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ORIGINAL ARTICLE

Morphometrics and Mitochondrial DNA Genes Analysis Suggest a New Species of *Penaeus* (Crustacea: Penaeidae) from the Persian Gulf

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Abstract

There are two morphotypes of *Penaeus semisulcatus* described hitherto in the Persian Gulf, namely the banded and non-banded antennae morphotypes. In this study, we used morphometric measurements and two mitochondrial genes (16S rRNA and cytochrome oxidase subunit I—COI) to assess relationships between the two morphotypes of *P. semisulcatus*. Out of 25 morphological characters examined, 10 characters were found significantly different between the two morphotypes when tested against separate sexes or both sexes combined. Results from the 16S rRNA and COI sequence analysis of two morphotypes of *P. semisulcatus* morphotype showed up to 6% and 17% sequence divergence, respectively. The 16S rDNA and COI sequences of the non-banding morphotype were not only very different to those of the banding morphotype but was also very different to all other *Penaeus* species (i.e., *P. monodon, P. merguiensis*, and *P. indicus*) included in the study. Both parsimony and Neighbor-Joining trees based on 16S rDNA and COI sequences provide similar tree topology that clearly separated the two morphotypes into two distinct groups. Based on these findings, we propose the two morphotypes of *P. semisulcatus* to be relegated as two sympatric species.

Keywords *Penaeus semisulcatus* · mtDNA · Morphometric · Persian Gulf · 16S rRNA · COI

Introduction

Marine shrimps of the super family Penaeidae represent approximately one-third of the world's commercially important shrimp species and account for over 80% of the wild catch (Baldwin et al. 1998). In 2012, shrimp production from marine capture fisheries reached a record high at 3.5 million tons. This catch was mainly

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Extended author information available on the last page of the article from the Northwest and Western Central Pacific, although catches also increased in the Indian Ocean and the Western Atlantic (FAO 2016). In Iran, shrimp farming started about 25 years ago and is considered as a good way of developing unproductive salty coastal flats. The perennial problem of over-fishing and infectious diseases has severely affected many shrimp's aquaculture industries, causing heavy economic losses. Highly pathogenic viruses like WSSV (White Spot Syndrome Virus) are capable of killing over 90% of hatchery-reared shrimps in Bushehr and Khuzestan provinces, thereby resulting in a decrease in the main cultured species such as *Penaeus indicus* broodstocks. The Green Tiger prawn or Grooved tiger prawn, *Penaeus semisulcatus* (De Haan 1844), is regarded as one of the most common and commercially important marine shrimps in the Indo-West Pacific regions (Grey et al. 1983; Holthuis 1980) and also south and east Africa to India and Sri Lanka, including the Red Sea, Persian Gulf, and Western Madagascar (Holthuis 1980; Hayashi 1992; Yu and Chan 1986). It has recently colonized the east Mediterranean through the Suez Canal and now forms the basis of an important fishery in that region. This species inhabits mud, sandy-mud, or sandy-grit substrates on the continental shelf from the coastline to depths of about 130 m, but is most abundant in waters shallower than 60 m (Yu and Chan 1986; Hayashi 1992). *Penaeus semisulcatus* can form small shoals and is predominantly nocturnal, and buries itself in the substrate during daytime. This species is mostly fished at night, but in some areas it is also fished during the day (Holthuis 1980).

This species is distinguished from the other species of *Penaeus* by having characteristic teeth on the rostrum (rostrum armed with 5–8 teeth on dorsal, and 2–4 teeth on the ventral margin), the special crests, and grooves on the carapace as well as white and red transverse stripes present as bands in the antennae (Holthuis 1980). There are at least two morphotypes of the green tiger prawn, described hitherto from the Persian Gulf; the *P. semisulcatus* with banded antenna and pronounced body transverse lines (De Haan 1844), which is mentioned here as the banded antennae morphotype and another morphotype with non-banded antenna, pail greenish body color, and faint body transverse lines. The main fishing areas for *P. semisulcatus* in Iran are Bushehr (non-banded morphotype) and Hormozgan (banded morphotype) provinces. In this study, the endemic morphotype (non-banded morphotype) did not show remarkable transverse body linage and the antennae are not banded. This morphometric feature has not been reported from the major *P. semisulcatus* distribution area, including the Indo-Pacific regions. Also, the non-banded morphotype is yet to be described as distinct species.

Recently, the taxonomic status of the morphotypes of *P. semisulcatus* has been debated in order to select the best lineage for brood stocking and related aquaculture program. A study of the genetic characteristics of shrimp species and the analysis of genetic relationships between broodstocks is a useful tool for their management because genetic identification and discrimination of broodstocks are essential for successful rearing programs (Allegrucci et al. 1998; Sekino et al. 2002).

Mitochondrial DNA (mtDNA) shows a high level of polymorphism with an evolutionary rate which is 10 times faster than nuclear genes and is subjected to maternal inheritance (Awadalla et al. 1999). These characteristics make mtDNA a very useful tool for genetic studies in aquaculture species (Allegrucci et al. 1998).

Mitochondrial genes such as the large subunit ribosomal DNA (16S rRNA) and cytochrome c oxidase subunit I (COI) are popular markers used for the study of crustaceans at the species and population levels (Sarver et al. 1998; Chu et al. 1999; Tong et al. 2000; Schubart et al. 2000). The sequence analyses of mtDNA have been widely used in phylogenetic studies of natural shrimp populations (Baldwin et al. 1998; Gusmão et al. 2000; Maggioni et al. 2001) and for the evaluation of genetic divergence between captive populations (Sekino et al. 2002).

Over the years, using mitochondrial genes, several phylogenetic studies have been targeted on *Penaeus* species, in order to address their taxonomy and evolution (Lavery et al. 2004). Palumbi and Benzie (1991) reported surprisingly high genetic differentiation between two morphologically and ecologically similar species belonging to the subgenus *Litopenaeus*. Such genetic divergence between morphologically similar species can be a recurring pattern within the family Penaeidae. In these circumstances, a comparison of molecular genetics can provide a valuable framework for re-examination of the systematic relationships of Penaeid shrimp.

This study utilized both mitochondrial DNA (16S rRNA and COI genes) and morphometrics data, in order to find differentiation signature between two morphotypes of *P. semisulcatus* and assess the phylogenetic relationship between both morphotypes. Currently, *P. semisulcatus* is being investigated for future major aquaculture species by Iranian Fisheries Sciences Research Institute. Investigations on both morphometric and phylogenetic studies will enable us to better understand the taxonomic status of both the banded antenna and non-banded antenna of *P. semisulcatus* morphotypes present in the Persian Gulf.

Materials and Methods

Morphometric Analysis

The morphometric analyses performed on all specimens (30 samples male and 30 samples females) collected from two morphotypes of *P. semisulcatus* (banded and non-banded antennae morphotypes) are as described by Tzeng and Yeh (1999) and Tsoi et al. (2005).

