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GENETIC STUDIES OF RELATIONSHIP BETWEEN PENAEUS SEMESULACATUS, PENAEUS JAPANICUS AND META PENAEUS MONOCEROS USING BIOCHEMICAL AND MOLECULAR TOOLS

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ABSTRACT

A comparative study of three shrimp populations (Penaeus semesulacatus, Penaeus japonicus, Meta penaeus monoceros) was carried out. Biochemical and the molecular genetic structure of the relevant population was investigated using SDS- PAGE, RAPD and ISSR Techniques. A high degree of similarity was detected by SDS-PAGE within each population and the similarity values were equal to 0.87 when calculated in .semesulacatus population, o.9 in *P. Japonicus* population and o.85 in M. P. Monoceros population. The data inferred from molecular markers obtained by ISSR and RAPD–PCR were used to calculate the genetic distances, and showed that M.P. Monoceros was distantly related from P.japonicus and P.semesulacatu. Also, the P.semesulacatus and P.japonicus had relatively high level of genetic relationship. The present study revealed that the ISSR and RAPD Techniques are powerful tools in detecting genetic variations.

KEYWORD: ***SDS-PAGE, RAPD, ISSR, Relationship and Shrimp.***

INTRODUCTION

The world needs to increase the sources of food permanently to meet the continuous demographic increase. In this respect freshwater fish species are of the main and important sources for food security. Shrimps contribute about 20% by volume of the world seafood market (Bhavan PS et al. 2010; Abde-Sallam HA, 2013) and some species of shrimps are also cultivated in aquaculture in tropical countries (Gobal PM et al 2009). Penaeid shrimp belong to the largest phylum in the Animal Kingdom, the Arthropoda,which is characterized by the presence of jointed appendages and an exoskeleton orcuticle that is periodically molted. There are thousands of terrestrial species in thisphylum, and a large, predominately aquatic subphylum, the Crustacea. The morehighly evolved crustaceans (Class: Malacostraca) include the penaeid shrimp (OrderDecapoda), Suborder: Natantia, Superfamily: Penaeoidea, Family: Penaeidae, Genus: Penaeus, Species: monodon, japonicus, indicus, merguiensis, vannamei (Bailey-Brock JH and Moss SM, 1992). There are a number of different molecular methods which have been designed to study fish populations. The use of biochemical methods such as Isozymes and protein electrophoretic techniques for species identification has been widely applied in fish. Species identification problem fall into three categories: identification of early life stage (eggs and larvae), separation of problematic species and introgressive hybridization. The identification of problematical species is similar to identification of distinct populations. It was necessary to know the estimates of intra and inter population variations and phylogenetic relationships among fish populations to help the breeders in the breeding programs and detect any genetic contamination (Puorto et al. 2001). RAPD polymorphisms are inherited in a Mendelian fashion and can be used as genetic markers (Bardakci F and Skibinski DOF, 1994). The majority of allozyme and RAPD loci segregate in a Mendelian fashion. Allozyme markers in general show co dominant inheritance patterns. While RAPD markers conform to expectations for band presence/absence under a dominant allele model (Appleyard SA and Mather PB, 2000). The

genetic variations, structure, and phylogenetic relations of some shrimp species have been a focus of debate. Some molecular techniques such as microsatellite DNA (Liu P et al. 2004; Meng Xian Hong a et al. 2007), AFLP or Amplified Fragment Length Polymorphism (Moore WW et al. 1999), RAPD or random amplification of polymorphic DNA (Meng XH et al. 2004) and analysis of some mitochondrial genes (Cui LCP et al. 2007) have been used to investigated the genetic variability among many shrimp species. The use of DNA markers can contribute significantly to develop of genetic improvement programs (Rashed MA et al. 2009; Saad YM et al. 2011) and species identifications (Saad YM 2013)**.** Molecular characterization using ISSR analysis was an attractive tool for species identification. These markers were recommended when coupled with appropriate statistical analyses in species identification and classification (Saad YM 2012)**.** The present study aims to Gain more information about the biochemical and genetic background of shrimp populations (P. Semesulacatus, P. Japanicus and Meta P. monoceros), and to contrast these populations at the molecular level by studying the intra and the inter biochemical and molecular genetic variations in order to infer the phylogenetic relationships among the relevant populations. Also, the work aim to investigate the existence of biochemical and molecular genetic markers which serve as selectable markers during selection programs.

