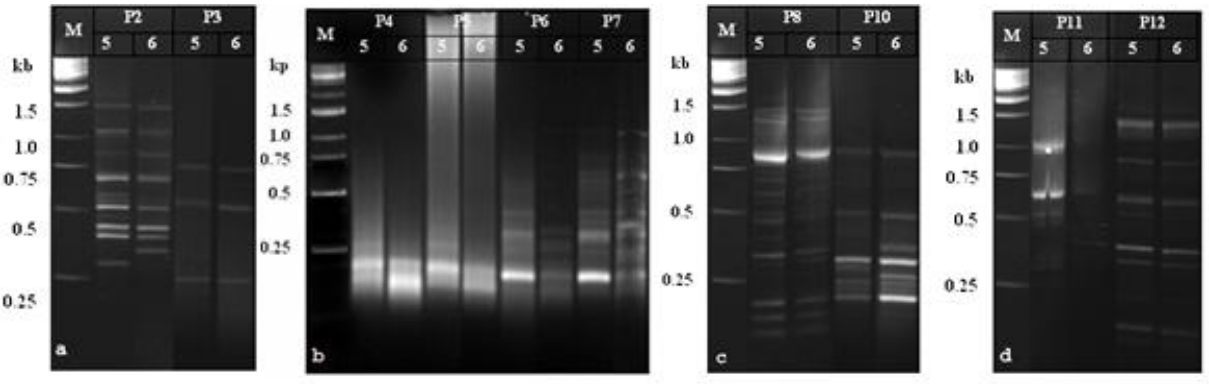

Fig. 3: ISSR amplification profiles using primers P2, P3, P4, P5, P6, P7, P8, P10, P11 and P12 respectively. *Mulloidichthys flavolineatus* sample codes are in lanes 5 is the control and 6 the exposure sample, DNA Marker (range 0.14 to 1.5 kb)

Table2: ISSR amplicons of species *Lethrinus borbonicus*

primer	Total bands	Polymorphic bands	Polymorphism %	Amplicon size range (kb)
P1	6	0	0	0.2-0.24
P2	5	1	20	0.23-0.26
P6	11	3	27.3	0.2-0.4
P10	21	1	4.8	0.24-0.85
P11	7	1	14.3	0.24-0.37
P14	22	2	9.1	0.2-1.0
P15	18	8	44.4	0.15-1.7
P16	18	0	0	0.23-1.3
P17	10	0	0	0.23-1.3
P18	13	3	23.1	0.2-1.2
P19	16	0	0	0.23-1.2
Sum.	147	19	12.9	

Table3: ISSR amplicons of species *Siganus rivulatus*

primer	Total bands	Polymorphic bands	Polymorphism %	Amplicon size range (kb)
P1	9	1	11.1	0.2-0.27
P2	7	1	14.3	0.21-0.26
P6	12	2	16.7	0.22-0.5
P7	10	0	0	0.21-0.4
P8	8	2	25	0.24-0.31
P9	19	1	5.3	0.22-1.5
P10	21	1	4.8	0.24-0.65
P11	27	1	3.7	0.22-1.25
P12	21	1	4.8	0.2-1.0
P13	18	0	0	0.27-1.2
Sum.	152	10	6.6	

Table4: ISSR amplicons of species *Mulloidichthys flavolineatus*

primer	Total bands	Polymorphic bands	Polymorphism %	Amplicon size range (kb)
P2	21	3	14.3	0.28-1.5
P3	8	0	0	0.25-0.7
P4	10	2	20	0.14-0.4
P5	9	1	11.1	0.15-0.4
P6	11	3	27.3	0.15-1.0
P7	20	2	10	0.16-1.0
P8	28	0	0	0.15-1.5
P10	22	2	4.5	0.22-0.8
P11	13	3	23.1	0.3-1.0
P12	24	0	0	0.15-1.5
Sum.	166	16	9.6	

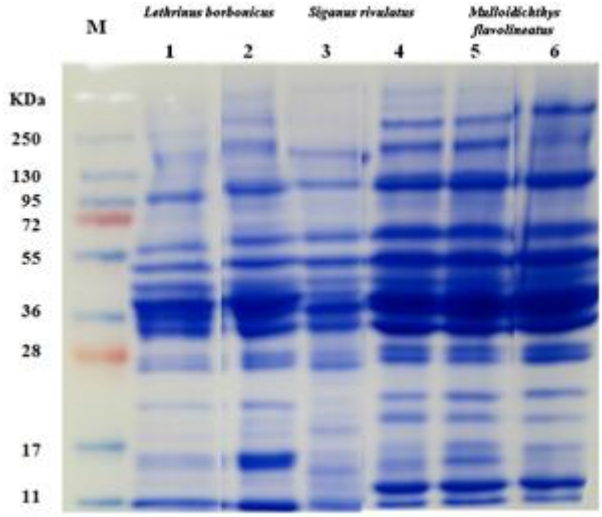

Figure 4: Changes in protein subunits in muscle tissues of the three species of fishes, *Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus* exposed to petroleum oil, lanes 1, 3 and 5 muscle control and 2, 4 and 6 petroleum oil exposed respectively for the three species.

