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Molecular Genetics Characterization of *Plectropomus areolatus* Samples Collected from Jeddah Coast Using ISSR Analysis

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Abstract: Six samples of *Plectropomus areolatus* (coral reef guide) collected from different locations of Jeddah coast were identified. ISSR analysis using five primers showed that 18 of 33 total amplified fragments were polymorphic with 55% polymorphism, whereas 83% polymorphism in primer 17898A was higher than that observed for the other four primers. The fish samples 2 and 6 revealed species-specific markers in primer 814B with 800 and 850 bp, respectively. Two other samples 4 and 5 were characterized by uniquely 3 fragments 680, 1000 and 700 bp one fragment in each the three primers HB12, HB14 and 814B respectively. UPGMA dendrogram characterized the six *P. areolatus* samples into two main clusters. The first cluster contained three samples, whereas samples 4 and 5 were closely related with the highest similarity with sample 5 (93%) and sample 6 that showed high similarity with the previous two samples (77 and 83%, respectively). The second cluster contained three samples, 1 and 2 displayed the second highest similarity (92%), however the sample 3 showed low similarity percentages with the two samples (66 and 72%, respectively). In addition, samples 3 vs. 4 and sample 2 vs. 5 showed the lowest genetic similarities among samples (66 and 67%, respectively).

Key words: *Plectropomus areolatus* fish samples · ISSR fingerprinting · Jeddah · Saudi Arabia

INTRODUCTION

A PCR-based, semi-arbitrary marker called inter simple sequence repeat (ISSR) [1, 2] assesses variation in the microsatellite regions. Simple sequence repeats (SSR) or microsatellites are short hypervariable stretches of DNA which are dispersed throughout the genome of eukaryotes. The amplification of the regions between two inversely orientated microsatellites with primers anchored within these elements leads to multilocus and highly polymorphous patterns. Like RAPD-PCR, this method does not need prior genomic information and only small quantities of template DNA are required. In addition, ISSR showed greater robustness in repeatability experiments across a wide range of PCR parameters [3]. The utility of this technique had been established for a wide range of applications from conservation biology to molecular ecology and systematic. Liu *et al.* [4] performed inter-simple sequence repeat (ISSR) analysis in order to evaluate one common population and two selected hatchery populations of Japanese flounder *Paralichthys olivaceus*. A total of 105 individuals belonging to three populations were screened using 12 different ISSR primers. A total of 112 loci were produced in the three studied populations. The total number of loci and polymorphic loci detected by single primer was ranged from six to twelve and two to five. The ISSR fingerprinting technique used was confirmed to be a reproducible and sensitive tool for the study of population genetics of Japanese flounder. They suggested that the genetic variability of domestic hatchery populations has implications to the conservation of natural Japanese flounder resources. Maltagliati *et al.* [5] used ISSR technique to obtain species-specific molecular markers for the cyprinodontiform fish *Valencia hispanica*, *Valencia letourneuxi* and *Aphanius fasciatus*. The aims were to assess the effectiveness of ISSRs in discriminating the three species and to identify tissues of two unidentified fish suspected to belong to one of the three above species by comparing ISSR genotypes. Hou *et al.* [6] investigated the genetic structure of seven different populations of the surf clam *Mactra veneriformes* along the coast of China was by ISSR fingerprinting of 210 individual clams. Of the 240 ISSR loci tested, 235 (97.9%)

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were polymorphic. The seven different geographic populations were divided into three subgroups: Liaoning, Qingdao/Lianyungang and Ningbo based on the UPGMA dendrogram of Nei's genetic distance. These results indicated that isolation of geographic distance played an important role in population differentiation.

Pazza *et al.* [7] used RAPD and ISSR molecular markers to complement the study of chromosomal polymorphism in *Astyanax fasciatus* (Teleostei, Characidae) from the Mogi-Guaçu River (Southeastern Brazil), analyzed in three collection sites along the river. Liu *et al.* [8] performed ISSR analysis in order to evaluate the genetic diversity of wild and hatchery samples of half-smooth tongue sole *Cynoglossus semilaevis*. A group of 200 genotypes belonging to four wild samples, Laizhou (LZ), Weihai (WH), Qingdao (QD), Rizhao (RZ) and one hatchery sample, Mingbo (MB) were screened using 15 different ISSR primers.