In this study, we measured 16 morphometric characters namely antennal spine width (ASPW), body length (BL), carapace length (CL), diagonal CL (DCL), the first abdominal somite height (ASH1), length (ASL1), and width (ASW1), second abdominal somite height (ASH2), second abdominal somite length (ASL2), second abdominal somite width (ASW2), the sixth abdominal somite height (ASH6), length (ASL6), width (ASW6), and hepatic spine width (HSW). In addition, rostrum length (RL) and telson length (TL) were measured. These 13 morphometric parameters were measured with a ruler or a vernier caliper to 0.01 mm.

A total of 25 variables (ASPW/BL, HSW/BL, CL/BL, DCL/BL, ASW1/ASH1, ASW1/BL, ASH1/BL, ASH6/BL, ASW6/BL, ASL6/ASH6, ASL6/BL, RL/BL, TL/BL, ASPW/CL, HSW/CL, DCL/CL, ASW1/CL, ASH1/CL, ASH6/CL, ASW6/CL, RL/CL, TL/CL, HSW/ASPW, ASL6/CL, and ASL6/TL) were estimated in order to find morphological differentiations between two morphotypes. The significance of each individual variable was calculated using two-factor analysis of variance (ANOVA) with morphotype and sex as major factors as suggested by Zar (1996).

Samples for Mitochondrial DNA Sequence Analysis

Eight individuals of the banded antennae morphotype of *P. semisulcatus* were collected from Iran (two individuals from Hormoz and two individuals from Jask areas) and Malaysia (four individuals from Penang). In addition, four individuals of the non-banded antennae morphotype (two individuals from Bushehr, one individual from Jask and one individual from Hormoz) were collected for this part of study (Table 1 and Fig. 1).

The other species of Penaeidea family namely *P. monodon* from Iran (one individual from Chabahar) and from Malaysia (one individual from Penang), *P. indicus* from Iran (two individuals from Jask) and *P. merguiensis* from Iran (two individuals from Hormoz) were collected and included in this study for comparative purposes (Table 1).

Total Genomic DNA Extraction

Total genomic DNA was extracted from 0.1 to 0.2 g of frozen muscle tissue (swimming legs) mixed with 600 µl DNA extraction buffer (10 mM Tris–HCl, pH 7.5, 100 mM EDTA, 1 µg/ml proteinase K and 0.05 µg/ml RNase) and subsequently ground using a mortar and pestle. The homogenate was incubated for 12 h at 55 °C. DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1), and precipitated in 100% cold ethanol following Sambrook et al. (1989). Extracted genomic DNA was diluted to a working concentration of 50 ng/µl in deionized water and stored in −20 °C.

PCR Amplification and DNA Sequencing

Polymerase Chain Reaction (PCR) for the amplification of 16S rRNA was conducted using universal primers 16SarF (5′-GCCTGTTTAACAAAAACAT-3′) and 16SbrR (5′-CCGGTCTGAACT CAGATCATGT-3′) following Simon et al. (1991). PCR amplification of COI gene was performed using universal primer pair of COIP4 (5′-AGGAAATG TTGAGGGAAGAAATAA-3′) Tong et al. (2000) and COIf (5′-TAA CCTGCAGGAGGAGGAG AYCC-3′) of Palumbi and Benzie (1991). The PCR amplification was carried out in a standard 25 or 50 µl reaction volume with 2 µl of total genomic DNA, 1 picomol of each primer, 2.5 mM. $MgCl_2$ (optimized from 1.5 mM up to 3.0 mM), 2 mM dNTPs (Promega), and 5U/µl *Taq* DNA polymerase (Promega). Amplification was performed in a PTC-200 Peltier Thermal Cycler, (MJ Research, Watertown MA) with a profile of precycle denaturation at 94 °C for 4.30 min, followed by 30 cycles of 1.30 min at 94 °C, 1 min at 52–56 °C (optimizing annealing temperature), 1 min at 72 °C (extension temperature), and a final extension of 5 min at 72 °C. Amplified DNA from individual shrimp was purified

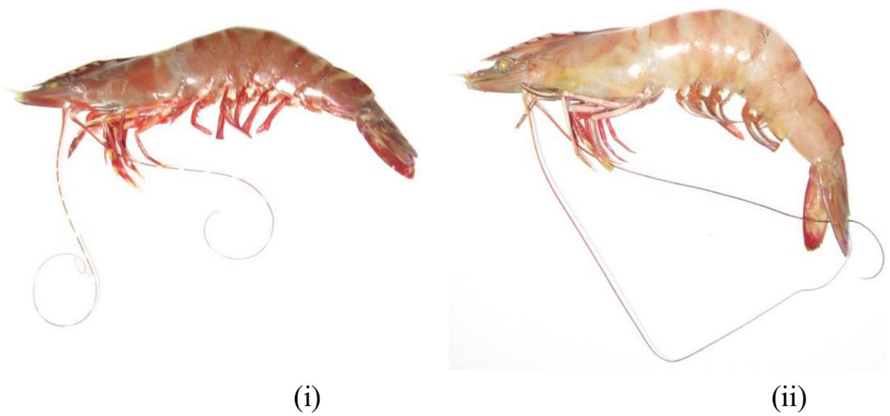
Table 1 Sample size and sites of studied species

Species	*N**	Site (area)	Labels	NCBI Rec. 16SrRNA	NCBI Rec. COI
P. semisulcatus (Banded type Iran, (11M, 14F)	2	Hormoz	B.HO11IR, B.HO14IR	KC315744 KC315743	KC525192 KC525195
P. semisulcatus (Banded type Iran) (28M, 30F)	2	Jask	B.JA28IR, B.JA30IR	KC315742 KC315745	KC525193 KC525194
P.semisulcatus (Banded type Malaysia) (17&25 M, 03&06F)	4	Penang	B.PE17MA,B.PE03MA,B.PE25MA, B.PE06MA	KC315746 KC315747 KC315748 KC315749	KC525188 KC525189 KC525190 KC525191
P. semisulcatus (non-Banded type Iran) (10 M, 12F)	2	Bushehr	NB.Bu10IR,NB.Bu12IR	KC315750 KC315753	KC525184 KC525185
P. semisulcatus (non-Banded type Iran) (M)	1	Jask	NB.JA12IR	KC315751	KC525187
P. semisulcatus (non-Banded type Iran) (F)	1	Hormoz	NB.HO04IR	KC315752	KC525186
P. monodon (Iran)	1	Chabahar	MO.CH49IR	KC315758	KC525200
P. monodon (Malaysia)	1	Penang	MO.PE33MA	KC315759	KC525201
P. merguiensis (Iran)	2	Hormoz	ME.HO44IR, ME.HO34IR	KC315756 KC315757	KC525198 KC525199
P. indicus (Iran)	2	Jask	IN.JA46IR,IN.JA47IR	KC315754 KC315755	KC525196 KC525197
*P. indicus*16S & COI	2	NCBI	P.IND.AF335279, P.IND. AY395242	Rungsithum J. 2001	Querci j. 2003
P. merguiensis 16S & COI	2	NCBI	P.MER.FJ002573, P.MER. AY143988	Shekhar M.S 2008	Chu KH 2002
M. affinis.16S & COI	2	NCBI	AFF.AY622203, M.AFF. AY264889	Tong JG. 2004	Quan J. 2003
P. semisulcatus 16S & COI	2	NCBI	P.SEM.AY744267, P.SEM. AF014379	Kumar N 2004	Baldwin JD. 1997

