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ISOLATION OF SAFE TISSUE GLUE AND MOLECULAR FINGERPRINTING OF HAMMOUR (*GREASY GROUPER*) FISH

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ABSTRACT

Saudi Arabia fisheries resources are quite rich with diverse fish species. This diversity of fish species is important to changes in aquatic environment (e.g. pollution, global warming and overfishing...*etc)* which in turn might led to numerous consequences for fish populations. Collagen is a major structural protein of connective tissues. It can be used as a prosthetic biomaterial applicable to artificial skin, tendon ligaments and development collagen implants. In the present study, an attempt was made to isolate and characterize collagen from hammour fish. Hammour, or grouper, is the most popular eating fish in Saudi Arabia due to its deliciousness and rich source of protein. During its processing, the scales, Fins, Skins etc are expelled out as waste acid solubilized collagen. Initial extraction by this method acid yielded 20-30% of collagen on dry weight basis. SEM micrograph showed the fibrous nature of this collagen. Moreover, the molecular characterization of Saudi Arabia grouper fish was done using RAPD, rep-PCR and ISSR markers. Specific results for fingerprinting were obtained by the several primers of each RAPD, rep-PCR and ISSR analysis produced different fragment patterns with varied number of bands. The primers yielded a total of 176 distinct bands 72.2% were considered as polymorphic bands and 27.8% were considered as monomorphic bands. The Dendrogram based on RAPD, rep-PCR and ISSR results grouped the twelve grouper fish samples into two different clusters with about 82% genetic similarity.

KEYWORDS: RAPD, rep-PCR and ISSR markers, grouper fish, Saudi Arabia.

INTRODUCTION

Collagen is the most ubiquitous protein polymer in all multicellular animals. It is particularly rigid and inextensible extracellular matrix protein that serves as a major constituent of many connective tissues. It is distributed in skin, bones, cartilage, tendons, ligaments, blood vessels, teeth, cornea and all other organs of vertebrates and constitutes approximately 30% of total animal protein (Muyonga et al., 2004; Senaratne et al., 2006). It is also regarded as one of the most useful biomaterials, mainly due to its non-toxicity, biocompatibility, immunological properties, and well documented structure (Ho et al. 1997). Collagen has a wide range of applications in leather and film industries, pharmaceutical and biomedical fields. The food utilization of collagen, in pharmaceutical applications, includes production of wound dressings, and vitreous implants and it also act as carrier in drug delivery. Moreover, collagen is used for the production of cosmetics because it has a good moisturizing property (Swatschek et al., 2002).

Generally, genetic markers provide the needed information for management of aquatic species such as fish in Saudi Arabia (Rashed et al., 2008, Rashed et al., 2009, Saad et al. 2011, Saad et al., 2012), shrimp (Saad et al., 2013). The advantage of Randomly Amplified Polymorphic DNA (RAPD) to generate molecular characterization is the production of molecular markers without any previous genomic information on the target species. RAPD assays have been used for estimating genetic diversity among different fishes (Saad et al., 2013, Saad et al., 2014). RAPD is a quick and effective method that can be applied to generate genotype with specific banding patterns (Hassan et al., 2014; Ahmed et al., 2014). RAPD was used for the analysis of genetic diversity in Saudi Arabia fish species, which had a significant impact on the fish genetic resources in Saudi

