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Original Article

A Study on the Specialization of Anemone Fishes Using Molecular Genetics Techniques

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ABSTRACT

This research investigated the specialization of anemone fishes and the phylogenetic relationships among 12 species of the genus *Amphiprion* and between two species of the genus *Premnas* using the mtDNA D-LOOP sequence. Following DNA extraction from caudal fins of 14 fish samples, a 450 bp fragment of the D-LOOP mitochondrial (mt) DNA was amplified through polymerase chain reaction (PCR) using a pair of specific primer. The amplified products were tested on agarose gel (1%). The obtained sequences were registered in the Genbank. The arrays of nucleotides obtained from the samples were corresponded to those available in Genbank using BLAST tool in NCBI database. The results showed significant correlations with D-loop nucleotide arrangements of common anemone fish (*Amphiprion ocellaris*) and those of other fish types indicating the accurate selection of the desired fragment of mtDNA. Resultant phylogenetic tree revealed species abundance together with distribution of many species located in the end of the tree probably classified as older species. Accordingly, a region at a longitude between the Philippines and large sea cliffs at latitude between Sumatra and Melanesia was determined to be the possible area of origin for Amphiprioninae, which was identified as the most important center of biodiversity and evolution.

Keywords: D-LOOP, mtDNA, Nucleotide arrangement, Phylogenetic tree.

INTRODUCTION

Genetic materials either chromosomal or extra-chromosomal are subject to constant changes and mutations. Furthermore, physical and chemical agents inside the cell or outside the organism cause a shift in the sequence of nucleotides in DNA replication process. For this reason, different populations and strains develop within plant and/or animal species making their identification through morphological characters difficult and/or impossible. Hence, genetics and genetic markers particularly DNA markers have become reliable and affordable tools in genetic studies and population genetics (Lin *et al.*, 2002).

Anemone fish belong to one of four crowded family of coral reef fishes, the Pomacentridae, covering over 350 species commonly known as damselfishes and/or pomacentrids. Damselfishes are among the first fishes described by Carolus Linnaeus and later attracted other famous taxonomists such as Bleecker in 1877, and Cuvier and Valenciennes in 1830 (Fautin and Allen, 1997).

One of the most useful tools for evaluating dynastic relationships among populations of a species and even between individuals is nucleotides sequences of mtDNA. The control region or D-Loop, due to its features, is particularly important in molecular genetics studies. In this study, the genetic diversity of 14 species of anemone fishes is investigated using nucleotide sequencing.

MATERIALS AND METHODS

Fourteen species of anemone fishes were sampled from Iran, Indonesia and northern China in May 2013. Then 50 mg of pectoral and caudal fins were removed and stored in ependorf tubes (1.5 mL) containing 96% ethanol. The samples were transferred to the Molecular and Cellular Laboratory, University of Agricultural Sciences and Natural Resource (Sari, Iran). Following addition of 100 ml of sterile distilled water, the extracted DNA was stored at −20 °C until use. The quality and quantity of extracted DNA were determined using 1% agarose gel electrophoresis and spectrophotometry, respectively (Sambrok *et al.*, 1989). Universal primers (A and E; Table 1) were adopted for amplification of the D-LOOP locus (Lee, 1995). The primers specified above were synthesized by Sina Gene Co. (Tehran, Iran).

Table 1: Sequences and other features of the primers used for amplification of D-LOOP region

Primer	Sequence	Melting temperature	% GC	Size (bp)
Forward	5`-TTCCACCTCTAACTCCCAAAGCTAG-3`	57.18	48	450
Reverse	5`-CCTGAAGTAGGAACCAGATG -3`	60.02	50	

PCR reaction was conducted in a thermocycler (Eppendorf Co.) using 100 ng of the extracted DNA, 1.0 ml of the primers with 0.5 ml of dNTP, 0.2 ml Taq polymeras, 2.5 ml of PCR buffer, 0.8 ml of $MgCl_2$, and distilled water (final solution volume of 25 ml). The thermocycler was programmed respectively as: denaturation (95°C, 30 min), annealing (52-64 °C, 45 s), and extension (72°C, 1 min) all for 30 cycles. The amplification was verified by agarose gel electrophoresis (1.5 %, 85 volt, 30 min). To determine the size of the amplified fragment (band length), a DNA marker (Fermentas 6 Co.) was used, which yields bands of 100 to 10,000 bp. The sequences of PCR products obtained from the experimental samples were processed and observed in Bio Edit software. The nucleotide arrays were compared with those in the Gene Bank to detect their similarities. The sequences of PCR products were analyzed by DNASTAR program using Megalign and SeqMan II softwares to realize the nucleotide differences.

The species were compared with each other by phylogenetic trees and to obtain evolutionary relationships. The relationships between the sequences obtained were analyzed using MEGA6 software, the phylogenetic trees drawn, and the results examined. The phylogenetic tree was acquired based on the mtDNA (450 bp) from 14 samples tested.

RESULTS AND DISCUSSION

The outline of the resultant tree was consistent with that of Elliott *et al.*, (1999), but it was less consistent with the findings of Quenouille *et al.*, (2004). This is likely because the two surveys were conducted specifically for the phylogeny of the Pomacentridae, and not for the Amphiprioninae. The ultimate end of the tree is *ocellaris*/*percula*, which was determined as a sub-genus *Actinicola* by Allen (1972), who confirmed that both the genus and sub-genus were derived from *P. biaculeatus*, which is of the same origin as *Amphiprion* recognized by Elliott *et al.* (1999), Tang (2001), Jang-Liaw *et al.* (2002), and Quenouille *et al.*, (2004).

