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Molecular characterization of four Mullet species based on SCoT and ISSR markers

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ABSTRACT

Molecular markers were used as indicators in assessing the genetic diversity of a fish, they allow for direct observation of genetic information and estimation of genetic relationships between the population and species. In the present study, eleven SCoT primers and ten ISSR primers were used to estimate the genetic diversity among the four mullet species namely *Mugil cephalus, Liza ramada, Liza grana,* and *Valamugil seheli* collected from four different Egypt governorates Alexandria, Ismailia, Port Said and Damietta. The two markers SCoT and ISSR successfully amplified amplicons with a total number of 176 and 132 of which 153 and 111 were polymorphic, representing a percentage of polymorphism of 86.9% and 84.1% polymorphic amplicons/ primer, respectively. The similarity indices ranged from 0.47 to 0.84, 054 to 0.92 and 0.52 to 0.86 for SCoT, ISSR and combined, respectively. Cluster analysis based on similarity matrices of SCoT, ISSR and combined dendrograms revealed some similarities; for example, the grouping of the *Mugile cephallus* (Alexandria, Ismailia and Port Said) together and the grouping *Valamugil seheli* (Ismailia and Damietta) based on the three dendrograms. Also, *Liza ramada*, *Liza garana* (Damietta) and *Mugile cephallus* (Damietta) were clustered together based on the three dendrograms. From gene sequencing and NCBI Blast analysis identified of gene location in fish genomes. The NCBI blast tool was used to align our gene with the NCBI database and it revealed that this sequence is highly similar to a sequence located on fish species called *Dicentrarchus labrax* (the European bass). Also, the sequence is located on Chr1 near a gene called "Type II keratin E3".

INTRODUCTION

Fish production play a great role in the nutritional foods of the human because of its high source polyunsaturated fatty acids (PUFAs) especially omega-3, omega-6, vitamins and minerals, and relatively low caloric content. These properties could limit atherosclerosis and thrombosis. Also, fishs have different minerals,i.e iron (Fe), Calcium (Ca), Zinc (Zn), Phosphorus (P), Selenium (Se), Fluorine (F) and Iodine (I). These minerals are with high

bioavailability; they can easily be absorbed by the body (**Pal *et al.*, 2018**). Mullet (Family: Mugilidae) are very important fish, which are cultured in many countries, due to their high quality flesh, superior growth and wide salinity and temperature tolerance. The family Mugilidae currently comprises 20 genera and 74 species (**Eschmeyer and Fong, 2016)** The world fish production is 178.5 million tons, only 156.4 million tons are used for human consumption and remaining are used as the non-food purpose and discarded as waste material **(FAO, 2018**).The family has approximately 26 genera. As of 2017, there are 20 genera and 75 valid species within the order of Mugiliformes in the world. Species identification is uncertain in some parts of the world with morphological methods (**Labastida *et al.*, 2015**). So aquaculture outputs are needed many aspects to be increased in order to meet the rising demands for fish in the coming years. Biotechnology can provide the means to increase the intensity and capacity of the operation (**Sabry *et al.*, 2015**). Molecular markers are useful tools for assessing the genetic diversity for individuals, populations and species. The genetic diversity data have varied applications in research on evolution, conservation and management of natural resources and the genetic improvement programms (**Frankham *et al.*2004; Mahrous *et al.*, 2011)**. **Al-Soudy (2018)** studied the genetic diversity, relationships and population structure of sixty Egyptian camels derived from four breeds using ISSR (inter simple sequence repeat) and SCoT (Start codon targeted marker) primers. The results of the genetic relationship based on both markers (microsatellites and SCoT) confirmed the close relationship between the two breeds. The molecular markers research has made wide possible genetic characterization and biodiversity studies in fish populations (**Sugama *et al.*, 2012; El-Tarras *et al.*, 2017**). Different molecular markers have been used for studying the genetic diversity for fish such as Inter Simple Sequence Repeat (ISSR), SCoT and barcoding. Moreover, the molecular markers were used to identify sex-associated genomic regions (**Luzio *et al.*, 2015; Ibraheem *et al.*, 2018**). The sequence targets of ISSR are available in a eukaryotic genome, thus its revealed a much higher number of polymorphic fragments per primer than the RAPD markers (**Esselman, 1999**). ISSRs markers are very useful tools for population differentiation because of its longer length of the primer, highly reproducibility and easy quick handle (**Bornet and Branchard, 2001)**.It has been widely used to detect intraspecific polymorphisms in different species such as chickpea and Grevillea (**Pharmawati *et al.*, 2004**). ISSR marker is the most accurate tool for assessing genetic variation. Furthermore, the ISSR marker does not need to DNA sequence information of the species (**Maheswaran, 2004).** Newly, genetic diversity has been evaluated using the SCoT (start codon targeted marker) among female fish in early development stages (**Ibrahim, 2018**). Start Codon Targeted (SCoT) polymorphism has been developed as a new functional marker system depended on the short conserved region flanking the ATG start codon. SCoT markers are generally highly reproducible, targeted marker and could be used for genetic diversity searches, quantitative trait loci (QTLs) mapping and bulk segregation analysis (**Nolte *et al.*, 2005; Mehta and Sahani.,**

2014).The microsatellite markers are targeting repetitive sequences, while the SCoT markers were employed to target the polymorphism in sequences near the genes (**Penedo *et al.*, 1999; Nouairia *et al.*, 2015).** The SCoT Polymorphism technique is similar to RAPD (Randomly Amplified Polymorphic) DNA and ISSR (Inter Simple Sequence Repeat) because it uses a single forward and reverses primer. So the SCoT markers are expected to be linked to functional genes and corresponding traits, **(Bhattacharyya *et al.*, 2013)**. Studying the evolution and phylogenetic relationships of the aquatic organism including fish species were created by analysis of partial sequencing of certain mitochondrial genes such as12s rRNA, 16s rRNA, COI, D-Loop and cytochrome b.(**Carvalho and Hauser, 1994; Healey *et al.*, 2017**) and concerning mullet fishes among some Mugilidae fishes (Abudefduf vaigiensis, Labracinus cyclophthalmus and Oryzias latipes **(Durand *et al.*, 2012**). **Saad *et al.* (2019)** studied the sequencing of Sox14 gene to identify the molecular variations among three mullet fishs (*M. seheli, L. carinata and M. cephalus*) comparatively with COI barcoding system. The complete phylogenetic position of teleost fishes can be done by using the sequenced genomes in a large mitogenomic context for the whole genome (**Martin *et al.*, 2017).** The present study was focused on the molecular variability among the four Mullet species (*M. cephalus, L.ramada,V. seheli, L.grana*) using two molecular markers (SCoT and ISSR). Also, to assess the genetic relationship among the four genotypes. In addition, to identify the unique markers related to some species traits which can differentiate among the studied genotypes by bioinformatics tools.

MATERIALS AND METHODS

Materials:

Sixteen samples of Mullet fish species (four replicates for each species) used in the present investigation(*Mugil cephalus, Liza ramada, Liza grana* and *Valamugil seheli)* were collected from four different locations which be: Alexandria, Ismailia, Port Said and Damietta.