De Marco *et al.* [9] analyzed the effectiveness of ISSR technique in detecting specific molecular markers to genetically discriminate some species of teleost fish. As model species, they used four Mediterranean sea bream species (i.e. *Diplodus annularis*, *D. puntazzo*, *D. sargus sargus* and *D. vulgaris*) and the common Mediterranean dentex *Dentex dentex*. The results obtained evidence the usefulness of ISSR markers in specimen and species identification, particularly when the morphological traits of fish make the classification problematic. Moysés *et al.* [10] carried out an analysis of the polymorphism of ISSR in specimens sampled from populations of the Upper Paraná, São Francisco and Amazon river basins (Brazil) of genus *Eigenmannia* (Teleostei: Gymnotiformes). Bignotto *et al.* [11] analyzed the polymorphisms in ISSR fragments of *P. corruscans* and *P. reticulatum* populations from the Paraná River Basin. The interspecific polymorphisms within the mtDNA control region and ISSR fragments were suitable as diagnostic molecular markers and could be used to discriminate the two species. Antunes *et al.* [12] studied the molecular polymorphism of *Brycon* species; *Brycon orbignyanus*, *B. hilarii*, *B. cf. pesu*, *B. cephalus*, *B. falcatus* and *B. gouldingi* using ISSR technique. According to the phylogenetic tree obtained from the data, these *Brycon* species can be divided into two clades: one comprised only *B. cf. pesu* and the second comprised the remaining *Brycon* species. The authors concluded that ISSR primers can be used for the identification of species-specific bands in fish, such as *Brycon* spp.

The objectives of the present study are focused on the assessment of genetic variation, phylogenetic relationship and DNA fingerprinting using ISSR markers between six samples of *Plectropomus areolatus* (coral reef guide) that collected from different locations along Jeddah coast.

MATERIALS AND METHODS

Six samples of *Plectropomus areolatus* (coral reef guide) were collected from Jeddah coast and were identified phenotypically.

Samples Preparation for DNA Extraction: About 0.5 gm fresh muscle were taken and stored at - 20°C. About 80 mg of frozen tissues were thawed and homogenized in o.5 ml ice-cold bi-distilled water using glass homogenizer. Tissue homogenates were centrifuged at 10,000 rpm at 4°C for 5 min

DNA Extraction from Fish Muscle Samples: DNA extraction was performed following the method of Li *et al.* [13]. Samples were ground to fine powder in liquid nitrogen and 1 ml of the extraction buffer was added. Lysis buffer (4 mM NaCl, 0.5 mM EDTA, 0.1% SDS and 0.02 NP 40) and 0.01% proteinase K were added and incubated at 55°C 60 min for complete lysis of cells. A mix of chloroform and phenol were added, mixed gently and centrifuged at 10,000 rpm for 5 min. A volume of chloroform: isoamyl alcohol (24:1) was added to the supernatants and mixed by gentle agitation for 2 min. Ammonium acetate and two volumes of ice-cold ethanol were added, centrifuged at 10,000 rpm for 1 min and the pellets washed twice with 70% (v/v) ethanol. The DNA dried and re-suspended in an appropriate volume of TE buffer.

PCR Amplification Conditions of ISSR Primers: Inter simple sequence repeats (ISSR) analysis was applied according to Ziêtkiewicz *et al.* [1]. The ISSR primers procured from UBC (Univ. of British Columbia, Vancouver, Canada) based on core repeats anchored at the 5' or 3' end as shown in Table 1. Amplification reactions were used in a final volume of 25 µl containing: 2.5 µl 10x PCR buffer, 1.25 µl 50 mM $MgCl_2$, 2.5µl 2 mM dNTP, 0.625µl 10 mM primer, 2-3µl 50 ng DNA, 1µl 5µ *Taq* polymerase (Promega, USA) and 16 µl Millipore H_2O. PCR reactions were performed using a Thermal Cycler (Biometra, biomedizinische Analytik GmbH). Amplification

