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Phylogeny of Iranian Coastal Lobsters Inferred from Mitochondrial DNA Restriction Fragment Length Polymorphism

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Abstract: There are five commercial lobsters in the Iranian coastal waters including three spiny lobsters (*Panulirus homarus* (Linnaeus, 1758) , *P. versicolor* (Latreille, 1804)*, P. polyphagus* (Herbst, 1793)) and two slipper lobsters (*Thenus orientalis* (Lund, 1793) and *Scyllarides squamosus* (H. Milne Edwards, 1837)). Reconstruction of evolutionary history or phylogeny is crucial for revealing stock identity to be used for fishery management. Therefore, DNA was extracted using phenol- chloroform method from muscle tissues and the COI gene was studied using PCR- RFLP technique. Totally 67 molecular data from digestion of PCR products using 11 restriction endonuclease enzymes were analyzed. Then the homoplasy rate of the molecular characters was considered and evolutionary distance and the role of related speciation events were discussed. Finally the divergence time for each species is estimated and the cladograms were compared. The resulted phylogenetic trees supported the monophyly of these species.

Key words: COI gene, PCR-RFLP, Iran, Gulf of Oman, Evolution

Introduction

Palinuridae (spiny lobsters), Scyllaridae (slipper lobsters) and Synaxidae (furry or coral lobsters) are families of a monophyletic group of lobsters namely Achelata (Palero *et al.* 2009). According to Ptack *et al.* (2001) and Holthuis (1991), geographic distribution of lobsters is very broad from warm shallow to cold deep waters of all oceans. Based on Holthuis (1991), lobsters of Indo-West Pacific belong to five families and almost 47 species. Of these, three species of Palinurid (*P. homarus*, *P. versicolor* and *P. polyphagus*) and two species of Scyllarid (*S. squamosus* and *T. orientalis*) lobsters are usually found in Iranian shallow waters of the Persian Gulf and the Gulf of Oman (Sari, 1991). According to Ferraris and Palumbi (1996) lobsters (or their larvae) are transferred to water bodies by various oceanic circulations, such as southwestern monsoon. The north Indian Ocean is affected by this circulation (Suryanarayana *et al*. 1992).

In addition to extensive geographic distribution of lobsters, their high species diversity and economical importance in fishery industry make them an interesting subject to evolutionary and phylogenetic studies.

From phylogeny point of view some classic studies provided important data for on evolution-

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ary history of Palinuridae. Firstly, George and Main (1967) estimated the lobster relationships based on their ability in sound producing and hence called them strident or silent accordingly. Then, Baisre (1994) and McWilliam (1995) considered the evolution and phylogeny of Palinuridae using characters on phyllosoma larvae. The speciation of genera *Jasus* and *Panulirus* was considered by Pollock (1990, 1992) and George (1997) using paleooceanographic events and tectonic plate movement, respectively. Later, the speciation of spiny lobsters with respect to climatical changes using ocean circulation was proposed by Pollock (1993) and finally, evolution of life- history patterns in some genera of Palinuridae was demonstrated by Pollock (1995). The most important cladistic analyses using molecular data were carried out by Ovenden *et al.* (1997) and Palero *et al.* (2009) on *Jasus* and Achelata lobsters, respectively. The studies on *Panulirus* were carried out by Ptacek *et al.* (2001) and Groeneveld *et al.* (2007). The phylogeny and evolution of lobsters were analyzed by Patek *et al.* (2006) using morphological and molecular data.

Two hypotheses about Achelatan evolution have been proposed based on morphological and paleontological evidences. According to Forster (1973), Scyllaridae and Palinuridae are Paraphyletic while Palero *et al.* (2009) believed that they are monophyletic.

Due to lack of phylogenetic studies on Iranian lobsters, the objective of this study was to provide a preliminary phylogeny reconstruction of the Iranian lobsters using molecular data and to evaluate their monophyly. The divergence time of taxa using molecular data was also estimated.

Material and Methods

Five species of commercial lobsters were used in the present study. These belong to family Palinuridae (*Panulirus homarus, P. versicolor* and *P. polyphagus*) and Scyllaridae (*Thenus orientalis* and *Scyllarides squamosus*). Adult specimens of *T. orientalis* were collected by fishing trawler vessel Ferdows II. Other species were collected by lobster fishermen mostly using gill net. But in the case of *P. homarus* baited trap was also used. Collection sites and there coordinates are given in Table 1.