Arabia (Rashed et al., 2009, Saad et al. 2011). Genetic divergence has been fully examined by Inter Simple Sequence Repeat (ISSR). The ISSR marker technique involves polymerase chain reaction (PCR) amplification of DNA using a single primer composed of a microsatellite sequence, the ISSR has mild technical difficulty, good reproducibility and reasonable cost, permitting its use for genetic studies of population (Chen et.al, 2005 and Li and Xia, 2005). ISSR was reported as more preferable tool than other markers for studying genetic divergence between quite near individuals (Fang et al., 1997; Ullah et al., 2015). Many studies showed that this approach could be used as a useful tool for the genetic diversity monitoring in different populations of animals (AhaniAzari et al., 2007; Kol and Lazebny, 2006; Sabir et. al 2013 and Ahmed and Rezk, 2015). Latterly, ISSR marker tool has been applied to determine genetic variety and DNA polymorphism of some molluscs. Varela et al. (2007) assessed the genetic differentiation among Mytilusedulis mussels' complex collected from six sampling localities distributed along the European Atlantic coast by microsatellite markers. Sabry et al. (2015) evaluated the genetic divergence of different Saudi fish populations using RAPD and ISSR analysis in Saudi Arabia. The other molecular techniques were used by numerous researchers to study genetic diversity among different populations such as in *Tilapia* species (Rashed et al, 2011) and grouper fish (Sabry et al., 2015). The main objective is to molecularly characterize grouper fish (*Greasy grouper*), in Saudi Arabia using different genetic markers RAPD, ISSR and Rep-PCR. Determining true genetic dissimilarity between individuals is a decisive point for clustering and analyzing diversity within this specie, because different dissimilarity indices may yield conflicting outcomes.

MATERIALS AND METHODS

Sample collection

The in grouper fish (*Greasy grouper*) samples of this experiment was conducted to isolate the collagen and perform the genetic fingerprinting using RAPD, rep-PCR and ISSR markers for generating polymorphism. Grouper fish was collected based on their morphological characterization from Jeddah, KSA fish Market. Twelve grouper fish was sampled for molecular markers. From each samples, approximately 1 x 1 cm of caudal fin tissue was excised, placed in a 70 % ethanol and held at 4°C for subsequent DNA extraction. The laboratory work was performed in the Biotechnology and Genetic Engineering Unit and Scientific Research Deanship, Taif University, KSA. DNA extraction genomic DNA extraction and purification were performed according to DNA Qiagen Kit (QIAamp DNA tissue kit), following the instructions of the manufacturer. DNA quality was checked by electrophoresis in a mini-gel.

Extraction of Collagen

Collagen was extracted from fish according to Sujithra et al (2013) with some modification as follows: Fish skins, fins and scales were cut into small pieces (0.3 x 0.3 cm) then soaked in 0.1 M NaOH solution (1:30 W/V) for 48 hours to remove non-collagenous proteins, then the sample was soaked into 0.5 M acetic acid and kept for 42 hours. After that the samples were centrifuged at 30000*xg* for 60 min. The supernatant was then collected and precipitated by adjusting the pH to around 7. The obtained precipitate was collected as pellet by centrifugation at 20000*xg* for 30 min, then the precipitate was dissolved in 0.5 M acetic acid and then dialyzed against 0.1 M acidic acid and deionized water in a dialysis membrane and finally lyophilized. The dialysate was referred to as acid solubilized collagen.

SEM Morphological Analysis

Morphological analysis was undertaken using aJoel Jem-1200 EX II Electron microscope at an accelerating voltage of 25KV to get images of morphological observations.

RAPD analysis

For RAPD analysis, seven 10-mer random primers were used according to Sabry et al., (2015). Following the experiments for optimization of component concentrations, PCR amplification of random primers were carried out according to Williams et al. (1990) and Saad et al., (2012). in 25 µl volume containing 1µl (20 ng) of genomic DNA, 12.5µl of Go Taq® Green Master Mix, Promega, USA. 1µl of primer (20 p.mol), deionized distilled water (up to a total volume of 25 µl). For DNA amplification, the C1000TM Thermo Cycler Bio-Rad, Germany, was programmed under the conditions involving denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 Sec, primer annealing at 35°C for 1.5 min and primer extension at 72°C for 2.5 min; final extension step at 72°C for 7 min. Amplified DNA products were analysed by electrophoresis in 1.5% agarose gel run in TBE. The gels were stained with ethidium bromide (5 µg ml-1). 100 pb. DNA Ladder RTU, (Gene Direx®) was used as a standard. DNA was visualized by UV illumination and then photographed by a Bio-Rad Gel Doc 2000 device.

