Molecular genetics and divergence time study of the cone snail species in the Persian Gulf

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(Received: 7 July 2017; Accepted: 10 September 2017)

Marine gastropod genus' Conus diversified rapidly during the Miocene includes the most species-rich modern marine genus. The aim of this study is an expanded molecular phylogenetic analysis and divergence time of two dominant cone snail species from the Persian Gulf. The mitochondrial cytochrome oxidase subunit I sequence data of Conus coronatus and Conus frigidus species from the Persian Gulf were used to compare with the other Conus species in Viroconus and Virgoconus clades from the different regions. Moreover, divergence time of the Persian Gulf clusters was estimated from the substitution rate of the genome. Results showed, low differences between the Conus species of the Persian Gulf and Indo-Pacific, and divergence time of the Persian Gulf Conus species was about 2 million years ago. So, the Persian Gulf Conus species originated from the Indo-Pacific parallel to the geological events and after the Ice Age. Then, these species were transferred through the Indian Ocean to the Persian Gulf. These findings give use knowledge of the origin and the evolution of these species in the Persian Gulf.

Key words: Beast Software, Conus, Divergence Time, DNA Barcoding, Molecular Genetics

INTRODUCTION

DNA barcode (a short DNA sequence), used for the species identification (Bandyopadhyay et al., 2006; Brauer et al., 2012). The mitochondrial cytochrome oxidase subunit I (COI) gene, codifies part of an enzyme that is necessary for the cell respiration in eukaryotes (Bouchet et al., 2011). COI sequences are available for a wide range of species, such as Conus species. So it is accessible to use these gene sequences for phylogenetic relationships (Bandyopadhyay et al., 2006 & 2008; Bouchet et al., 2011).

Conus species with 803 valid species are one of the most diverse species in the marine environment (WoRMS, 2014). According to the molecular phylogenetic analysis, there are three major lineages: one located at Indo-Pacific (IP) and East Pacific (EP), and another at West Atlantic (WA). The third one has a single species that only restricted to the East Pacific (EP) (Bouchet et al., 2011). The identification of these diverse species are done by using different mitochondrial gene (Duda & Palumbi, 1999; Duda & Kohn, 2005; Cunha et al., 2008; Duda, 2008; Espino et al., 2008; Nam et al., 2009; Pereira et al., 2010; Kraus et al., 2012; Biass et al., 2015). Moreover, a major cause that contributes to the success of these species is the remarkable biochemistry of their venoms (Hu et al., 2011; Violette et al., 2012; Dutertre et al., 2013; Rodriguez et al., 2015), that can paralyze different types of prey. So, the venom of each Conus species acts on special receptors and ion channels and is great interest in molecular neuroscience (Kaas et al., 2010; Lewis et al., 2012; Craik et al., 2013). Moreover, these species are interesting for molecular phylogeny and evolution studies (Cunha et al.,

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The fossil record revealed that the origin of the *Conus* species was in the Lower Eocene and major radiations in the Miocene and Pleistocene (Duda, 2008). Duda and coworkers applied fossil and biogeographic data as time scales to estimate the times of origination of clades with distinct feeding modes in *Conus* species (Duda et al., 2001). Other researchers used the rate of synonymous substitutions of a genome (Duda & Palumbi, 1999). The most comprehensive study in molecular phylogeny includes 320 species of the 761 recognized valid *Conus* species (Puillandre et al., 2014).

*C. coronatus* and *C. frigidus* belong to the clades of *Conus* species that called Virgiconus and Viroconus, respectively (Following the classification of Puillandre et al., 2014). Both are dominant cone snail from the Persian Gulf that was identified for the first time. The Persian Gulf is one of the critical marine ecosystems, despite these ecosystem conditions, the Persian Gulf supports a range of geographic diversity. Molecular Phylogenetic is important because it realizes our understanding of genes, genomes evolutionary relationship between living things, through DNA barcoding. Therefore, due to the fact that there has not been a study on the identification of Persian Gulf cone snails, this study for the first time presents Persian Gulf samples and their divergence times study by using different genetic analysis softwares.

**MATERIAL AND METHODS**

**Sampling area**

*Conus* specimens were collected from the intertidal zone of the Larak and Qeshm Islands. Larak Island is located in the south of the Strait of Hormuz in the PG (26° 51′ N, 56° 21′E), covering an area of 49 km$^2$. Qeshm Island is the largest Island of the PG, with 120 km length and up to 30 km width, situated in the western part of the Strait of Hormuz (26 56′ 57 N, 56 16′ 08 E).

