Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 24(7): 619 – 628 (2020)
www.ejabf.journals.ekb.eg

Molecular Discrimination Among Three Fish Species of Family Sparidae Using ISSR and SDS-PAGE Techniques

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ARTICLE INFO
Article History:
Received: Oct. 14, 2020
Accepted: Nov.11, 2020
Online: Nov. 13, 2020

Keywords:
Fish,
ISSR,
Bands,
SDS-PAGE,
Sparidae.

ABSTRACT

Genetic variability and protein analysis of three fishes of family sparidae were studied using Inter-simple sequence repeated (ISSR) markers and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Species of *Sparus aurata, Diplodus sargus and Diplodus cervinus* were collected from the Mediterranean Sea in Port Said in Egypt for the present study. In ISSR analysis, twelve ISSR primers (ISSR1, ISSR2, ISSR5, ISSR6, ISSR7, ISSR8, ISSR9, ISSR10, ISSR13, ISSR14, ISSR15 and ISSR20) were used and DNA segments with different lengths were amplified ranging from 180bp with primer ISSR6 to 1000bp with primers ISSR9 and ISSR13. Polymorphism percent was the highest with ISSR-5 (83%) and the lowest with ISSR-20 (20%). Genetic similarity was the highest between *Sparus aurata* and *Diplodus cervinus* (73%) and the lowest was between *Sparus aurata* and *Diplodus sargus* (67%). SDS-PAGE analysis produced bands ranging from 19 to 200KD and a total of twenty four bands. Twenty three monomorphic bands and one polymorphic band were obtained.

INTRODUCTION

Microsatellites consist of nucleotide sequence (di, tri, or tetra-nucleotides) that are repeated beside each other at different distance on DNA molecule **(Rahman *et al*., 2000; Hayden and Sharp, 2001)**; conserved sequences flanking these nucleotides and are about 100-300bp are called ISSRs that can be amplified using PCR technique exploiting microsatellite sequences as primers **(Chambers and Mac Avoy, 2000)**. They are found in coding and noncoding sequences. Microsatellites can distinguish species that are closely related or populations that are close geographically. Microsatellites are professional genotyping molecular markers because of high allelic variation, inheritance has a co-dominant way and all living organisms have genome. They are also used for population genetics, management/conservation of genetic resources and DNA fingerprinting **(Cordeiro *et al*., 2003)**.

ISSR has a great advantage; recognition of genome sequences is not required where most plant and animal species can be studied by the same primers. A wide range of applications can be provided with genomic information through this technique; also, this

method finds abundance of polymorphisms in many systems **(Wink, 2006)**. Furthermore, few experimental steps are demanded for this method and costs are relatively law **(Huang and Sun, 2000; Le Roux and Wieczorek, 2009)**.

Based on studies that are related to ISSR markers (inter simple sequence repeat), it is obvious that natural population can be professional studied using ISSR technique. The amplified DNA segments involve the nucleotide sequence located between two microsatellites blocks, giving a multi-locus marker system that is useful for analysis of genetic diversity **(Maltagliati *et al*., 2006)**; The amplified product can be detected by agarose gel electrophoresis **(Leroy *et al*., 2000)**. Electrophoresis is a technique used for biochemical systematics analysis in various taxa. Isoelectric focusing and high-resolution starch or polyacrylamides are means used in identification of each species for proteins that are species- specific. Protein electrophoresis is a method used for determination of phylogenetic relationships and studding of genetic structure of species **(Pineiro *et al*., 2001)**.

The present study aims to measure the genetic relationship between three fish species of family Sparidae (*Sparus aurata*, *Diplodus sargus* and *Diplodus cervinus*); ISSR technique is used as a tool for measuring this relationship. This study also aims to analyze tissue proteins of the three fish species by SDS-PAGE.

MATERIALS AND METHODS

Samples collection

Three species of family Sparidae (*Sparus aurata*, *Diplodus sargus* and *Diplodus cervinus*) were collected from Mediterranean Sea, Port Said, Egypt. Appropriate size of muscle tissue was extracted from the three fish species and was frozen at -20˚C.