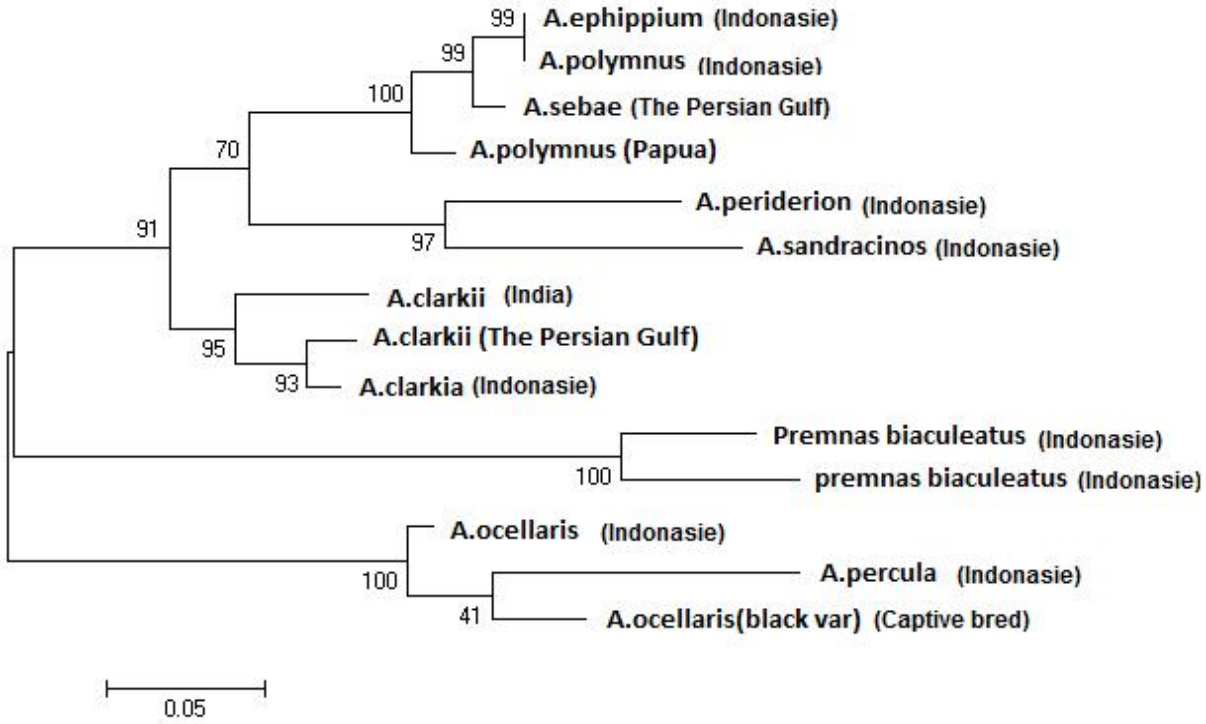

Fig. 1: Phylogenetic tree obtained from the control region of mtDNA (450 bp).

Interestingly, the position of *P. biaculeatus* is not related to other sea anemones in our reconstruction, whereas this species has been derived more as *ocellaris*/*percula* group based on our fairly reliable results. It is also noteworthy that Elliott *et al.* (1999) and Jang-Liaw *et al.* (2002), instead of the terminal ramifications created by *A. ocellaris*, *A. percula*, and *P. biaculeatus*, presented results that confirm some of our findings. All these reconstructions were rejected by various authors in order to explain the relationship between *A. ocellaris*/*percula* and *P. biaculeatus*. Accordingly, our results on the position of *P. biaculeatus* in Amphirioninae should not be considered decisive, though its reclassification as *Amphiprion* seems to be the most appropriate and most conservative option.

The Melanesian peninsula is the habitat for *A. ocellaris*/*percula* and *P. biaculeatus*. The simultaneous and general presence of *A. clarkia* species located in the bottom half of the pedigree together with their local types means that they are neither among the most ancestral species nor among the most derivative species, but they are an average level of development in Amphiprioninae.

The *Perideraion* and *Sandaracinos* species occupy the central part of the pedigree, and there is a close relationship between these two species, which also have very similar

appearances. *Perideraion* and *Sandaracinos* species have a wide distribution in the Pacific particularly from Sumatra Island to Solomons Island to the north-west coast of Australia. It appears that Sumatra Island shows the connection point between two distribution limits, a conclusion drawn by the presence of *A. akallopisos* in the Indian Ocean coast and that of *A. perideraion*/*sandaracinos* in the Pacific coast. In addition, *A. polymnus* and *A. sebae* have a very close relationship with a very similar appearance; however, the adults are distinguishable. In fact, *A. sebae* is distributed almost all coastal areas of the Indian Ocean, while *A. polymnus* is dispersed throughout the East Pacific Coast.

CONCLUSIONS

The distribution and abundance of species found in the bottom of the tree being classified as likely older species indicate that a region within a longitude between the Philippines and large sea cliffs, and at latitude between Sumatra and Melanesia was determined to be the possible area of origin for Amphiprioninae. This conclusion corroborates that of Roberts *et al.* (2002). The above area was identified as the most important center of biodiversity and evolution.

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