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Full Length Research Paper

Phylogenetic analysis of anemone fishes of the Persian Gulf using mtDNA sequences

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Anemone fishes are a group of 28 species of coral reef fishes belonging to the family, Pomacentridae; subfamily, Amphiprioninae and all have an obligate symbiotic relationship with sea anemones. Two species of these small ornamental fishes have been identified in the Persian Gulf including *Amphiprion clarkii* and *Amphiprion sebae*. The phylogenetic relationship between *Amphiprion* species of the Persian Gulf was studied by collecting 15 samples from three Iranian islands, Larak, Farur and Kish. DNA was extracted from each sample and a part of mtDNA was amplified. Two pairs of primers were designed to amplify a final target of 400 bp by nested-PCR. Each amplican was sequenced, aligned and genetic diversity among samples was investigated by phylogenetic analysis. Results show that there is no significant genetic variation among *A. clarkii* individuals; however, *A. sebae* individuals from Larak were different from other fishes of the same species. Most probably this is due to the ability of *A. clarkii* to be symbiotant with all 10 species of host sea anemones which enables it to spread its own population in the 3 islands. However, *A. sebae* is observed to be symbiotant only with one host in the sea, therefore, has one option that reduces its distribution.

Key words: *Amphiprion*, mtDNA, Persian Gulf, phylogeny.

INTRODUCTION

Anemone fishes (also called clown fishes) are members of the family, Pomacentridae; subfamily Amphiprioninae. 28 species of these fishes have been identified (Elliot et al., 1999). They are unique because all of the species have an obligate symbiotic relationship with sea anemones (Jang-Liaw et al., 2002). Anemone fishes are one of the most popular and best marine ornamental fishes and have been bred successfully in captivity (Hoff, 1996). They are widely distributed, ranging from the Caroline Islands westward to the Red Sea (Bell et al., 1982). Two species of them have been identified in the Persian Gulf which are *Amphiprion clarkii* and *Amphiprion sebae* (Field, 2005). The Persian Gulf is an important military, economic and political region owing to its oil and gas resources and is one of the busiest waterways in the world (Kämpf and Sadrinasab, 2006). The Gulf extends between 24-30º*N* and 48-56º*E* and is *L*- shaped. It is a shallow semi-enclosed basin (of average depth 35 m) connected to Oman Sea through the strait of Hormoz. The general orientation of the two gulfs is northwest-southeast. The region is arid leading to substantial evaporation and higher salinity in comparism with open marine waters (Pous et al., 2004). The studies about anemone fishes of the Persian Gulf are restricted to a few titles of identifying the fishes of this region including anemone fishes (Heemstra, 1984; Dipper and Woodward, 1993; Randall, 1995; Carpenter, 1997; Field, 2005; Zahran and Al-Abdessalam, 1995) and no exclusive study on the anemone fishes of this basin has been carried out (including molecular studies). The molecular studies on anemone fishes are scarce all over the world. These studies are mainly focused on taxonomy and evo-

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Table 1. Sample code and sampling area descriptions.

Taxa	No. of samples	Collecting locations	Latitude and Longitude	Sample code	Accession Number
Pomacentridae					
Amphiprioninae					
Amphiprion clarkii	2	Larak island	26˚N056˚E	CL1, CL2	EF990151, EF990152
A. clarkii	2	Farur island	26˚N054˚E	CF1, CF2	EF990147, EF646856
A. clarkii	3	Kish island	26˚N054˚E	CK1, CK2, CK3	EF990148, EF990149, EF990150
Amphiprion sebae	2	Larak island		SL1, SL2	EU113312, EU113313
A. sebae	3	Farur island		SF1, SF2, SF3	EU113314, EU113315, EU113316
A. sebae	3	Kish island		SK1, SK2, SK3	EU113317, EU113318, EU113319

lution of the anemone fishes based on phylogenetic analysis of nuclear and mtDNA (Elliott et al., 1999; Tang, 2001; Jang-Liaw et al., 2002; Quenouille et al., 2004; Santini and Polacco, 2006; Mabuchi ea al., 2007). There is only one report about anemone fish populations using genetic and morphological variations among them (Bell et al., 1982). A molecular research on anemone fishes has been carried out for their ability to be a symbiotant of different sea anemones (Elliott et al., 1999). A similar study is carried out with more anemone fish species to survey their unusual life style (Santini and Polacco, 2006). Both of these later studies explained the hypotheses on the ancestral of the anemone fishes. It seems that these three studies are the only ones that dedicated to anemone fishes using morphological, genetic or molecular methods. More recent studies have focused on phylogenetic relationships and molecular systematic of the members of Pomacentridae (Tang, 2001; Jang-Liaw et al., 2002; Quenouille et al., 2004; Mabuchi et al., 2007).