ISSR and Rep-PCR analysis

For ISSR and Rep-PCR analysis, Go Taq® Green Master Mix, Promega, USA was used. The final total volume of each reaction was 25 µl; contained 0.625 units of Taq DNA polymerase, 2 mM MgCl2 and 0.2 mM of each dNTPs. 50 ng of DNA and 1000 p.mol of the primer were added to the reaction. PCR amplification of rep-PCR and ISSR primers were designed according to (Hassan et al., 2014 and Gaber et al., 2015). The thermocycler was programmed by an initial standard denaturation cycle at 94°C for 5 min. The following 40 cycles were composed of: denaturation step at 94°C for 30 sec, annealing step was programmed at different temperatures according to the primer used (40 to 58 °C) for 1.5 min and elongation step at 72°C for 2.5 min. The final cycle was polymerization cycle performed at 72°C for 7 min. The PCR products of each reaction were analyzed by electrophoretic separation in 1.5% agarose

gel. 100 pb. DNA Ladder RTU, (Gene Direx®) was added on one side of the gel to determine the DNA patterns. Gel was stained by ethidium bromide (0.5 mg/ml). After electrophoresis, the ISSR patterns of the PCR products were visualized by UV illumination and then photographed by a Bio-Rad Gel Doc 2000 device.

Data analysis

The amplification products of RAPD-PCR were scored for the presence "1" or absence "0" and missing data as "9". The genetic associations between isolates were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers. The dissimilarity matrix was generated using Neighbour joined method and hence dendrogram was reconstructed. The computations were performed using the program NTSYS-PC version 2.01 (Rohlf, 2000). The Jaccard's similarity matrix was subjected to principal component analysis.

RESULTS

Isolation of grouper collagen and SEM analysis

The investigation of this study in the first reporting of isolation of collagen from grouper (*Greasy grouper*) fish. The protein estimation resulted good amount of protein in acid solubilized collagen (ASC). The value of ASC was about 25-30% based on the lyophilized dry weight. SEM was performed to characterize the micro-architecture of grouper collagen. The analysis of this collagen under moderate and higher magnification revealed that it was highly porous, interconnected with scaffolds and their surface was rough and uneven and some rod-like spicule structure appeared (Figure 1a,b). The fibres of the scaffold contained spicules which were attached or embedded within the fibrous network in a mixture of orientation (Figure 1a,b).

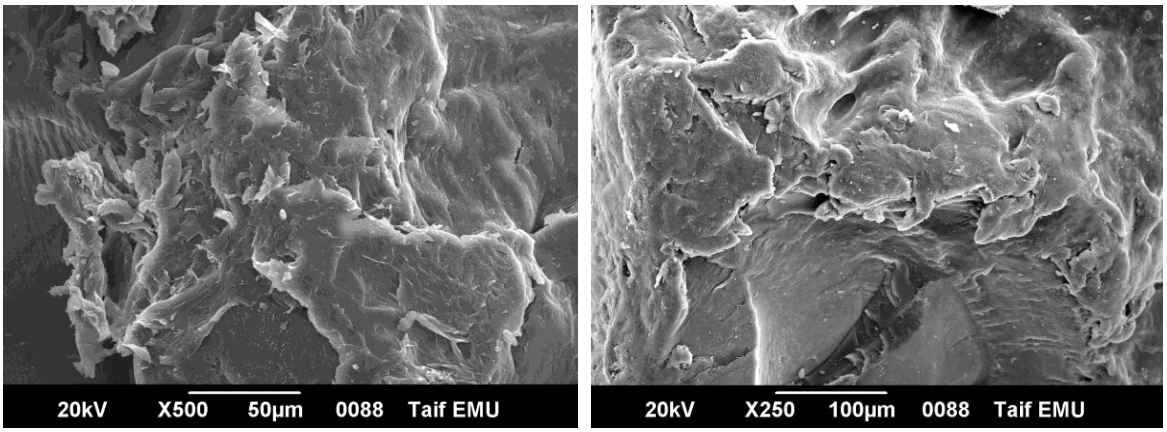

Figure 1: SEM micrograph fibrous layers of Acid Solubilized collagen of grouper fish (*Greasy grouper*) with X250 and X500 zoom power.