The ISSR primers reacted successfully with the genomic DNA of the three samples (*Lethrinus borbonicus*, *Siganus rivulatus* and *Mulloidichthys flavolineatus*) generated 19, 10 and 16 polymorphic bands respectively which life in pollutant environment with petroleum oil compounded (Ras-Gharep) site compared to control species (El-Shalateen) site. The polymorphic bands variant between decrease or increase in the DNA fragment compared to the control samples, *Lethrinus borbonicus* showed disappear nine bands and appear ten new bands compared to control species, *Siganus rivulatus* showed disappear four bands and appear six bands and in *Mulloidichthys flavolineatus* species in contrast to the previous species revealed decrease in twelve bands to compare to control and appear four new bands only. These results agree with (Sharaf-Eldeen *et al*., 2006) reported that both agricultural and industrial water pollution of *Tilapia zillii* caused an increase in the percentage of DNA fragmentation but the percentage of industrial polutio was higher than in areas of agricultural pollution. (Mahrous *et al*., 2006) mentioned that, the genotoxic effects were indicated by appearance of some changes in polymorphism band patterns including lost of stable bands occurrence of new bands. (Hasheesh *et al*., 2011) reported the exposure to genotoxic agent will give rise to alteration of DNA structure that can lead to abnormal changes of DNA fingerprints. The results of ISSR analysis showed that 9 primers of 19 primers didn't observe

any polymorphic bands that reacted with the three DNA of samples, primers (P1, P16, P17 and P19) for *Lethrinus borbonicus* species, (P7 and P13) for *Siganus rivulatus* and (P3, P8 and P12) for *Mulloidichthys flavolineatus* which produced completely common bands compared the control samples. There are evident that the DNA fragments at these nine primers were not affected by petroleum oil. The amount of genetic diversity of the sample from control site (El-Shalateen) and the sample from exposure pollution (Ras-Gharib) may reduce their ability to suffer in a changing environment, these results agree with (Imtiaz *et al*., 2011) who reported that, genetic diversity plays an important role in the survival and adaptability of a species. When a species environment changes, slight gene variations are necessary for it to adapt and survive.

The petroleum oil may inhibit the expression of some genes (or) activate the others to produce specific mRNAs which may subsequently be translated into specific proteins called stress induced proteins (Frigo *et al*., 2004, Cheshenko *et al*., 2008 and Şentürk *et al*., 2009). An alteration of protein metabolism was observed in fish exposed to various types of environment stresses like metals and pesticides (Becker *et al*., 2009 and Shwete and Gopal 2009). Tripathi and Shukla (1990a and 1990b) performed SDS-PAGE of the cytoplasmic proteins of liver and the skeletal muscle of *Clarias batrachus* exposed to endosulfan and methyl parathion for 1 to 28 days. The appearance of new proteins after exposure of the pesticide, demonstrated clearly alteration in the cytoplasmic proteins. In the present SDS polyacrylamide gel electrophorsis was performed for the muscle tissues of the three species of red sea fishes exposed to petroleum oil. When compared to control the protein subunits of petroleum oil exposed tissues showed increase in intensity and new protein subunits were appeared and some protein subunits were disappeared varies in percentage among the three species, where, *Lethrinus borbonicus* species was showed 13.6% of polymorphism protein subunits, all of new proteins compared to the control, *Siganus rivulatus* showed 34.9% of polymorphism protein subunits 30.2% of new proteins and 4.7% of protein subunits were disappeared compared to the control, and in *Mulloidichthys flavolineatus* revealed 4% only of new protein subunits and 8% of proteins were disappeared compared to control. The variation in protein subunit band patterns may be due to change in the turn over (Synthesis/degradation) of various proteins. This study indicates that petroleum oil may be increase protein subunit band patterns in fishes exposure to this environment than the decrease in protein subunit band patterns.

A number of authors have reported similar observations (Sharaf-Eldeen and Abdel-Hamid, 2002 and Kamalam *et al*., 2013) recorded that, the exposure of some fishes to pollutants showed change levels of proteins. As a result of DNA and protein differences, the three species of red sea fishes from Ras-Gharib were effected by a different degree by petroleum oil, this means that, fishes were sensitivity to environmental contaminants, (Saravanan *et al*., 2011) reported that fish are used as excellent indicator of aquatic pollution due to their high sensitivity to environmental contamination which may damage certain physiological and biochemical processes when contact with the organs of fishes. These results proved that, petroleum oil affected on the genetic structure of the three species from the red sea fishes which lead to change in the structure of proteins of these fishes, (Bye and Ponnaiah, 1983) mentioned that, protein is a translated phynotypic expression of genetic code and the variations in the genome usually result in change in the structure of proteins. Our results will be useful in studying the environmental changes and biochemical structure on Red sea fishes. The present study was showed that, both SDS-protien and ISSR primers were informative in detecting species specific DNA markers. Results revealed that, the three fish species represent various degree of sensitivity in monitoring genetic damage. This is indicated by variations in averages of DNA and protein fragments among species and its control.

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