DNA Extraction and ISSR-Technique

Twelve primers were used in the study (ISSR1, ISSR2, ISSR5, ISSR6, ISSR7, ISSR8, ISSR9, ISSR10, ISSR13, ISSR14, ISSR15 and ISSR20) as illustrated in Table (1). DNeasy Mini Kit (Qiagen) was used in DNA extraction from fish samples. The concentration of genomic DNA which estimated by NanoDrop. Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) was used for performing PCR amplification. It was programmed to fulfill 40 cycles after an initial denaturation cycle that lasted for 5 min at 94ºC; each cycle programed to three steps, a denaturation step for 1 min at 94ºC, an annealing step for 1 min at 50ºC, and an elongation step for 1.5 min at 72ºC.

Detection of the PCR Products:

Agarose gel (1.5%) with ethidium bromide was used for running the amplified products and the buffer used was 1X TBE buffer; the run was at 95volts. UV light was used for visualizing PCR products and a Gel Documentation System (BIO-RAD 2000) was used for photographing.

Data Analysis

Gene profiler computer software program was used for analyzing the banding patterns of the DNA fragments, products of the amplification were scored as ‘1’ for presence and ‘0’ for absence of the bands. The cluster analysis was made through the similarity matrix. The cluster analysis was used for organizing the resulted data into meaningful structures developing the taxonomies. The distances between accessions are showed by Dice coefficient through PAST program after each accession represented its own cluster.

SDS-PAGE analysis

Muscular proteins were separated based on their molecular weight by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins of muscle tissue were extracted according to **Fadda *et al*., (1999).**

One gram of muscular tissue was homogenized with 9 ml PBS (phosphate buffer solution), and then the sample was centrifuged for 15 min at 10000 rpm; 4ºC. The supernatant containing proteins was transferred to a clean Eppendorf. Treatment buffer (1% SDS, 10% glycerol, 10 mM Tris-HCL PH 6.8, 1mM EDTA, DTT (dithiolthreitor) and pinch of bromophenole blue) was added to the protein sample and boiled for 5 min at 90ºC. Protein solution and protein marker were loaded into polyacrylamide gel and the run was carried out at 100 volts in 1x Tris/glycine-SDS running buffer. After electrophoresis, staining of the gel was occurred using 50 ml of staining solution (10% acetic acid, 0.125% coomassie blue R-250 and 50% methanol), and then gel was dried and photographed. SDS-PAGE was performed according to (**Laemmli, 1970).** Molecular weight of protein patterns was stated according to **Weber *et al*., (1972).**

Table 1. The sequence of ISSR primers, A: Adenine, T: Thymine, G: Guanine, C: Cytosine, Y: (C or T), R (A or G), H: (A or C or T)

Primer Name	Sequence
ISSR-1	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR-2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR -5	5'-GTGTGTGTGTGTGTGTYG-3'
ISSR -6	5'-CGCGATAGATAGATAGAT-3'
ISSR -7	5'-GACGATAGATAGATAGATA-3'
ISSR -8	5'-AGACAGACAGACAGACGC-3'
ISSR -9	5'-GATAGATAGATAGATAGC-3'
ISSR -10	5'-GACAGACAGACAGACAAT-3'
ISSR -13	5'-AGAGAGAGAGAGAGAGYT-3'
ISSR -14	5'-CTCCTCCTCCTCCTCTT-3'
ISSR -15	5'-CTCTCTCTCTCTCTCTRG-3'
ISSR -20	5'-HVHTGTGTGTGTGTGTGT-3'

RESULTS

Inter-simple sequence repeated (ISSR) analysis

In this study, *Sparus auratus, Diplodus sargus and Diplodus cervinus* of family Sparidae were collected from Mediterranean Sea, Port Said, Egypt for studding the genetic variability among them using twelve ISSR primers. All primers generated strong amplification profiles with distinct bands that revealed DNA polymorphism among the three species under study as shown in (Figures 1, 2, 3 and 4). The twelve ISSR primers detected a total of 131 DNA fragments (Table 2), with an average of 11 fragments per primer. The total number of amplified fragments varied from 7 (ISSR10) to 14 (ISSR13) primers. Of the 131 amplified bands, 51 were monomorphic bands, 37 polymorphic and 43 unique bands with polymorphism ranged from 20% to 83%.

Following are the amplification results of three fish species obtained by the examined primers:

Sparus aurata

ISSR with this species produced different band patterns of 80 bands ranged in size from 180 bp in the primer (ISSR6) to 900 bp in (ISSR13). The generated bands ranged in number from 4 in (ISSR1) to 10 in (ISSR13 and ISSR20).