PCR amplification and RAPD analyses of grouper fish samples

Genomic diversity of grouper fish samples was investigated by RAPD analysis. The RAPD results illustrated in Table (1) and Figures (2 and 3) showed polymorphic numbers of the genetic bands, which were the electrophoretic products of PCR for grouper fish samples. RAPD-PCR reactions were performed with 12 grouper fish samples and seven different 10-mer primers, which were pre-selected for their performance with grouper fish samples DNA. Out of the seven primers four retained for RAPD analysis produced different fragment patterns with varied number of bands. The primers yielded a total of 81 distinct bands (RAPD markers), (79 %) of which were considered as polymorphic and 21% of which were considered as monomorphic. Table (1) record the number of amplified fragments scored for each grouper fish samples. The amplified products were highly polymorphic among the grouper fish samples. The total number of bands as shown in Table (1) varied from 7 bands with primer OPA-08 to 22 bands with mix primers OP-A2 + OP-D5. The total of monomorphic amplicons was 8 and the total of polymorphic amplicons was 22. The RAPD-PCR results using mix primers (OPA-06 + OPA-08) has showed the highest polymorphism, a total of 11 bands in these grouper fish samples ranged from 150 bp-1200 bp. In case of OP-A2 + OP-D5 primers mix has showed the lowest polymorphism a total of 22 fragments have showed 63.6 % polymorphism among the grouper fish samples.

Table 1: Polymorphic bands of each genetic primers and percentage of polymorphism in grouper fish samples.

Primers	Total Bands	No. of Monomorphic Bands	No. Polymorphic Bands	% Monomorphic bands	% Polymorphic bands
OP-A6	9	2	7	22.2	77.8
OP-A8	7	1	6	14.3	85.7
OP-A9	7	1	6	14.3	85.7
OP-A10	18	3	15	16.7	83.3
OP-A6 + OP-A8	11	1	10	9.00	91.0
OP-A2 + OP-D5	22	8	18	36.4	63.6
OP-A4 + OP-B1	7	1	6	14.3	85.7
BOXA1 + ERIC	14	4	10	28.5	71.5
(GTG)5 + rep-R1	14	5	9	35.7	64.3
Rep-12+ Rep-13	18	6	12	33.3	66.7
ISSR-1 + ISSR-3	20	6	14	30.0	70.0
ISSR-2 + ISSR-8	21	8	13	38.1	61.9
ISSR-18 + ISSR-19	8	3	5	37.5	62.5
Total	176	49	127	27.8	72.2

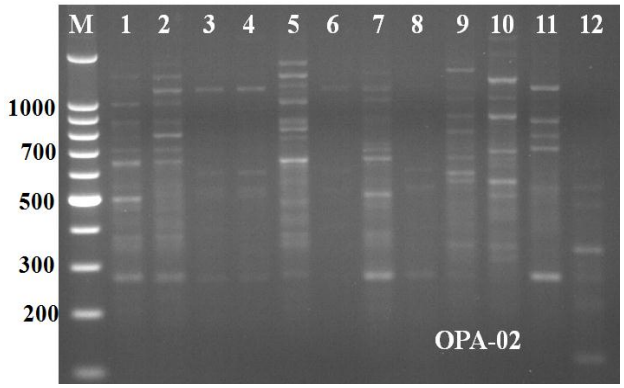

Figure 2: RAPD profile of 12 grouper fish samples (*Greasy grouper*) generated by primer OPA-02 mix.