The mitochondrial DNA (mtDNA) sequences are one of the most widely used molecular tools to assess phylogenetic relationships between similar species, populations of the same species or even between individuals (Aboim, 2005). The mtDNA control region (also called D-loop) has a large capacity to accumulate mutations that give it a faster rate of evolution than nuclear DNA in higher animals (Aboim, 2005); therefore we used this molecular tool for attaining our purposes. The most of the financial sources, researches and studies in the Persian Gulf are dedicated to underground sources in comparism with its wild life, so such studies can help to understand more about the living organisms found in this region. Many marine organisms are relatively sedentary as adults, dispersing primarily during the larval phase (Bay et al., 2006). Meanwhile previous reports showed that the species which have shorter larval durations, generally display stronger genetic differentiation than the species with longer larval durations (Doherty et al., 1995; Riginos and Victor, 2001). However there are many exceptions (Planes et al., 1998; Barber et al., 2000; Taylor and Hellberg, 2003). Larval duration is one of the effective traits that can affect the dispersal potential of such species and therefore the genetic homogeneity among geographically separated populations (Shulman and Bermingham, 1995). The aim of this study was to investigate the mtDNA diversity among the anemone fishes in three regions of the Persian Gulf.

MATERIALS AND METHODS

Sampling and DNA extraction

Between September till March 2005, 15 individuals of anemone fishes were collected at 3 locations in the Persian Gulf. The descriptions have been given in Table 1 and also the geographical locations of the Persian Gulf and sampling islands have been shown in Figure 1. Fishes were caught by hand-nets while SCUBA diving. Tissue samples were stored without preservation at -70ºC until needed. Total DNA was extracted from 50-100 mg of gill tissue using a DNA extraction method as described earlier (Sadeghi et al., 2006). Briefly, each tissue sample was homogenized in a pestle and mortar and was added to extraction solution. Samples were phenol-chloroform extracted and DNA was precipitated using equal volume of isopropanol. The DNA was washed by 70% ethanol. The semi-dried extracted DNA was resuspended in 20 µl of (deionized distilled water) ddH_2O and stored at -70ºC until use.

DNA amplification and sequencing

Since at the time of this study, there was no submitted sequence of anemone fishes mtDNA control region available in the GenBank, we used the partial sequence of cytochrome *b* and 12S rRNA (Accession no. AY208513.1 and AF285923.1) of related species to design forward and reverse primers. The primers (Cxt-b and 12S-RNA) were designed by Oligo6 software. Target region of the mtDNA was amplified using the polymerase chain reaction (PCR). PCR amplifications were performed on a thermocycler (TECHNE, England) in a 50 µl reaction containing: 5 µl of 10 x buffer, dNTps (10 mM) 1 , 1 µl of each primer (250 ng/µl), *Taq* polymerase (5 U/µl) 0.2 µl, $MgCl_2$ (50 mM) 1.5, 3 µl of total genomic DNA and 37 µl of ddH_2O. Target regions of mtDNA were amplified using the following thermal cycling profile: 94ºC initial denaturing for 3 min (one cycle) followed by 35 cycles of 94ºC denaturing for 60 s, 54ºC annealing for 60 s, 72ºC extention for 90 s and one cycle of 72ºC final extention for 10 min. Amplification products were visualized by running 5 µl of the PCR product on a 1% TBE agarose gel after staining by EtBr. The expected mtDNA fragment amplified by these primers was 2200 bp. When positive, the remaining 45 µl of each