Table 1 (continued)

Species	N^*	Site (area)	Labels	NCBI Rec. 16SrRNA	NCBI Rec. COI
P. monodon 16S & COI	2	NCBI	P.MON.AY744272, P.MON.EF646172	Kumar N 2004	Klinbunga S. 2007

N^* Number of individuals



(i) (ii)

Fig. 1 Body color pattern in the two morphotypes: **i** banded antennae morphotype; **ii** non-banded antennae morphotype (Color figure online)

by using a Spin Clean Gel Extraction kit (column). Samples were sent to service provider, 1st BASE Laboratories (Malaysia) for direct sequencing in both directions. Resulting PCR products were purified using ethanol precipitation and run using Automatic Sequencer 3730xl (Applied Biosystem, Foster City, CA).

Phylogenetic Analysis

EIectropherograms were edited and sequences aligned using Clustal 1.5C (Thompson et al. 1994) with adjustments made manually. Sequences were confirmed through BLAST analysis with Genbank sequences: 16S rRNA (*P. semisulcatus*; AY744267 *P. merguiensis*; FJ002573, *P. monodon* 16SAY744272, and *P. indicus* AF335279) and COI (*P. semis*ulcatus AF 014379, *P. merguiensis* AY143988, *P. monodon* EF466172, and *P. indicus* AY395242). DNA sequences of other *Penaeus* species were obtained from Genbank and included into the alignment for phylogenetic comparison. DNA sequences (both 16S rRNA and COI genes) from another shrimp genus *Metapenaeus affinis* (Gene Bank Accession No: AY622203) were used as the outgroup.

The distance matrix option of PAUP* 4.0b10 (Swofford 2002) was used to calculate genetic distance according to the Kimura 2-Parameter model of sequence evolution (Kimura 1980). A maximum parsimony (MP) tree was constructed with TBR branch swapping and 10 random taxon addition replicates under a heuristic search, saving no more than 100 equally parsimonious trees per replicate. To estimate branch support on the recovered topology, non-parametric bootstrap values were assessed with 1000 bootstrap pseudo-replicates. In addition, Neighbor-joining (Saitou and Nei 1987) tree was constructed based on pairwise genetic distance using the Kimura 2-Parameter algorithm. The relationship between the CO1 and 16S rRNA haplotypes was investigated by constructing unrooted networks using a median-joining algorithm in NETWORK v. 5.0.0.3 (Bandelt et al. 1999).

Results and Discussion

Morphometric Analysis

Out of 25 morphometric variables measured in this study, ten variables were significantly different between the two morphotypes, partitioned by sex ($P < 0.05$) (Table 2). Four variables were found significant between the males whereas five variables were significant between the females of banded and non-banded morphotypes. The discriminating variables for the male (banded versus non-banded antenna morphotypes) include ASW/BL, ASH6/CL, HSW/CL and ASW6/CL while for the females include HSW/BL, ASL6/BL, HSW/ASW, ASL6/CL and ASL6/TEL.

Results obtained from morphometric indices analysis for the total number of individuals showed that there are several variables which were significant between the banded and non-banded morphotypes according to sex (female banded compared to female non-banded and also male banded compared to male non-banded morphotypes).

Specifically, the data presented here showed significant differentiation between the morphometric patterns of the somatic parts of the body in females of the banded versus the non-banded morphotype, such as ASL6/BL, ASL6/CL and ASL6/TEL, from two separated studied areas. This study showed no extensive variation in morphometric indices between the combined sexes of banded and non-banded antennae morphotypes, but indicated the differences between the single sexes. Out of 25 morphological characters measured, ten characters showed significant differentiations between single sex and combined sex (see Table 2). Differences in somatic indices such as ASW1/BL, ASH1/CL and ASL6/TL were suggested by Tsoi et al. (2005) as differential variables for distinguishing two morphologically similar morphotypes of kuruma shrimps (*P. japonicas*).

Mitochondria DNA Base Compositions

Two partial mitochondrial DNA (mtDNA) genes, 16S rRNA and COI, were successfully amplified and sequenced. The DNA sequences for both genes were also obtained from other *Penaeus* species namely *P. merguiensis*, *P. monodon* and *P. indicus*. The PCR amplification of the 16S rRNA gene generated amplicons of approximately 530 base pairs (bp) for most of the samples analyzed. After editing, unambiguous sequences of 457 characters were aligned and used for phylogenetic tree reconstruction (Fig. 2).

In this study, a total of six haplotypes for the banded antennae morphotype and two haplotypes for the non-banded antennae morphotype were identified for the 16S rRNA gene. The highest pairwise genetic divergences of 6.6% was observed between the banded antennae morphotype from Penang (B.PE25MA) and the non-banded antennae morphotype from Jask (NB.JA12IR). The lowest nucleotide divergence of 3.9% was recorded between individuals of the same morphotypic

Table 2 Morphometric analysis of 10 relative growth variables of *Penaeus semisulcatus* specimens from Persian Gulf area characterized as banded and non-banded antennae morphotypes