Methods

DNA extraction and purification

DNA extraction and purification was carried out according to DNeasy Kit (Qiagen)**.**

DNA concentration

The concentration of genomic DNA was estimated by comparing the size and intensity of each sample band with those of a sizing standard marker. The DNA samples was diluted (l:5) (2 µl of DNA + 8ul dd. H2O). The DNA samples were loaded on 1% agarose gel in comparison to 10 µl of a DNA size marker (lambda DNA Hind III digest Phi X 174/HaeIII digest). To estimate DNA concentration, compare the degree of fluorescence of the DNA sample with the different bands in the DNA size marker.

To detect the genetic variability among the four fish species by eleven **SCoT** markers(Table **1**). The analysis reaction was conducted according to **Collard and Mackill (2009).**

The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgC_{l2}, 0.2 mM dNTPs, 1 µM primer, 1 U *Taq* DNA polymerase and 30 ng templates DNA. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems). The thermocycling profile was as follows: an initial primary denaturation cycle at 94 °C for 3 min; followed by 35 cycles of 94°C/50 s, 50°C/1 min and 72°C/2 min; the final extension at 72°C/5 min.The PCR products were resolved on 1.5% agarose gel electrophoresis in 1X TBE buffer, stained with ethidium bromide (0.5 mg/mL) and visualized under UV light.

Table (1): The sequence of the eleven and names of SCoT primers used to detect the genetic variability among the four Mullet fish species.

No.	Primer name	Sequence
1	**SCoT-2**	5'- ACCATGGCTACCACCGGC -3'
2	**SCoT-3**	5'- ACGACATGGCGACCCACA -3'
3	**SCoT-4**	5'- ACCATGGCTACCACCGCA -3'
4	**SCoT-5**	5'- CAATGGCTACCACTAGCG -3'
5	**SCoT-11**	5'- ACAATGGCTACCACTACC -3'
6	**SCoT-12**	5'- CAACAATGGCTACCACCG -3'
7	**SCoT-13**	5'- ACCATGGCTACCACGGCA -3'
8	**SCoT-14**	5'- ACCATGGCTACCAGCGCG -3'
9	**SCoT-16**	5'- CCATGGCTACCACCGGCA -3'
10	**SCoT-20**	5'- CAACAATGGCTACCACGC -3'
11	**SCoT-22**	5'- CCATGGCTACCACCGCAC -3'

2- ISSR -PCR Reactions:

A set of ten ISSR primers (Table 2) was used in the detection of polymorphism among four fish species according to **Hills *et al.* (1996**).The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 µM primer, 1 U Taq DNA polymerase and 40 ng templates DNA. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 35 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 50ºC for 1 min, and an elongation step at 72ºC for 1 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle. PCR products were visualized using the same procedure as in SCoT.

Table(2) : Primers code, nucletiode sequence of the eleven, names of ISSR primers used to detect the genetic variability among the four Mullet fish species.

Primer code	Sequence
ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR- 4	5'-ACACACACACACACACYG-3'
ISSR- 8	5'-AGACAGACAGACAGACGC-3'
ISSR- 10	5'-GACAGACAGACAGACAAT-3'
ISSR- 14	5'-CTCCTCCTCCTCCTCTT-3'
ISSR- 15	5'-CTCTCTCTCTCTCTCTRG-3'
ISSR- 16	5'-TCTCTCTCTCTCTCTCA-3'
ISSR- 18	5'-HVHCACACACACACACAT-3'
ISSR- 19	5'-HVHTCCTCCTCCTCCTCC-3'

Data Analysis for SCOT and ISSR markers:

The banding patterns generated by SCoT and ISSR markers were compared to determine the genetic relationships among the four Mullet fish species. Clear and distinct amplification products were scored as (1) for present and (0) for absent bands for all samples. Bands of the same mobility were scored as identical. SCoT and ISSR banding patterns were compared to determine the genetic relationships among the different species, using Phortix nonlinear dynamics (UK) software Version 10.

3- DNA sequencing for SCOT 5' products :

Purification of PCR Products:

PCR products of SCoT were purified using EZ-10 spin column PCR products purification. PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes was added in binding buffer 1 after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 minutes after that centrifuge, 750 ul of wash solution was added to the column and centrifuge at 10.000 rpm for two minutes, repeated washing, 10.000 rpm was spine for an additional minute to remove any residual wash solution. The column was transferred into a clean 1.5 ml microfuge tube and adds 50 ul of elution buffer, incubated at room temperature for 2 minutes and when store purified DNA at -20°C.

Fragment SCoT sequencing analysis:

The purified fragment DNA was sent to Microgen Company, Germany, for sequencing. The sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).Sequences were aligned using Align Sequences Nucleotide BLAST and Sequences were aligned with MEGA5 program

Mullet fish species (four replicates for each species) used in the present investigation (*Mugil cephalus*, *Liza ramada*, *Liza grana* and *Valamugil seheli)* were collected from four different Egypt governorates Alexandria, Ismailia, Port Said and Damietta.

Methods

DNA extraction and purification

DNA extraction and purification was carried out according to DNeasy Kit (Qiagen)**.**

Estimation for the DNA concentration

The concentration of genomic DNA was estimated by comparing the size and intensity of each sample band with those of a sizing standard marker. The DNA samples was diluted (l:5) (2 µl of DNA + 8ul dd. H2O). The DNA samples were loaded on 1% agarose gel in comparison to 10 µl of a DNA size marker (lambda DNA Hind III digest Phi X 174/HaeIII digest). To estimate DNA concentration, compare the degree of fluorescence of the DNA sample with the different bands in the DNA size marker.

1- SCoT-PCR Reactions

To detect the genetic variability among the four fish species by eleven **SCoT** markers (Table 1). The analysis reaction was described in detail in according to **Collard and Mackill (2009)**. The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgC_{l2}, 0.2 mM dNTPs, 1 µM primer, 1 U *Taq* DNA polymerase and 30 ng templates DNA. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems). The thermocycling profile was as follows: an initial primary denaturation cycle at 94 °C for 3 min; followed by 35 cycles of 94°C/50 s, 50°C/1 min and 72°C/2 min; the final extension at 72°C/5 min.The PCR products were resolved on 1.5% agarose gel electrophoresis in 1X TBE buffer, stained with ethidium bromide (0.5 mg/mL) and visualized under UV light

Table 1. The sequence of the eleven and names of SCoT primers used to detect the genetic variability among the four Mullet fish species.

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7	**SCoT-13**	5'- ACCATGGCTACCACGGCA -3'
8	**SCoT-14**	5'- ACCATGGCTACCAGCGCG -3'
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2- ISSR -PCR Reactions:

Aset of ten ISSR primers (Table 2) was used in the detection of polymorphism among four fish species according to **Hills *et al*. (1996**). The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 µM primer, 1 U Taq DNA polymerase and 40 ng templates DNA. PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 35 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 50ºC for 1 min, and an elongation step at 72ºC for 1 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle. PCR products were visualized using the same procedure as in SCoT.

