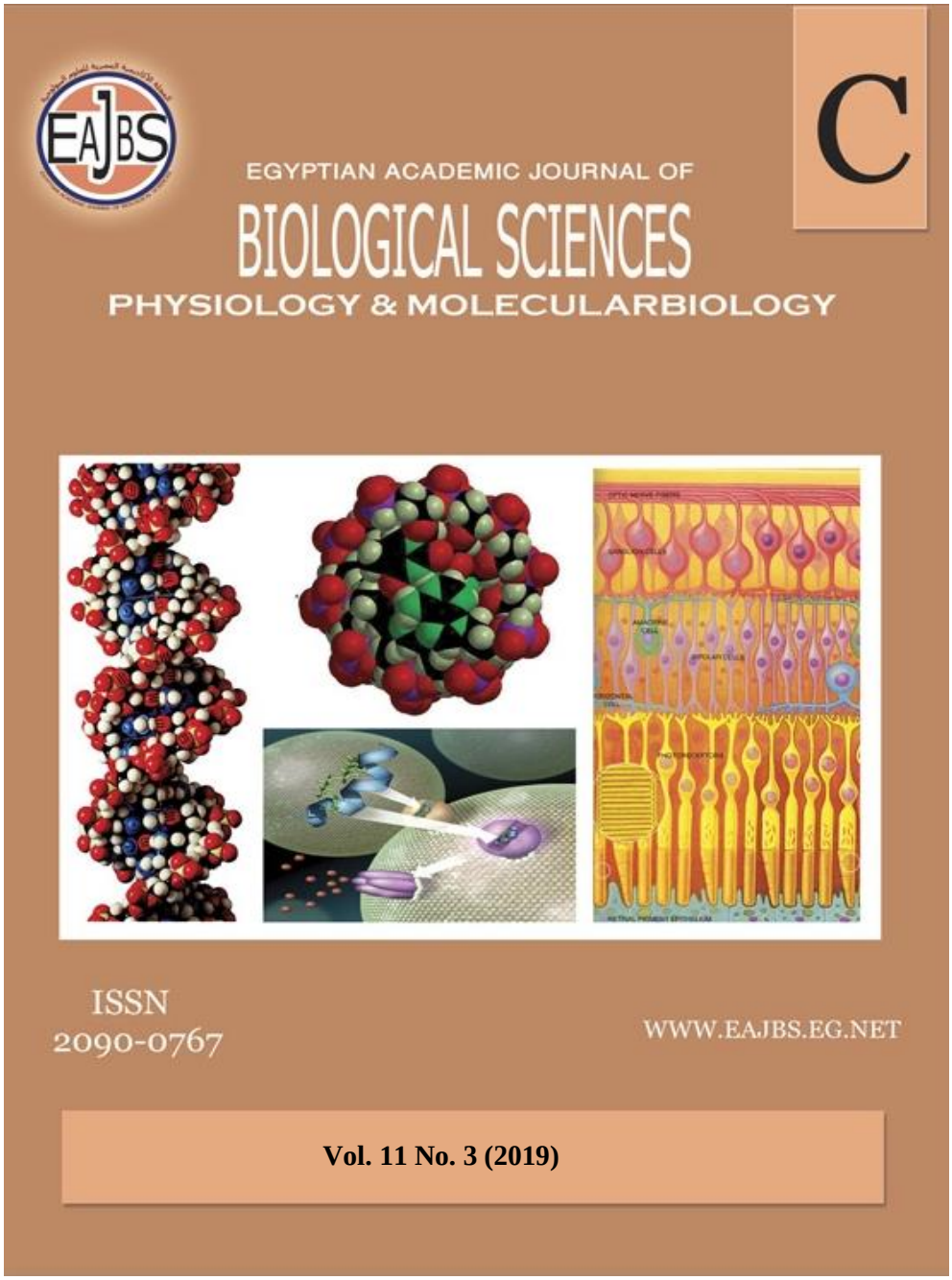

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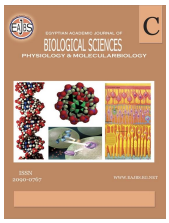

Usage of Inter Simple Sequence Repeat (ISSR) Marker in Assessing the Genetic Variation of Six Parrotfish Species from the Egyptian Red Sea

Zeinab A. Mar'ie [1,2] and Mohammad Allam [1]

1- Zoology Department, Faculty of Science, South Valley University, Egypt
2- Science Departments, Faculty of Education, Hurghada , South Valley University, Egypt.
E.Mail: **Mohammad_allam10@sci.svu.edu.eg**