Each *Conus* specimen was crushed and repeatedly washed with distilled water. The foot of the specimens was cut and preserved in 95% ethanol.

**DNA extraction and sequence determination**

The foot of the specimens was cut into small pieces and then was added 700 µl digestive solution (1% CTAB, 50mM Tris-HC1 (pH 8.0), 10mM EDTA (pH 8.0), 10% SDS and 10g/L proteinase K). After overnight incubation at 60°C, subsequent phenol/chloroform extractions and alcohol participations were performed (Palumbi, 1996). Finally, DNA was resuspended in 50 µl water and 5 µl of the resuspension was electrophoresed and visualized on 1% agarose gel. The obtained DNA sample was stored at -20°C until used for PCR amplification. PCR was performed using 0.3 µl of Taq DNA polymerase (5 U/µl), 2.5 µl of 10× PCR buffer, 1 µl of MgCl2, 0.5 µl of dNTPs (25 mM), 1 µl of each primer (50 pmol/µL) and 1 µl of template DNA in 25 µl final volume of reaction under the following protocol: initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 45 S, followed by extension at 72 °C for 1 min. The final extension was at 72 °C for 5 min (Bouchet et al., 2011).

Two conserved primers were used to amplify the mtDNA coding for partial COI gene (Folmer et al., 1994):

Forward: 5´-GGTCAACAAAATCATAAAGATATTGG-3(LCO1490)
Reverse 5´-TAAACTTCAGGGTGACCAAAAAATCA-3 (LCO2198)

Finally, the identified COI genes from 5 *Conus* specimens, registered in the GenBank. GenBank accession numbers are present in Table 1. These data were combined with published sequences from GenBank and BOLD (Barcode of Life Data system) sites. Non-conoidean Borsoniidae (*Bathytona neocaledonia*) as out group was chosen according to Puillandre et al. (2014).
DNA BARCODING STUDY OF CONE SNAILS

TABLE 1. List of PG Conus species used in the data set for phylogenetic analyses.

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank Accession Number</th>
<th>Sampling area</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. coronatus</td>
<td>LC121597.1</td>
<td>Qeshm Island</td>
</tr>
<tr>
<td>C. coronatus</td>
<td>LC101448.1</td>
<td>Qeshm Island</td>
</tr>
<tr>
<td>C. coronatus</td>
<td>LC126014</td>
<td>Larak Island</td>
</tr>
<tr>
<td>C. frigidus</td>
<td>LC126015</td>
<td>Qeshm Island</td>
</tr>
<tr>
<td>C. frigidus</td>
<td>LC126016</td>
<td>Larak Island</td>
</tr>
</tbody>
</table>

Mitochondrial COI gene analysis

Molecular phylogeny of Conus species of PG

Sequences were automatically aligned using Clustal W multiple alignments, implemented in Bio Edit v.7.0.5.3. Preliminary analyses were performed for each gene separately using the Neighbor-Joining algorithm (with a Kimura-2-parameters model (K2P)) implemented in MEGA v.6 (Stamatakis, 2006; Tamura et al., 2013) and J Model test v. 3.06 was used to choose the best model to construct phylogenies with posterior probability with MrBayes. Bayesian analyses (BA) were performed running two parallel analyses in MrBayes (Ronquist & Huelsenbeck, 2003) consisting of 4 Markov chains of 1,300,000 generations each with a sampling frequency of one tree each thousand generations and Tracer 1.4.1 was evaluated the convergence of each analysis (Rambaut & Drummond, 2007), and analyses were terminated when Effective Sample Size (ESS) values were all higher than 200 and after omitting the first 25% trees as burn-in.

Genetic Distances

The most similar sequences from the C. coronatus and C. frigidus were computed by using MEGA v. 6, with a K2P model and Bathytoma neocaledonia as an out group to obtain the genetic distances.

Divergence time and molecular clock

Beast 1.8.2 software was used to estimate the rate of synonymous substitutions (0.63–1.8% per million years) within the nuclear genome of this genus (Duda et al., 2001; Duda & Kohn, 2005). This rate was used for the obtained sequences to estimate the times of the divergence of these species in PG compared to the other regions. Convergence of analysis was evaluated by using Tracer 1.4.1 and was terminated when ESS values were superior to 200 (Rambaut & Drummond, 2007).