MATERIALS AND METHODS

Sample collection

Three shrimp populations (P. semesulacatus, P. japonicus and Meta p. Monoceros) were used in the present study. Individuals of shrimp samples were collected from Mediterranean Sea in north Sinai in Egypt. Each sample comprises at least five individuals.

Organs sampling

Appropriate pieces of skeletal muscles were cut from immediately killed alive shrimp individuals of the three different populations and put in eppendorf tubes with a saline solution (0.85 % of NaCl). The same individuals were cut and preserved with 70% ethanol into eppendorf tubes. Then, both muscle samples were frozen at -20 ˚C.

Protein extraction

One piece of skeletal muscle (0.2 g) from each individual was powdered in a mortar, and extracted two times in appropriate volume of extraction saline, respectively (0.85 % of NaCl. The homogenized samples were centrifuged at 12000 rpm /15 min /4˚C. The supernatants were transferred to new eppendorf tubes, and kept in deep freezer until use (Gorinstein S 1999).

Protein electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique was used to compare the studied populations by their protein patterns. Protein Samples were applied to a 15 % polyacrylamide gel. Gel preparation, electrophoresis conditions, staining and destaining gels were done according to (Laemmli UK 1970)**.**

DNA extraction and amplification

The DNA extraction was performed using DNeasy Mini Kit (QIAGEN). The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94º C for 4 min followed by 35 cycles of 1 min at 94º C, 1 min at 57º C for RAPD, 37 º C for ISSR, and 1 min at 72º C. the final extension at 72º C for 10 min using RAPD and ISSR primers as shown in Tables1and2:

Table 1

List of the primer names and sequences used in RAPD PCR.

No	Name	Sequence	No	Name	Sequence
1	AO9	5'- GGG TAA CGC C -3'	6	B07	5`- GGT GAC GCA G3
2	A10	5- GTG ATC GCA G-3	7	B09	5- TGG GGG ACT C3
3	A18	5 AGG TGA CCG T-3	8	B11	5`-GTA GAC CCG T3`
4	B01	5- GTT TCG CTC C-3	9	C19	5- GTT GCC AGC C-3`
5	B06	5- TGC TCT GCC C3			

Table 2
PCR List of the primer names and sequences used in ISSR.

No	Name	Sequence	No	Name	Sequence
1	14A	5 CTC TCT CTC TCT CTC TTG 3	6	HB-09	5-GTG TGT GTG TGT GC-3
2	98A	5 CAC ACA CAC ACA CA 3	7	HB-12	5 CAC CACCAC GC- 3
3	44B	5 CTC TCT CTC TCT CTC TTG 3	8	HB-13	5` GAG GAGGAG GC-3`
4	49B	5 CAC ACA CAC ACA GG 3			
5	98B	5 CAC ACA CAC ACA GT 3			

STATISTICAL ANALYSIS

All gels protein and DNA electrophoresis were analyzed using gel analyzer and SPSS 10 software. The banding protein profiles were scored in a binary data (1 if the band present and 0 if absent). This data was introduced to SPSS software package in order to infer similarities, and genetic distances for intra and inter population relationships. Within population similarity s (homogeneity) was calculated as average of sxy across all possible comparison between individuals within such population (Bardakci F and Skibinski DOF, 1994). To construct the dendrogram which describes the phylogenetic relationships between studied populations, the data of sample number one derived from all DNA patterns of each population was combined together and introduced to SPSS software package, the dendogram was constructed according to (Sneath PHA and Sokal RR, 1973), using Unweighted pair-Group Method of Analysis (UPGMA).

RESULTS

SDS-PAGE analysis was used to examine the polymorphism and determine relationship between three shrimp species at protein level. RAPD and ISSR analysis were used to detect species-specific DNA markers, to examine the genetic polymorphism, and to determine the variability among the three studied shrimp species. The Molecular sizes (bp) of SDS-PAGE, RAPD and the specific ISSR markers for the three studied shrimp species were presented in Tables 3,5 and 6.