Table 2. Primers code, nucletiode sequence and annealing tamprature of the eleven, names of ISSR primers used to detect the genetic variability among the four Mullet fish species.

Primer code	Sequence
ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR- 4	5'-ACACACACACACACACYG-3'
ISSR- 8	5'-AGACAGACAGACAGACGC-3'
ISSR- 10	5'-GACAGACAGACAGACAAT-3'
ISSR- 14	5'-CTCCTCCTCCTCCTCTT-3'
ISSR- 15	5'-CTCTCTCTCTCTCTCTRG-3'
ISSR- 16	5'-TCTCTCTCTCTCTCTCA-3'
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Data Analysis for SCOT and ISSR markers:

The banding patterns generated by SCoT and ISSR markers were compared to determine the genetic relationships among the four Mullet fish species. Clear and distinct amplification products were scored as (1) for present and (0) for absent bands for all samples. Bands of the same mobility were scored as identical. SCoT and ISSR banding patterns were compared to determine the genetic relationships among the different species, using Phortix nonlinear dynamics (UK) software Version 10.

3- DNA sequencing for band 3' SCOT 5':

Purification of PCR Products:

Amplified products for all PCR were purified using EZ-10 spin column PCR products purification PCR reaction mixture was transferred to 1.5 ml microfuge tube and three volumes was added of binding buffer 1 after that the mixture solution was transferred to the EZ-10 column and let it stand at room temperature for 2 minutes after that centrifuge, 750 ul of wash solution was added to the column and centrifuge at 10.000rpm for two minutes, repeated washing, 10.000 rpm was spine for an additional minute to remove any

residual wash solution. The column was transferred into a clean 1.5 ml microfuge tube and adds 50 ul of elution buffer, incubated at room temperature for 2 minutes and when store purified DNA at -20°C.

Fragment SCoT sequencing analysis:

The purified fragment DNA was sent to Microgen Company, Germany, for sequencing. The sequences were analyzed using the BLAST program (http://www.ncbi. nlm. nih.gov/BLAST).Sequences were aligned using Align Sequences Nucleotide BLAST and Sequences were aligned with MEGA5 program.

RESULTS

Assessment of the genetic diversity and relationship among the four mullet species by molecular markers :

The molecular markers research has made wide possible to genetic characterization and biodiversity studies in fish populations **(El-Tarras *et al.*, 2017**). Many properties can be obtained with different molecular marker platforms uses including reliability, reproducibility, coverage, cost and automation (**Agrawal *et al.*, 2008**).

(1) Genetic relationships as revealed by by SCoT markers

In the present study, eleven SCoT and ten ISSR markers were used to assess the genetic diversity among the four mullet species. As shown in table (3) the total number of DNA generated by eleven SCoT primers was 176 with an average of 16 amplicons/primer. The highest number of amplicon was 21generated by SCoT-16 and the lowest number was 13 generated by SCoT-2.The total number of polymorphic amplicons was 153 with an average 13.9 / primer. The polymorphic amplicons ranged from 12 to 21 amplicons. Therefore, the eleven SCoT primers expressed different levels of polymorphism, ranging from 75% with the primer SCoT-20 to 100 % with the primer SCoT-16. The molecular size of amplified fragments varied all eleven SCoT primers, ranging from 100 to 1,500 bp (Fig. 1).

(2) Genetic relationships as revealed by ISSR markers:

The polymorphic among the four species was investigated using the ten ISSR primers (Table 4). Fig. (2) shows the ISSR profiles amplified with the ten ISSR primers. The total number of DNA fragments amplified generated by ten ISSR primers was 132 with an average of 13.2 amplicons / primer. The ISSR profiles amplified by the different primers contained multiple bands ranging from 10 with ISSR 8 to 17 with ISSR 14 amplicons. The total number of polymorphic amplicons that generated by ten primers 111 amplicons with an average of 11.1. The polymorphic amplicons was ranged from 7 with ISSR 8 to 15 with ISSR 14. The highest number of amplicons was 17 amplicons generated by primer ISSR-14, and the lowest number was 10 amplicons

generated by primer ISSR -8. Therefore, all ten ISSR primers expressed different levels of polymorphism, ranged from 70% with the primer ISSR -8 to 100 % with the primer ISSR-16 with an average of 83.5 %. In addition, the highest percentage (100%) of polymorphism was exhibited by ISSR-16 primer. The molecular size of amplified fragments varied in all ten ISSR primers, ranging from 100 to 1,500 bp (Fig. 2).

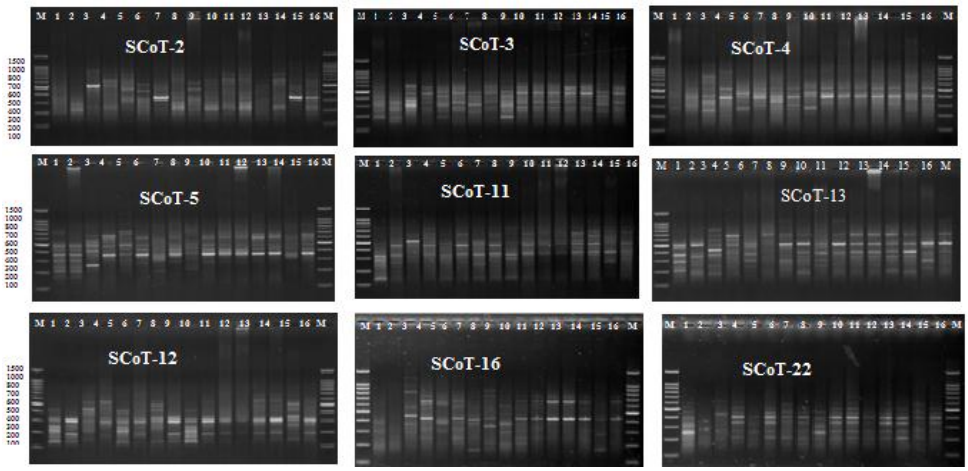

Fig. 1. SCoT profiles of the four mullet species as revealed by SCoT primers. lane. 1.5.9.13 *Mugile cephlus* (Alexanderia, Ismailia, Port Said, Damietta) respectively, 2.6.10.14 *Liza ramada* (Alexanderia,Ismailia, Port Said, Damietta) respectively.,3.7.11.15 *valamugil sehili* (Alexanderia, Ismailia, Port Said, Damietta) respectively. and 4.8.12.16 *Liza garana* (Alexanderia,Ismailia, Port Said,Damietta) respectively. M=1 Kb marker.

Table 3. Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphic as revealed by SCoT markers among the four mullet species.