RESULTS

The results of the gene amplification of five specimens of Conus coronatus and Conus frigidus were visible on the agarose gel. After sequencing PCR products, three sequences of C. coronatus and two for C. frigidus were obtained. These sequences are the first time reported of the PG, and were recorded in NCBI database: Available on www.ncbi.nih.gov (Fig. 1).

Molecular phylogeny of the Conus species of the PG

The dataset was limited to the species belonging to the both Viroconus and the Virgiconus, with additional species from closely related clusters (Chelyconus, Phasmoconus and Lividoconus) and more distantly related Conus species. In the resulting tree (Fig. 2), the Viroconus, clustered from the Chelyconus and Phasmoconus clades (Posterior Probability (PP) = 0.83 and ML=71), and the C. coronatus clustered from the C. jadaeus (PP=0.9, ML=67).

Conus species of the PG were grouped in a highly supported clade (PP = 0.75 and ML= 72) with other Conus species from IP. The phylogenetic tree showed the specimens of C. frigidus (LC126016 and LC126015) and KJ549914.1 had a close genetic relationship. They were clustered in a monophyletic and highly supported clade (PP=0.99, ML=83).
Genetic Distances
The genetic distances between the Conus coronatus specimens from the PG (LC121597.1, LC101448.1 and LC126014) and the other regions, was very low. (The lowest value was 0.3% for CONO1650-14). But, the specimens of the C. frigidus from the PG (LC126016 and LC126015) and KJ549914.1 had the same clade by genetic distances about 0.3%. Correspond to the genetic distances; the other clades of these species had a larger amount (2.2%-3.6%).

Divergence time and molecular clock
To estimate the times of origination of the PG clades, substitution rate about 0.63–1.8% per million years in Conus genes was applied as the time scale. Correspond to these rates, the average divergence time of Viroconus and Virgoconus clusters, is about 18.76-26.89 and 22.15-30.3 million year ago (MYA), respectively. Likewise, the average time of the divergence of the C. coronatus cluster from C. judaicus, and C. frigidus clusters from C. flavidus, is about 4.79-7.93 and 5.86-9.6, respectively. Also, clustered rates of the C. coronatus and C. frigidus clades of the PG from the other regions were estimated about 0.8-1.84 and 0.75-2.05, respectively.

DISCUSSION
Molecular phylogeny of the specimens of the PG
A molecular phylogeny can help us to guess the evolutionary patterns of the species (Bandyopadhyay et al., 2008; Bouchet et al., 2011) and a phylogenetic tree based on molecular data can help to estimate diversification rates, divergence times, ancestral distributions, and community compositions (Espino et al., 2008; Nam et al., 2009). So, Mitochondrial DNA analysis, and specially COI, is a good DNA barcoding to show the variation in DNA sequences of different species (Bandyopadhyay et al., 2006, 2008; Puillandre et al., 2014).

Puillandre and coworkers analyzed the evolution of the diet, the biogeography, and the toxins of Conus species, based on three mitochondrial genes (COI, 16S rDNA, and 12S rDNA) to illustrate the usefulness of molecular phylogenies in addressing specific evolutionary. About 85% of the species clustered in the single Large Major Clade. C. coronatus and C. frigidus were in Large Major Clade and also located in Viroconus and Virgiconus clades, respectively (Puillandre et al., 2014). According to our study, the PG species are grouped in a highly supported cluster of the C. coronatus (PP = 0.99, ML=83), and also with the other C. coronatus specimens from IP (PP = 0.77, ML=82).
The genetic distance between these groups was very low about 0.3-0.5, except the specimen number (CONO1650-14 (0.9). The *C. frigidus* specimens from the PG (LC126016 and LC126015), and KJ549914.1 had a close genetic relationship (0.3). They were clustered in a monophyletic and highly supported clade (PP=1, ML=99), too.

The K2P genetic distances between the same specimens are very low (0.2–0.3%) and correspond to the genetic distances, generally considered as intraspecific distances in *Conus* species. Conversely, all the genetic distances with other known *Conus* species are large (> 10%) and correspond to genetic distances generally considered as interspecific distances in *Conus* species (Duda & Kohn, 2005; Lorenz & Puillandre, 2015).

