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Original Article

Evaluation of 5.8S rRNA to identify *Penaeus semisulcatus* and its subspecies, *Penaeus semisulcatus persicus* (Penaeidae) and some Decapoda species

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Abstract: The green tiger prawn, *Penaeus semisulcatus* is one of the most important members of the family Penaeidae in the Persian Gulf. Based on the morphological characteristics, two groups, including *P. semisulcatus* and its subspecies viz. *P. s. persicus* are recognized. This study was conducted to investigate the genetic distance between *P. semisulcatus* and *P. s. persicus* by analyzing partial sequence of 5.8S rRNA. Another objective of this study is to evaluate the ability of 5.8S rRNA to identify the species of Decapoda. The results indicated that the 5.8S rRNA gene of both *P. semisulcatus* and *P. s. persicus* were exactly identical, and sequence variation was not observed. The results also indicated that 5.8S rRNA sequences between species of the same genus of analysed species of Decapoda are conserved, and no genetic distance was observed in species level. The low evolutionary rate and efficient conservation of the 5.8S rRNA can be attributed to its role in the translation process.

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Introduction

Penaeid shrimps are the most important economic resource in the world's crustacean fishery industry (Voloch et al., 2005; Holthuis, 1980; Dall et al., 1990). The genus *Penaeus* has 27 species (Holthuis, 1980) and among them, the green tiger shrimp (*Penaeus semisulcatus*) is included more than 90% of shrimp fishing in the Persian Gulf (Hosseini et al., 2004). Based on the morphological characteristics, two groups of *P. semisulcatus* are distinguished in the Persian Gulf. The first group (I) is characterized by a reddish body color with deep red or brown transverse bands, and cream and brown striped color of the whip antenna. Second group (II) i.e. subspecies of *P. s. persicus*, is characterized by a creamy pink body color without distinct transverse stripes, and its whip antenna has a cream color without stripes (Rahnama et al., 2010). The group I is the main species in the coast of Hormozgan Province but group II is found in the coast of Bushehr Province. The subspecies of *P. s. persicus* has been described based on the carapace morphology and protein electrophoresis patterns (Matinfar, 1999). The identification of shrimps traditionally are relied on morphometric analysis; however, it is well-known that such characteristics are influenced by environmental conditions (Bowman et al., 1982).

To overcome this problem, molecular markers e.g. nuclear and mitochondrial DNA have been developed in the past two decades for study of the phylogenetic relationship and genetic diversity of such an aquatic organism (Ferguson and Danzmann, 1998; Liu and Cordes, 2004; Chauhan and Rajiv, 2010; Askary et al., 2013). Both nuclear and mitochondrial sequences are used for species identification and genetic diversity evaluation. The mitochondrial genes of 16S rRNA and subunit I of cytochrome oxidase (COI) were extensively used for

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molecular study of the crustaceans especially shrimps of the family Penaeidae (Lavery et al., 2004; Chan et al., 2008; Nayak and Umadevi, 2012). Of the nuclear genes, 28S ribosomal RNA, 18S rRNA, 5.8S rRNA, phosphoenolpyruvate carboxy kinase and sodium-potassium ATPase α-subunit have been considered for the study of the phylogenetic relationships among shrimp (Porter et al., 2005; Calomata et al., 2009; Ma et al., 2009).

The molecular comparison of *P. semisulcatus* in Persian Gulf with its subspecies, *P. s. persicus* using mitochondrial 16S rRNA showed a significant difference (Rahnema et al., 2010). The genetic distance between them, based on a 561 bp section of the mitochondrial 16S rRNA was calculated as 3.3% (Rahnema et al., 2010). The high mutation rate of the mitochondrial DNA limits its utility in the phylogenetics of deep divergences. Furthermore, the highly A/T-biased mitochondrial DNA, especially at the third codon position of the protein coding genes, suffers from high levels of homoplasy and thus exhibits strong negative effects in phylogenetic analyses (Chu et al., 2009). Therefore, it is necessary to evaluate the genetic distance between *P. semisulcatus* and the subspecies of *P. s. persicus* based on a proper and robust molecular marker. Of the nuclear marker, the 5.8S rRNA is considered for the study of the phylogenetic relationships among shrimps (Calomata et al., 2009) and other organisms (Gulling and Voglers, 1998). Hence, in the present study, we investigated the genetic distance between *P. semisulcatus* and *P. s. persicus* by analyzing partial sequence of 5.8S rRNA. Another objective of this study is to evaluate the ability of 5.8S rRNA to identify the different species of Decapoda.