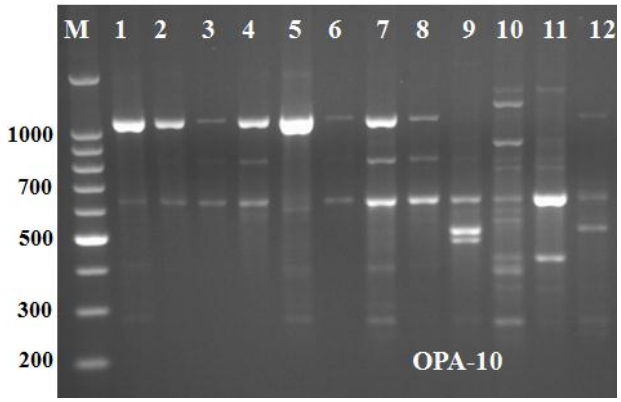

Figure 3: RAPD profile of 12 grouper fish samples (*Greasy grouper*) generated by primer OPA-10.

PCR amplification and ISSR, rep-PCR of grouper fish samples

The results of PCR analysis done on the genomic DNA of grouper fish samples using ISSR and rep-PCR primers were summarized in table (1) and shown in Figures (4, 5, 6 and 7). The polymorphic and monomorphic bands were produced from the PCR amplification. A total of 95 bands were obtained from the ISSR and rep-PCR primers. Out of them, 63 bands were polymorphic with a polymorphism average of 66.3 %. The number of total bands varied from 8, with primers mixISSR-18 + ISSR-19, to 21, with primers mix ISSR-2 + ISSR-8 with band size ranging from 180 to 1700 bp. The highest polymorphism among populations was revealed by BOXA1 + ERIC primers mix (71.5 %), followed by that revealed by ISSR-1 + ISSR-3 primers mix (70 %). However, the lowest polymorphism was 61.9% resulted from application of ISSR-2 + ISSR-8 primers mix.

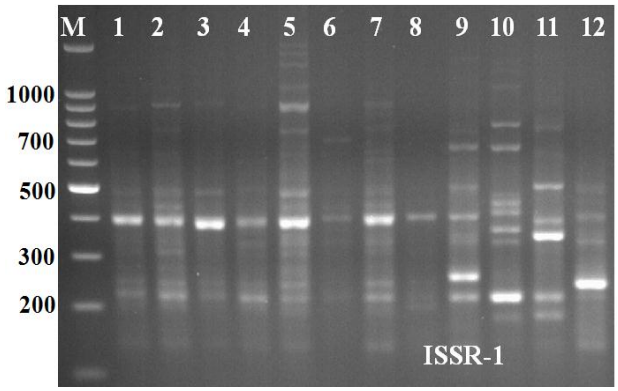

Figure 4: ISSR profile of 12 grouper fish samples (*Greasy grouper*) generated by primer ISSR-1.

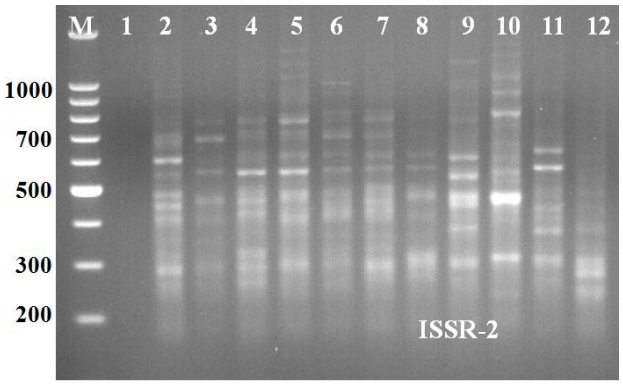

Figure 5: ISSR profile of 12 grouper fish samples (*Greasy grouper*) generated by primer ISSR-2.