Muscle tissues of pleopods, antenna and preiopods were removed from exoskeleton, preserved with 70º ethanol (Barber and Erdmann 2000, Ovenden *et al*. 1997, Silberman and Walsh 1992) or with a NaCl- saturated solution containing 250 mM EDTA (PH=7.5) and 20% dimethylsulfoxide (Baldwin *et al*. 1998, Van Syoc 1995) and shipped to the laboratory. Five individuals of each species were used for molecular analysis to ensure presence of a specific band among individuals of each species. Total DNA was isolated using standard phenol-chloroform extraction protocol (Hillis and Moritz 1990). The polymerase chain reaction was used to amplify a 700-800 bp fragment of mitochondrial cytochrome oxidase subunit I gene selected from deposited sequences in the GenBank (accession numbers AF339457, AF339472 and AF339469). Two primers (f: 5´-GGAGCATGAGCTGGGATAGT and r: 5´- GGATCTCCCCCCCCTACTGG) were developed for this study. PCR amplifications were conducted in a Amersham Pharmacia Biotechthermal cycler TC 341 using a 5 min pre-denaturation at 95ºC followed by 30 cycles of 30 sec at 95ºC, 1 min at 55ºC, 2 min at 72ºC and a final 5 min at 72 ºC. All amplification products were digested separately with 15 restriction endonuclease enzymes: *Alu I*, *BamH I*, *Bcl I*, *Bgl II*, *EcoR I*, *Hae III*, *Hinc II*, *Hind III*, *Hinf I*, *Mbo I*, *MsP I*, *Pvu I*, *Pvu II*, *Rsa I* and *Taq I*. The digested mtDNA was electrophoresed in 6% acrylamide gel in 10X TBE buffer. The gels were stained using silver staining (Sambrook *et al*. 2001). The sizes of restricted fragments were estimated in comparison with a 50bp standard DNA size marker. The bands with similar distance from the gel source were considered to present homologous sequences. All characters are weighted equally in the analysis and scored as present or absent.

Data sets were analyzed using REAP software (Restriction Enzyme Analysis Package, Version 4.0: McElroy *et al*. 1992) to determine nucleotide diversity and divergence. Data sets were modified to phylogenetic ones. An exhaustive analysis was performed using Version 3.1 PAUP software (Swofford 1993). Based on previous works on *S. squamosus* (Maddison *et al*. 1984, George and Griffin 1972, Jones 1990, Basire 1994) which shows this is an older species than others, this was considered as the outgroup.

Table 1. Sampling localities for specimens used in the present study. Distances between sampling locations are as follows: Pozm and Ramin 51.22 Km; Pozm and Tang 40.19 Km; Pozm and Cape of Pozm 12.53 Km; Ramin and Tang 89.61 Km; Ramin and Cape of Pozm 40.18 Km and Tang and Cape of Pozm 49.50 Km.

Locations	***Panulirus homarus***	***Panulirus versicolor***	***Panulirus polyphagus***	***Thenus orientalis***	***Scyllarides squamosus***
Pozm (25º 21´N, 60º 17´E)	+		+		
Ramin (25º 10´N, 60º 45´E)					+
Tang (25º 21´N, 59º 53´E)		+			
Cape of Pozm (25º 16´N, 60º 22´E)				+	

Results

In present study at first the efficiency of these 15 restriction endonuclease enzymes was considered. In most species all five individuals show similar banding pattern. However in *Panulirus versicolor*, different banding patterns were observed among the individuals for *MboI* and therefore, the character state was scored as 'missing'. Then characters were analyzed and finally phylogenetic relationship of species was reconstructed.

The digestion of PCR products by restriction endonuclease enzymes produced polymorphic patterns excluding *BamH I*, *EcoR I*, *Hind III* and *Pvu II* in different species. Digestion of PCR products using other 11 restriction endonuclease enzymes yielded 67 molecular characters. Of these seven characters show homoplasy, 28 were constant and 32 were parsimony- informative. Their CI, HI, RI and RC are given in Table 2.

A total of 55 positions (6.9% – 7.7% of original 710-790 bps alignment in different species) were analyzed in this survey. Nucleotide diversities and divergences are presented in Table 3.