Primer code	Total number of amplicons	Monomorphic amplicons	Polymorphic amplicons	% polymorphic
SCoT 2	13	0	13	100
SCoT 3	15	2	13	86.7
SCoT 4	15	2	13	86.7
SCoT 5	17	4	13	76.5
SCoT 11	15	2	13	86.7
SCoT 12	16	2	14	87.5
SCoT 13	15	2	13	86.7
SCoT 14	18	2	16	88.9
SCoT 16	21	0	21	100
SCoT 20	16	4	12	75.0
SCoT 22	15	3	12	80
Total	176	23	153	86.9
average	16	2.09	13.9	

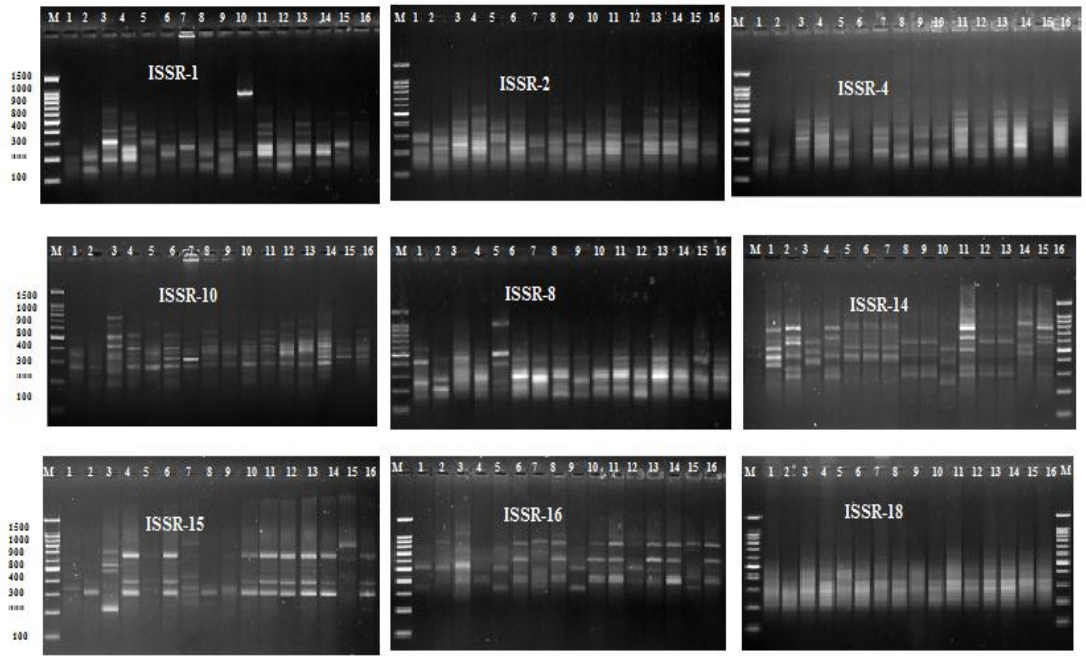

Fig. 2. ISSR profiles of the four mullet species as revealed by ISSR**.** Lane: 1.5.9.13 *Mugile cephlus* (Alexanderia,Ismailia, Port Said,Damietta) respectively, 2.6.10.14 *Liza ramada* (Alexanderia,Ismailia, Port Said,Damietta) respectively.,3.7.11.15 *valamugil sehili* (Alexanderia,Ismailia, Port Said,Damietta) respectively.and 4.8.12.16 *Liza garana* (Alexanderia,Ismailia, Port Said,Damietta) respectively. M=1 Kb marker.

Table 4. Primer code,total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphic as revealed by ISSR marker among the mullet species.

Primer code	Total number of amplicons	Monomorphic amplicons	Polymorphic amplicons	% polymorphic
ISSR 1	14	1	13	92.9
ISSR 4	16	3	13	81.3
ISSR 8	10	3	7	70.0
ISSR 10	14	3	11	78.6
ISSR 14	17	2	15	88.2
ISSR 15	13	2	11	84.6
ISSR 2	13	2	11	84.6
ISSR 16	11	0	11	100
ISSR 18	11	3	8	72.7
ISSR 19	13	2	11	84.6
Total	132	21	111	84.1
average	13.2	2.1	11.1	

In this respect, **Ibrahim *et al.*, (2018**) used ISSR and SCoT markers to sex sequences determination of an early age of fish. The total number of amplicons generated by the SCoT and ISSR markers was 575 and 190 amplicons, respectively. **Marie and Allam (2017**) used twelve Start Codon Targeted (SCoT) markers for studying the genetic diversity and relationships among three species of Red Sea fishes, carangids, (*Carangoides bajad*, *Caranx sexfasciatus* and *Caranx melampygus*).

A total of 325 fragments were generated by the twelve SCoT primers in the three species. 192 amplicons generated in the two species (*Carangoides bajad* and *Caranx sexfasciatus*), out of these amplicons 98 (51%) were common between the two species, while 248 amplicons were in the two species (*Carangoides bajad* and *Caranx melampygus*), out of the 162 (65%) were common between the two species, 210 amplicons were generated by the Twelve primers and only 98 (47%) amplicons of them were common between the two species (*Caranx sexfasciatus* and *Caranx melampygus***).** Also, **Luzio *et al*. (2015)** stated that the ISSR marker could use for screening and identification of potential sex-associated sequences in *Danio rerio*. All 100 ISSR primers on both bulks by PCR were tested, confirmed the differences in all individual samples of male or female. Obtained a total of 516 bands, with an average 8.32 bands per ISSR primer. A total of 36 reproducible bands were obtained by five primers, of which nine were polymorphic (25%). When used these five primers to generate profiles of 50 individuals, did not produce a predominant band in individuals of the same sex. Moreover, **Sabry *et al*., (2015),** who reported that three ISSR and six RAPD markers are useful tools to determining molecular characterization of seven popular Saudi fish species, Morgan (*Nemipteru sjaponicus*), Mousa (*Solea solea*), Hamor (*Greasy grouper*), Shour (*Lethrinus lentjan*), Dennis (*Caranxsex fasciatus*), Harid (*Scarus arabicus*) and Black surgeon (*Acanthurus gahhm*).These markers generated 187 distinct bands 89.4% bands were considered as polymorphic and 10.6% were considered as monomorphic bands.

(3) Genotype identification by unique SCoT and ISSR markers:

Unique markers are bands that define specifically an individual/samples from the others, by their presence or absence. Positive unique markers (PUM) for the bands that are present in one species but not found in the others, in contrast the negative unique markers (NUM) are called for the bands that absent in a specific genotype. The genotype could be identified by these bands.

As shown in tables (5 and 6) the SCoT and ISSR markers permitted the identification of the four species of mullet fish genotypes by unique positive and negative markers. The total number of unique markers produced by SCoT and ISSR primers was 23 and 21 positive respectively. All species showed a positive unique marker. Among the four spesies two species were characterized by 2 negative unique markers. *Mugile cephallus* collected from Ismailia exhibited one negative unique marker by SCoT- 22 primer, while, *Liza grana* collected from Port Said and Damietta was distinguished by two negative unique markers one by SCoT-11 primer and ISSR-8, respectively. In addition, *Valamugil.seheli* was characterized by the highest number of unique positive (14) by SCoT and (12) by ISSR markers.