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ABSTRACT

The report compared six parrotfish species (*Scarus ferrugineus, Scarus psittacus, Scarus frenatus, Chlorurus sordidus, scarus genazotus and Cetoscarus bicolor*) for molecular diversity and phylogenetic using eleven ISSR primers, the obtained results showed a total of 235 DNA fragments; 11 were common bands, 65 were specific bands and 159 were polymorphic bands. Additionally, the results indicated the largest similarity degree 58 between (*Chlorurus sordidus* and *scarus genazotus*), while the lowest similarity degree 35 was found between (*Scarus frenatus* and *Chlorurus sordidus*). The results that occurred from ISSR analysis found to be more reliable for differentiating species of fishes particularly parrotfish.

INTRODUCTION

Parrotfish are among the most colorful and numerous dominant inhabitants of coral reefs and seagrass beds (Sale, 1991). Individuals of parrotfish have beak-like oral jaws of fused teeth used to take off algae and detritus from the substratum. As such, scarids have been famed as important agents of marine bioerosion (Bellwood and Choat, 1990 and Bellwood, 1995 a&b) and have essential roles in the determination of benthic community structure (Lewis and Wainwright, 1985). Parrotfish are a diversified group of marine fishes, including about 90 species in 10 genera and more than half of all species are in a single genus *Scarus*, they are nested within the family Labridae (Streelman *et al*., 2002 and Westneat and Alfaro, 2005).

Assessment of the genetic variety using DNA polymorphisms of an organism, as well as of the popularization of molecular techniques, has to support progress in population genetics studies (Antunes *et al*., 2010 and Moresco *et al*., 2013). Genetic markers are significant tools for evaluating fish populations (Rashed *et al*., 2008) and fish species genetic diversity (Saad *et al*., 2009). The identification of parrotfish by using the genetic studies and evolution is not sufficient, so it is important to use the genetic variability in parrotfish species as economic genetic resources using a highly polymorphic and easy molecular technique such as Inter Simple Sequence Repeats ISSR (Saad *et al*., 2013).

Zietkiewicz *et al*. (1994) stated that the ISSR strategy involves amplification of DNA segments between two identical microsatellite repeat regions oriented in the opposite direction using primers designed from microsatellite core regions. This method uses primers, usually, 16-25 bp. long of di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide repeats to target multiple genomic loci.

The Inter Simple Sequence Repeat technique is favorite because it does not need to carry out costly cloning and sequencing inherent in the original microsatellite-based approach (Nagaraju *et al*., 2002). ISSR methods have possibility as dominant markers for studying genetic diversity of several fishes (Tong *et al*., 2005). Inter Simple Sequence Repeats marker detects many polymorphisms in numerous systems and provides genomic information for a broad range of applications (Maltagliati *et al*., 2006 and Wink, 2006). ISSR method has been used due to it produces excellent results additional to it has very low costs compared to other markers (Moresco *et al*., 2013).

The Inter Simple Sequence Repeats analysis has been successfully used for studying the genetic diversity among vertebrate animals; in Amphibian (Moresco *et al*., 2013) reported that the ISSR considered being an efficient and valuable molecular marker for the genetic diversity in amphibian populations. The study of (El-Sabrout and Aggag, 2015) showed the usefulness of the ISSR-PCR method to understand the genetic variability in some productive traits in rabbits. Particular ISSR technique was used in several studies in fishes like some cyprinodontiform fish (Maltagliati *et al*., 2006), *Diplodus spp*. and *Dentex dentex* (Casu *et al*., 2009), some Tilapia species (Saad *et al*., 2012). In the same context (Zhigileva *et al*., 2013) compare the population differentiation of three species of cyprinids using ISSR-PCR-markers. Also (Vitorino *et al*., 2015) estimated the genetic diversity of *Arapaima gigas* by mean of the ISSR marker. Similarly (de Queiroz *et al*., 2016) tested specimens from three tambaqui fry production. Additionally (Oliveira *et al*., 2019) estimate the genetic variation in natural populations of *Colossoma macropomum* based on ISSR markers.

The variable lengths of these amplified DNA sequences allow for the identification of differences between closely related species, so the present investigation was carried out to study the genetic diversity and phylogenetic relation among six parrotfish species from the Egyptian Red Sea and assessing the usefulness of ISSR technique to genetically identify of parrotfish species.