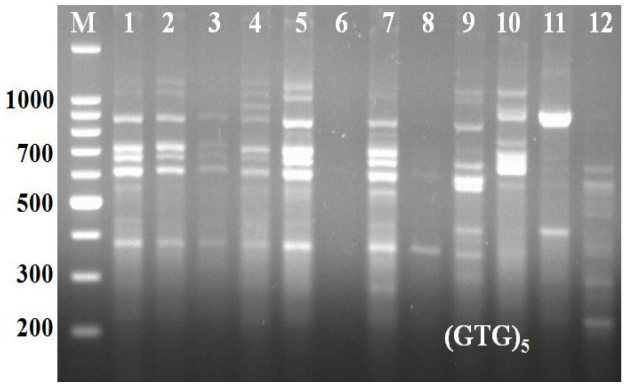

Figure 6: Rep-PCR profile of 12 grouper fish samples (*Greasy grouper*) generated by primer $(GTG)_5$.

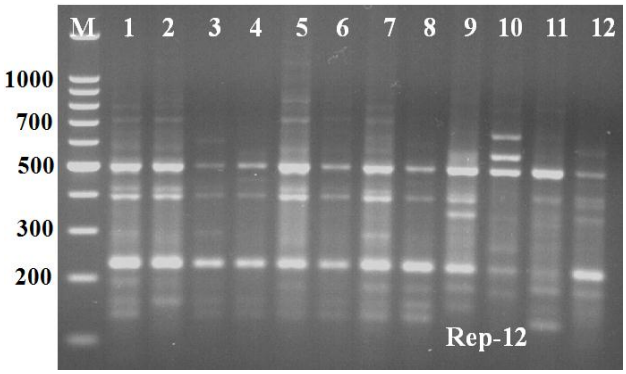

Figure 7: Rep-PCR profile of 12 grouper fish samples (*Greasy grouper*) generated by primer Rep-12.

Genetic distances and the cluster dendrogram

A total of 176 fragments from all analysis were enough for the identification and the evaluation of genetic similarities and designing the phylogenetic tree for these grouper fish samples. According to genetic similarity and intra-species differentiation and the dendrogram constructed using UPGMA based on Jaccard's similarity coefficients that ranged from 0.16 to 0.98 (Figure 8), the grouper fish samples were grouped into two different clusters with about 82% genetic similarity. The first cluster contained grouper fish sample G10, while the second cluster contained other grouper fish samples. The second cluster contained two sub-cluster, the first sub-cluster contained G1, G2, G3, G4, G5, G6, G7, G8, G9 and G12. While the second sub-cluster contained G11 only. The dendrogram showed a high relationship between G1 and G2 and low genetic similarity between G10 and other grouper fish samples. The dendrogram constructed using UPGMA based on Jaccard's similarity coefficients (Figure 8) indicated that G2 and G1 were in the same sub-cluster and appeared more similar to each other than G12.

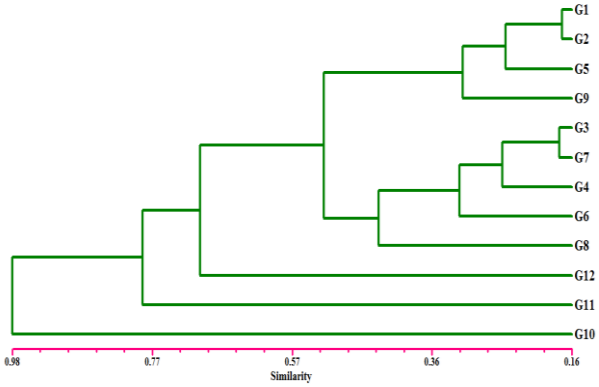

Figure 8: Dendrogram analysis among the twelve grouper fish samples based on RAPD, rep-PCR and ISSR markers.

DISCUSSION

The grouper (Greasy grouper) fish could be found ubiquitously in gulf area and contains a lot of collagen. It is a characteristic feature of collagen from grouper, which is composed of a fibrous organic network (collagen), a non-structural ground substance of a glycoprotein nature and inorganic skeletal components. Isolation of fish collagen is a new era. Limited studies are available in collagen from fish. We yielded about 20-30% of collagen on dry weight basis using acid solubilized method.