In this respect, **Marie and Allam (2017**) reported that several amplified fragments were clearly distinguishable bands from the total number of polymorphic and appearance of unique bands marker between the three fish species (*Carangoides bajad*, *Caranx*

sexfasciatus and *Caranx melampygus*). **Ibrahim *et al*, (2018**) detected About 33 SCoT and 30 ISSR markers for *S. aegyptiaca* species were unique bands for this species at the male and female levels, gender specific markers for *S.aegyptiaca*. and for *S.vulgari* were detected by ISSR-15_{590}, ISSR-71_{410}, ISSR-9_{550}, and about 11 SCoT and 15 ISSR PCR-bands were unique for this species. Moreover, SCoT-3_{740}, SCoT-24_{740}, SCoT-21_{800} and SCoT-21482, ISSR-9_{300}, ISSR-9_{850} ISSR-10_{300}, ISSR-14_{400}, ISSR-14_{500} and ISSR-14_{950} were gender specific markers for *S. vulgaris*. Also, **Abou-Gabl *et al*. (2018)** stated that, the both unique and polymorphic RAPD markers can be used to differentiate for the mullet species.

(4) Genetic relationships and cluster analysis as revealed by SCOT markers:

The estimated similarities among the four species ranged from 0.47 between *Liza grana* (Damietta) and *Mugile cephallus* (Alexandria) to 0.84 between *Liza grana* (Damietta) and *Liza ramada* (Damietta). The highest genetic similarity was (0.84) between *Liza grana* (Damietta) and *Liza ramada* (Damietta).This was followed by 0.83 between *Liza garana* (Damietta) and *Mugile cephallus* (Damietta). The UPGMA clustering analysis was carried out in order to represent the genetic distances among the 4 mullet species **(Fig. 3).**

Table 5. Genotypes characterized by unique positive and / or negative SCoT markers, marker size and total number of bands identifying each genotype.

Unique positive				**Unique negative**			
Species	**Primer Name**	**Size of Band**	**Total Marker numberof / species**	**Primer Name**	**Size of Band**	**Total of number / species**	**Grand Total**
Mugil.cephalus	**SCoT-5** **SCoT-11** **SCoT-13** **SCoT-22**	190 370 560 740&520	5	SCoT-22	180	1	6
Liza. Ramada	**SCoT-3** **SCoT-4** **SCoT-20**	290&150 260 600	4				4
Valamugil.seheli	**SCoT-2 SCoT-4** **SCoT-12** **SCoT-14** **SCoT-16**	920 840 700&460 430&330&300&190 &170&110970& 420	12				12
Liza. grana	**SCoT-14** **SCoT-12**	220 1050	2	SCoT-11	250	1	3
Total			23			2	25

Table (6): Genotypes characterized by unique positive and / or negative ISSR markers, marker size and total number of bands identifying each genotype

Unique positive				Unique negative			
Species	**Primer Name**	**Size of Band**	**Total number of band/ species**	**Primer Name**	**Size of Band**	**Total number of band/ species**	**Grand Total**
Mugil.cephalus	ISSR-8	1118	1				1
Liza. Ramada	ISSR-1 ISSR-10	1046&434 947	3				3
Valamugil.seheli	ISSR-1 ISSR-4 ISSR-10 ISSR-14 ISSR-15 ISSR-2 ISSR-16 ISSR-18 ISSR-19	460 198 202 881,678,225 555,222,204 251 1118&523 607 288	14				14
Liza. grana	ISSR-4 ISSR-8 ISSR -11	194 396 809	3	ISSR-8	420	1	4
Total			21			1	22

The Phylogenetic tree grouped the four mullet species into two main clusters. The first cluster was divided into two subclusters. The first subcluster contained *Mugile cephallus (*Alexandria, Ismailia and Port Said), while the second subcluster included *Valamugil seheli* (Ismalia and Damietta).The second cluster was divided into two subclusters. *Valamugil seheli* (Alexandria) was separated from the other remaining (10) genotypes. On the other hand, the second subcluster was divided into two groups. *Liza ramada* (Alexandria) was separated into one group. While, the second group was divided into two subgroups. the first subgroup was divided into two classes,the first one contains *Liza grana* (Ismalia) and *Liza ramada* (Port Said). Meanwhile, the second class contains *Valamugil seheli* (Port Said) and *Liza grana* (Ismalia). The second subgroup was divided into two classes, *Liza ramada* (Ismalia) was separated in one class.While the second class included *Liza grana* (Alexandria), *Mugile cephallus* (Damietta), *Liza ramada* (Damietta) and *liza garana* (Damietta). *Liza ramada* (Damietta) and *liza garana* (Damietta) were clustered together.

Table 7. Genetic similarity matrix computed from ISSR data for the four mullet species

Speies	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1															
2	0.69	1														
3	0.53	0.53	1													
4	0.47	0.55	0.67	1												
5	0.70	0.56	0.56	0.52	1											
6	0.56	0.63	0.61	0.79	0.58	1										
7	0.62	0.56	0.60	0.63	0.63	0.70	1									
8	0.60	0.73	0.61	0.70	0.59	0.76	0.62	1								
9	0.72	0.62	0.52	0.54	0.77	0.62	0.64	0.65	1							
10	0.59	0.63	0.59	0.70	0.60	0.72	0.68	0.77	0.65	1						
11	0.54	0.60	0.61	0.78	0.56	0.77	0.65	0.71	0.58	0.79	1					
12	0.54	0.68	0.54	0.66	0.52	0.71	0.54	0.76	0.60	0.75	0.81	1				
13	0.55	0.57	0.63	0.77	0.61	0.70	0.62	0.68	0.58	0.75	0.80	0.71	1			
14	0.50	0.60	0.66	0.80	0.58	0.77	0.60	0.76	0.57	0.78	0.81	0.76	0.81	1		
15	0.62	0.57	0.60	0.62	0.65	0.62	0.71	0.65	0.63	0.65	0.63	0.59	0.67	0.59	1	
16	0.52	0.63	0.64	0.80	0.63	0.74	0.64	0.79	0.61	0.79	0.78	0.71	0.83	0.84	0.68	1

Speies(1.5.9.13 *Mugile cephlus* (Alexanderia, Ismailia, Port Said, Damietta), 2.6.10.14 *Liza ramada* (Alexanderia,Ismailia, Port Said, Damietta). 3.7.11.15 *valamugil sehili* (Alexanderia, Ismailia, Port Said, Damietta) and 4.8.12.16 Liza garana (Alexanderia, Ismailia, Port Said, Damietta)