The protein polymorphism

The protein banding pattern for P.semesulacatus, P.Japonicus and Meta p. monoceros populations is shown in Figure (1,2 and3) respectively and The number of detected bands and number of polymorphic bands in the studied shrimp species were presented in Table (3).

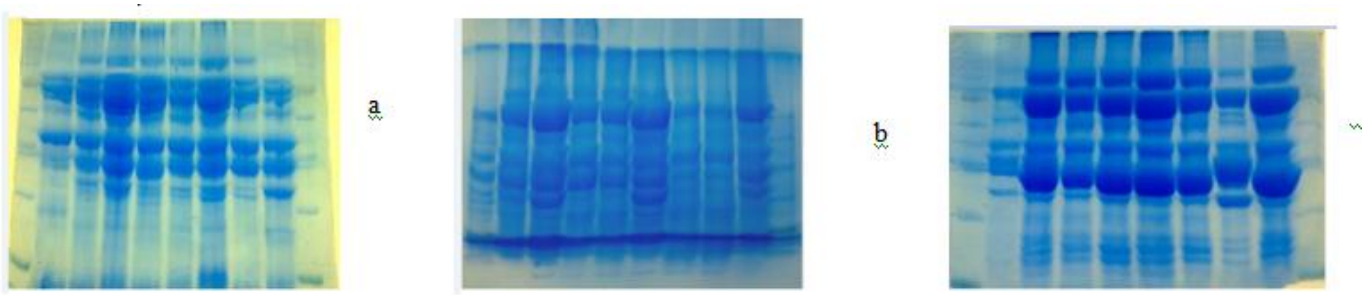


Figure 1
SDS-PAGE profile of (a) P.semesulacatus, (b) P.japonicus and (c) Meta p. monoceros populations saline soluble

Table 3

Average of band frequencies (bf), number of: sample, detected loci, polymorphic, monomorphic loci, polymorphic bands, detected bands and percentage of polymorphic in the applied Shrimp species.

Populations	P. semesulacatus	P. Japonicus	Meta P.Monoceros
No. of sample	8	8	8
Aver. of bf	0.7	0.8	0.61
detected loci	15	10	16
polymorphic loci	9	4	12
monomorphic loci	6	6	4
detected bands	84	64	78
polymorphic bands	34	16	46
%of polymorphism	40.47%	25%	58.97%
Aver. Of similarity	0.87%	0.9%	0.85%

The percentages of polymorphism were ranged from 0% to100%. The percentages of polymorphism using SDS-PAGE markers (34/84=40.47%, 16/64=25% and 46/78=58.97%) were calculated within, P.semisulcatus, P.Japonicus and Meta p. monoceros respectively. These percentages were inferred from data presented in Table (3). The averages of band frequencies were calculated. It ranged from 0.1 to 1, the lowest average of band frequency values was 0.61 in Meta p. monoceros and the highest average of band frequency value was 0.8 in P.Japonicus (Table 3). A maximum of 12 banding were dispersed according to their relative fronts along the gel and the positioned for P. Semesulacatus in 15 loci. Out of these fifteen band loci, six were monomorphic loci while the rest 9 band loci were polymorphic. The average similarity value in P.semesulacatus population was 0.87; while P. Japonicus populations recorded 10 banding as a maximum in 10 loci. Out of these ten band loci, six were monomorphic loci while the rest 4 band loci were polymorphic ones. The average similarity value with P. japonicus population was 0.9 and Meta p. monoceros recorded 11 banding as a maximum in 16 loci. Out of these sixteen band loci, 4 were monomorphic loci while the rest 11 band loci were polymorphic ones. The average similarity value with Meta p. monoceros population was 0.85 (Table3).

Analysis of SDS-PAGE markers

The molecular sizes (bp) of SDS-PAGE marker for the three studied shrimp species were presented in Table (4). P.semesulacatus given common bands at six molecular weight markers (89.172, 75.802, 66.125, 38.265, 30.502, 27.279bp), while P. japonicas given common bands at 552.004, 138.182, 55.674, 38.495, 23.039, 16.626bp) and Meta p. monoceros given common bands at four molecular weight (160.354, 140.365, 93.580, 45.588bp).