Variables	Type	Female ($N=30$)	Male ($N=30$)	Male and female
ASPW/BL	B	0.023 ± 0.005	0.022 ± 0.008	0.976 ± 0.219
	NB	0.020 ± 0.002	0.021 ± 0.004	1.062 ± 0.238
	P	0.159	0.024*	0.358
HSW/BL	B	0.166 ± 0.011	0.178 ± 0.068	1.004 ± 0.074
	NB	0.166 ± 0.011	0.170 ± 0.013	0.960 ± 0.097
	P	0.047*	0.540	0.238
ASL6/BL	B	0.216 ± 0.016	0.215 ± 0.073	0.979 ± 0.129
	NB	0.199 ± 0.012	0.203 ± 0.015	1.021 ± 0.098
	P	0.003*	0.351	0.318
ASH6/CL	B	0.463 ± 0.024	0.443 ± 0.035	0.940 ± 0.137
	NB	0.461 ± 0.054	0.468 ± 0.038	1.026 ± 0.125
	P	0.924	0.011*	0.099
HSW/ASW	B	7.702 ± 1.782	8.289 ± 0.822	1.077 ± 0.260
	NB	9.092 ± 1.323	8.390 ± 1.350	1.077 ± 0.260
	P	0.019*	0.726	0.146
ASL6/CL	B	0.591 ± 0.052	0.563 ± 0.060	0.979 ± 0.129
	NB	0.544 ± 0.040	0.582 ± 0.049	1.021 ± 0.098
	P	0.009*	0.168	0.318
ASL6/TEL	B	0.959 ± 0.101	0.898 ± 0.134	0.982 ± 0.244
	NB	0.878 ± 0.083	0.888 ± 0.098	1.022 ± 0.163
	P	0.024*	0.731	0.583
HSW/CL	B	0.455 ± 0.015	0.462 ± 0.026	1.020 ± 0.049
	NB	0.486 ± 0.043	0.489 ± 0.040	1.013 ± 0.118
	P	0.051	0.003*	0.871
ASW1/CL	B	0.511 ± 0.025	0.489 ± 0.037	0.932 ± 0.052
	NB	0.501 ± 0.020	0.505 ± 0.039	1.010 ± 0.096
	P	0.253	0.110	0.034*
ASW6/CL	B	0.279 ± 0.029	0.280 ± 0.019	1.012 ± 0.116
	NB	0.274 ± 0.041	0.304 ± 0.061	1.123 ± 0.255
	P	0.790	0.047*	0.243

Data expressed as mean ± SD, $N=30$, CL, DCL, BL, ASH6, ASL6, ASW6, HPW, ASPW and TEL represent, carapace length, diagonal carapace length, body length, the sixth abdominal somite height, the sixth abdominal somite length, the sixth abdominal somite width, hepatic spine width, antennal spine width and Telson length

*Denotes the significant characters ($P<0.05$)

group from Busher (NB.BU12IR and NB.BU10IR), Hormoz (NB.HO04IR) and those from Hormoz area (B.14 and B.11) and Jask (B.28 and B.30IR) (Table 3).

Amplification of COI resulted in approximately 600 bp of PCR products. The final multiple aligned consensus sequences resulted in a data matrix of 577 characters. Seventy six variable sites were observed between the two morphotypes of

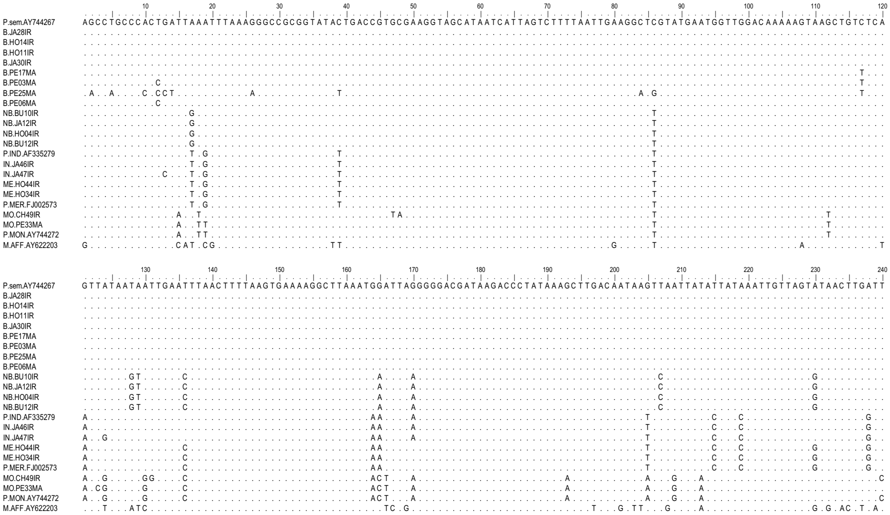


Fig. 2 Aligned sequences of 16S rRNA of 23 taxa including both morphotypes of *P. semisulcatus, P. merguiensis, P. indicus* and *P. monodon* from Persian Gulf (IR) and Penang (PE). Dots denote identity to the top sequence. Details on name of individuals are given in Table 1

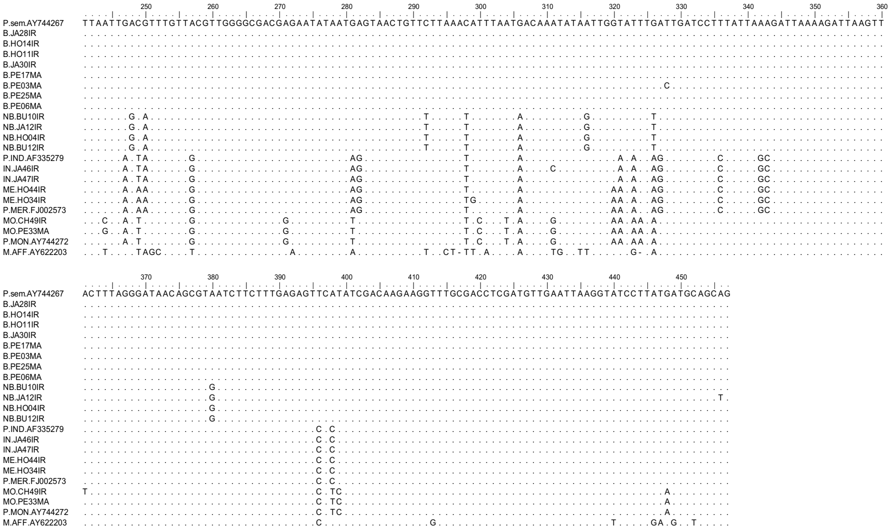


Fig. 2 (continued)

P. semisulcatus (Fig. 3). A total of nine haplotypes for banded antennae morphotype and four haplotypes for non-banded antennae morphotype were recorded for COI gene. Table 4 summarized the nucleotide divergences between the banded and non-banded antennae morphotypes ranging from 17.3% between B.PE03MA

Table 3 Pairwise genetic distance among banded and non-banded antennae morphotypes of *Penaeus semisulcatus*, and four other *Penaeus*, and the outgroup (*M. affinis*), revealed from partial 16S rRNA gene sequences