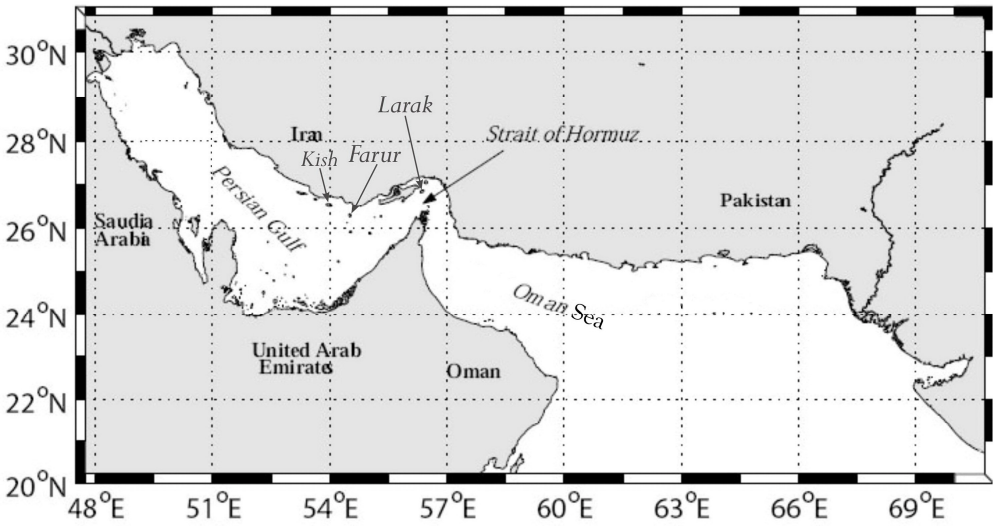

Figure 1. The geographical locations of the Persian Gulf and sampling islands; Larak, Farur and Kish (modified from Pous et al., 2004).

Table 2. Sequencing and amplification primers used in the study.

Primer	Sequence (5′-3′)
Cxt-b	AAC GAA GCC TAA CCT TCC GAC
12S-RNA	TGT GAA GCA CCG CCA AGT C
NPCR-F	TCT TTC CTT GAC CCT T
NPCR-R	AAG TAA GTT TTT GGG TAT TTA

reaction were run on a 2% TBE agarose gel in the presence of EtBr. The amplification product was cut from the gel, melted at 60ºC for 6 min and then was purified, using the High Pure PCR Purification Kit (Roche, Germany). Two of the 2200 bp PCR products from CF1 and SK2 samples were cloned into pTZ57R/T plasmid and sequenced. The results of sequencing were recorded and used for designing nested primers in order to amplify the desired mtDNA control region. Eventually, a pair of primers (NPCR-F and NPCR-R) were designed to amplify approximately a 400 bp of mtDNA D-loop region, using following thermal cycling profile: 94ºC denaturing for 30 s, 54ºC annealing for 30 s, 72ºC extention for 30 s for 30 cycles and a 72ºC final extention for 10 min. Each PCR reaction was preceded by a hot start at 94ºC for 3 min. The sequences of primers that were utilized in this study are given in Table 2. Amplification of the main target region of D-loop was carried out through nested PCR method, using 0.2 µl of 2200 bp PCR product as template DNA and other PCR components were the same as described before.

Sequence alignment and phylogenetic analysis

All sequences were aligned and compared together by DNASTAR software (option; MegAlign) to denote the nucleotide variations among the individuals. Phylogenetic analysis was performed based on sequenced fragments, representing a part of mtDNA control region.

The phylogenetic analysis was carried out by DNASTAR and ClustalX software and results were recorded.

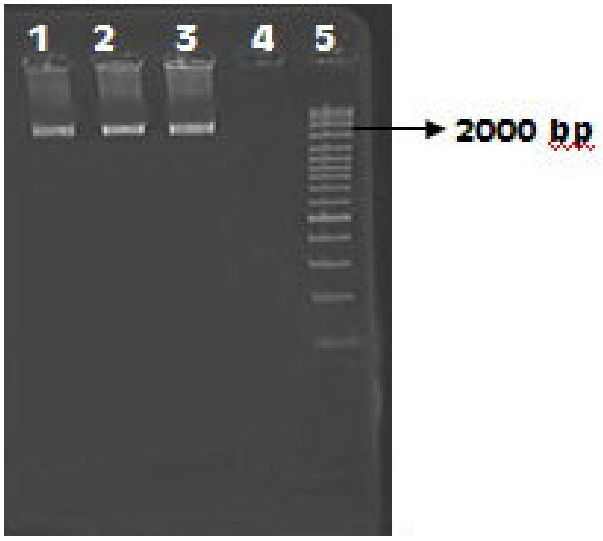

Figure 2. The 2200 bp PCR product of amplified mtDNA with primers Cxt-b and 12S-RNA on 1% TBE agarose gel. Lanes 1 to 3: amplified DNA fragment of 2200 bp length. Lane 4: PCR negative control, Lane 5: 100bp DNA ladder (Fermentas, Lithuania).

