Evolutionary study on two closed *Gammarus* (Crustacea, Amphipoda) species from Zagros Mountains (IRAN) using molecular methods

Azin Fahim A.[1]; Rezvani Gilkalaie S.[2]*

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Abstract

Mitochondrial Cytochrome Oxidase Subunit I DNA sequences are a good discriminative marker for phylogenetic studies in crustaceans and especially in amphipoda. In the present study, molecular and morphological data were analyzed to test whether *Gammarus lobifer* authority and *Gammarus balutchi* authority which one or two geographically separated but morphologically similar species. The analyses proved that there are two species and that uplift of the Zagros Mountains was probably the most important cause of Allopatric speciation in this region during the Miocene period.

Keywords: Molecular phylogeny, COI, *Gammarus lobifer*, *Gammarus balutchi*, Zagros Mountains

1-School of Biological Sciences, University College of Science, University of Tehran.
2-Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran.
*Corresponding author's Email:srgilkolaei@gmail.com

Introduction

The genus *Gammarus* (Crustacea, Amphipoda), with 204 described continental species, are distributed mainly in Europe, with the range extending to China and North America (Väinölä *et al.*, 2008). Because of this widely distribution, researchers have faced lots of taxonomic problems. Thereby since 1785 multiple modifications of the systematics have been used based on morphological characters (Meyran *et al.*, 1997). Species taxonomy is an important part of estimating biodiversity and designing conservation strategies. Improvement and lower costs in molecular techniques has allowed DNA sequencing to become the most popular choice for the taxonomy of animals at species and population levels (Hou *et al.*, 2007). Consequently, many systematists incorporate molecular techniques into their studies when morphological surveys are not sufficient to answer their study's questions (Hou *et al.*, 2007). Among the various molecular approaches, mitochondrial cytochrome oxidase subunit I (COI) DNA sequences have been chosen as a suitable marker for identification gammarids at both inter- and intraspecific levels (Siegismurd and Müller, 1991; Meyran *et al.*, 1997; 1998; Müller, 2000; Hou *et al.*, 2007). In Iran, before 1982 few studies have been done on Iranian amphipods but in 1998 and in a wide amphipod survey many specious were identified from Iran by Stock *et al.* (1998). Following their work, a series of studies including Yavari (2000), Banakar (2001), Amraii (2001), Khalaji-Pirbalouty (2002), Pourmohammadi-Sarbanani (2002), Naghib (2002), Khalaji-Pirbalouty and Sari (2004); Khalaji-Pirbalouty and Sari (2006), Zamanpoore *et al.* (2009) and Hekmatara *et al.* (2011were started from 1999 in the Department of Zoology, University of Tehran, and on some occasions in collaboration with other universities. In these studies, various species of freshwater *Gammarus* reported only from Iran, various species of freshwater *Gammarus* reported only from Iran (Stock *et al.* 1998; Khalaji-Pirbalouty and Sari 2004; 2006; Zamanpoore *et al.*, 2009; Hekmatara *et al.*, 2011). Two of these Iranian species, *Gammarus lobifer* (Stock *et al.*, 1998) from Kohgyloye-va-Boyer Ahmad and *Gammarus balutchi* (Khalaji-Pirbalouty and Sari, 2006) from adjacent province, Charmahal-va-Bakhteyari, at lower magnification have close morphological affinities with each other (Khalaji-Pirbalouty and Sari, 2006). Based on light and electron microscopy, some distinguishable characteristics were found. For example, *G. lobifer* has a truncate lateral head lobe and one subangular seta on the postero-ventral corner of pereopod 7, but *G. balutchi* has a round lateral head lobe and lacks subangular seta. Also as it has been shown in Fig. 1, scanning electron microscopes (SEM) studies revealed that the lateral head lobes are truncate in *G. lobifer* (Fig.1A) but rounded in *G. balutchi* (Fig.1B). Head microsculpturing shows some Marked

differences in the number and patterns of pore distribution (Fig.1 A and B) (Khalaji-Pirbalouty and Sari, 2006). Nevertheless the presence of more than 95% morphological similarities using light and SEM caused these two species be controversial. In the present study, we examine the molecular differences between these two species using molecular COI sequence data and try to estimate the time of divergence of these species to assess patterns of diversity and how they related to geography and past natural events.