MATERIALS AND METHODS

a) Samples Collection and DNA Extraction*:*

Six Parrotfish species of Family Scaridae, Order Perciformes (*Scarus ferrugineus*, *Scarus psittacus*, *Scarus frenatus*, *Chlorurus sordidus*, *scarus genazotus* and *Cetoscarus bicolor*) were collected from the Red Sea, Hurghada, Egypt and then were identified morphologically according to (Randall, 1982) and the FishBase website. The muscle tissues were isolated and preserved at -80ºC until DNA extraction. DNA was extracted from the preserved muscle tissues using the DNA extraction method of QIAamp DNA Mini kit (Qiagen, Hidden, Germany) by following the manufacturer's guidelines.

b) PCR Amplification and ISSR Primers:

A set of 11 primers ISSR (Tab. 1) was used in the detection of polymorphism. The reaction of amplification 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 30 ng template DNA. PCR amplification was performed to fulfill 35 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle involved the following steps; a denaturation step at 94ºC for 1 min, an annealing step at 45ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

Amplification products were resolved by electrophoresis in a 1.5% agarose gel with ethidium bromide

(0.5ug/ml) and visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000). The banding patterns generated by ISSR-PCR marker analyses were compared to determine the genetic relatedness of the samples under study. Present bands were scored as '1' and absent bands were scored as '0'. The genetic similarity coefficient was estimated according to the Dice coefficient (Sneath and Sokal, 1973).

Tab. 1 Names and sequences of 11 ISSR primers used in this study.

Primer Name	Sequence
ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'
ISSR- 3	5'-ACACACACACACACACYT-3'
ISSR- 4	5'-ACACACACACACACACYG-3'
ISSR- 5	5'-GTGTGTGTGTGTGTGTYG-3'
ISSR- 6	5'-CGCGATAGATAGATAGATA-3'
ISSR- 7	5'-GACGATAGATAGATAGATA-3'
ISSR- 8	5'-AGACAGACAGACAGACGC-3'
ISSR- 9	5'-GATAGATAGATAGATAGC-3'
ISSR- 10	5'-GACAGACAGACAGACAAT-3'
ISSR- 11	5'-ACACACACACACACACYA-3'

RESULTS AND DISCUSSION

All the ISSR primers were amplified successfully with the genomic DNA of the samples (*Scarus ferrugineus, Scarus psittacus, Scarus frenatus, Chlorurus sordidus, scarus genazotus* and *Cetoscarus bicolor*) and generated 235 DNA fragments (Fig. 1). The total numbers of amplified bands addition to common, polymorphic and unique bands in each species were shown in (Tab. 2).

Although there were several amplified fragments shared among these six species, most of the fragments were polymorphic. The results revealed about 5% monomorphism and 95% polymorphism among the samples. The high polymorphism percentage 95% was concordant with (Vitorino *et al*., 2015) who found 165 of 168 loci (98.21%) were polymorphic in their study on *Arapaima gigas*, also similar results were observed by (de Queiroz *et al*., 2016) who noticed about 146 out of 152 (96%) loci were polymorphic when applied ISSR marker on tambaqui fry.

Diagnostic bands (Luque *et al*., 2002) are significant for the differentiation among species, particularly fish species (Maltagliati *et al*., 2006 and Casu *et al*., 2009). The identification of species by using the ISSR marker always reveals a high number of diagnostic bands (Maltagliati *et al*., 2006 and *Casu et al*., 2009). These were agreeing with our results that revealed 65 (28%) specific bands.

The generated bands ranged in size approximately from 130 bp. by the primer (10) to 2500 bp. by the primer (9) in *Scarus ferrugineus*, from 130 bp. by the primer (10) to 2000 bp. by the primer (3) in *Scarus psittacus*, 140 bp. by the primer (5) to 2000 bp. by the primer (3) in *Scarus frenatus*, 170 bp. by the primer (3) to 1800 bp. by the primer (1) in *Chlorurus sordidus*, 140 bp. by the primer (5) to 3000 bp. by the primer (9) in *Scarus genazotus*, 130 bp. by the primer (10) to 3000 bp. by the primer (9) in *Cetoscarus bicolor*.

The calculation of band frequency in each fish species reflects the homogeneity and heterogeneity levels (Rashed *et al*., 2008). The averages of band frequencies in (*Scarus ferrugineus, Scarus psittacus, Scarus frenatus, Chlorurus sordidus, scarus genazotus* and *Cetoscarus bicolor*) were 0.49, 0.34, 0.37, 0.38, 0.49 and 0.46 respectively.