Materials and methods

Sample collection and genomic DNA extraction: 10 and 8 specimens of *P. semisulcatus* and *P. s. persicus* were collected from Bushehr and Dayyer (Bushehr Province, South of Iran), respectively. Total genomic DNA was extracted from 100-150 mg muscle tissue from the ethanol-preserved samples according to Brandfass and Karlovsky (2008).

DNA amplification: The partial sequence of 5.8S rRNA and complete sequence of internal transcribed spacer-II were amplified using ITS2F (5' GATCACTTGGCTCGTGCGTC 3') and ITS2R (5' GCTCTTCCCGTTTCGGTCGC 3') primers. These primers have been designed based on 5.8S rRNA sequence of *P. merguiensis* (AY331590) and *P. vannamei* (AF 124597) and 28S rRNA of *P. semisulcatus* (DQ079809) and *P. vannamei* (AF 124597), respectively. In fact, the ITS2F was designed to amplify of a 150 bp (93%) fragment of 3' end of 5.8S rRNA. In the first stage, PCR conditions were optimized using DMSO (0, 2, 4 and 6%) and annealing temperature gradient. Polymerase chain reactions (PCRs) were performed in 50 µl volume, containing 5 µl Mg^{2+} free-PCR buffer (10X), 3 mM $MgCl_2$, 0.4 mM of dNTP mix, 20 pico mole of each primer, 2.5 U Taq DNA polymerase (Fermentas), 200-400 nanogram DNA, and ddH_2O. The PCR reaction was carried out according to the following thermal program: 4 min at 94°C for initial denaturation, followed by 30 cycle with 94°C for 1 min, 59°C for 45 sec and 72°C for 1.5 min. The final extension was at 72ºC for 5 min. The negative control reactions were also used. The size and quality of PCR products were visualized on 1% agarose gel.

DNA sequencing and analysis: Three samples of *P. semisulcatus* (Sem3, Sem4 and Sem6) and three samples of *P. s. persicus* (Per3, Per4 and Per6) were selected for sequencing. PCR products were sequenced in both strands, using the same primer pairs for PCR. The sequencing was performed using ABI 370 automated sequencer (Seq/Teqh/California, USA). Chromatograms of each of the forward and reverse sequences were checked using ChromasPro and Chromas LITE softwares (Technelysium Pty Ltd, Australia). The sequences confirmation and homologies were searched using Blast (NCBI). The sequences were aligned using the multiple–alignment program ClustalW2 (Larkin et al., 2007). Base composition was calculated using MEGA6 software (Tamura et al., 2013). The sequences distance matrix was calculated using Kimura 2-Parameter (K2P) (Kimura, 1980) and subjected to

Table 1. Accession numbers of the materials of decapod species retrieved from GenBank database.

Taxonomic designation	Abbreviation	Accession number	Region used
Penaeus vannamei	P.vann	AF124597	853-967
Macrbrchium rosenbergii	M.ros	HM804252	1180-1294
Macrbrchium nipponense	M.nipp	GQ369796	1519-1633
Exopalaemon carinicauda	E.car	GQ369794	469-583
Exopalaemon cf. modestus	E.cfmod	GQ369793	685-799
Pandalus goniurus	Pa.gon	EF035129	450-564
Pandalus hypsinotus	Pa.hip	AB193480	970-1021
Pandalus eous	Pa.eou	AB193477	790-904
Eriocheir japonica	E.jap	AF316381	382-496
Eriocheir leptognathus	E.lept	AF316385	385-499
Eriocheir formosa	E.for	AF316375	389-503
Epilobocera sinuatifrons	Ep.sin	FN395447	616-779
Sesarma meridies	S.mer	FN396099	457-571
Sesarma dolphinum	S.dol	FN396039	468-582
Chionoecetes japonicus	Ch.jap	HQ909101	866-980
Chionoecetes opili	Ch.opi	HQ909100	942-1056