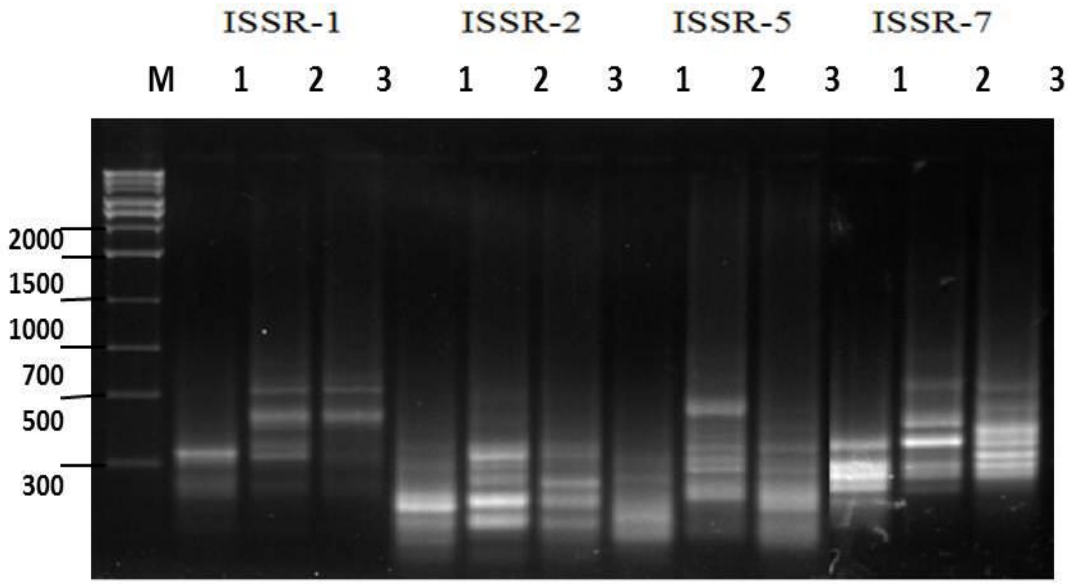

Fig. 1. ISSR profile of three fish species using ISSR primers: (ISSR1, ISSR2, ISSR5 and ISSR7). M refers to DNA marker (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*).

Table 2. Percentage of polymorphism, molecular weight and number of total bands, monomorphic bands, polymorphic and unique bands generated by twelve ISSR primers with three fish species (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*).

Primer code	No. of amplified bands			Total amplified bands	Size of amplified bands	NO. of monomorphic bands	No. of polymorphic bands	No. of unique bands	Polymorphic and unique bands	Polymorphism%
	1	2	3							
ISSR1	4	8	8	10	250-550bp	2	6	2	8	80%
ISSR2	6	9	7	10	200-550bp	4	4	2	6	60%
ISSR5	5	10	6	12	210-800bp	2	5	5	10	83%
ISSR6	8	11	7	13	180-700bp	6	1	6	7	54%
ISSR7	5	6	8	10	250-700bp	4	1	5	6	60%
ISSR8	7	7	6	9	230-500bp	4	3	2	5	55%
ISSR9	5	6	11	12	240-1000bp	4	2	6	8	67%
ISSR10	5	5	6	7	220-500bp	4	1	2	3	43%
ISSR13	10	8	12	14	260-1000bp	6	4	4	8	57%
ISSR14	9	8	8	13	240-800bp	3	6	4	10	77%
ISSR15	6	8	7	11	210-700bp	4	2	5	7	64%
ISSR20	10	9	9	10	200-550bp	8	2	0	2	20%
Total	80	95	95	131	180-100bp	51	37	43	80	61%

Diplodus sargus

The number of different ISSR band patterns in this species was 95 bands ranged in size from 180 bp in the primer (ISSR6) to 800bp in (ISSR5). The generated bands ranged in number from 5 in (ISSR10) to 11 in (ISSR6).