RESULTS

PCR

The primers, Cxt-b and 12S-RNA amplified a 2200 bp fragment. There was no DNA fragment in negative control reaction (Figure 2). Using the nested-PCR with primers NPCR-F and NPCR-R for amplification of mtDNA target region, a 400 bp was amplified as expected (Figure 3).

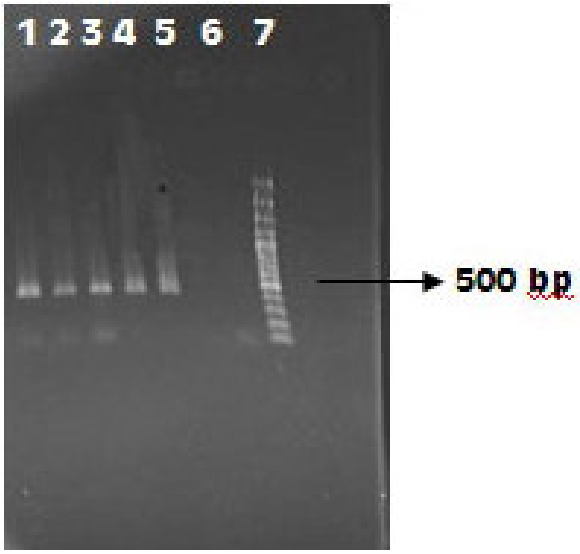

Figure 3. The PCR product of amplifying the final target region of mtDNA D-loop with the primers N-PCR-F and N-PCR-R, on 1% TBE agarose gel. Lanes 1 - 5: 400 bp amplified fragment from 5 different samples. Lane 6: N-PCR negative control. Lane 7: 100 bp DNA ladder (Fermentas, Lithuania).

Sequence analysis

The sequence of a 352 bp segment of the D-loop was determined for 15 individual of anemonefishes (seven *A. clarkii* and eight *A. sebae*) submitted to GenBank according to sampling locations. The sample code, sampling location and corresponding accession numbers are listed in Table 1. The phylogenetic tree obtained by analyzing D-loop sequences of the samples and related partial D-loop sequences of *Amphiprion ocellaris* and *Haplochromis* sp. as out groups is shown in Figure 4.

The phylogenetic tree clearly shows that the *Haplochromis* sp. is very different from the other species and falls into a completely separate phylogenetic branch as expected. *A. ocellaris* is classified in the same genus with test samples and is mainly grouped with them. However it is still genetically different enough to be located in a different branch.

The other two species (*A. clarkii* and *A. sebae*) clustered together; however, SL1 and SL2 are different according to their sequence analysis. Among the 15 samples, there is no evidence that shows significant genetic difference regarding geographical distribution of the two *Amphiprion* species. However, *A. sebae* samples (SL1 and SL2) collected from Larak Island appeared to be different from others.

DISCUSSION

To our knowledge, at the time of this study, there was no information on the anemone fishes mtDNA control region sequence available in the GenBank. The sequences that are submitted through this study are the first ones, particularly for the *A. clarkii* and *A. sebae*. Probably, deficiency of the data about these fishes is due to limited number of the individuals in nature and the difficulty of acquiring them. Moreover, since these small ornamental fishes are not consumed by human, they are not considered as economic fishes. As a result, funds for studying these fishes are restricted and, therefore, the number of research studies in this field is very limited. Since anemone fishes are hosted only by sea anemones, the distribution of their populations in every region including the Persian Gulf is mainly influenced by existence and distribution of host sea anemones. Therefore, in order to study *Amphiprion* population, it would be necessary to get a better understanding of factors that might influence the existence of sea anemones in the Persian Gulf.