Table 2. Maximum composite likelihood estimation of the pattern of nucleotide substitution.

	A	T	C	G
A	-	*5.46*	*7.51*	**8.81**
T	*5.99*	-	**18.41**	*6.92*
C	*5.99*	**13.38**	-	*6.92*
G	**7.63**	*5.46*	*7.51*	-

Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics.

the construction of neighbor-joining tree with 1000 bootstrap replicates (Tamura et al., 2013). Other conditions, for calculation of the genetic distance and tree construction include: transition and transversion substitutions, uniform rate among sites, homogeneous (same) pattern among lineages and complete deletion.

For phylogenetic evaluation, the 5.8S rRNA of other available species of the decapoda were retrieved from GenBank database following Blast search. At least two species of each genus were selected (Table 1). Then, their 114 bp of 5.8S rRNA 3' end were selected for phylogenetic reconstruction. All molecular analyses include sequence alignment, nucleotide composition, the pattern of nucleotide substitution, pairwise sequence distance and phylogenetic tree were conducted in MEGA6 (Tamura et al., 2013).

Results and Discussion

The nuclear DNA marker has been widely recruited for studies of phylogenetic relationship of crustacean (Ahyong et al., 2007; Liu and Cordes, 2004; Porter et al. 2005). The DNA-based nuclear molecular markers can be classified into two types, the nuclear ribosomal RNA (rRNA or rDNA) genes and protein –coding genes (Ma et al., 2009; Tsang et al., 2008; Blanck et al., 2013). The nuclear ribosomal DNA has three rRNA genes (5.8S, 18S and 28S rRNA) and two internal transcribed spacers (ITS-I and ITS-II). The ITS-I and ITS-II are located between 18S and 5.8S rRNA and 28S rRNA, respectively (Gillespie et al., 2006).

Analysis of 5.8S rRNA between P. semisulcatus and P. semisulcatus persicus: The 5.8S rRNA section of PCR products were well-sequenced using ITS2F primer pairs, and reverse sequencing was failed. Therefore, only 114 bp section of the 3' end of 5.8S rRNA, corresponds to more than 70% of the 5.8S rRNA, was obtained from 6 studied samples. The base composition of the 5.8S rRNA fragment of *P. semisulcatus* and *P. s. persicus* samples was as