From, exhaustive analysis of 67 molecular characters, two most parsimonious cladograms were obtained (Fig. 1) with length (L) = 46, consistency index (CI) = 0.848, retention index (RI) = 0.562, and the rescaled consistency index (RC) = 0.477. Reweighing of characters based on their rescaled consistency indices improved the tree indices as follows: CI = 0.921, RI = 0.750 and RC = 0.691, but had no influence on tree topologies. The cladograms show the following topologies: *P. homarus* and *P. versicolor* form the most inner clade on both trees, but the position of *P. polyphagus* and *T. orientalis* is altered as the sister to these taxa (Fig. 1). Both cladograms illustrate the monophyly of these species.

Discussion and Conclusion

The monophyly of Palinuridae and Scyllaridae is apparent from genetic datasets. Based on resulted phylogenetic trees, the clade of *Panulirus homarus* and *P. versicolor* is the shortest (Fig. 1). Therefore, it seems these are derived more recently. This is completely agree with George and Main (1967) founding. They indicated that *P. homarus* and *P. versicolor* (belong to group IV of their classification), appeared following the last glacial period of the Pleistocene. It seems that the *P. versicolor* derived more recent than *P. homarus* because the latter species diverged to three subspecies (see Berry 1974 and Holthuis 1991) in the Indo-West Pacific region namely *P. homarus homarus* (throughout the range of the species), *P. homarus megasculptus* (limited to western Indian Ocean consist of Socotra, South coast of Arabia and west coast of India) and *P. homarus rubellus* (in southeast coast of Africa including Natal, South Africa, South Mozambique, South East Madagascar) in response to patterns of marine currents formed by the monsoon southwestern and northeastern winds (Pollock 1993).

According to Baisre (1994) and George and Griffin (1972), *S. squamosus* and *T. orientalis* diverged earlier. This is also demonstrated in figures 1 A and B in which the *S. squamosus* and *T. orientalis* were derived earlier than others and placed in the outer clades. Similarly *P. polyphagus* derived later than *S. squamosus* and *T. orientalis* but earlier than *P. versicolor* and *P. homarus*. This is agreed with finding of George and Main (1967) concerning their

Table 2. Molecular characters indices list. Consistency index (CI), retention index (RI), homoplasy index (HI) and rescaled consistency index (RC).

RC	RI	HI	CI	Maximum Steps	Tree Steps	Minimum Steps	Character
0/0	0/0	0/0	0/0	0	0	n/a	1
0/0	0/0	0/0	0/0	0	0	n/a	2
1.000	1.000	0.000	1.000	2	1	1	3
0.000	0.000	0.500	0.500	2	2	1	4
0.000	0.000	0.500	0.500	2	2	1	5
0/0	0/0	0/0	0/0	0	0	n/a	6
0/0	0/0	0/0	0/0	0	0	n/a	7
0.000	0.000	0.500	0.500	2	2	1	8
0/0	0/0	0/0	0/0	0	0	n/a	9
0/0	0/0	0/0	0/0	0	0	n/a	10
0/0	0/0	0.000	1.000	1	1	1	11
0.000	0.000	0.500	0.500	2	2	1	12
0/0	0/0	0.000	1.000	1	1	1	13
0/0	0/0	0.000	1.000	1	1	1	14
0/0	0/0	0.000	1.000	1	1	1	15
0/0	0/0	0.000	1.000	1	1	1	16
0/0	0/0	0.000	1.000	1	1	1	17
0/0	0/0	0.000	1.000	1	1	1	18
1.000	1.000	0.000	1.000	2	1	1	19
1.000	1.000	0.000	1.000	2	1	1	20
0/0	0/0	0.000	1.000	1	1	1	21
0/0	0/0	0.000	1.000	1	1	1	22
0/0	0/0	0.000	1.000	1	1	1	23
0/0	0/0	0.000	1.000	1	1	1	24
0.000	0.000	0.500	0.500	2	2	1	25
0.000	0.000	0.500	0.500	2	2	1	26
0/0	0/0	0/0	0/0	0	0	n/a	27
0/0	0/0	0/0	0/0	0	0	n/a	28
0/0	0/0	0/0	0/0	0	0	n/a	29
0/0	0/0	0/0	0/0	0	0	n/a	30
0/0	0/0	0/0	0/0	0	0	n/a	31
0/0	0/0	0/0	0/0	0	0	n/a	32
0/0	0/0	0.000	1.000	1	1	1	33
1.000	1.000	0.000	1.000	2	1	1	34
1.000	1.000	0.000	1.000	2	1	1	35
0/0	0/0	0.000	1.000	1	1	1	36
0/0	0/0	0.000	1.000	1	1	1	37
0/0	0/0	0.000	1.000	1	1	1	38
0/0	0/0	0/0	0/0	0	0	n/a	39
1.000	1.000	0.000	1.000	2	1	1	40
0/0	0/0	0/0	0/0	0	0	n/a	41
0/0	0/0	0.000	1.000	1	1	1	42
0/0	0/0	0/0	0/0	0	0	n/a	43
0/0	0/0	0/0	0/0	0	0	n/a	44
0/0	0/0	0/0	0/0	0	0	n/a	45
0/0	0/0	0/0	0/0	0	0	n/a	46
0/0	0/0	0/0	0/0	0	0	n/a	47
0/0	0/0	0/0	0/0	0	0	n/a	48
0/0	0/0	0/0	0/0	0	0	n/a	49
0/0	0/0	0/0	0/0	0	0	n/a	50
0/0	0/0	0/0	0/0	0	0	n/a	51
0/0	0/0	0/0	0/0	0	0	n/a	52
0/0	0/0	0.000	1.000	1	1	1	53
0/0	0/0	0.000	1.000	1	1	1	54