	(A,B,C,D,E)	(F,G)	(H)	(I)	(J)	(K)	(L&M)	(N)	(O)	(P)	(Q)	(R)	(S)	(T)	(U)	(V)	(W)
P.SEM.AY744267,B.28,B.14,B.11&B.30IR(A,B,C,D,E)																	
B.PE17MA&B.PE06MA (F&G)	0.002																
B.PE03MA (H)	0.007	0.004															
B.PE25 MA (I)	0.025	0.023	0.023														
NB.BU10IR (J)	0.039	0.041	0.046	0.064													
NB.JA12IR (K)	0.041	0.044	0.048	0.066	0.002												
NB.HO04IR,NB.BU12 IR (L&M)	0.039	0.041	0.046	0.064	0	0.002											
P.IND.AF335279 (N)	0.068	0.071	0.076	0.09	0.076	0.079	0.076										
IN.JA46 IR (O)	0.071	0.073	0.078	0.093	0.079	0.082	0.079	0.002									
IN.JA47 IR (P)	0.073	0.076	0.081	0.09	0.082	0.084	0.082	0.004	0.007								
ME.HO44IR (Q)	0.073	0.076	0.081	0.095	0.077	0.079	0.077	0.011	0.013	0.016							
ME.HO34IR (R)	0.076	0.079	0.083	0.098	0.079	0.082	0.079	0.013	0.016	0.018	0.002						
P.MER.FJ002573 (S)	0.073	0.076	0.081	0.095	0.077	0.079	0.077	0.011	0.013	0.016	0	0.002					
MO.CH49IR (T)	0.098	0.101	0.106	0.127	0.113	0.116	0.113	0.096	0.096	0.096	0.099	0.101	0.099				
MO.PE33 MA (U)	0.09	0.092	0.097	0.118	0.104	0.106	0.104	0.084	0.085	0.085	0.087	0.09	0.087	0.018			
P.MON.AY744272 (V)	0.087	0.089	0.095	0.115	0.101	0.104	0.101	0.082	0.082	0.082	0.085	0.087	0.085	0.013	0.007		
M.AFF.AY622203 (W)	0.157	0.16	0.166	0.185	0.154	0.154	0.154	0.177	0.177	0.181	0.181	0.181	0.181	0.212	0.197	0.197	

Details on name of individuals are given in Table 1

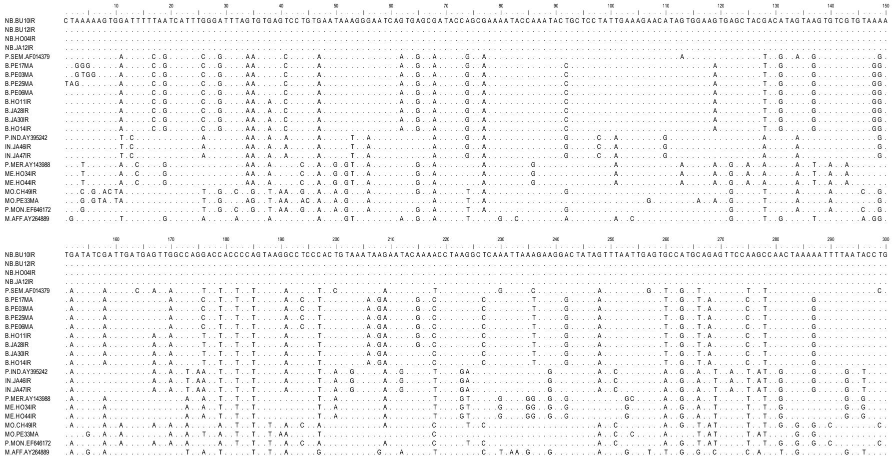


Fig. 3 Sequence alignment of partial COI gene of 23 taxa including both morphotypes of *P. semisulcatus, P. merguiensis, P. indicus* and *P. monodon* from Persian Gulf (IR) and Penang (PE). Dots denote identity to the top sequence. Details on name of individuals are given in Table 1

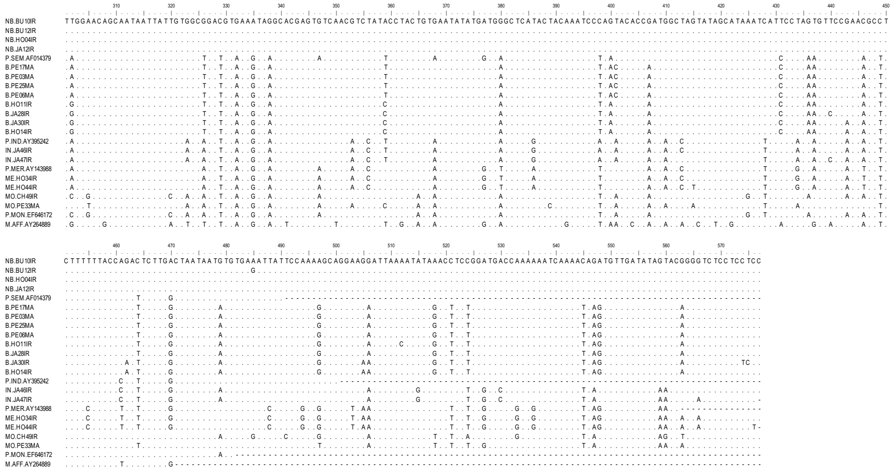


Fig. 3 (continued)

(Penang) and NB.HO04IR (Hormoz), to 16.0% between B.PE06MA (Penang) and NB.HO04IR (Hormoz).

It should be noted that in this study, the genetic distance between the COI sequences of *P. semisulcatus* (non-banded antennae morphotype) with more than 17% sequence divergence was not only very different from the banded antennae morphotype but was also very different from all other known *Penaeus* species reported in other regions.

The 16S rRNA analysis among the *P. semisulcatus* morphotypes was able to distinguish several haplotypes and 18 variable sites. The 16S rRNA region is known to

Table 4 Pairwise genetic distances between the banded and non-banded antennae morphotypes of *Penaeus semisulcatus*, four other shrimp species, and the outgroup taxon (*M. affinis*), revealed from partial COI gene sequences

	A&B&D	C	E	F	G	H	I	J	K	L &M	N & O	P	Q	R &S	T	U	V	W
NB.BU10&12IR&NBJA12IR (A,B.D)																		
NB.HO04IR C	0.002																	
P.SEM.AF014379 E	0.154	0.157																
B.PE17MA F	0.168	0.17	0.069															
B.PE03MA G	0.171	0.173	0.072	0.004														
B.PE25MA H	0.168	0.171	0.07	0.009	0.011													
B.PE06MA I	0.162	0.16	0.065	0.009	0.011	0.009												
B.HO11IR J	0.162	0.162	0.062	0.024	0.026	0.024	0.017											
B.JA28IR K	0.165	0.165	0.065	0.026	0.028	0.026	0.019	0.002										
B.JA30IR &HO14IR (L&M)	0.162	0.165	0.062	0.028	0.03	0.028	0.024	0.009	0.011									
P.IND.AY395242&IN. JA46IR(N&O)	0.201	0.204	0.156	0.179	0.182	0.179	0.173	0.171	0.174	0.165								
IN.JA47IR P	0.201	0.204	0.156	0.179	0.182	0.179	0.173	0.171	0.168	0.165	0.004							
P.MER.AY143988 Q	0.208	0.211	0.165	0.202	0.201	0.205	0.199	0.196	0.199	0.19	0.136	0.141						
ME.HO34&ME.HO44IR (R&S)	0.208	0.208	0.165	0.202	0.201	0.206	0.196	0.19	0.193	0.19	0.136	0.141	0.004					
MO.CH49IR T	0.208	0.21	0.169	0.174	0.171	0.178	0.171	0.172	0.175	0.166	0.182	0.182	0.209	0.21				
MO.PE33MA U	0.201	0.198	0.186	0.19	0.19	0.196	0.184	0.181	0.184	0.183	0.184	0.184	0.205	0.209	0.102			
P.MON.EF646172V	0.192	0.195	0.16	0.162	0.168	0.168	0.162	0.163	0.166	0.157	0.17	0.17	0.2	0.201	0.013	0.107		
M.AFF.AY264889W	0.246	0.249	0.212	0.214	0.218	0.211	0.209	0.206	0.206	0.206	0.227	0.227	0.248	0.249	0.264	0.287	0.25	