Table 3. The 5.8S rRNA gene distance among some decapoda species analyzed by pairwise distance calculation using Kimura two-parameter model. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. Sem3		0.000	0.000	0.000	0.000	0.000	0.000	0.119	0.119	0.132	0.132	0.106	0.106	0.106	0.101	0.101	0.101	0.101	0.104	0.104	0.093	0.090
2. Sem4	0.000		0.000	0.000	0.000	0.000	0.000	0.119	0.119	0.132	0.132	0.106	0.106	0.106	0.101	0.101	0.101	0.101	0.104	0.104	0.093	0.090
3. Sem6	0.000	0.000		0.000	0.000	0.000	0.000	0.119	0.119	0.132	0.132	0.106	0.106	0.106	0.101	0.101	0.101	0.101	0.104	0.104	0.093	0.090
4. Per3	0.000	0.000	0.000		0.000	0.000	0.000	0.119	0.119	0.132	0.132	0.106	0.106	0.106	0.101	0.101	0.101	0.101	0.104	0.104	0.093	0.090
5. Per4	0.000	0.000	0.000	0.000		0.000	0.000	0.119	0.119	0.132	0.132	0.106	0.106	0.106	0.101	0.101	0.101	0.101	0.104	0.104	0.093	0.090
6. Per6	0.000	0.000	0.000	0.000	0.000		0.000	0.119	0.119	0.132	0.132	0.106	0.106	0.106	0.101	0.101	0.101	0.101	0.104	0.104	0.093	0.090
7. P.vann	0.000	0.000	0.000	0.000	0.000	0.000		0.119	0.119	0.132	0.132	0.106	0.106	0.106	0.101	0.101	0.101	0.101	0.104	0.104	0.093	0.090
8. M.ros	0.639	0.639	0.639	0.639	0.639	0.639	0.639		0.000	0.022	0.022	0.028	0.028	0.028	0.058	0.058	0.058	0.058	0.060	0.060	0.062	0.065
9. M.nip	0.639	0.639	0.639	0.639	0.639	0.639	0.639	0.000		0.022	0.022	0.028	0.028	0.028	0.058	0.058	0.058	0.058	0.060	0.060	0.062	0.065
10. E.car	0.707	0.707	0.707	0.707	0.707	0.707	0.707	0.049	0.049		0.000	0.038	0.038	0.038	0.060	0.060	0.060	0.060	0.062	0.062	0.064	0.067
11. E.cfmod	0.707	0.707	0.707	0.707	0.707	0.707	0.707	0.049	0.049	0.000		0.038	0.038	0.038	0.060	0.060	0.060	0.060	0.062	0.062	0.064	0.067
12. Pan.gon	0.572	0.572	0.572	0.572	0.572	0.572	0.572	0.080	0.080	0.136	0.136		0.000	0.000	0.056	0.056	0.056	0.056	0.058	0.058	0.059	0.062
13. Pan.hyp	0.572	0.572	0.572	0.572	0.572	0.572	0.572	0.080	0.080	0.136	0.136	0.000		0.000	0.056	0.056	0.056	0.056	0.058	0.058	0.059	0.062
14. Pan.eous	0.572	0.572	0.572	0.572	0.572	0.572	0.572	0.080	0.080	0.136	0.136	0.000	0.000		0.056	0.056	0.056	0.056	0.058	0.058	0.059	0.062
15. Er.lept	0.553	0.553	0.553	0.553	0.553	0.553	0.553	0.251	0.251	0.276	0.276	0.237	0.237	0.237		0.000	0.000	0.000	0.010	0.010	0.017	0.019
16. Er.jap	0.553	0.553	0.553	0.553	0.553	0.553	0.553	0.251	0.251	0.276	0.276	0.237	0.237	0.237	0.000		0.000	0.000	0.010	0.010	0.017	0.019
17. Er.for	0.553	0.553	0.553	0.553	0.553	0.553	0.553	0.251	0.251	0.276	0.276	0.237	0.237	0.237	0.000	0.000		0.000	0.010	0.010	0.017	0.019
18. Ep.sin	0.553	0.553	0.553	0.553	0.553	0.553	0.553	0.251	0.251	0.276	0.276	0.237	0.237	0.237	0.000	0.000	0.000		0.010	0.010	0.017	0.019
19. S.dol	0.573	0.573	0.573	0.573	0.573	0.573	0.573	0.264	0.264	0.290	0.290	0.250	0.250	0.250	0.010	0.010	0.010	0.010		0.000	0.020	0.022
20. S.mer	0.573	0.573	0.573	0.573	0.573	0.573	0.573	0.264	0.264	0.290	0.290	0.250	0.250	0.250	0.010	0.010	0.010	0.010	0.000		0.020	0.022
21. Ch.jap	0.515	0.515	0.515	0.515	0.515	0.515	0.515	0.277	0.277	0.303	0.303	0.263	0.263	0.263	0.029	0.029	0.029	0.029	0.039	0.039		0.010
22. Ch.opi	0.495	0.495	0.495	0.495	0.495	0.495	0.495	0.293	0.293	0.319	0.319	0.277	0.277	0.277	0.039	0.039	0.039	0.039	0.049	0.049	0.010	