Table 2. Continued.

0/0	0/0	0/0	0/0	0	0	n/a	55
0/0	0/0	0/0	0/0	0	0	n/a	56
0.000	0.000	0.500	0.500	2	2	1	57
0/0	0/0	0.000	1.000	1	1	1	58
0/0	0/0	0.000	1.000	1	1	1	59
0/0	0/0	0.000	1.000	1	1	1	60
0/0	0/0	0.000	1.000	1	1	1	61
0/0	0/0	0.000	1.000	1	1	1	62
1.000	1.000	0.000	1.000	2	1	1	63
1.000	1.000	0.000	1.000	2	1	1	64
0/0	0/0	0/0	0/0	0	0	n/a	65
0/0	0/0	0/0	0/0	0	0	n/a	66
1.000	1.000	0.000	1.000	2	1	1	67

*n/a= not applicable

Table 3. Nucleotide diversity (upper diagonal) and divergence (lower diagonal) among five species of the present study

	P. homarus	***P. versicolor***	***P. polyphagus***	***T. orientalis***	***S. squamosus***
P. homarus		0.048774	0.029492	0.029492	0.000000
P. versicolor	0.011647		0.086229	0.086229	0.000000
P. polyphagus	0.018586	0.060008		0.030738	0.000000
T. orientalis	0.018586	0.060008	0.030738		0.000000
S. squammosus	-0.010905	-0.026222	0.000000	0.000000	

