Development of ISSR and multiplex ISSR markers for reconstructing phylogenetic relations among some shrimp species

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Abstract: Some Inter-simple sequence repeats (ISSR) and multiplex ISSR markers were developed for reconstructing phylogenetic relations among four shrimp species [*Penaeus* (*Melicertus*) *latisulcatus*, *Penaeus semisulcatus*, *Penaeus monodon* and *Penaeus indicus*]. Some DNA markers were detected as species-specific for the applied shrimp species. The effectiveness of resulted DNA markers were tested for discriminating among the four shrimp species. The percentage of genetic polymorphism within each estimated shrimp species was calculated. Some of the studied loci were informative in detecting the genetic variations in the applied shrimp species. ISSR and multiplex ISSR analysis were an attractive tools for shrimp species identification. *P.indicus* was distantly related from the other estimated shrimp species [*Penaeus* (*Melicertus*) *latisulcatus*, *Penaeus semisulcatus*, *Penaeus monodon*]. This work should be useful to those using PCR technology in both the research Laboratories and farms for producing hybrids and/or local shrimp breeds. Using the developed DNA markers were recommended (when coupled with appropriate statistical analyses) in shrimp species identification, classification and estimation of homogeneity and/or inbreeding levels. ISSR and multiplex ISSR techniques offered low cost and fast analysis for reconstructing phylogenetic relations among the four applied shrimp species.
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Key wards: Shrimp, Molecular, ISSR, Multiplex, Characterizationand Phylogeny.

1. Introduction

Shrimps contribute about 20% by volume of the world seafood market (Bhavan *et al.,* 2010 and Abdel –Salam 2013). The shrimp especially *Penaeus*, are economically important genus around the world.

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 *et al.,* 2004 and Meng *et al.,* 2007), AFLP or Amplified Fragment Length Polymorphism (Moore *et al.,* 1999), RAPD or random amplification of polymorphic DNA (Meng *et al.,* 2004) and analysis of some mitochondrial genes (Cui *et al.,* 2007) have been used to investigate the genetic variability among many shrimp species.

The use of DNA markers can contribute significantly to develop of genetic improvement programs (Rashed *et al.,* 2009 and Saad *et al*., 2011) and species identifications (Moore *et al.,* 1999, Saad *et al.,* 2012 and Saad *et al*., 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 *et al.,* 2012).

To explore the genetic variations within and among four shrimp species (*Penaeus latisulcatus, Penaeus semisulcatus, Penaeus monodon and Penaeus indicus*), ISSR and multiplex ISSR were used. Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction (Henegariu *et al*., 1997).

Relatively little has been published about the important experimental factors and the common difficulties frequently encountered with multiplex PCR in shrimp identification.

In this work, we developed some ISSR and multiplex ISSR markers to investigate the genetic variability and reconstructing phylogenetic relations among some shrimp species (*Penaeus latisulcatus, Penaeus semisulcatus, Penaeus monodon and Penaeus indicus*). The developed DNA markers could provide more convincing data with regard to the genetic diversity, homogeneity and/or inbreeding of the applied shrimp species.

2. Material and Methods

Shrimp [*Penaeus* (*Melicertus*) *latisulcatus, Penaeus semisulcatus, Penaeus monodon and Penaeus indicus*] samples individuals were obtained from project (Inferring of inbreeding and heterozygosity levels in the shrimp genetic resources

in Saudi Arabia based on molecular markers) funded from DSR, King Abdulaziz Univ., KSA during year of (2013). The *Melicertus latisulcatus* is formerly Penaeus *la*tisulcatus.

From each specimen, approximately 0.2g of shrimp muscle tissue was excised, placed in a 70 % isopropanol and held at 4°C for subsequent DNA extraction. DNA of 20 individuals from each estimated shrimp species were extracted as described by (Hills *et al.,* 1996).

ISSR analysis:

Ten ISSR primers (Table 1) were originally selected (Biotechnology Laboratory, University of British Columbia) to measure the genetic variability among the applied shrimp samples.

Table (1): ISSR primer names and sequences.

Code	Sequence	Code	Sequence
HB8	[GA]6GG	17899B	5' [CA]6GG 3'
John	5' [AG]7YC 3'	HB9	[GT]6GG
HB15	5' [GTG]3GC 3'	HB11	5' [GT]6CC 3'
17898A	5' [CA]6AC 3'	HB12	5'[CAC]3GC 3'
17898B	5' [CA]6GT 3'	HB13	5'[GAG]3GC3'

Multiplex ISSR analysis:

From all available ISSR primer combinations (10 X 10), only 10 multiplex primer combinations were succeeded for matching a clear and informative banding patterns.These primer combinations were HB8 /17898A, HB8/17898B, HB12 /17898B, HB11/HB9, HB15/John, 17899B/ John, 17899B/Hb8, 17898B/hb13, 17899B/Hb12 and HB13/17898A.