The obtained result was higher to that of Crassostrea gigas (11%) (Mizuta et al. 2005) and almost similar to that of Jelly fish (25-35%) (Nagai and Suzuki 2000), Baltic cod (21.5%), but lower than Japanese sea bass (51.4%), chub mackerel (49.8%) (Nagai and Suzuki 2000), and Bull head shark waste (50.1%) (Sujithra et al. 2013). Thus, this suggests that the process wastes of grouper was potential source of alternative natural collagen.

ISSR technique was used because it is simple and reliable tool for assessing the molecular genetic variability within and among many living organisms with highly reproducible results and abundant polymorphism (Kol and Lazebny, 2006; Lalhruaitluanga and Prasad, 2009; Saad et al., 2013). Moreover, the potential applications of ISSR analysis for diverse aims are depend on the variety and frequencies of microsatellites within the specific genomes. (Chunjiang et al., 2005; Hassan et al., 2014). In addition, variable ISSR patterns have potentials as dominant markers for studying genetic diversity of many fishes (Tong et al., 2005; Saad *et al*., 2012). In the present study, ISSR analysis was offered some species-specific markers. The numbers of these molecular markers were varied among grouper fish samples. These DNA markers will be useful value, especially in fish breeding programs, which use genetic markers as marker-assisted selection to improve the fish performance (Rashed et al., 2009). RAPDs were proved to be useful as genetic markers and fingerprinting (Salem *et al*., 2005; Rabie and Abdou, 2010; Nikkhoo *et al*.,

2011; Ghanem *et al*., 2012; Ahmed and Rezk, 2015). It can be concluded from our study that RAPD markers are effective in detecting similarity among grouper fish samples and they provide a potential tool for studying the inter-species genetic similarity and the establishment of genetic relationships. Although major bands from RAPD reactions are highly reproducible, minor bands can difficult to repeat due to random priming nature of this PCR reaction and potential confounding effects associated with co-migration with other markers. The same idea was tested by Rashed et al., (2011) and Sabry et al., (2015). They used RAPD marker to detect the genetic variations among some fish species. The molecular genetic markers are widely used to identify lines or strains, define stock diversity, monitor inbreeding, diagnose simply inherited traits and even to improve stocks (Rashed *et al*., 2008; Rashed *et al*., 2009). The application of DNA-based genetic analysis as marker-assisted selection in fish research and stock development and management is still not fully maximized (Kocher *et al*., 1998 and Rashed *et al*., 2009). This conclusion was previously confirmed using another analysis such as RAPD. However, Saad *et al*. (2012) and Rashed *et al*, (2011) used bulked segregate analysis to reconstruct the phylogenetic relationships among three fish species. They found that *T. zillii* species was distantly related from both *O. aureus* and *O. niloticus* species. Liu *et al*., (2006) studied the genetic diversity in three *Paralichthys olivaceus* populations using ISSR analysis, which was confirmed to be a reproducible and sensitive tool for the study of population genetics of these fish. The genetic variability of domestic hatchery populations has implications to the conservation of natural *Paralichthys olivaceus* resources (Yun-Guo *et al*., 2006). The use of ISSR and RAPD primers consisting of degenerate anchors or degenerate motifs increased the number of amplified markers. Since ISSR analysis is an easy to perform, high flow-through technique may represent it an alternative for the RAPD, better reproducibility was characterized due to the elevated annealing temperatures. An especially attractive feature of ISSR analysis is its flexibility in terms of experimental design, where the number of generated amplicons may be optimized by changing the number of the core repeat units and anchoring bases (Liu and Wendel, 2001; Hassan et al., 2014; Ullah et al., 2015). We suggest that ISSR analysis should be a stand by choice for genome mapping or gene tagging and marker-assisted selection. For its high simplicity, ISSR analysis should be the first choice for genome mapping or gene tagging for organisms (which genomic knowledge is limited). The above-mentioned exploitation and further studies would be significant for the basic and applied research on fisheries and aquaculture genetics and extend the knowledge of microsatellite conservation and evolution in Saudi fish species.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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