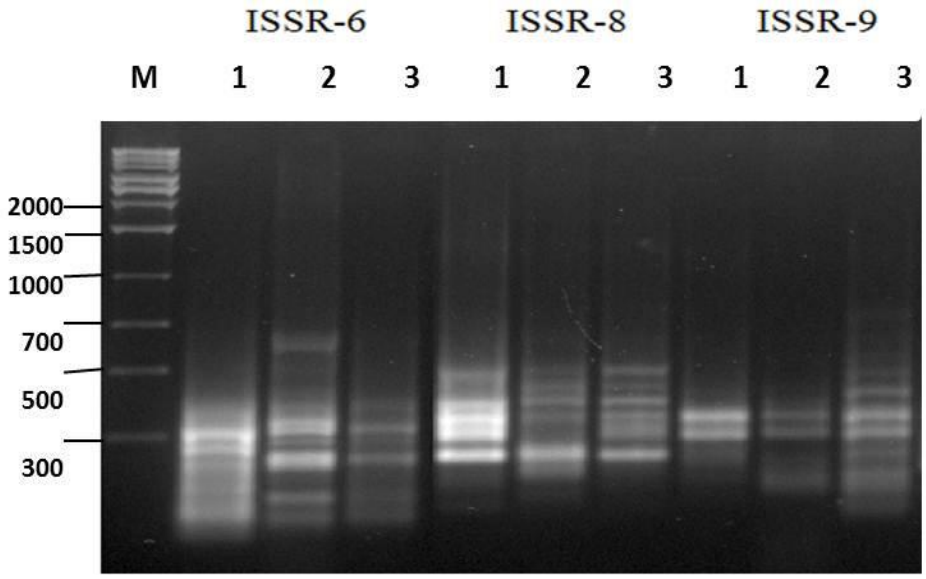

Fig. 2. ISSR profile of three fish species using ISSR primers: (ISSR6, ISSR8 and ISSR9). M refers to DNA marker (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*).

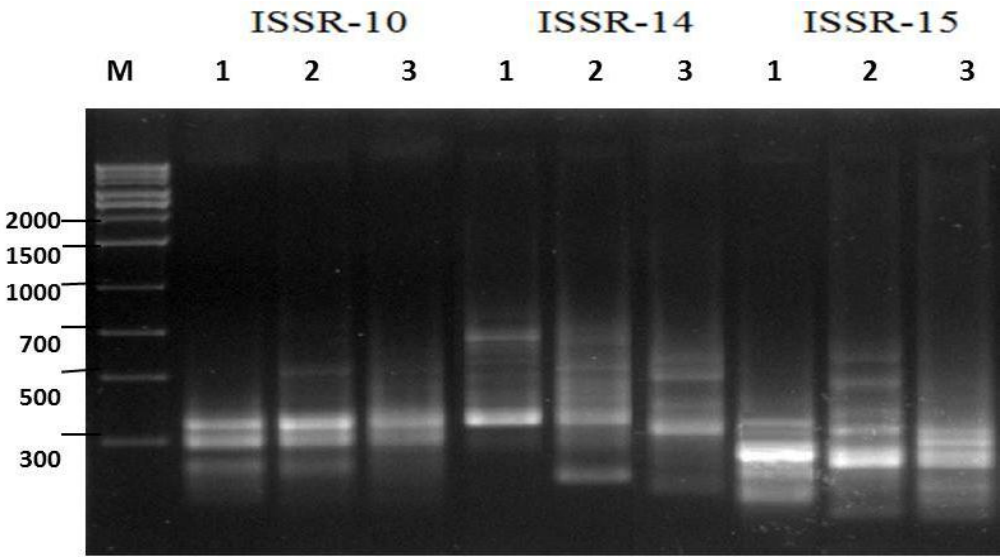

Fig. 3. ISSR profile of three fish species using ISSR primers: (ISSR10, ISSR14 and ISSR15). M refers to DNA marker (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*).

Diplodus cervinus

This sample produced different ISSR band patterns number of 95 bands ranged in size from 180 bp in the primer (ISSR6) to 1000 bp in (ISSR13 and ISSR9). The generated bands ranged in number from 6 in (ISSR5, ISSR8 and ISSR10) to 12 in (ISSR13).