PCR reaction was prepared in a 10 µl contained a 50 ng of DNA, a 0.3 µM of primer, a 0.2mM of dNTPs, a 25 mM of $MgCl_2$, a 0.5 unit of Taq polymerase and a 1 X buffer. PCR program was consisted of one cycle for 2 min. at 94°C, 35 cycles for (30 sec. at 94°C, 45 sec. at 44°C & 1.5 min. at 72°C) and one cycle for 10 min. at 72°C.The amplification products were size-separated by standard horizontal electrophoresis in 1.5% agarose (Sigma) gels and stained with ethidium bromide (0.3ug/ml), then visually examined with UV trans illuminator and photographed using a CCDcamera (UVP, UK).

Data score and analyzing:

Gel images were analyzed using GelAnalyzer3 software to determine molecular sizes, presence (1) or absence (0). In addition, frequencies and polymorphism type of the amplified fragments, the mean of band frequency and the polymorphism percentage for each primer were calculated.

Data were analyzed as described by **Rashed *et al.*, (2011)** with some modifications. NTSYSpc2.01b and SPSS (10, and 15) software were used to estimate the similarity percentages between the four shrimp species and reconstructing the phylogenetic relationships using Dice coefficients (Dice, 1945).

3. Results

ISSR and multiplex ISSR analysis were used to detect species-specific DNA markers, to examine the genetic polymorphism, and to determine the variability among the four applied shrimp species.

The Molecular sizes (bp) of the specific ISSR and multiplex ISSR markers for the four applied shrimp species were presented in Table (2).

Analysis of ISSR markers:

A total of 207 ISSR band were detected and analyzed in the four applied shrimp species. These ISSR bands were divided into 54, 48, 51 and 54 bands in *Penaeus latisulcatus*, *Penaeus semisulcatus*, *Penaeus monodon* and *Penaeus indicus* respectively.

The Molecular sizes (bp) of the specific ISSR markers for the four applied shrimp species were presented in Table (2). *Penaeus latisulcatus* had only one specific ISSR marker around 578bp (generated by primer 17898B). *Penaeus semisulcatus* had four specific ISSR marker around 140bp, 450bp, 650bp and 652bp generated by John, HB15, 17898B and 17899B respectively. *Penaeus monodon* had five specific ISSR markers generated by primers HB15 (346bp), HB9 (206, 222&234bp) and HB11 (474bp). *Penaeus indicus* had seven specific ISSR markers generated by primers HB18 (200bp), 17898A (106bp), 17898B (190bp), HB12 (272&230bp) and HB13 (116&128bp).

The genetic polymorphism within each applied shrimp species:

The number of detected bands and number of polymorphic bands in the applied shrimp species were presented in Table (3).The percentages of

polymorphism were ranged from 0% to100%. The percentages of polymorphism using ISSR markers (13/54=24%, 14/48=29%, 13/51=25.4 and 10/54=18.5%) were calculated within *P.latisulcatus, P.semisulcatus, P.monodon and P.indicus* respectively. These percentages were inferred from data presented in Table (3).

The average of band frequencies were calculated. It ranged from 0.56 to 1 (monomorphic pattern). The lowest averages of band frequency values were 0.58 (primer John), 0.56 (primer HB12), 0.65 (primer 17898A) and 0.6 (primer 17898A) in *Penaeus latisulcatus*, *Penaeus semisulcatus*, *Penaeus monodon* and *Penaeus indicus* respectively (Table 3).

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

The genetic similarity and dissimilarity values among the applied shrimp species were calculated. The genetic similarity values were 0.673, 0.655, 0.650, 0.760, 0.502 and 0.556 between (*P.latisulcatus* and *P.semisulcatus*), (*P.latisulcatus* and *P.monodon*), (*P.latisulcatus* and *P.indicus*), (*P.semisulcatus and P.monodon), (P.semisulcatus* and *P.indicus)and* (*P.monodon* and *P.indicus*) shrimp pairs, respectively (Table 4).

Analysis of multiplex ISSR markers:

A total of 205 multiplex ISSR band were detected and analyzed in the four applied shrimp species. These bands were divided into 54, 58, 45 and 48 bands in *Penaeus latisulcatus*, *Penaeus semisulcatus*, *Penaeus monodon* and *Penaeus indicus* respectively.