Table 4

The common molecular weight (bp) of the detect protein markers in three populations

Populations	P. semesulacatus	P. Japonicus	Meta P. monoceros
common molecular weight	89.172	552.004	160.354
	75.802	138.182	140.365
	66.125	55.674	93.580
	38.265	38.495	45.588
	30.502	23.039	
	27.279	16.626	

Analysis of RAPD markers

A total of 521 RAPD bands were detected and analyzed in the three studied shrimp species. These RAPD bands were divided into 119, 216 and 186 bands in P. semisulcatus, P. japonicus and Meta p. monoceros respectively. The Molecular sizes (bp) of the specific RAPD markers for the three studied shrimp species were presented in Table (5). P. semisulcatus had sixten specific RAPD marker generated by primers A9 (485,359&285bp), A18

(117bp), B1 (173&349bp), B6 (241&440pb), B7 (309bp), B9 (577&351bp), B11 (868bp and C19 (953,706,329&210bp) while P. japonicas had eleven specific RAPD marker generated by primers A10 (302&123bp), A18 (80bp), B1 (173bp), B6 (241&440), B9 (465), B11 (347bp) and C19 (953,272&210bp) and Meta p. monoceros had six specific RAPD marker generated by primers A9 (285.1bp), B1 (173bp), B6 (241bp), B9 (465bp) , C19 (378&210), respectively.

Table 5

The Molecular sizes (bp) of the detected specific DNA bands in the three studied shrimp species.

	P. semesulacatus	**P. Japonicus**	**Meta P. monoceros**
Code of RAPD primers			
A9	485, 359, 285.1	…..	285.1
A10	……	302, 123	……
A18	117	80	……
B1	173, 349	173	173
B6	241, 440	241,440	241
B7	309	…….	……
B9	577,351	465	465
B11	868	347	……
C19	953, 706, 329, 210	953, 272, 210	378, 210
Code of ISSR primers			
HB12	596, 374, 282	596, 374	……..
HB13	182	278	278

The genetic polymorphism within each tested shrimp species based on RAPD technique

The number of detected bands (db) and number of polymorphic bands (pb) in the tested shrimp species were presented in the Table (6).The percentages of polymorphism were ranged from 0% to100%. The percentages of polymorphism using RAPD markers (91/199=45.7%, 141/216=65.3% and 131/186=70.4) as calculated within P.semisulcatus, P.japonicus and Meta p. monoceros populations, respectively (Table6). The averages of band frequencies were calculated. The lowest averages of band frequency were 0.22 (primer A10), 0.28 (primer A10) and 0.21(primer B9) in the three studied species, respectively (Table 6).

Table 6
Average of band frequencies (bf), number of detected bands (db) and number of polymorphic bands (pb) in the studied Shrimp species.