Details on name of individuals are given in Table 1 be conserved and have a low rate of evolution (Meyer 1994) which means that it can be more accurate in discriminating between species rather than within species.

In shrimp, high genetic variations of mitochondrial 16S rRNA gene have been observed within *P. notialis*, as well as between *P. notialis* and *P. schmitti* (Machado et al. 1993). It has been found that the 16S rRNA sequences are able to characterize a morphotype which is identified as *F. subtilis* but which displays a different genetic pattern and could be regarded as a new species (Maggioni et al. 2001). However, the low evolutionary rate of the 16S rRNA gene means that there is little or no differentiation between sequences from specimens belonging to the same species, the homogeneity of the *P. semisulcatus* broodstocks (1 morphotype) found in the present study, could probably be due to the conserved nature of this gene.

In contrast to the 16S rRNA gene, the genetic divergences detected by COI gene analysis were consistently higher. In the genus *Penaeus*, although insufficient studies on mtDNA have been reported, large genetic variations of the COI gene have been detected among 15 species (Baldwin et al. 1998; Palumbi and Benzie 1991). The COI region sequences exhibited sufficient nucleotide diversity to be able to determine the occurrence of one haplotype per morphotype and the other morphotypes for *P. semisulcatus*, with three divergent sites being detected among the five non-banded antenna morphotype and 73 divergent sites between two morphotypes of *P. semisulcatus.*

The substitution rate presented by the COI gene was somewhat higher, allowing the detection of genetic differences between different populations of the same species (Gusmão et al. 2000). Intraspecific variation of 0–3% was observed in a sequence analysis of the 558 bp COI gene in 13 *Penaeus* species (Baldwin et al. 1998). In that study, three or more individuals of each species were sampled. In this current study the genetic distances for banded *P. semisulcatus* morphotype were close to zero (0.0–0.3%) and the values were similar to those published by Gusmão et al. (2000) for some penaeid species investigated using COI gene sequencing. In addition, in our study the mean genetic distance between two morphotypes of *P. semisulcatus* was 17.3%, which was higher than the 8.1% genetic distance obtained by Gusmão et al. (2000) for *Farfantepenaeus paulensis* and *F. subtilis* and 10.9% genetic distance obtained by De Francisco and Galetti (2005) for *L. vannamei* and *F. subtilis*.

Also, Tsoi et al. (2005) found high sequence divergence using mtDNA markers between the two morphotypes of *P. japonicus*. This species is extensively dispersed throughout the Indo-West Pacific region. Two morphological morphotypes, (known as morphotypes I and II), are found in the South China Sea.

The two morphotypes are distinguished by different color banding patterns on the carapace. However, there were no separate differences in morphometric traits between them based on measurement of 13 characters. In that study, sequence information of the mitochondrial genes showed that the two morphotypes were characterized by separate clades, with nucleotide divergences of about 1% (473 bp) for 16S rRNA and 6–7% (504 bp) for cytochrome oxidase I.

The haplotype network showed high variation of the mtDNA haplotypes from banded and non-banded morphotypes and also the existence of two distinct clusters for COI gene: cluster I non-banded morphotypes (Iran) and cluster II banded

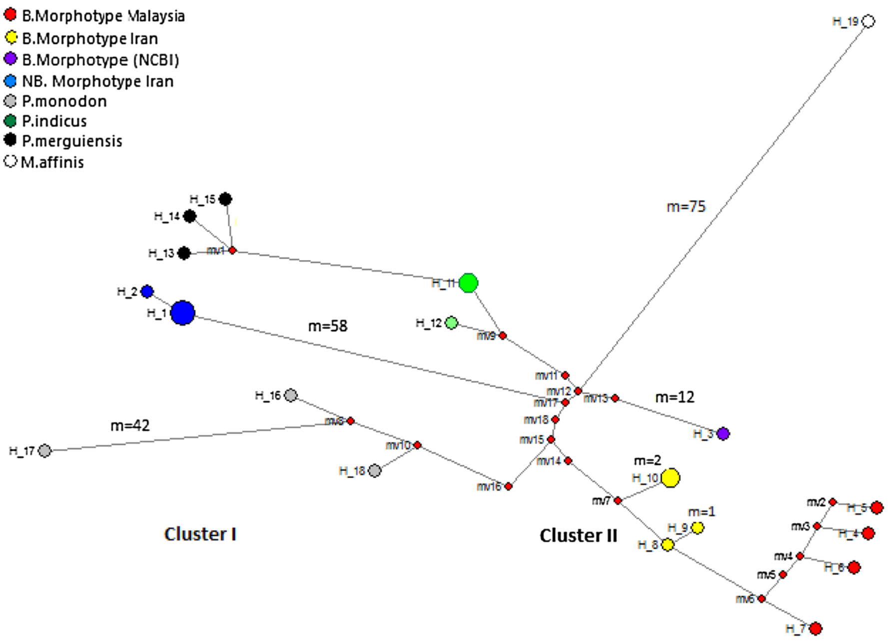


Fig. 4 Median-joining haplotype network based COI mtDNA of *Penaeus semisulcatus* sampled from Iran (B and NB morphotypes) and Malaysia (B morphotype), as well as GenBank sequences. The NB morphotypes sequences form cluster I and the B morphotypes from cluster II. Each pie represents a haplotype and its size reflects the frequency of samples. Distances between pies correspond to number of mutations (*m*) between the haplotypes (Color figure online)

morphotypes (Iran and Malaysia), separated by 58 mutations (Fig. 4). The other mtDNA marker 16S rRNA, support the split but showed a lower ratio, possibly due to slower mutation rate in the conserved region of 16S rRNA (Fig. 5).