It was acceptable that the specimen of the PG had a close genetic relationship of the species of the IP region. IP is the ancestral source of the Conidae and has the most *Conus* species than the other
region (Duda et al., 2001), so the Conus species of the PG have low genetic distance to the IP, since the PG is located in the subtropical region on the northwest of the Indian Ocean (IO) through the Strait of Hormuz (Akbari & Masoudian, 2009). AL-Khayat, suggested that the molluscan fauna of Qatari waters (south of the PG), the Gulf of Oman and the Red Sea have already been observed among the widespread IP species that found throughout the tropical IO and WP Oceans (examples: Pinctada radiata), some of the species restricted in distribution to the Northern IO, the PG and the Red Sea (examples: Fusinus townsendianus), and the last one was the endemic species to the species PG (examples: Strombus persicus) (AL-Khayat, 2008). Moreover, the species-level phylogenetic hypotheses of 138 Conus species indicated that one clade originated in the IP and the other in the EP + WA. Obstacles to dispersal in these regions may have promoted this early separation of IP and EP + WA lineages of Conus (Duda & Kohn, 2005).

**Divergence time and molecular clock**

To estimate the divergence time of the species, the data of fossil record (Duda & Kohn, 2005) and the rate of synonymous substitutions within the nuclear genome of this genus can be used as time scales (Duda & Palumbi, 1999). Duda et al. (2001), estimated the dates of the divergence of lineages from the mitochondrial genome data based on the divergence of the C. lividus and C. quercinus, at 11 MYA by the fossil record. In this study as the time scale, substitutions rate of the genome of the Conus was used (Duda & Palumbi, 1999). Application of a molecular clock to the phylogenies of our specimens of Conus suggested that this genus appeared about 50.09-73.98 MYA. According to the other studies, about 55 MYA in the Lower Eocene, Conus has evolved into the most species-rich marine animal genus, with well over 500 extant species throughout the world’s tropical oceans (Kraus et al., 2012; Puillandre et al., 2014). The Viroconus clade clustered from the Chelyconus and the Phasmoconus clades, at lower Miocene. And the Virgiconus, from the Lividoconus at upper Oligocene (Fig. 3). C. coronatus and C. frigidus both appeared at upper Miocene similar to other reports (Lorenz & Puillandre, 2015). Conus species of the PG (C. coronatus and C. frigidus) have been segregated in about 0.8-1.84, and 0.75-2.05 MYA, respectively. According to geological events, the PG is a geological subsidence at the southern edge of the Zagros Mountains, and was formed in the late Tertiary time. At the end of the Pliocene, the water level of the PG was 150 meters above the current level that today, in the form of marine terrace and Sabkha on the Arabic site of PG was seen (Al-Khayat, 2008; Akbari & Masoudian, 2009). During the Ice Age, the Pleistocene, the water level was low, and the most areas were dry. There was only a small channel of water. So, The PG fauna presumably was occupied from the IP region after the Ice Age, and via the connection of the IO (Akbari & Masoudian, 2009). This study was revealed that the cone snail species from the PG, mainly belong to the IO which displaced at ancient times. The Conus species from the PG have been not studied very well. It is necessary to disclose the relationship between native and migrant species.
FIGURE 3. Molecular Phylogenetic tree and divergent time of *Conus* species from the Persian Gulf. The number of nodes were indicated the time of the divergent of the clusters.

The evolutionary history of the *Conus* species revealed by the substitution rate was highlighted by its origin in the Eocene and the major radiations in the Miocene. The phylogenetic tree being constructed according to partial COI gene sequence data of the PG specimens confirmed that the *C. coronatus* and *Conus frigidus* belong to the Viroconus and the Virgoconus clades, respectively, also in the same cluster of the IP region. Moreover, the PG specimens were clustered lately about 2 MYA. The Persian Gulf with specific circumstances has remarkable geographic diversity. So, phylogenetic identification of the species through DNA barcoding and its relationship with the Indian Ocean, are important both economically and in terms of obtaining information of ecological water sources.
Acknowledgments
This study supported by the department of marine biology, faculty of marine sciences and oceanography, Khorramshahr University of marine science and technology. It is declared that authors have no conflict of interest.

LITERATURE CITED


Tamura, K., Stecher G., Peterson, D., Filipski, A.m, Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.