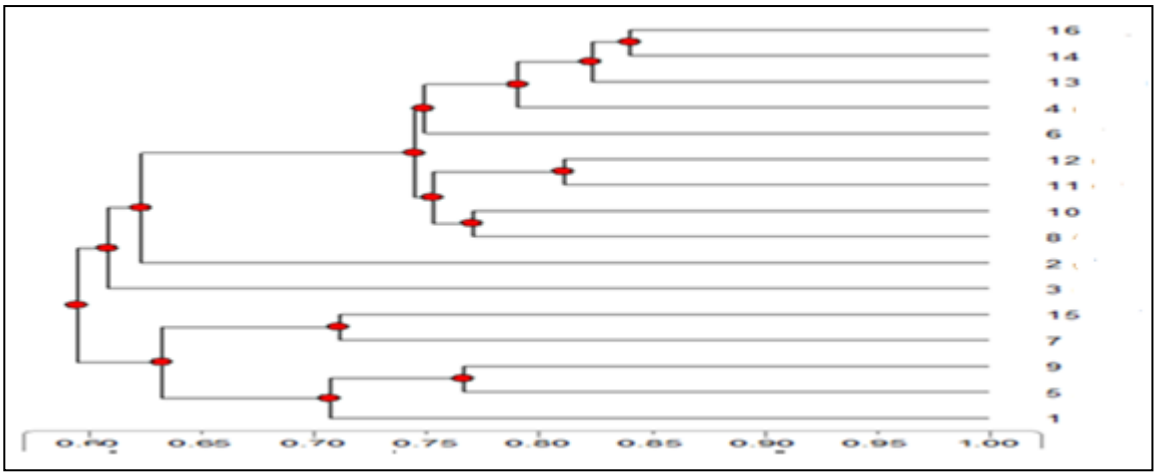

Fig. 3. Dendrogram revealing the genetic relationships among the four mullet species based on the genetic similarity computed from SCoT data using the UPGMA algorithm. 1.5.9.13 *Mugile cephlus* (Alexanderia, Ismailia, Port Said, Damietta), 2.6.10.14 *Liza ramada* (Alexanderia, Ismailia, Port Said, Damietta), 3.7.11.15 *valamugil sehili* (Alexanderia, Ismailia, Port Said, Damietta) and 4.8.12.16 *Liza garana* (Alexanderia, Ismailia, Port Said, Damietta).

(5) Genetic relationships and cluster analysis as revealed by ISSR markers:

Table (8) showed the genetic similarity by the ISSR marker among the four mullet species. The genetic similarity ranged from 0.54 to 0.92. The highest genetic similarity revealed by ISSR analysis (0.92) was among Mugile cephallus (Dimmiate) and Liza.ramada(Dimmiate).While, the lowest (0.54) was among the Mugile

cephallus(Alexandria) and Liza garana (Damietta). The genetic relationships among the four mullet species were estimated by the UPGMA cluster analysis to generate a dendrogram (Fig. 4). The dendrogram revealed that Valamugil sehili (Alexandria) was separated from the other remaining (15) genotypes in one cluster.The other cluster was divided into two main subclusters, the first subcluster was divided into two groups, Liza ramada (Alexandria) was separated from one group, while the other group collecting Mugile cephallus (Alexandria, Ismailia and Port Said). On the other hand, the second subcluster was divided into two groups, the first group contains Valamugil seheli (Ismailia and Damietta),and the second group was divided into two subgroups, one containing Liza.ramada and Liza.grana (Ismailia), and the other subgroup was divided into two classes. Liza grana (Damietta) separated in one class while, the 6 remaining genotypes in the other class, Liza grana and Liza ramada (Port Said) clustered together. Meanwhile, Liza garana (Alexandria), Valamugil seheli (Port Said), Mugile cephallus (Damietta) and Liza ramada (Damietta) were grouped together.

Table 8. Genetic similarity matrix computed from ISSR data for the four mullet species.

species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1															
2	0.69	1														
3	0.55	0.56	1													
4	0.57	0.70	0.68	1												
5	0.72	0.65	0.66	0.67	1											
6	0.55	0.68	0.59	0.78	0.65	1										
7	0.63	0.65	0.55	0.64	0.67	0.75	1									
8	0.55	0.61	0.55	0.71	0.56	0.82	0.68	1								
9	0.76	0.71	0.64	0.75	0.80	0.65	0.72	0.63	1							
10	0.61	0.71	0.60	0.83	0.66	0.81	0.66	0.79	0.76	1						
11	0.58	0.67	0.61	0.83	0.64	0.79	0.65	0.75	0.67	0.80	1					
12	0.62	0.70	0.63	0.78	0.69	0.78	0.69	0.74	0.71	0.83	0.82	1				
13	0.61	0.63	0.68	0.82	0.63	0.76	0.60	0.72	0.69	0.79	0.89	0.81	1			
14	0.59	0.65	0.65	0.83	0.63	0.76	0.62	0.72	0.69	0.80	0.85	0.81	0.92	1		
15	0.57	0.57	0.62	0.68	0.67	0.64	0.67	0.59	0.66	0.63	0.71	0.62	0.69	0.69	1	
16	0.54	0.62	0.61	0.76	0.67	0.76	0.63	0.70	0.69	0.75	0.79	0.80	0.81	0.85	0.69	1

Speies (1.5.9.13 *Mugile cephlus* (Alexanderia, Ismailia, Port Said, Damietta), 2.6.10.14 *Liza ramada* (Alexanderia, Ismailia, Port Said, Damietta) ,3.7.11.15 *valamugil sehili* (Alexanderia, Ismailia, Port Said, Damietta) and 4.8.12.16 Liza garana (Alexanderia, Ismailia, Port Said, Damietta)

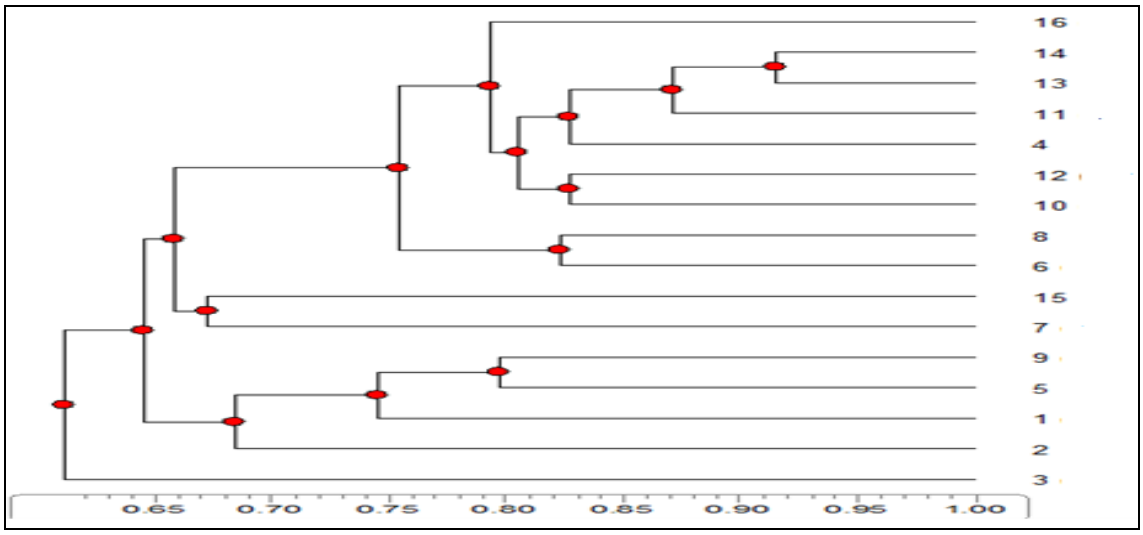

Fig. 4. Dendrogram revealing the genetic relationships among the four mullet species based on the genetic similarity computed from ISSR data using the UPGMA algorithm. 1.5.9.13 *Mugile cephlus* (Alexanderia, Ismailia, Port Said, Damietta), 6.10.14 *Liza ramada* (Alexanderia, Ismailia, Port Said, Damietta), 3.7.11.15 *valamugil sehili* (Alexanderia, Ismailia, Port Said, Damietta) and 4.8.12.16 *Liza garana* (Alexanderia, Ismailia, Port Said, Damietta).