Figure 1. Alignment of *Penaeus senisulcatus*, and its subspecies based on a 114 pb fragment of 5.8S rRNA (Sem3, 4 and 6= *P. senisulcatus* and Per3, 4 and 6= *P. s. persicus*).

follows: A:17.5, T:24.6, G:26.3 and C:31.6. The multiple sequence alignment (Fig. 1) indicated that the 5.8S rRNA gene of both studied taxa were exactly identical, and no variation was observed.

Analysis of 5.8S rRNA variation among Decapoda: The pattern of nucleotide substitution between analyzed decapoda is shown in Table 2. The rate of substitution of Thymine by Cytosine was 18.41%. The nucleotide frequencies were 23.16% (A), 21.08% (T/U), 29.00% (C), and 26.75% (G). The transition/transversion rate ratios were k_1 = 1.273 (purines) and k_2 = 2.453 (pyrimidines). The overall transition/transversion bias is R = 0.915, where R = [A*G*k_1 + T*C*k_2]/[(A+G)*(T+C)]. The alignment of 5.8S rRNA resulted 129 sites. The aligned sequences showed that the GC content was more than AT content (50.4% to 57.9% in *Pandalus* and *Penaeus*, respectively) and GC content average was calculated as 55.7%. The 129 sites of the 5.8S rRNA gene were containing 74 conserved and 55 variable and parsimony informative sites. The average distance between all taxa was 0.339 and ranged from 0.00 between the species of one genus to 0.707 between *Penaeus* and *Exopalaemon* (Table 3).

The phylogenetic tree was inferred using the neighbor-joining method (Saitou and Nei, 1987) in

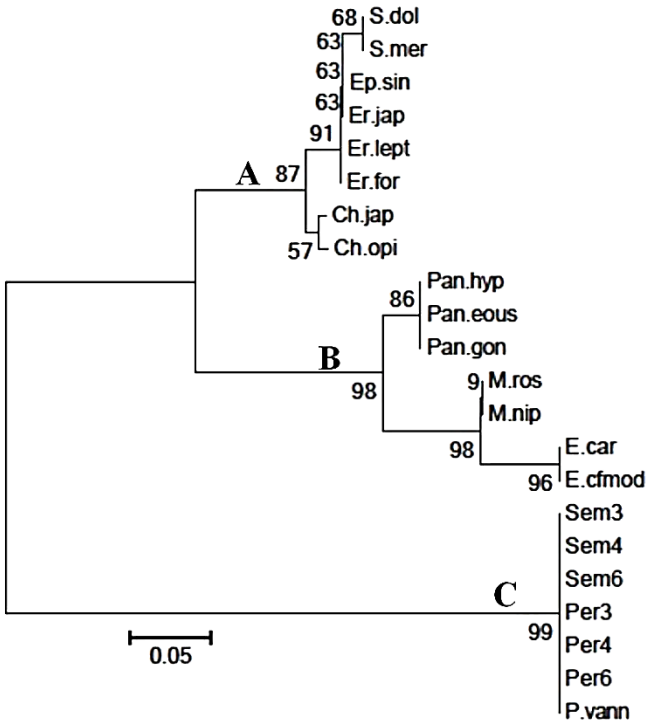

Figure 2. Topologies resulting from the neighbor-joining analysis of the nucleotide sequences of the 114 bp 5.8S RRNA genes in all species of the Decapoda (Numbers above and below branches indicate bootstrap values from NJ analysis. A, B and C refer to three main clades in the tree belongings to Brachyura, Caridea and Penaeadea infraorder, respectively).

MEGA6 software (Tamura et al., 2013) based on K2P (Fig. 2). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Fleckenstein, 1985). The same tree topology was obtained by minimum evolution, UPGMA and maximum likelihood (Tamura et al., 2013). The results of molecular analyses indicated that except for *Chionoecetes*, no genetic variation observed between species of same genus among the studied members of Decapoda.