The Molecular sizes (bp) of the specific multiplex ISSR markers for the four applied shrimp species were presented in Table (2). *Penaeus latisulcatus* had two specific multiplex ISSR marker generated by HB8/17899B (450bp) and 17898B/Hb13 (296 bp). *Penaeus monodon* had three specific multiplex ISSR markers generated by HB11/ HB9 (64bp) and HB8/17899B (640 & 428bp). *Penaeus indicus* had one specific multiplex ISSR markers generated by primers HB15/John (140bp).

The genetic polymorphism within each applied shrimp species:

The percentages of polymorphism were ranged from 0% to100%. The percentages of polymorphism using multiplex ISSR markers (19/54=35.18%, 26/58=44.82%, 3/45=6.6% and 15/48=31.25%) were inferred within *P.latisulcatus, P.semisulcatus, P.monodon and P.indicus* respectively (Table 3).

The average of band frequencies were calculated. It ranged from 0.52 to 1 (monomorphic pattern). The lowest average of band frequency values were calculated in the applied shrimp species. It were 0.6, 0.52, 0.65 and 0.54 in *Penaeus latisulcatus*, *Penaeus semisulcatus*, *Penaeus monodon* and *Penaeus indicus* respectively (Table 3).

Table (2): The Molecular sizes (bp) of the detected specific DNA markers in the four studied shrimp species.

species	*P. latisulcatus*	*P. semisulcatus*	*P. monodon*	*P. indicus*
ISSR code				
HB8	-	-	-	200bp
John	-	140bp	-	-
HB15	-	450bp	346 bp	-
17898A	-	-	-	106 bp
17898B	578bp	650 bp		190 bp
17899B	-	652bp	-	-
HB9	-	-	206, 222&234bp	-
HB11	-	-	474 bp	-
HB12	-	-	-	272&230bp
HB13				116&128bp
Multiplex ISSR code				
HB11/ HB9	-	-	64 bp	-
HB8/17899B	450bp	-	640&428bp	-
HB15/John	-	-	-	140bp
17898B/HB13	296 bp	-	-	-

Table (3): Average of band frequencies, standard deviation, number of detected bands and number of polymorphic bands in the applied Shrimp species.

species	*P.latisulcatus*				*P.semisulcatus*				*P.monodon*				*P.indicus*			
	xbf	sd	db	pb	xbf	sd	db	pb	xbf	sd	db	pb	xbf	sd	db	pb
ISSR primer																
HB8	1	0	2	0	1	0	4	0	0.92	0.18	5	1	1	0	3	0
HB15	0.95	0.1	4	1	0.9	0.24	6	1	1	0	5	0	0.88	0.27	5	1
17899B	1	0	2	0	0.68	0.33	5	3	1	0	2	0	1	0	4	0
HB13	1	0	8	0	0.87	0.33	6	1	0.73	0.41	6	2	1	0	9	0
HB12	0.84	0.22	9	4	0.56	0.39	10	6	0.73	0.27	11	7	0.85	0.24	12	5
HB11	1	0	6	0	0.83	0.29	7	2	1	0	8	0	1	0	4	0
17898A	0.8	0.4	4	1	1	0	2	0	0.65	0.34	4	3	0.6	0.28	5	4
HB9	1	0	5	0	1	0	3	0	1	0	7	0	1	0	3	0
17898B	0.9	0.24	6	1	0.85	0.3	4	1	1	0	2	0	1	0	2	0
John	0.58	0.27	8	6	1	0	1	0	1	0	1	0	1	0	7	0
Total			54	13			48	14			51	13			54	10
	abf	Sd	db	pb	xbf	sd	db	pb	xbf	sd	db	pb	xbf	sd	db	pb
Multiplex																
HB8 /17898 A	0.71	0.36	7	3	0.52	0.36	10	7	0.88	0.27	5	0	0.8	0.33	6	2
HB8/17898B	1	0	5	0	0.6	0.46	4	2	1	0	6	0	0.93	0.12	3	1
HB12 /17898B	0.67	0.33	6	4	0.7	0.24	6	4	1	0	4	0	0.77	0.27	7	4
HB11/HB9	0.9	0.24	6	1	1	0	5	0	1	0	6	0	1	0	5	0
HB15/John	0.69	0.4	7	3	0.7	0.35	6	3	0.65	0.41	4	2	0.86	0.3	7	2
17899B/ John	0.6	0.57	2	1	0.7	0.38	4	2	1	0	1	0	1	0	3	0
17899B/Hb8	0.85	0.3	4	1	0.8	0.4	4	1	1	0	4	0	1	0	2	0
17898B/hb13	0.88	0.18	5	2	0.76	0.36	5	2	1	0	3	0	1	0	4	0
17899B/Hb12	0.78	0.29	9	4	0.87	0.2	9	3	0.93	0.2	9	1	0.54	0.25	7	6
HB13/17898A	1	0	3	0	0.72	0.39	5	2	1	0	3	0	1	0	4	0
Total			54	19			58	26			45	3			48	15

abf= Average of band frequency, Sd= Standard deviation, db= Number of detected bands and pb= Number of polymorphic bands