(6) Genetic relationships and cluster analysis as revealed by the combined of SCoT and ISSR markers.

In the present study, eleven SCoT primers, and ten ISSR primers were used to characterize the four mullet species. The genetic similarity by combined SCoT and ISSR markers among four mullet species ranged from 0.52 to 0.86 (Table 9).The highest genetic similarity (0.86) revealed by the combined marker analysis was among Liza ramada(Damietta) and Mugile cephallus (Damietta).While, the lowest similarity (0.52) was among the Liza garana (Alexandria) and Mugile cephallus (Alexandria). The dendrogram was based on the combined data from SCoT and ISSR markers which separated the Valamugil seheli (Alexandria) genotype from all the other remaining genotypes.The cluster containing 16 genotypes was divided into two main subclusters one of these subclusters was divided into two groups. Liza ramada (Alexandria) separated in one group while, the second group containing Mugile cephallus species (Alexandria,, Ismailia and Port Said). The second subcluster was divided into two groups, the first group contains Valamugil seheli (Ismailia and Damietta).While, the second group was divided into two subgroups, the first subgroub containing Liza garana and Liza ramada (Ismailia).The second subgroup was divided into two classes, the first class contains Liza garana and Liza ramada (Port Said), the secondr class included the 5 remaining genotypes, Liza grana (Alexandria) Valamugil seheli (Port Said), Liza grana (Damietta), Mugile cephallus (Damietta) and Liza ramada (Damietta)**.**

Table 9. Genetic similarity matrix computed from combined SCoT and ISSR data for the four mullet species.

species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1															
2	0.69	1														
3	0.54	0.54	1													
4	0.52	0.61	0.67	1												
5	0.71	0.60	0.60	0.58	1											
6	0.56	0.66	0.60	0.79	0.61	1										
7	0.62	0.60	0.58	0.63	0.65	0.72	1									
8	0.58	0.68	0.58	0.70	0.58	0.79	0.65	1								
9	0.73	0.66	0.57	0.62	0.78	0.63	0.68	0.65	1							
10	0.60	0.67	0.59	0.75	0.62	0.76	0.67	0.78	0.69	1						
11	0.56	0.63	0.61	0.80	0.60	0.78	0.65	0.73	0.62	0.80	1					
12	0.58	0.69	0.58	0.71	0.60	0.74	0.61	0.75	0.65	0.78	0.81	1				
13	0.58	0.60	0.65	0.79	0.62	0.73	0.61	0.70	0.63	0.77	0.85	0.76	1			
14	0.54	0.62	0.65	0.81	0.60	0.76	0.61	0.74	0.62	0.79	0.83	0.78	0.86	1		
15	0.60	0.57	0.61	0.65	0.66	0.63	0.69	0.62	0.64	0.64	0.67	0.60	0.68	0.64	1	
16	0.53	0.62	0.63	0.78	0.65	0.75	0.63	0.75	0.64	0.77	0.79	0.75	0.82	0.84	0.68	1

Speies(1.5.9.13 *Mugile cephlus* (Alexanderia,Ismailia, Port Said,Damietta), 2.6.10.14 *Liza ramada* (Alexanderia,Ismailia, Port Said Damietta).,3.7.11.15 *valamugil sehili* (Alexanderia,Ismailia, Port Said,Damietta) and 4.8.12.16 Liza garana (Alexanderia, Ismaili, Port Said, Damietta)

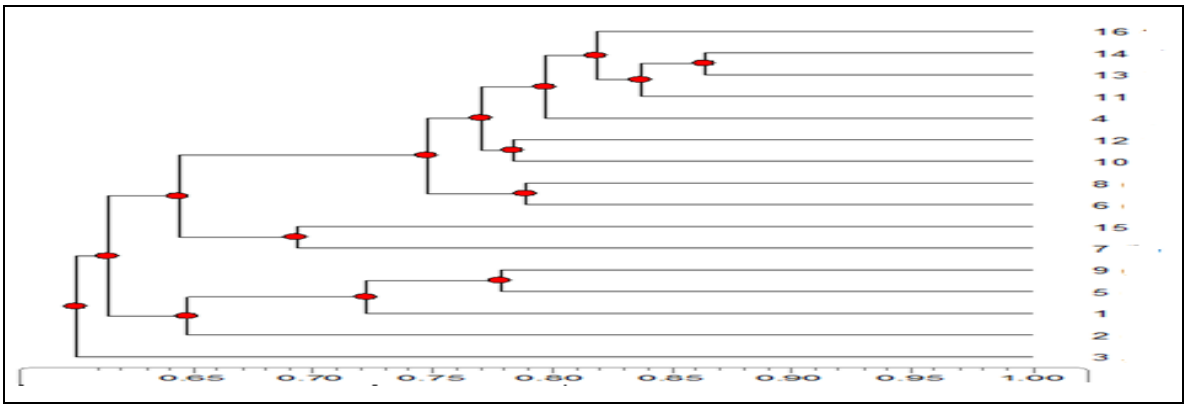

Fig. 5. Dendrogram revealing the genetic relationships among the four mullet species based on the genetic similarity computed from SCoT and ISSR marker data using the UPGMA algorithm. 1.5.9.13 Mugile cephlus (Alexanderia, Ismailia, Port Said, Damietta), 2.6.10.14 Liza ramada (Alexanderia,Ismailia, Port Said, Damietta), 3.7.11.15 valamugil sehili (Alexanderia, Ismailia, Port Said, Damietta) and 4.8.12.16 Liza garana (Alexanderia, Ismailia, Port Said, Damietta).