Table 1: Names and sequences of ISSR primers

No	Primer names	Primer sequences
1	HB12	5′ CAC CAC CAC GC ′3
2	17899A	5' CAC ACA CAC ACA AG '3
3	17898A	5' CAC ACA CAC ACA AC '3
4	814B	5' CTC TCT CTC TCT CTC TTG '3
5	HBI4	5 CTC CTC CTC GC'3

conditions were as follow: Initial denaturation of 2 min at 94°C, followed by 30 cycles of 94°C for 30 sec, annealing at 52°C for 45 sec, extension at 72°C for 2 min and a final extension at 72°C for 7 min. PCR amplification products were analyzed using 1.2% agarose gel electrophoresis in 1X TBE buffer staining with ethidium bromide (1µg/ml). The bands of amplified DNA were visualized under UV transilluminator and photographed using Gel Doc 2000 (Bio-Rad system). The sizes of the fragments were estimated based on a DNA ladder of 100 to 2000 (MBI, Fermentas) base pair which run with each gel.

Statistical Genetic Analysis: Data generated from ISSR analysis was analyzed using the Nei genetic similarity index [14] which excludes common negative data on the basis of the equation:

Similarity = $2N_{ab}/ (N_a+ N_b)$, where N_{ab}= number of scored amplification fragments with the same molecular weight shared between genotype a and b; N_a= number of scored amplification fragments in genotype a, N_b= number of scored amplification fragments in genotype b. A dendrogram was constructed on the basis of the similarity matrix data by un-weighted pair group method with arithmetic average (UPGMA) cluster analysis using the software MEGA program.

RESULTS AND DISCUSSION

ISSR Analysis of *P. areolatus* Muscle Samples: Five inter-simple sequences repeat (ISSR) primers; HB12, HB14, 17899A, 17898A and 814B were used to detect the genetic variations of the six fish samples. Primer 17899A displayed 7 fragments with similar molecular sizes and unable to differentiated the six samples. The results of ISSR analysis using primer HB12 showed 8 amplified fragments with molecular sizes ranged from 680 to 150 bp (Fig. 1 and Table 2), whereas 3 fragments were polymorphic and the residual fragments were ordinary appeared among the samples. Primer 17898A revealed 6 fragments with molecular sizes ranged from 800 to 350 bp (Fig. 1). Similarity in banding numbers and positions are quite similar except in band with 800 bp that disappeared

Table 2: Analysis of six *P. areolatus* samples using four ISSR primers

				Fish samples					
Item	T	P	Bs (bp)	1	2	3	4	5	6
HB12			680				+	+	
			600	+	+	+	+	+	+
			550	+	+	+	+	+	+
	8	5	420	+	+	+	+	+	+
			350	+	+		+	+	
			270			+		+	+
			210	+	+	+	+	+	+
			150	+	+	+	+	+	+
		63%		6	6	6	7	8	6
			800	+	+	+			
17898A			740	+	+	+	+	+	+
			630	+	+	+	+	+	+
	6	5	570	+	+	+	+	+	+
			460	+	+	+	+	+	+
			350	+	+	+	+	+	+
		83%		6	6	6	5	5	5
			1000				+	+	
			850				+	+	+
HB14			800	+	+	+	+	+	+
			700			+		+	+
			600	+	+	+	+	+	+
	9	5	400			+	+	+	+
			300	+	+	+	+	+	+
			260	+	+	+	+	+	+
			170	+	+	+	+	+	+
		56%		5	5	7	8	9	8
			1400				+	+	+
			1000	+	+	+	+	+	+
			850						+
			800		+				
			700				+	+	
814B	10	3	600	+	+	+	+	+	+
			400	+	+		+	+	+
			320	+	+	+	+	+	+
			290	+	+		+	+	+
			190	+			+	+	+
		30%		6	6	3	8	8	8
	33	18	T	23	23	22	28	30	27

Polymorphic % =55

T = Total number of amplified bands, Bs = Molecular size by base pair, (+) Present of amplified bands, P = Numbers of polymorphic band.