Phylogenetic Analysis

Phylogenetic trees were constructed based on the 457 bp for 16S rRNA and 577 bp for COI genes. Trees were generated using MP, a character-based algorithm and neighbor joining (NJ) a distance-based algorithm for phenetic analysis. Overall the tree topology for both phylogenetic and phenetic trees for 16S rRNA was similar. For 16S rRNA gene, 18 haplotypes observed were clustered into six groups on both trees (Figs. 6, 7). In MP, all 17 haplotypes of the five *Penaeus* species clustered into five clades with reasonably high bootstrap values (> 64%) with the *M. affinis* outgroup clustered as the sixth clade. In both trees, all haplotypes of banded antennae of *P. semisulcatus* (Iranian and Malaysian samples) were clustered in one clade while all of the non-banded antennae morphotype individuals clustered in another distinct clade, supporting the differentiation between the two morphotypes. The two morphotypes are sister taxa in the 16S rRNA tree. There is a hint of wide scale geographical differentiation as all banded individuals from Iran clustered in one clade

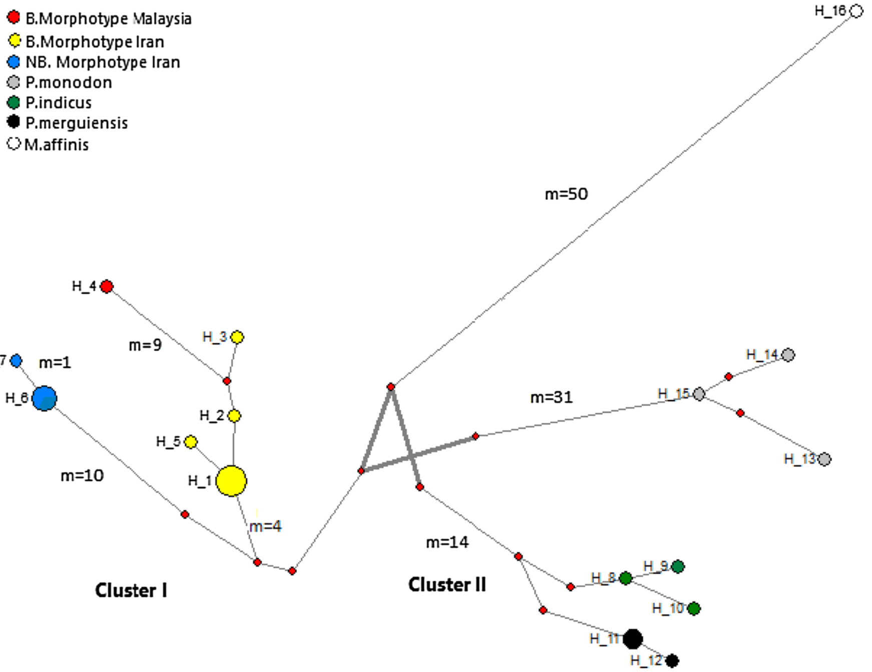


Fig. 5 Median-joining haplotype network based 16S rRNA mtDNA of *Penaeus semisulcatus* sampled from Iran (B and NB morphotypes) and Malaysia (B morphotypes), as well as GenBank sequences. The B and NB morphotypes sequences form cluster I and The other species from cluster II. Each pie represents a haplotype and its size reflects the frequency of samples. Distances between pies correspond to number of mutations (*m*) between the haplotypes (Color figure online)

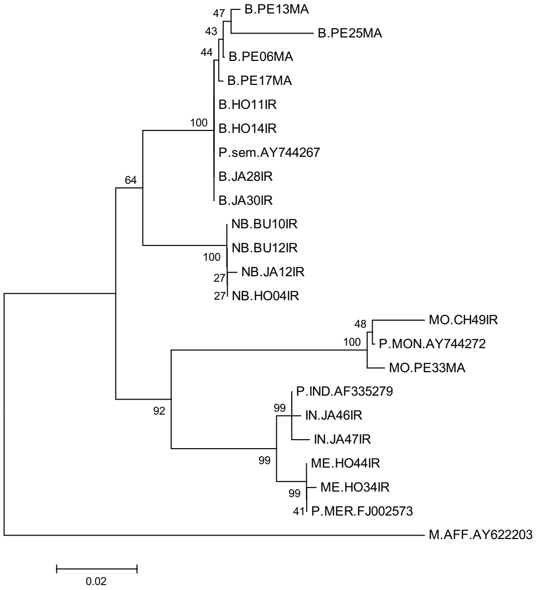


Fig. 6 A neighbor-joining tree based on genetic distance analysis of 16S rRNA sequences showing the genetic relationships of *Penaeus* sp. *M. affinis* was used as outgroup. Scale shown refers to genetic distance based on nucleotide substitutions. Numbers at branching points are bootstrap support. Details on name of individuals are given in Table 1

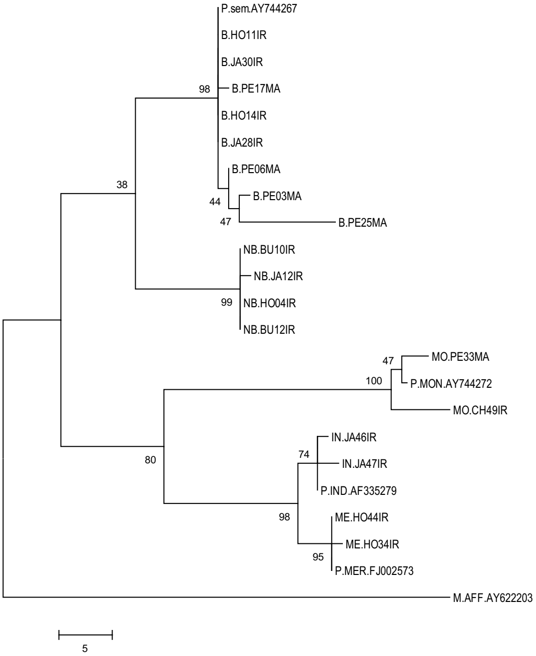


Fig. 7 A maximum parsimony tree based 16S rRNA sequences showing the relationships within *Penaeus*. Individuals with accession numbers were sequences retrieved from GenBank. The scale bar shows the number of substitutions per sequence position

while the Penang individuals in another. However, within the non-banded antennae individuals analyzed at two sites in Iran, no spatial clustering was observed.

Overall tree topology for both phylogenetic and phenetic trees generated from COI was similar to the 16S rRNA gene. A total of 19 haplotypes were detected for all studied species, which clustered into five separate clades/groups (Figs. 8, 9). All seven haplotypes of the banded antennae morphotype of *P. semisulcatus* were clustered into a single distinct major clade with two minor clades, while the two haplotypes of the non-banded antennae morphotype were clustered into another clade, and showed clear differentiation between the two morphotypes. Again, as in the 16S rRNA analysis, there is a trend of wide scale geographical differentiation where all banded antennae individuals from Iran clustered in one clade while the Penang individuals in another. However, within the non-banded antennae individuals analyzed at the two sites in Iran, no spatial clustering was observed. Neighbor-joining analysis based 16S rRNA and COI sequences further revealed the genetic distinctiveness of the banded and non-banded antennae morphotypes, and their genetic relationships with the other *Penaeus* species.