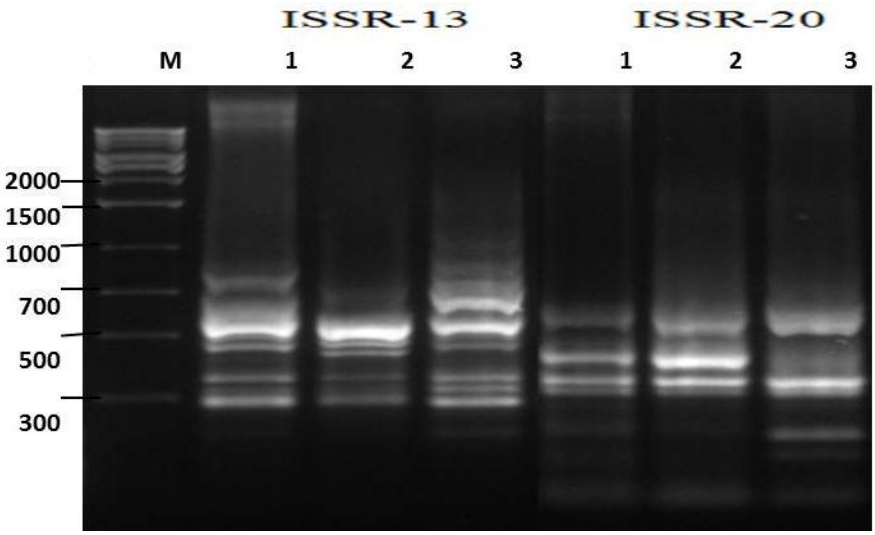

Fig. 4. ISSR profile of three fish species using ISSR primers: (ISSR13 and ISSR20). M refers to DNA marker (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*).

Genetic similarity was the highest between *Sparus aurata* and *Diplodus cervinus* (73%) and the lowest was between *Sparua aurata* and *Diplodus sargus* (67%) as shown in (Table 3).

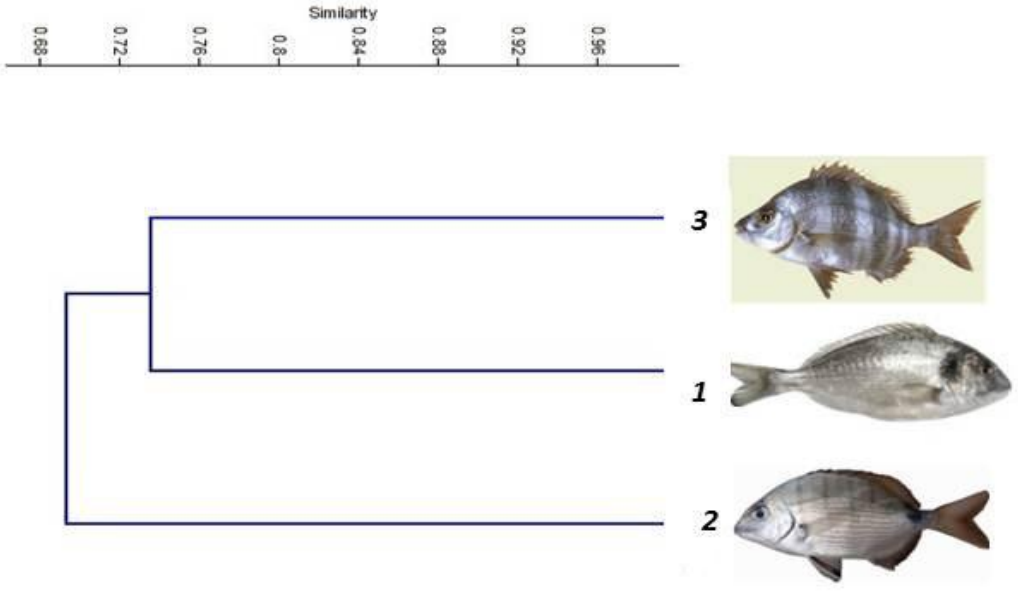

Fig. 5. Dendrogram showing Cluster analysis for three fish species (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*). based on ISSR molecular markers.

Table 3. Averages of genetic similarities (%) estimated by molecular ISSR primers, adopting the arithmetic complement of Jaccard coefficient for three fish species of Sparidae. (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*).

	1	2	3
1	**100**		
2	**67**	**100**	
3	**73**	**72**	**100**

SDS-PAGE analysis

The generated bands were with molecular weight ranging from 19 to 200 KD and a total of 24 bands were produced for three fish species by SDS-PAGE. 23 monomorphic bands were resulted and only one polymorphic band with polymorphism 4.16%. *Sparus aurata* and *Diplodus sargus* had 24 bands while *Diplodus cervinus* had 23 bands as illustrated in figure (6) and Table (4).