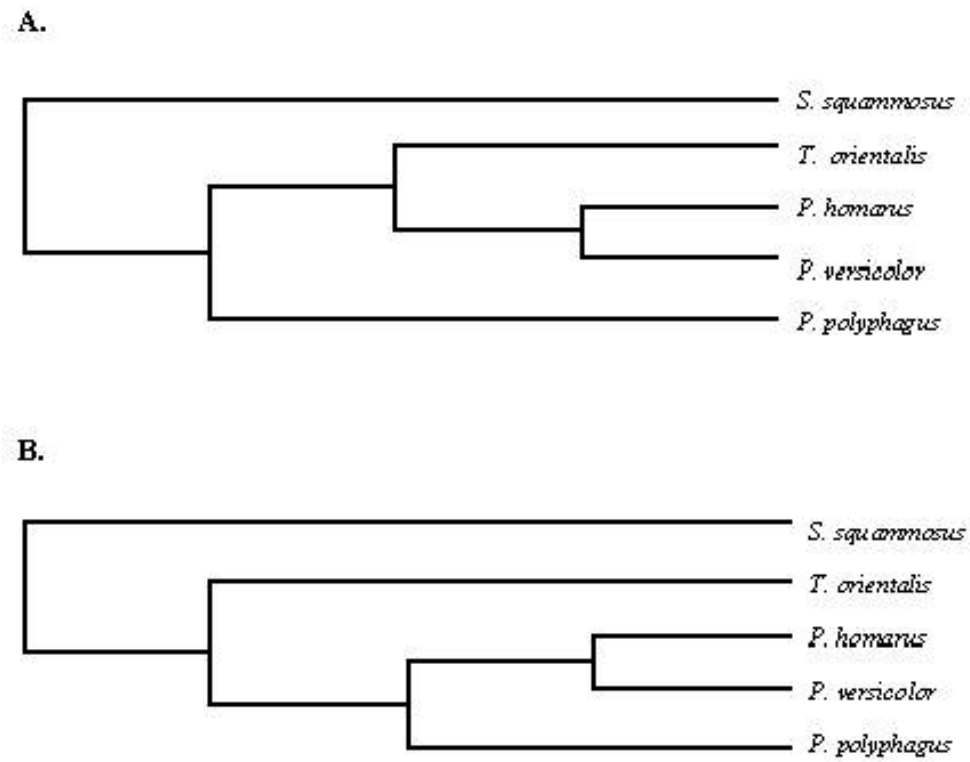

Fig. 1. Two most parsimonious cladograms generated by exhaustive analysis of all molecular characters. *Scyllarides squamosus* is used as an outgroup. Both cladograms indicate the monophyly of studing species. *Panulirus homarus* and *P. versicolor* locate on the most inner clade of both phylogenetic trees. (A) Shows that *P. polyphagus* is the most similar taxon to the outgroup. (B) Shows that *T. orientalis* is the outer clade.

origin in the third glacial of the Pliocene. George (1997) attributed the speciation events to tectonic plate movement. He mentioned that the Gondwana fragmentation (20 mya) produced Antarctic Circle resulted in temperature changes, ecological conditions and speciation events which affected the ancestor of *Jasus* and *Panulirus*. According to George (1997) probably the Himalayan formation and Monsoon provided conditions for origination of *P. polyphagus* and *P. homarus megasculptus*, respectively. It seems speciation of *Panulirus* might be affected by sea current system (George and Main 1967) and as Pollock (1992, 1995) also stated, the evolution of *P. homarus megasculptus* in North Africa seems to be due to this system. In arthropods, rate of mutation in mitochondrial DNA was estimated 1.1-1.2% per million year (Brower 1994) and in genus *Jasus* this is equal to 1.1% per million year (Ovenden *et al.* 1997). On this basis, it seems that the *P. homarus* and *P. versicolor* were derived about 1.7 and 1.06 million years ago, respectively. These values agree with Macaranas *et al.* (1995) and George (1997) findings. The estimated time of divergence for *P. polyphagus* is about 5.4 million years ago in this study which is agree with timing given by George (1997) for this species. As shown in figure 1A, the *P. polyphagus* is diverged earlier than the *T. orientalis* but George and Griffin (1972) and also Basire (1994) mentioned that genera of family Scyllaridae are older than *Panulirus*, therefore cladogram A (Fig. 1) seems to be inappropriate. In contrast cladogram B (Figure 1) agrees with maximum likelihood analysis of 16S rDNA gene and maximum- parsimony analysis of COI gene reported by Ptacek *et al.* (2001) for Palinurid species. This cladogram also agrees with George and Main (1967) classification and their opinion about the *P. polyphagus* which is diverged earlier than the *P. homarus* and *P. versicolor*. Therefore it seems the latter cladogram better reconstructed the phylogeny of these five species.

In summary, the overall finding of present study supports previous hypotheses based on both morphological and molecular characters. Generally, it can be inferred from present study that. *T. orientalis* is the most similar species to outgroup (*S. squamosus*). Later, *P. polyphagus* and finally *P. homarus* and *P. versicolor* were derived. Further analyses using other mitochondrial and nuclear genes may shed light to current knowledge on Palinuroid lobsers of North West Indian Ocean.

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Филогения на Ирански крайбрежни раци, установена чрез митохондриален ДНК рестрикционен фрагментен полиморфизъм

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(Резюме)

В крайбрежните води на Иран са намерени пет вида с раци с търговско значение (*Panulirus homarus* (Linnaeus, 1758) , *P. versicolor* (Latreille, 1804), *P. polyphagus* (Herbst, 1793), *Thenus orientalis* (Lund, 1793) and *Scyllarides squamosus* (H. Milne Edwards, 1837). Възстановяването на тяхната еволюция и филогения е от важно значение за тяхната идентификация в рибовъдството. И така, ДНК е екстрахирана от мускулни тъкани чрез фенол- хлороформ метода и COI гена е изследван чрез PCR-RELP техника. Общо 67 молекулярни данни са анализирани чрез смилане на PCR продуктите при използване на 11 рестрикционни ендонуклеазни ензима. Хомоплазната стойност на молекулярните характеристики е разглеждана и ролята на близки еволюционни събития е дискутирана. Установено е времето на дивергенция на всеки вид и са сравнени кладограмите. Полученото филогенетично дърво подкрепя монофилетичният произход на видовете.