Sea anemones such as other corals belong to the phylum Cnidaria or Coelenterata (Fautin and Allen, 1997). Corals had been traditionally considered to be highly stenotopic organisms with very limited endurance of changes in temperature, salinity, turbidity and nutrient concentrations typical of clear, oligotrophic open ocean water (Coles, 2003). Corals are believed to live close to fixed upper temperature limits usually considered to be around 30ºC and lower limits around 16ºC, with little chance of survival outside of this limited temperature range (Coles, 2003). The coral community of the Persian Gulf is a subset of the great Indo-Pacific tropical faunas. The estimates for the Persian Gulf and the Oman Sea indicate that of the 656 species among 109 genera of zooxanthellate corals for the Indo-Pacific (Cairns, 1999), only about 10%, or 68 species among 28 genera, occur in the Persian Gulf (Coles, 2003). This situation is same for the fish species because according to fish base records 705 fish species have been identified in the Persian Gulf while this number is 5429 for Indian Ocean. With only about 10% of Indo-Pacific coral species occurring in the Persian Gulf, some combination of factors has limited the recruitment, settlement, survival and growth of reef corals in the region, eliminating many species and perhaps favoring a few that are adapted to the uniquely harsh conditions of this Gulf. Potential limiting factors include: temperature extremes above and/or below usual coral tolerance limits, high salinities, macro algal competition, past and present isolation of this basin from Indo-Pacific, oil production and pollution (Coles, 2003). The entrance to the Persian Gulf at the strait of Hormoz is only about 50 km wide, restricting circulation of water to the Gulf from the Oman Sea. During the last glacial period at about 17,000 years ago sea level lowering reduced water in the Gulf to a minimum and it was largely a dry basin with only a small area of sea water extending in from the Oman Sea (Coles, 2003). So, all coral settlement and growth in the Gulf has occurred since the beginning of the present interglacial period about 15,000 years ago. The Persian Gulf is one of the few areas in the world where corals occur in a re-

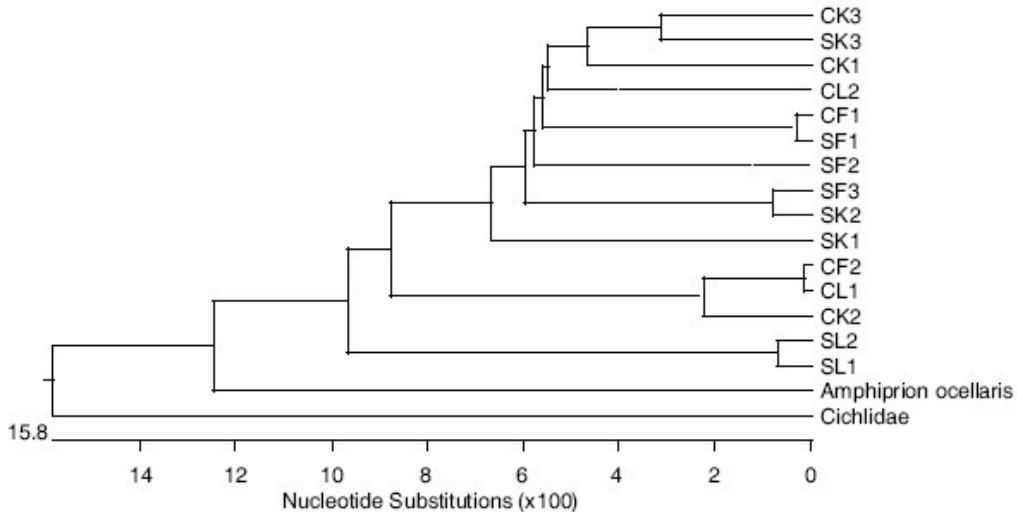

Figure 4. The phylogenetic tree based on analyzing 352bp of D-loop sequence of 15 anemonefishes. The *Amphiprion ocellaris* (Accession number: AP006017) and *Haplochromis* sp. (Accession number: AY929957) D-loop sequences were included in the tree out group.

gion of elevated salinity (Coles, 2003). The average salinity of the Gulf is around 42 ppt in open water (Coles, 2003). It is believed that salinity tolerance is a major factor in limiting the coral species diversity of reef corals within the Gulf (Coles, 2003). In addition to elevated salinity, the temperature environment of the Gulf region presents one of the harshest challenges in the world to resident corals. The averages of minimum and maximum temperature of the southern parts are 12.2 and 36ºC and the northern parts, 13 and 31ºC, respectively (Coles, 2003). All of these limiting factors have restricted the diversity and even number of the suitable host sea anemones and anemone fishes that are found in the Persian Gulf.