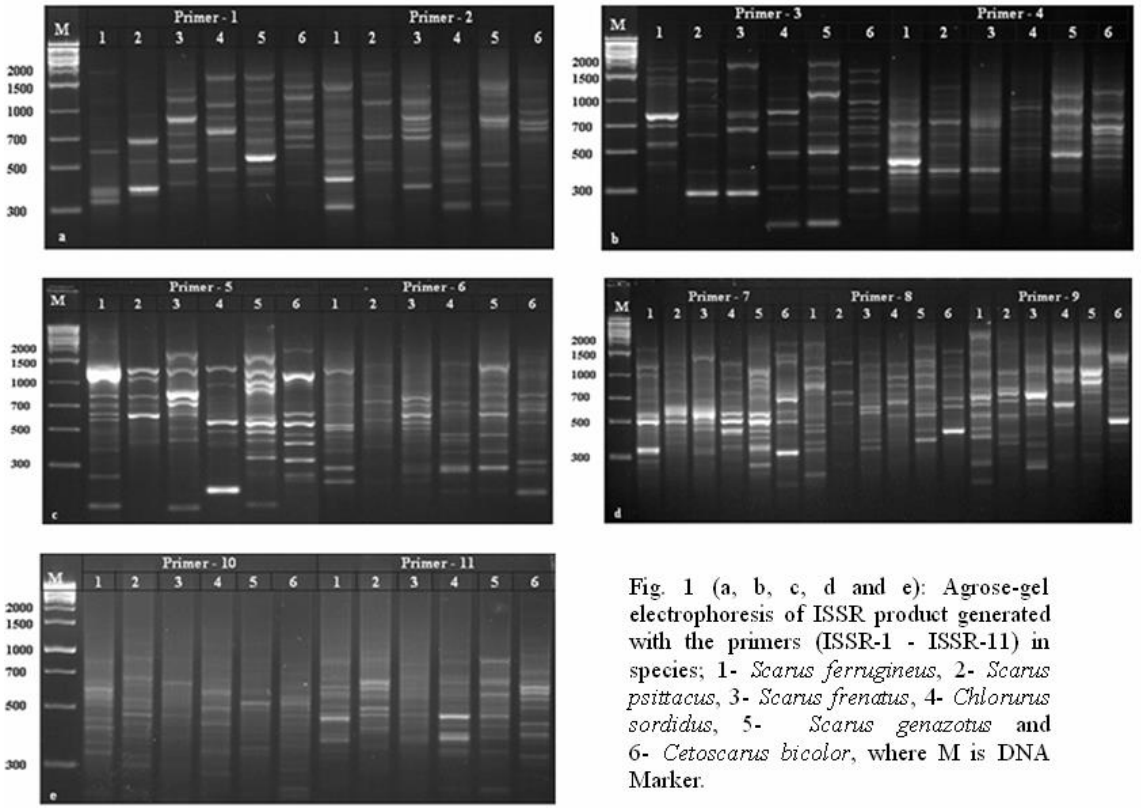

Fig. 1 (a, b, c, d and e): Agrose-gel electrophoresis of ISSR product generated with the primers (ISSR-1 - ISSR-11) in species; 1- *Scarus ferrugineus*, 2- *Scarus psittacus*, 3- *Scarus frenatus*, 4- *Chlorurus sordidus*, 5- *Scarus genazotus* and 6- *Cetoscarus bicolor*, where M is DNA Marker.

Tab. 2 Number of amplified, Common, unique and polymorphic DNA fragments using 11 ISSR primers in six Parrot fishes species; 1- *Scarus ferrugineus*, 2-*Scarus psittacus*, 3- *Scarus frenatus*, 4- *Chlorurus sordidus*, 5- *scarus genazotus* and 6- *Cetoscarus bicolor*.

No.	Primer code	No. of total bands	No. of amplified bands						No. of common bands	No. of specific bands	No. of polymorphic bands	Polymorphism%
			1	2	3	4	5	6				
1	ISSR- 1	22	8	5	7	7	10	10	0	10	12	100%
2	ISSR- 2	20	13	7	8	12	10	7	1	3	16	95%
3	ISSR- 3	23	9	7	7	7	12	10	0	5	18	100%
4	ISSR- 4	20	11	9	8	6	9	10	1	4	15	95%
5	ISSR- 5	23	10	6	9	6	13	11	1	8	14	96%
6	ISSR- 6	20	10	7	10	10	10	12	0	2	18	100%
7	ISSR- 7	22	9	6	8	8	11	9	2	8	12	91%
8	ISSR- 8	22	14	5	9	8	12	8	1	6	15	95%
9	ISSR- 9	24	12	9	11	9	10	12	2	7	15	92%
10	ISSR- 10	19	10	10	3	7	8	9	0	4	15	100%
11	ISSR- 11	20	10	9	6	10	10	11	3	8	9	85%
Sum.		235	116	80	86	90	115	109	11	65	159	95%

The genetic similarity among the six species was calculated based on data of the presence/absence of DNA fragments of these species (Tab. 3). The lowest genetic similarity (35) found between *Scarus frenatus* and *Chlorurus sordidus*, while the highest value (58) was observed between *Chlorurus sordidus* and *scarus genazotus*.