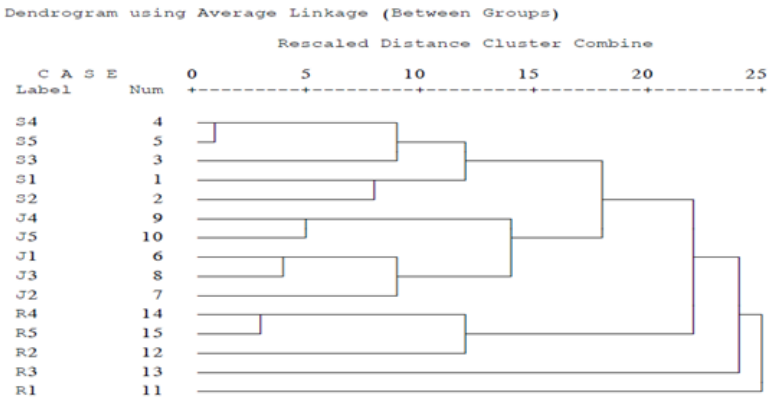
	P. semesulacatus			P. Japonicus			Meta P. monoceros		
Code of RAPD primer	$\bar{b}_f$	db	pb	$\bar{b}_f$	db	pb	$\bar{b}_f$	db	pb
A9	0.5	25	8	0.4	20	19	0.4	20	13
A10	0.22	18	12	0.28	22	9	0.3	23	18
A18	0.26	12	6	0.31	14	8	0.37	17	15
B1	0.31	19	4	0.3	18	10	0.33	20	14
B6	0.53	24	14	0.44	20	9	0.46	21	15
B7	0.37	26	16	0.35	25	22	0.25	18	16
B9	0.24	21	6	0.38	37	31	0.21	19	9
B11	0.31	27	19	0.31	27	17	0.22	19	12
C19	0.36	27	6	0.44	33	16	0.38	29	19
	199	91		216	141		186	131	
Code of ISSR primer	$\bar{b}_f$	db	pb	$\bar{b}_f$	db	pb	$\bar{b}_f$	db	pb
94B	0	9		0.085	9	2	0.11	12	2
98A	0.11	17	6	0.13	20	7	0.96	14	4
98B	0.13	22	13	0.096	14	5	0.084	14	6
44B	0.085	23	6	0.096	26	4	0.088	24	4
HB11	0.31	14	12	0.22	10	5	0.28	13	11
HB12	0.43	29	12	0.38	23	9	0.26	16	14
HB13	0.73	11	6	0.73	11	6	0.8	12	7
	125	55		113	38		105	48	

Genetic similarity and dissimilarity values based on RAPD markers

The genetic similarity and dissimilarity values among the studied shrimp species were calculated and the dendrogram showed phylogenetic relation between the three populations (Figure2). The average of genetic similarity values were 0.58, 0.47 and 0.46 between each pair of the following population (P.semesulacatus & p. japonicus), (P.semesulacatus. & Meta. p. monoceros) and (Meta p.monoceros & P. Japonicas), respectively (Table 7).

Table 7
The average of similarity values between three populations

population	P. semesulacatus	P. japonicas	Meta p.Monoceros
P. semesulacatus	1	0.585	0.471
P. japonicas	0.585	1	0.462
Meta p. monoceros	0.471	0.462	1



When: S= P. semisulcatus, J= P. japonicas and R= Meta p. monoceros

Figure 2
Dendrogram of genetic relationship among relevant populations based on DNA polymorphism

Analysis of ISSR markers

A total of 343 ISSR band were detected and analyzed in the three studied shrimp species. These ISSR bands were divided into 125, 113 and 105 bands in P. semisulcatus, P. japonicus and Meta p. monoceros, respectively. The Molecular sizes (bp) of the specific ISSR markers for the three studied shrimp species were presented in Table (5). P. semisulcatus had four specific ISSR marker generated by primers HB12 (596,374&282bp), HB13 (182bp), while P. japonicas had three specific ISSR marker generated by primers HB12 (596&374bp), HB13 (278bp) and Meta p. monoceros had only one specific ISSR marker around 278bp generated by primer HB13.

The genetic polymorphism within each studied shrimp species based on ISSR technique:

The number of detected bands and number of polymorphic bands in the studied shrimp species were presented in Table (6).The percentages of polymorphism were ranged from 0% to100%. The percentages of polymorphism using ISSR markers (55/125=44%, 38/113=33.6% and 48/105=45.7) were calculated within P.semisulcatus, P.japonicus and Meta p. monoceros, respectively. These percentages were inferred from the data presented in Table (6). The averages of band frequencies were calculated. The lowest averages of band frequency values were 0.085 (primer 94B & 44B), 0.085 (primer 94B) and 0.084 (primer 98B) in P. semisulcatus, P. japonicas and Meta p. monoceros, respectively (Table 6).