Table 3: Genetic similarity percentages of six *P. areolatus* samples using four ISSR primers

	P. areolatus samples				
Item	1	2	3	4	5
2	92				
3	72	72			
4	76	70	66		
5	71	67	68	93	
6	72	74	74	77	84

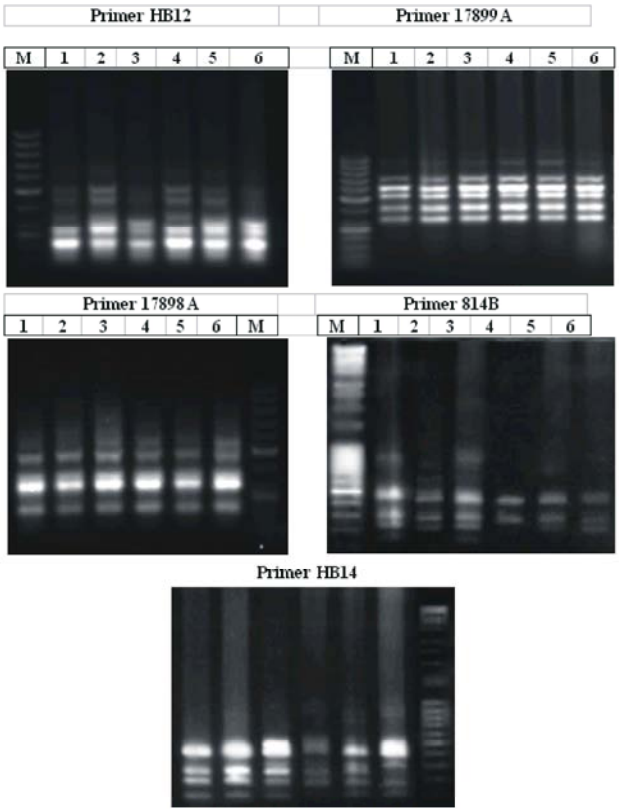

Fig. 1: ISSR amplified products of six *Plectropomus areolatus* samples using five ISSR primers

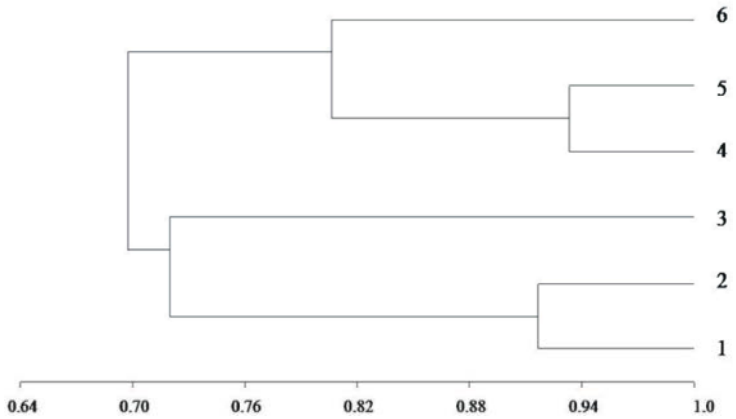

Fig. 2: Dendrograms represented the genetic relationships of six fish muscle samples using UPGMA cluster analysis of genetic similarity coefficients generated from the four ISSR primers

in three samples 4, 5 and 6. The six fish samples were genetically differentiated based on the presence of 5 polymorphic fragments obtained from ISSR amplified products. One of the nine fragments with molecular size 1000 bp was existed in samples 4 and 5, while they were absent in the four other samples. Three fragments with 850, 700 and 400 bp were absent in samples 1, 2 and 3 while existing in other three samples. Primer 814B showed 7 polymorphic fragments from 10, two species-specific markers with molecular weights 850 and 800 bp presented in samples 6 and 2 respectively and disappeared in the other samples. One fragment with 700 was uniquely existed in samples 4 and 5 while existed in the remaining samples. Overall results using the five primers showed that 18 of 33 total amplified fragments were polymorphic with 55% polymorphism, whereas 83% polymorphism in primer 17898A was higher than that observed for the other four primers.