The constructed neighbor-joining tree separated taxa into three major clades, including clade A consisting *Sesarma* (Sesramidea), *Epilobocera* (Pseudothelphusidae), *Eriocheir* (Varunidae) and *Chionocetes* (Oregoniidae), clade B consists of *Pandalus* (Pandalidae), *Macrobrachium* and *Exopalaemon* (Palaemonidae) and clade C consists of *Penaeus* (Penaeidae). The results indicated that clades A and B are closer to each other than to clade C. This result predictable, because, the members of the clades A and B taxa belong to suborder Pleocyemata and clades C to suborder Dendrobranchiata.

The divergences within clades A, B, and C were 0.0-0.039, 0.0-0.136 and 0.0, respectively. The highest genetic diversity was estimated between the genus *Penaeus* and *Exopalaemon* (Table 3), and the average distance between all taxa was 0.339. The maximum genetic distance (0.515) between the genus *Penaeus* and *exopalaemon*, and minimum genetic distance (0.00) between the genus *Eriocheir* and *Epilobacera* were calculated, and the average genetic distance was calculated to be 0.185.

When the full length of 5.8S rRNA was used, compared with 114 bp section of the 3' end of 5.8S rRNA gene, genetic diversity is decreased. This suggests that 5.8S rRNA 5' end, compared with 3' end, is the most conserved. Similar to other nuclear genes, the 5.8S, 18S and 28S rRNA genes evolve relatively slowly and are useful in addressing broad phylogenetic hypotheses involving a broad range of organisms i.e. a high level taxonomy (Gulling and Voglar, 1998). Molecular studies using nuclear protein-coding genes indicated that they are highly informative for phylogeny estimation across all taxonomic levels of Decapoda (Chu et al., 2009).

In addition, this study suggests that evolutionary rate of protein-coding genes are more than rRNA genes. This phenomenon could be due to the fundamental role played by rRNA in translation. The 5.8S rRNA plays an important role in mRNA translation (Elela and Nazar, 1997; Graifer et al., 2005). Studies on the inhibition of protein synthesis by specific anti 5.8S rRNA oligonucleotides have suggested that 5.8S rRNA plays an important role in eukaryotic ribosome function (Elela and Nazar, 1997).

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چکیده فارسی

ارزیابی ژن 5.8S rRNA در تشخیص میگوی سبز ببری *Penaeus semisulcatus* و زیرگونه آن، *Penaeus semisulcatus persicus* (Penaeidae) و برخی گونه‌های Decapoda

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چکیده:

میگوی سبز ببری، *Penaeus semisulcatus* یکی از اعضای بسیار مهم خانواده Penaeidae در خلیج فارس است. این مطالعه برای بررسی فاصله ژنتیکی بین *P. semisulcatus* و *P. s. persicus* به‌وسیله آنالیز توالی بخشی از ژن 5.8S rRNA به اجرا درآمد. هدف دیگر این تحقیق ارزیابی قابلیت ژن 5.8S rRNA برای تشخیص گونه‌های ده‌پایان است. نتایج نشان داد که ژن 5.8S rRNA هر دو گروه *P. semisulcatus* و *P. s. persicus* کاملاً مشابه هستند و تنوعی در توالی آنها مشاهده نشد. نتایج همچنین نشان داد که که توالی‌های ژن 5.8S rRNA بین گونه‌های جنس‌های مشابه گونه‌های آنالیز شده ده‌پایان حفاظت شده بوده و هیچ فاصله ژنتیکی در سطح گونه‌ای مشاهده نمی‌شد. نرخ پایین تکاملی و حفاظت موثر ژن 5.8S rRNA می‌تواند به نقش آن در فرایند ترجمه نسبت داده شود.

کلمات کلیدی: تنوع ژنتیکی، نشانگر هسته‌ای، سخت پوستان، ده‌پایان.