Table (4): Similarity values among applied Shrimp species based on ISSR (below diagonal) and dissimilarity values (above diagonal).

species	*P.latisulcatus*	*P.semisulcatus*	*P.monodon*	*P.indicus*
P.latisulcatus		0.327	0.345	0.35
P.semisulcatus	0.673		0.24	0.498
P.monodon	0.655	0.760		0.444
P.indicus	0.650	0.502	0.556	

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

The genetic similarity and dissimilarity values among the applied shrimp species were calculated. The genetic similarity values were 0.771, 0.766, 0.694, 0.760, 0.718 and 0.649 between (*P.latisulcatus* and *P.semisulcatus*), (*P.latisulcatus* and *P.monodon*), (*P.latisulcatus* and *P.indicus*), (*P.semisulcatus and P.monodon), (P.semisulcatus* and *P.indicus) and* (*P.monodon* and *P.indicus*) shrimp pairs, respectively (Table 5).

Table (5): Similarity values among applied Shrimp species based on multiplex ISSR (below diagonal) and dissimilarity values (above diagonal).

Species	*P.latisulcatus*	*P.semisulcatus*	*P.monodon*	*P.indicus*
P.latisulcatus		0.229	0.234	0.306
P.semisulcatus	0.771		0.24	0.282
P.monodon	0.766	0.760		0.351
P.indicus	0.694	0.718	0.649	

The homogeneity, genetic similarity and dissimilarity values based on combined data for the applied shrimp species:

The genetic similarity and dissimilarity values among the applied shrimp species were calculated. The genetic similarity values were 0.729, 0.717, 0.674, 0.760, 0.621 and 0.606 between (*P.latisulcatus* and *P.semisulcatus*), (*P.latisulcatus* and *P.monodon*), (*P.latisulcatus* and *P.indicus*), (*P.semisulcatus and P.monodon), (P.semisulcatus* and *P.indicus)* and (*P.monodon* and *P.indicus*) shrimp pairs, respectively (Table 6). The homogeneity value within each applied shrimp species was estimated. The averages of these values were presented in diagonal of Table (6).

Table (6): Similarity (below diagonal), dissimilarity (above diagonal) and homogeneity values (diagonal) based on combined ISSR and multiplex ISSR data for the applied shrimp species:

Species	*P.latisulcatus*	*P.semisulcatus*	*P.monodon*	*P.indicus*
P.latisulcatus	0. 91±0.04	0.271	0.283	0.326
P.semisulcatus	0.729	0.86±0.047	0.24	0.379
P.monodon	0.717	0.760	0.96±0.009	0.394
P.indicus	0.674	0.621	0.606	0.94±0.013

Values among applied Shrimp species based on ISSR and Multiplex ISSRcombined data and average of similarity values within each studied shrimp species.

The phylogenetic relationships among the applied shrimp species

The phylogenetic relationships among the applied shrimp species based on ISSR, multiplex ISSR polymorphism and combined data was presented in Figure (1a, b and c) respectively. *P.indicus* was distantly related from the other estimated shrimp species (*P.latisulcatus, P.semisulcatus*and *P.monodon*). The distance values among these three shrimp species were relatively low. The lowest distance were detected between *P.monodon* and *P.semisulcatus* based on ISSR, multiplex ISSR polymorphism and combined data (Table 6 and Figure 1).