Genetic Similarity of the Eight *Bt* Isolates Using RAPD Analysis: Genetic similarity between the six fish samples was calculated from the amplified fragment data using un-weighted pair group method with averages (UPGMA). UPGMA dendrogram constructed on the basis of genetic distance between each two pairs of the six fish samples was performed using the Nei similarity index (Table 3). The dendrogram of the five primers revealed clearly two main clusters that classified the six samples into different subgroups with various value percentages of bootstraps as shown in Fig. 2. Accordingly, the first cluster contained three samples, whereas samples 4 and 5 were closely related with the highest similarity with sample 5 (93%) and sample 6 that showed high similarity with the previous two samples (77 and 83, respectively). The second cluster contained three samples, 1 and 2 displayed the second highest similarity (92%), however the sample 3 showed low similarity percentages with the two samples (66 and 72, respectively). In addition, samples 3 vs. 4 and sample 2 vs. 5 showed the lowest genetic similarities among samples (66 and 67%, respectively) as shown in Table 3.

DISCUSSION

Genetic variation is a key tool for assessing biological potential of an organism. A population with high level of genetic variation may be better capable of dealing with changes in its surroundings such as fluctuations in water temperature, epidemics etc [15]. Reduction of genetic variability may cause greater sensitivity to environmental changes and eventually lead to extinction of a species [16]. Moreover, it may affect growth and reproduction [17]. Therefore, the maintenance of genetic variability is very important for the conservation of a species [18]. It is necessary for individuals to have the ability to survive environmental variations and develop fully. Therefore, genetic monitoring is ideal for use in a reproduction program with the aim of genetic conservation (i.e., stocking). Molecular markers are a realistic and useful tool for the investigation and monitoring of genetic conditions both in native populations and in captive lots [19]. Molecular markers may be used for the genetic characterization of native fish populations and fish lots, species identification and also to study the effects of environmental variation upon genetic variability [20].

Microsatellites or simple sequence repeats (SSR) are short DNA sequence stretches consisting of motifs of one to six bases that are tandemly repeated. Owing to their ubiquity, hypervariability, abundance and genome-wide distribution, SSR loci represent a new generation of powerful genetic markers for eukaryotes. Use of this marker system, however, is hampered by the requirement for sequence information from flanking regions, from which primers are designed for polymerase chain reaction (PCR) amplification. Discovery and characterization of a large number of SSRs is therefore time-consuming and expensive for many taxa. The developed modification of SSR-based marker systems, i.e. ISSR (inter-simple sequence repeat) analysis, circumvents this requirement for flanking sequence information and thus has found wide applicability in a variety of plants. Wolfe *et al.* [21] stated that ISSR analysis involves PCR amplification of genomic DNA using a single primer that targets the repeat *per se*, with 1-3 bases that anchor the primer at the 3' or 5' end. In addition to freedom from the necessity of obtaining flanking genomic sequence information, ISSR analysis is technically simpler than many other marker systems. The method provides highly reproducible results and generates abundant polymorphisms in many systems.

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REFERENCES

1. Ziêtkiewicz, E., A. Rafalski and D. Labuda, 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, 20: 176-183.
2. Gupta, M., Y.S. Chyi, J. Romero-severson and J.L. Owen, 1994. Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple sequence repeats. Theoretical and Applied Genetics, 89: 998-1006.