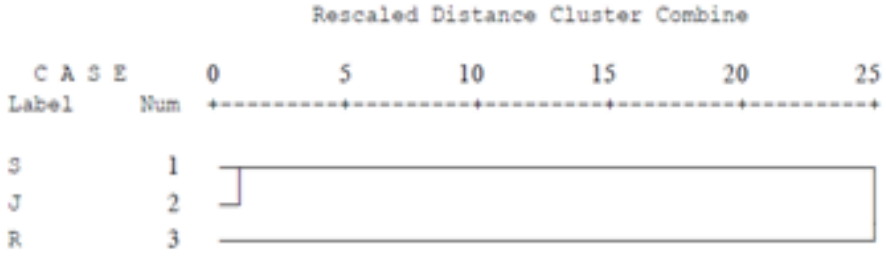
Genetic similarity and dissimilarity values among studied shrimp species based on ISSR markers:

The genetic similarity and dissimilarity values among the studied shrimp species were calculated and the dendrogram showed phylogenetic relation between the three populations (Figure3). The average of genetic similarity values were 0.9, 0.56 and 0.56 between each pair of the following populations (P.semesulacatus & p. japonicus), (P.semesulacatus. & Meta p. monoceros) and (Meta p. monoceros & P. Japonicas), respectively (Table 8).

Table 8

The average of similarity values between P. semesulacatus, P. japonicas and Meta p. monoceros

population	P. semesulacatus	P. japonicas	Meta p.Monoceros
P. semesulacatus	1	0.889	0.556
P. japonicas	0.889	1	0.556
Meta p. monoceros	0.556	0.556	1



When: S= P. semisulcatus, J= P. japonicas and R= Meta p. monoceros

Figure 3

Dendrogram of genetic relationship among relevant population based on ISSR polymorphism

DISCUSSION

In the present study, SDA-PAGE, RAPD and ISSR techniques were used for reconstructing phylogenetic relations among the studied shrimp species because they are attractive, simple, and reliable tool for assessing species characterization on molecular level. In addition, they offered highly reproducible results and abundant polymorphism (Kol L and Lazebny O, 2006; Lalhruaitluanga H. and Prasad MNV, 2009)**.** Understanding the aquatic species (such as shrimp) characterization (Saad et al. 2012)**,** phylogenetic relations (Saad YM et al. 2011) and population structure (Rashed M et al. 2008) of these organisms will provide essential practical guidance to design an innovative breeding program (Rashed MA et al. 2009) for genetic improvement and conservation (Saad YM et al. 2011)**.** Many studies on aquatic species have been carried out on the level of proteins electrophoretic polymorphism at 30 protein loci in 27 wild and cultured populations in two tilapia species O.niloticus, and T.zillii (RoguenX, 1996), plasma and muscle protein patterns were used to assess the intra and the inter population variation and to infer the phylogenetic relationships in catfish (Rashed MA et al. 2000)**.** Alcohol and saline soluble skeletal muscle proteins were involved the study the population structure in some Tilapia species. The study revealed tow T. zilli species protein markers (14.3 and 16.9 KDa), Also detected a high level of homogeneity within studied T. zilli and O. aureus population based on saline and alcohol soluble proteins 0.98, 0.89, 0.95 and 0.98 respectively (Saad YM et al. 2002)**.** The previous studies compatible results of the present study where the average similarity value in P.semesulacatus, P. japonicas and Meta p. monoceros populations were 0.87, 0.9 and 0.85, respectively. Some of the studied loci (in the present study) were informative in detected the genetic variations of the studied shrimp species. Out of 855 estimated bands (512RAPD and 343 ISSR bands) 41 bands are considered as DNA specific markers (Table 5). These markers could be useful in shrimp classifications. In addition, the application of DNA-based genetic analysis as marker-assisted selection in aquatic organisms such as in fish (Kocher TD et al. 1998; Saad YM etal. 2012) and shrimp research for stock development and management is still not fully maximized. So, the detected specific DNA markers (in the present study) will be useful value, especially in breeding programs which use genetic markers as marker-assisted selection to improve the shrimp production. The similarity values among three shrimp species (P.semisulcatus, P. japonicus and Meta p. monoceros) were calculated. The highest similarity was detected between P.semisulcatus and P.japonicusd based on ISSR. In conclusion, Genetic markers should be conducted to provide the information needed for a sound management of farming and the wild shrimp stocks. ISSR and RAPD analysis are confirmed to be reproducible and sensitive tools for shrimp species identification and phylogenetic analysis. The use of ISSR and RAPD DNA markers revealed that the

present study can contribute significantly for the development of shrimp genetic improvement programs and species identifications.

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