The average of genetic similarity among *Chlorurus sordidus* and species of genus *scarus* was 44.5%, but the average of genetic similarity among *Cetoscarus bicolor* and species of genus *scarus* was 43% which reflect the close genetic relationship among

Chlorurus sordidus and genus *scarus*, these results were consistent with (Saad *et al*., 2013) that indicated smallest genetic distance among *Chlorurus sordidus* and some species of genus *scarus* than the genetic distance among *Cetoscarus bicolor* and the same species of genus *scarus*.

Dendrogram of the understudy species (Fig. 2) revealed 4 main features: (1) *Scarus ferrugineus* and *Scarus frenatus* found in sister clade, (2) *Chlorurus sordidus* and *scarus genazotus* also found in sister clade, (3) *Scarus psittacus* formed a separate cluster near the clad of *Scarus ferrugineus* and *Scarus frenatus* and (4) *Cetoscarus bicolor* also formed a separate cluster.

The potential applications of the ISSR marker for diverse studies depend on the variety and frequencies of microsatellites within the specific genomes (Ye *et al*., 2005 and Hassan *et al*., 2014). Zhigileva *et al*. (2013) reported that the ISSR-PCR method can detect a high level of genetic variation using a smaller sample, which is especially important for rare and small species.

The obtained results illustrated that, Inter Simple Sequence Repeats is a beneficial and reliable tool for estimating the genetic variation and degree of similarity among fishes as were shown in several studies likes (Maltagliati *et al*., 2006; Casu *et al*., 2009; Saad *et al*., 2013; Vitorino *et al*., 2015; de Queiroz *et al*., 2016 and Oliveira *et al*., 2019).

Tab. 3 Genetic similarity among six Parrot fishes species; 1- *Scarus ferrugineus*, 2- *Scarus psittacus*, 3- *Scarus frenatus*, 4- *Chlorurus sordidus*, 5- *scarus genazotus* and 6- *Cetoscarus bicolor*.

Genetic Similarity						
	1	**2**	**3**	**4**	**5**	**6**
1	**100**					
2	**46**	**100**				
3	**50**	**47**	**100**			
4	**46**	**40**	**35**	**100**		
5	**55**	**41**	**50**	**58**	**100**	
6	**41**	**42**	**43**	**41**	**46**	**100**

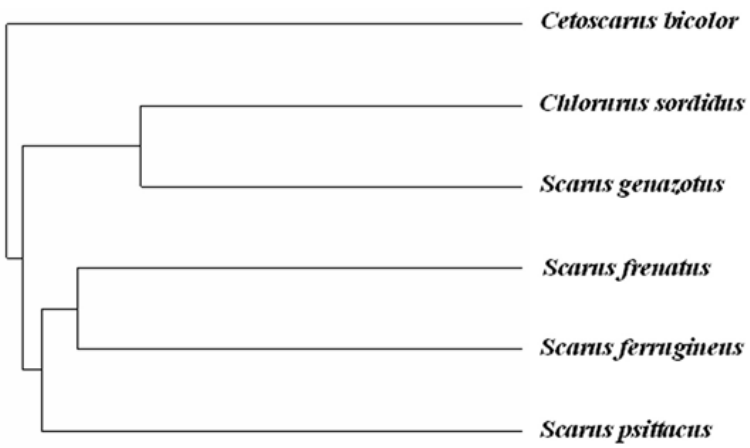

Fig. 2: Dendrogram demonstrating the relationship among six Parrot fishes species; 1- *Scarus ferrugineus*, 2- *Scarus psittacus*, 3- *Scarus frenatus*, 4- *Chlorurus sordidus*, 5- *scarus genazotus* and 6- *Cetoscarus bicolor*.

CONCLUSION

The present report was carried out to estimate the genetic relationship among six parrotfish species in the Egyptian Red Sea using DNA inter simple sequence repeat (ISSR) markers. The results showed different degrees of genetic similarity among the six species ranged from 35 to 58. The results also revealed that *Chlorurus sordidus* and *scarus genazotus* formed a sister clade, also *Scarus ferrugineus* and *Scarus frenatus* formed a sister clade, while both of *Scarus psittacus* and *Cetoscarus bicolor* formed separate clusters. The results described in the present study could be helpful understand the genetic relationship among parrotfish.

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