Two species of sea anemones have been already identified in the Persian Gulf including: *Entacmaea quadricolor* and *Heteractis aurora* (Fautin and Allen, 1997). Besides at least two more species were observed during sampling which probably are *Heteractis crispa* and *Stichodactyla haddoni* whereas 10 species of the host sea anemones are found in the Indo- Pacific (Fautin and Allen, 1997). The anemone fishes diversity in the Persian Gulf is also too restricted in compare with the Indo-Pacific region since 2 species live in the Persian Gulf (Field, 2005) out of 28 identified species in the Indo-Pacific (Fautin and Allen, 1997).

Relative isolation of genetic populations may be maintained by (1) localized larval dispersal resulting from a relatively short larval stage, and (2) current gyres tending to trap larvae, increasing the return of juveniles to their adult coastal habitat (Bell et al., 1982).

Anemone fishes have the shortest larval period of the damsels ranging from about 8 to 12 days (Fautin and Allen, 1997). This Characteristic has a remarkable effect on a certain species distribution and isolation of the geographically separated populations because one of the important effects of the distance that organisms travel during dispersal is its effect on gene flow and genetic differentiation within a species (Shulman and Birmingham, 1995). The dispersal abilities of organisms are likely to have strong effects on determining whether genetic connections exist between spatially segregated populations. If dispersal of larvae between populations is extensive enough, it can produce genetic homogeneity (Shulman and Birmingham, 1995). The D-loop partial sequence analysis of samples indicates that the distribution of the Clark's anemone fish is unisonous since the individuals show a monophyletic aggregation, but the Sebae's anemone fishes have a different configuration because the individuals caught from Larak Island showed significant genetic difference and located on a separate phylum of the phylogenetic tree. This must have occurred because of the 28 clown fish species, only *A. clarkii* naturally occurs with all 10 host anemones; since *A. sebae* has been observed to be symbiotant only with one host in nature (Fautin and Allen, 1997). *A. clarkii* is the least host-specific and most geographically widespread species of anemone fish. It owes its success, in terms of numbers and geographical extent, to its ability to occupy any host anemone (Fautin and Allen, 1997). So the Clark's anemone fish is superior to Sebae's anemone fish to occupy a host and spread its own population whereas its competitor only have one option that reduces its success and distribution. The distance between Larak and Farur islands is 195 km, while, Farur and Kish Island are 55 km apart. Therefore, the maximum distance between sampling sites would be 250 km. Surface currents attain typical speeds of 10-20 cm/s (Kämpf and Sadrinasab, 2006). The bottom flow attains typical speeds of 5-10 cm/s, but magnifies to 20-30 cm/s past

the strait of Hormoz (Johns and Olson, 1998). In addition the Persian Gulf exhibits a reverse estuarine circulation in which, due to geostrophy, the dense bottom outflow follows the coastline of United Arab Emirates, whereas inflow of Indian Ocean Surface Water follows the Iranian coastline (Chao et al., 1992; Reynolds, 1993) hence, the planktonic organisms (including clownfish larvae) at the Strait of Hormoz will be transported to upper areas along the Iranian coastline. If the average speed of surface currents is 15 cm/s (~ 0.6 km/h), so an anemone fish larvae needs two weeks to be transported from Larak to Farur which is near to its larval period (8-12 days according to Fautin and Allen, 1997). Due to these facts, *A. clarkii* has the chance to be settled in more diverse areas rather than *A. sebae* and this characteristic can be resulted in genetic variations among Sebae's anemone fish population in the Gulf rather than Clark's anemone fish population. Furthermore, *A. ocellaris* is separated from *A. clarkii* and *A. sebae* and most probably it might be due to their different geographical living locations. The distribution of *A. ocellaris* is usually limited to the South-east Asia and is about 5000 km far from living habitat of collected samples in the Persian Gulf. Therefore, observed genetic differences of *A. ocellaris* and *A. clarkii* / *A. sebae* might be due to such far and different living habitats.

The Persian Gulf hosts few species of sea anemones (Fautin and Allen, 1997) and two species of anemone fishes (Field, 2005). Most probably the Persian Gulf anemone fish population could be genetically different from other similar species due to unique environmental conditions in this region. All of the mentioned limiting factors have restricted the diversity, distribution and even the number of sea anemones and anemone fishes in the Persian Gulf. Therefore, further accurate study on the sea anemones and their symbiotants is required to assess their stocks and for any reconsideration in conservation laws if needed that most probably are.

To obtain more clear images of the Persian Gulf anemone fishes and sea anemones situation and to improve the results, we recommend the use of more samples, wider sampling area, and sequencing of longer regions of mtDNA and/or genomic DNA.

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