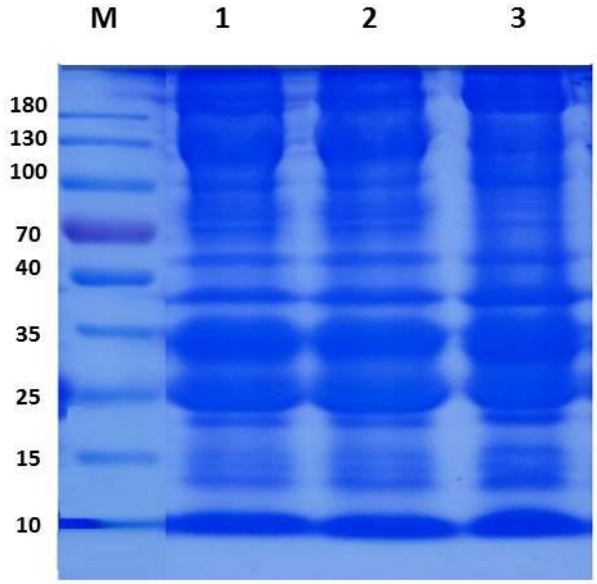

Fig. 6. Protein banding patterns of SDS-PAGE (1-*Sparus aurata*, 2-*Diplodus sargus* and 3-*Diplodus cervinus*). M refers to protein marker.

Table 4. Bands of SDS-PAGE protein of three fish species of Sparidae; (1-*Sparus aurata,* 2-*Diplodus sargus* and 3-*Diplodus cervinus*). MW refers to molecular

MW	1	2	3
200	**1**	**1**	**1**
178	**1**	**1**	**1**
169	**1**	**1**	**1**
137	**1**	**1**	**1**
127	**1**	**1**	**1**
124	**1**	**1**	**1**

109	**1**	**1**	**1**
99	**1**	**1**	**0**
92	**1**	**1**	**1**
85	**1**	**1**	**1**
79	**1**	**1**	**1**
71	**1**	**1**	**1**
59	**1**	**1**	**1**
46	**1**	**1**	**1**
42	**1**	**1**	**1**
36	**1**	**1**	**1**
33	**1**	**1**	**1**
32	**1**	**1**	**1**
30	**1**	**1**	**1**
27	**1**	**1**	**1**
26	**1**	**1**	**1**
24	**1**	**1**	**1**
22	**1**	**1**	**1**
19	**1**	**1**	**1**

DISCUSSION

The population structure is done using molecular techniques by analyzing colonization patterns, dispersal and gene flow among populations over a different of geographical scales. Deductions made from datasets can be impacted by the use of variable molecular techniques that based on nuclear DNA such as ISSR technique; or biochemical analysis such as Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) **(Duran *et al*., 2004)**.

Inter-simple sequence repeat (ISSR) is a DNA-based molecular technique that depends on the amplification of the nucleotide sequence found between two microsatellite blocks; this technique uses a single primer that contains the microsatellite repetitive sequence that the desired amplicon is flanked in between. ISSR technique is benefit for analysis of genetic diversity (**Maltagliati *et al*., 2006; Abu-Almaaty *et al*., 2020a)**.

Also, **Abu-Almaaty *et al*., (2017a)** investigated the molecular genetic taxonomic relationship among three species of Osphronemidae fishes using ISSR markers for first time in Egypt illustrating that this technique has high efficiency in generating polymorphism among the closely related varieties. DNA bands and Muscular protein profile of three species of family Sparidae were investigated in this study using ISSR markers and SDS-PAGE, respectively. Polymorphism percent were the highest with ISSR5 (83%) and the lowest with ISSR20 (20%). Genetic similarity was the highest between *Sparus aurata* and *Diplodus cervinus* (73%) and the lowest was between *Sparua aurata* and *Diplodus sargus* (67%).

This study is in contrast with **Ibrahim *et al*., (2020)** who used different ISSR primers in their study mentioning that the highest genetic similarity was between *Diplodus cervinus* and *Diplodus sargus.* So, the author recommends performing more DNA-based molecular analysis using more primers. Also, Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) is a molecular biomarker in which proteins are separated according to their molecular weight; it is an effective technique

illustrating the metabolic level of species under the study **(Muhammad *et al*., 2018)** and it can be used for differentiation between different fish species **(Abu-Almaaty *et al*., 2017b; 2020b)**.

In the present study this technique showed a similarity between *Sparus auratus* and *Diplodus sargus* in having the same number with the same molecular weight of protein bands that differed slightly from *Diplodus cervinus*.

CONCLUSION

In this study, ISSR analysis showed that *Sparus auratus* and *Diplodus cervinus* are very close to each other, *Sparus auratus* and *Diplodus sargus* are less related to each other. In SDS-PAGE analysis, *Sparus auratus* and *Diplodus sargus* were the closest in protein bands. In conclusion, these fish species are easily distinguished by ISSR and SDS-PAGE, but more studies using more ISSR primers and SDS-PAGE analysis should be performed.

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