3. Bornet, B. and M. Branchard, 2001. Nonanchored Inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Molecular Biology Reporter, 19: 209-215.
4. Liu, Y.G., S.L. Chen, J. Li and B.F. Li, 2006. Genetic diversity in three Japanese flounder (*Paralichthys olivaceus*) populations revealed by ISSR markers. Aquaculture, 255(1-4): 565-572.
5. Maltagliati, F., T. Lai, M. Casu, S. Valdesalici and A. Castelli, 2006. Identification of endangered Mediterranean cyprinodontiform fish by means of DNA inter-simple sequence repeats (ISSRs). Biochemical Systematics and Ecology, 34(8): 626-634.
6. Hou, L., H. Lü, X. Zou, X. Bi, D. Yan and C. He, 2006. Genetic characterizations of *Mactra veneriformis* (Bivalve) along the Chinese coast using ISSR-PCR markers. Aquaculture, 261(3): 865-871.
7. Pazza, R., K.F. Kavalco, M.A.P. Prioli, A.J. Prioli and L.A.C. Bertollo, 2007. Chromosome polymorphism in *Astyanax fasciatus* (Teleostei, Characidae), Part 3: Analysis of the RAPD and ISSR molecular markers. Biochem. Syst. and Ecol., 35(12): 843-851.
8. Liu, Y.G., Z.G. Yu, B.L. Bao, X.Q. Sun, Q.L. Shi and L.X. Liu, 2008. Population genetics studies of half-smooth tongue sole *Cynoglossus semilaevis* using ISSR markers. Biochem. Systematics and Ecology, 36(11): 821-827.
9. De Marco, E.G., L.O. Tacuatiá, L. Eggers, E. Kaltchuk-Santos and T.T. Souza-Chies, 2009. Genetic variability within Cypella fucata Ravenna in Southern Brazil. In: Mahoney CL, Springer DA, Editors. Genetic Diversity. Nova Science Publishers; New York, pp: 179-194.
10. Moysés, C.B., Z. Daniel-Silva, C.E. Lopes and L.F. De Almeida-Toledo, 2009. Cytotype-specific ISSR profiles and karyotypes in the Neotropical genus *Eigenmannia* (Teleostei: Gymnotiformes). Genetica, 138(2): 179-189.
11. Bignotto, T.S., A.J. Prioli, S.M. Prioli, T.C. Maniglia, T.A. Boni, L.C. Lucio, V.N. Gomes, R.A. Prioli A.V. Oliveira, H.F. Júlio Jr and L.M. Prioli, 2009. Genetic divergence between *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* (Siluriformes: Pimelodidae) in the Paraná River Basin. Braz. J. Biol., 69(2 Suppl.): 681-689.
12. Antunes, R.S., V.N. Gomes, S.M. Prioli, R.A. Prioli, H.F. Júlio Jr, L.M. Prioli, C.S. Agostinho and A.J. Prioli, 2010. Molecular characterization and phylogenetic relationships among species of the genus Brycon (Characiformes: Characidae) from four hydrographic basins in Brazil. Genet. Mol. Res., 13(2): 674-684.
13. Li, Y., Y. Gul, Z. Gao, W. Luo and W. Wang, 2013. A simple and affordable method of DNA extraction from fish scales for polymerase chain reaction. Biochemical Genetics, 51: 1-6.
14. Nei, M. and W.H. Li, 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Nat. Acad. Sci., 76: 5269-5273.
15. Alam, S., S. Islam and S. Alam, 2010. DNA fingerprinting of the freshwater mud eel, *Monopterus cuchia* (Hamilton) by randomly amplified polymorphic DNA (RAPD) marker. International J. of Biotech. and Biochem., 6(2): 271-278.
16. Evans, R.D., L. Van Herwerden, G.R. Russ and A.J. Frisch, 2010. Strong genetic but not spatial subdivision of two reef fish species targeted by fishers on the Great Barrier Reef. Fisheries Research, 102: 16-25.
17. Dixon, T.J., G.J. Coman, S.J. Arnold, M.J. Sellars, R.E. Lyons, L. Dierens, N.P. Preston and Y. Li, 2008. Shifts in genetic diversity during domestication of Black Tiger shrimp, Penaeus monodon, monitored using two multiplexed microsatellite systems Aquaculture, 283: 1-6.
18. Barroso, R., P. Wheeler, S. LaPatra, R. Drew and G. Thorgaard, 2008. QTL for IHNV resistance and growth identified in a rainbow (*Oncorhynchus mykiss*) x Yellowstone cutthroat (*Oncorhynchus clarki bouvieri*) trout cross. Aquaculture, 277: 156-163.
19. Alam, S. and S. Islam, 2005. Population genetic structure of Catla catla (Hamilton) revealed by microsatellite DNA markers. Aquaculture, 246: 151-160.
20. Ryman, N., 1991. Conservation genetics considerations in fishery management. Journal of Fish Biology, 39(Supplement A): 211-224.
21. Wolfe, A.D., Q.Y. Xiang and S.R. Kephart, 1998. Assessing hybridization in natural populations of Penstemon (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. Molecular Ecology, 7: 1107-1125.