In this respect, **Ibrahim *et al*, (2018**) used SCoT and ISSR analysis; to constructed the phylogenetic dendrogram to differentiate between the two different sole species (*S. aegyptiaca* and *S. vulgaris*). The phylogenetic tree was divided into two clusters. One cluster includes samples 1 to 10 belongs to S. *aegyptiaca* which has been divided into five sub clusters, where sample 1was clustered in one branch. The other cluster includes samples 11 to 20, which belongs to *S. vulgaris*. This cluster has been divided into four sub-clusters. **Marie and Allam (2017**) carried out cluster analysis to the estimation of the relationship between the red sea fish species. Concluded that the lowest genetic similarity 47 was obtained between *Caranx sexfasciatus* and *Caranx melampygus* also these two species belong to the same genus *Caranx*, while the highest value **65** observed

between *Carangoides bajad* and *Caranx melampygus*. They found that the least genetic distance was produced in *Carangoides Bajad* and *Caranx melampygus.*So genus *Carangoides* is genetically closer and show similarity with the genus *Caranx* which gave the possibility to combine all these species into one genus. **Al-Soudy *et al.* (2018**) reported that the genetic similarity among the four Egyptian camel breeds ranged from 0.70 (between Baladi and Maghrabi) to 0.93 (between Sudani and Somali). Using SCoT and ISSR analysis dataset for clustering algorithm. Four Egyptian camel breeds were clustered into 2 main clusters.Maghrabi was separated from the other breeds in a separate cluster. While, the second cluster was comprised of two groups, Baladi was in one group while, Sudani and Somali formed the second group. **Elizabeth *et al.* (2015)** determined that highly polymorphic between lionfish population resulted by use ISSR markers. The genetic profile of individuals in Mexico was compared with the genetic profile of specimens from Cuba. Nei's genetic distance and the unrooted tree do not show significant differences between both localities.

In the present investigation, the dendrograms revealed some similarities; for example, the grouping of the Mugile cephallus (Alexandria, Ismailia and Port Said) was clustered together in SCoT, ISSR, and combined dendrograms and the grouping Valamugil seheli (Ismailia and Damietta) based on the three dendrograms. Also, Liza ramada, Liza garana (Damietta) and Mugile cephallus (Damietta) were clustered together based on the three dendrograms.

Sequence analysis using Bioinformatics tools.

A total of five DNA fragments were characterized, isolated, purified, DNA sequencing was performed in Macrogen Company, Germany. Then, the sequence analysis was performed by blasting with the available sequences of the National Center for Biotechnology Information (NCBI) (http://www.NCBI.nlm.nih.gov/). The aligment of the nucleotide sequence of the five fragment from SCOT primers (11.5) and ISSR (1,8,15). only one fragment revealed sequence Fig. (6) that fasta format and contain 333 bases. The blasting revealed that this sequence is highly similar to a sequence located on fish species called *Dicentrarchus labrax* (the European bass), which is a primarily ocean-going fish native to the waters off Europe's western and southern and Africa's northern coasts, though it can also be found in shallow coastal waters and river mouths during the summer months. Additionally, the sequence is located on Chr1 near gene called “Type II keratin E3” (**Figure 7and 8**). Keratins I and II form the largest subgroups of mammalian intermediate filament (IF) proteins and account as obligatory heteropolymers for the keratin filaments of epithelia. All human type I genes except for the K18 gene are clustered on chromosome 17q21, while all type II genes form a cluster on chromosome 12q13, that ends with the type I gene K18. Highly related keratin gene clusters are found in rat and mouse. Since fish seem to lack a keratin II cluster we screened the recently established draft genomes of a bird (chicken) and an amphibian (Xenopus). The results show that keratin I and II gene clusters are a feature of all

terrestrial vertebrates. Because hair with its multiple hair keratins and inner root sheath keratins is a mammalian acquisition, the keratin gene clusters of chicken and Xenopus tropicalis have only about half the number of genes found in mammals **(Zimek and Weber, 2005**). Also, **Shibuya *et al*, 2019)** found that the mucus keratins of fugu Takifugu rubripes function as antimicrobial molecules. They suggest that fugu mucus keratin sequesters microbes into insoluble clumps, which are eventually eliminated from the mucus. Here, they present our finding of the novel function of keratin as a defense molecule in fish mucus.

>191002-039_E24_SC_SC.ab1 333

GGCATTACTTGTATGACCCTACTATTTTCCCGCTTTGAGACTTTGATATAACGTTTG
GATTACGTTACGATAGGATTGTGTACCGCTTTCTAATCA
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ATTGTTGAGTTG

Fig. 6. The nucleotide sequences for fragment SCoT -5.

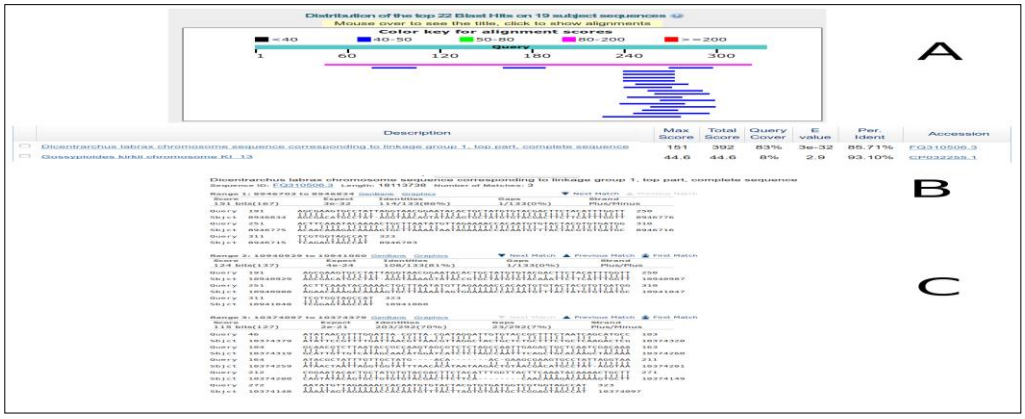

Fig. 7. The alignment of the amplified of the full nucleotides sequence of the fragment and the corresponding sequences in the database using BLAST

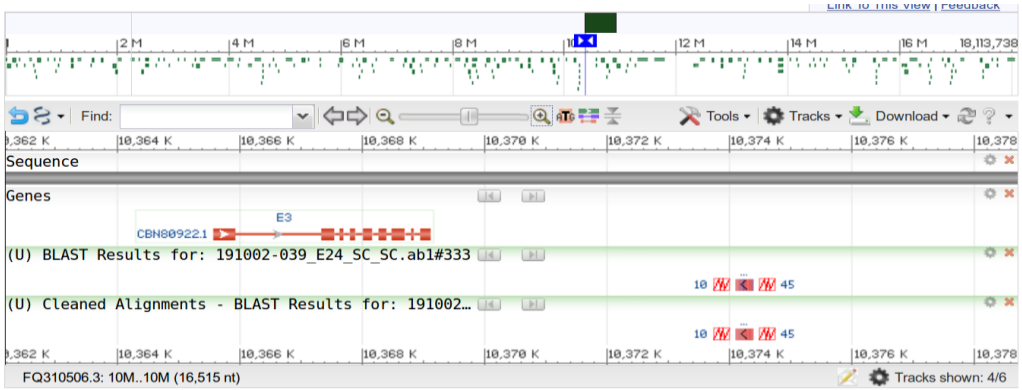

Fig. 8. The fra BLAST results on the NCBI

CONCLUSION

In the present study, we have used two different molecular markers, SCoT and ISSR to estimate the genetic relationships among four mullet species. The results obtained from the two dendrograms of ISSR and SCoT showed the presence of the two species of *Liza ramada* and *Liza granana* in the same group except for the *Liza ramada* (Alexandria). However, further studies are needed to elucidate this relationship at the molecular level.

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