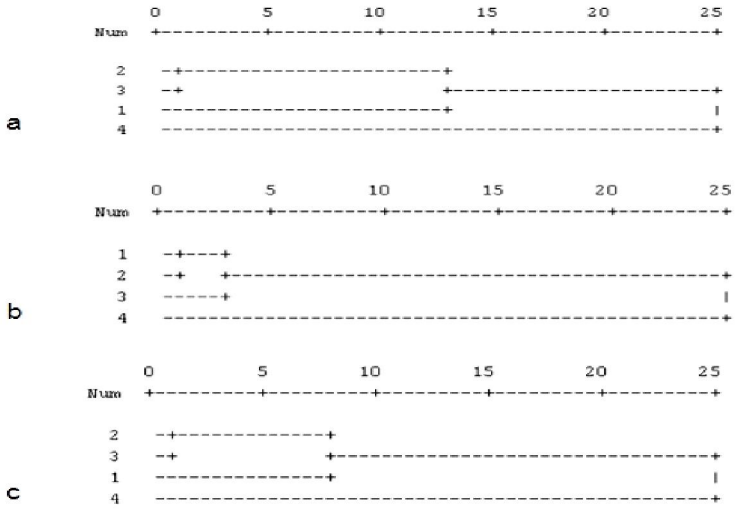

Figure 1. Phylogenetic relations among the applied shrimp species based on ISSR (a), Multiplex ISSR (b) and combined data analysis (c).1= *P.* (*Melicertus*) *latisulcatus*, 2=*P.semisulcatus*, 3= *P.monodon,* and 4= *P.indicus.*

4. Discussion:

In the present study, ISSR and multiplex ISSR techniques were used for reconstructing phylogenetic relations among the applied shrimp species because they are attractive, simple, and reliable tool for assessing species characterization based on molecular level. In addition, they offered highly reproducible results and abundant polymorphism (Tong *et al*., 2005, Kol and Lazebny, 2006, Lalhruaitluanga and Prasad, 2009 and Saad *et al*., 2012).

Understanding the aquatic species (such as shrimp) characterization (Saad *et al*., 2012), phylogenetic relations (Saad *et al*., 2011) and

population structure (Rashed *et al*., 2008) of these organisms will provide essential practical guidance to design an innovative breeding program (Rashed *et al.,* 2009) for genetic improvement and conservation (Saad *et al*., 2011).

The potential applications of ISSR (Chunjiang *et al*., 2005) and multiplex PCR (Henegariu *et al*., 1997) analysis for diverse aims is depend on the variety and frequencies of microsatellites within the specific genomes. The multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory (Henegariu *et al.,* 1997).

Some of the studied loci (in the present study) were informative in detecting the genetic variations of the applied shrimp species. Out of 412 estimated bands (207ISSR and 205 multiplex ISSR bands) 23 bands are considered as DNA specific markers (Table 2). 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 *et al*., 1998 , Rashed *et al.,* 2009 and Saad *et al.,* 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 performance.

In the present study, the average of band frequencies (based on ISSR analysis) were ranged from 0.56 (polymorphic pattern) to 1 (monomorphic pattern). The lowest average of band frequency values were calculated in the applied shrimp species. It were 0.58, 0.56, 0.65 and 0.6 in *Penaeus latisulcatus*, *Penaeus semisulcatus*, *Penaeus monodon* and *Penaeus indicus* respectively. Most average of band frequency values that revealed from ISSR and multiplex ISSR analysis equal (1). These values reflect the homogeneity levels within the applied shrimp samples especially as calculated in *P. indicus* and *P.monodon* samples.

ISSR analysis showed that, the HB12 was polymorphic in all studied shrimp species. On the other hand, multiplex ISSR analysis showed that, the primer combinations HB8/17898A, HB15/John and 17899B/HB12 were polymorphic in all estimated shrimp species. So, using the primer of these loci are recommended to reflect the genetic background in other shrimp species and subspecies in the future.

The homogeneity values within both *P.monodon* and *P.indicus* were relatively high. These values reflect the inbreeding levels within both of them. *P.monodon* and *P.indicus* are the most popular farmed species especially in Asian farms.

In India, *P. indicus* farming has had to take a back seat due to farmers' preference for *P. monodon*. Currently *P. indicus* is mainly cultured in Saudi Arabia (Abdel –Salam 2013) due to salinity resistance of this economic species.

The distance values among three shrimp species (*P.latisulcatus, P.semisulcatus* and *P.monodon*) were relatively low. The lowest distance were detected between *P.monodon* and *P.semisulcatus* based on ISSR, Multiplex ISSR polymorphism and combined data.

In conclusion, Genetic markers should be conducted to provide the information needed for a sound management of farming and wild shrimp stocks.

ISSR and multiplex ISSR analysis are confirmed to be a reproducible and sensitive tool for the study shrimp species identification and phylogenetic analysis.

The use of ISSR and multiplex ISSR DNA markers revealed from the present study can contribute significantly for development of shrimp genetic improvement programs and species identifications.

Using the developed DNA markers were recommended (when coupled with appropriate statistical analyses) in shrimp species identification, classification and estimation of homogeneity and/or inbreeding levels.

ISSR and multiplex ISSR techniques offered low cost and fast analysis for reconstructing phylogenetic relations among the four applied shrimp species.

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