All haplotypes of the banded antennae of *P. semisulcatus* (Iranian and Malaysian individuals) were grouped in a single major clade while all non-banded antennae morphotype individuals were grouped in another distinct clade. However, within the major banded antennae clade, there were two subclusters which distinctly grouped the Iranian from the Malaysian samples. Within the Iranian or Malaysian samples, such structuring was not observed. Thus suggesting homogeneity within the two populations. There was an indication of wide scale geographic variation between the banded populations of Iran and Malaysia. This is expected as both nations are separated by a

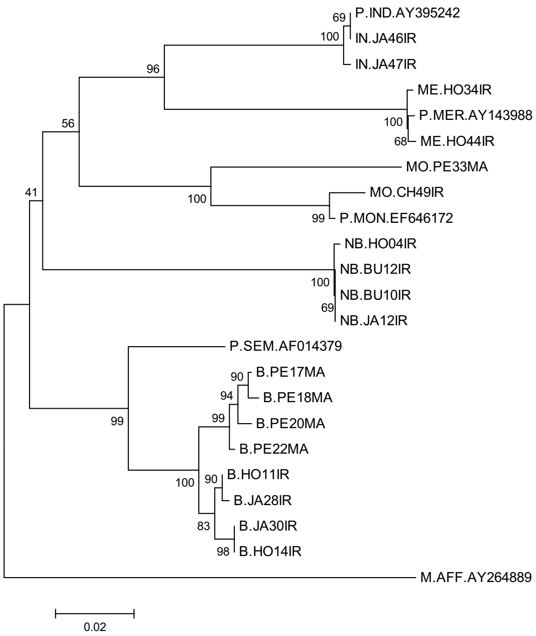


Fig. 8 Neighbor-joining tree based on the partial COI gene sequences showing the relationships of *Penaeus* sp. *M. affinis* was used as outgroup. Scale shown refers to genetic distance. Numbers below the branches are bootstrap values. Details on name of individuals are given in Table 1

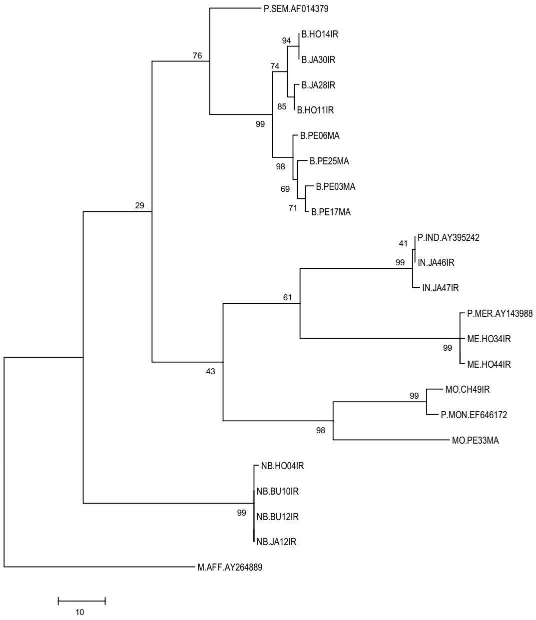


Fig. 9 Maximum parsimony tree based on partial COI sequences showing the relationships between *Penaeus* sp. Individuals with accession numbers are sequences retrieved from the GenBank. *Metapenaeus affinis* was used as outgroup. Scale shown refers to number of nucleotide substitutions. Numbers below the branches are bootstrap values

vast expanse of water with great possibilities of physical, oceanographic, hydrological barriers, among others. Such phenomenon has been widely documented in many aquatic marine species such as investigation on two morphotypes of *P. japonicus*

(Kuruma shrimp) sampled from South China Sea named as varieties I and II and the geographical distribution of the two varieties in the western Pacific area by analyzing individuals collected from Japan and Singapore. Previous results have shown that shrimps from Japan and Singapore belonged to varieties I and II, respectively, suggesting that both varieties have different geographical distributions (Tsoi et al. 2005). The presence of these two color variants of *P. semisulcatus* in the Persian Gulf could be due to natural selections which have supported different color morphotypes in this area. Endler (1983) mentioned that natural selection is one of the main factors for the observed color variants in several species. Dimmock et al. (2004) who investigated on the influence of environment on the morphology of *Macrobrachium australiense* reported that the morphometric differences and variability in morphological characteristics were adaptive responses to the environment in crustacean populations. Paugy and Leveque (1999) also demonstrated that populations (or morphotypes) of the same species originating from different geographical areas were morphologically different. The same factors could have influenced the manifestation between the banded and non-banded morphotypes in this study.

In this study, mitochondrial DNA variation in two studied morphotypes were shown by a large number of closely related haplotypes by constructing unrooted haplotype networks using a median-joining algorithm. Variation among the mtDNA markers varied from the lowest in the 16S rRNA to the highest in COI region. Such a high degree of genetic variation has also been reported for *Penaeus semisulcatus* populations. In that study the samples from China and the Philippines differed clearly from the samples from the Bay of Bengal and Iran (Alam et al. 2016).

This study clearly showed distinct differentiation between both morphotypes and indicated that the non-banded morphotype (new species) is probably endemic to the Persian Gulf. Another interesting fact is that the two morphotypes were sister taxa on the 16S rRNA tree. However, at a narrower geographical scale, the structuring is not clarified. This could be due to the small number of populations investigated as well as the limited amount of information. However, if the present data obtained is a true reflection of the genetic composition of these species, then several inferences can be made. The first one is that the banded antenna *P. semisalcatus* is homogeneous within the investigated range. There has been no previous record of inter location translocation. Therefore, the most likely explanation for this observation is that the populations share a common gene pool. If true, the populations could be managed as a single management or evolutionary unit. However, there is need to conduct further studies to confirm the homogeneity of the populations.

Conclusion

Mitochondrial DNA analysis provided a good resolution of genetic divergence among the populations. This type of analysis could be considered as an important tool to be used in broodstock selection in breeding programs. In this case, different management in broodstocking programs should be performed for two morphotypes of *P. semisulcatus* which were detected in Persian Gulf. Furthermore, the results of this study suggested that the two morphotypes of *P. semisulcatus* could be considered as two separate species from genetic aspects and the non-banded morphotype as a new species and warrant a new name.

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Compliance with Ethical Standards

Conflict of interest The authors do not have any conflict of interest.

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