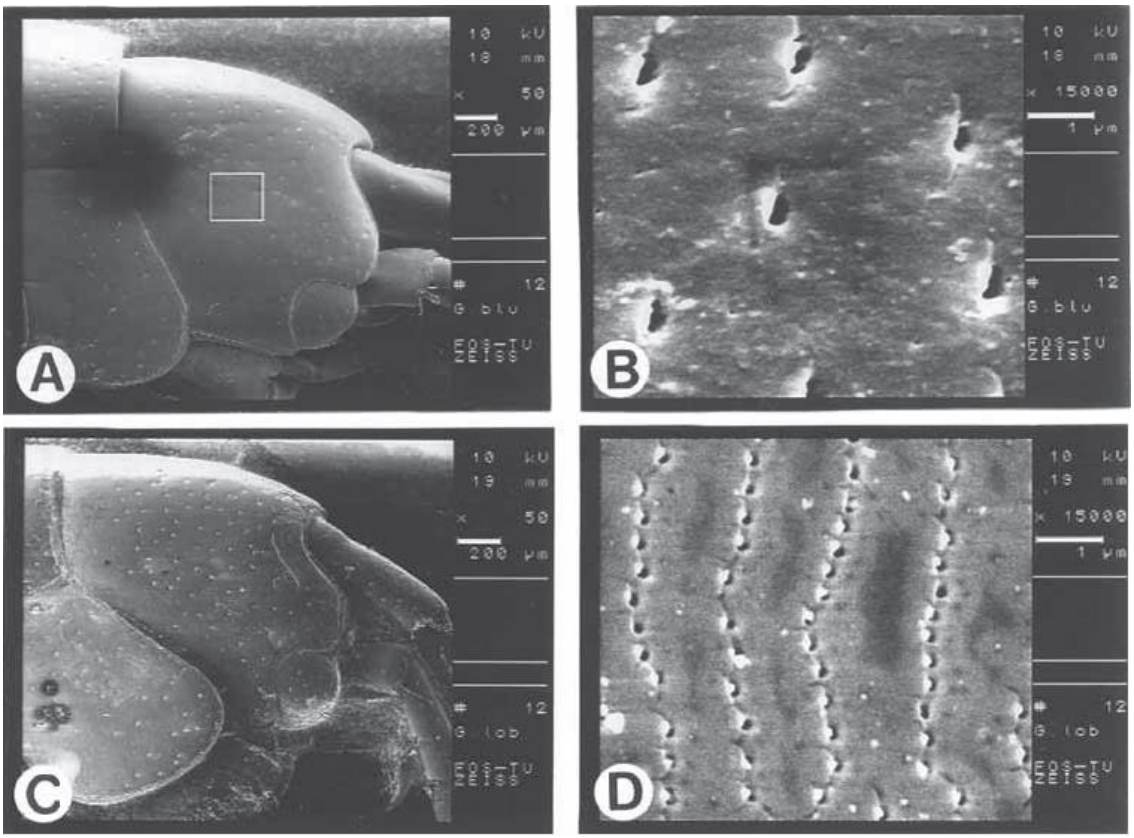

Figure 1: Scanning electron micrographs of head and details of head microsculpturing in Gammarus balutchi (A & B) and G. lobifer (C & D) (Khalaji-Pirbalouty & Sari 2006).

Material and methods

Sampling and DNA extraction

Samples of both species were obtained from two stations which they could have been found. *G. lobifer* specimens were collected from Cheshme-Belghais (N 30.722285, E 50.744905) near Yasouj in Kohgyloye-va-Boyer Ahmad Province and *G. balutchi* from Atashgah Fall (N31.245462, E51.006367) near Lordegan city, from adjacent province, Charmahal-va-Bakhteyari using 1mm mesh size sieves, small hand net. Samples were preserved in 96% ethanol for using genetic analysis while additional animals were kept in 70% ethanol for keeping in Zoological Museum, University of Tehran (ZUTC). The voucher numbers are ZUTC

Amph.2222 and ZUTC Amph.2221 for *G. lobifer* and *G. balutchi*, respectively. Analyses of morphological features confirmed that the proper species had been collected and total DNA was extracted from entire individuals using Chelex (Sigma) protocol (10-20% (w/v) of chelating resin in 10mM Tris/ 1mM EDTA solution with 20 mg/mL of proteinase K; (Walsh *et al.*, 1991).

PCR amplification and sequencing

A ca. 690bp fragment of mithochondrial COI was obtained using the universal primers HCO2198 (5´TAAACTTCAGGGTGACCAAAA AATCA3´) and LCO1490 (5´GGTCAACAAATCATAAAGATA TTGG 3´) (Folmer *et al.*, 1994). Polymerase chain reaction (PCR) was used to amplify COI gene.The amplification reaction mixture consists of 2µL DNA template 10ng, 0.5µL dNTPs10mM, 1µL of each primers (10 pmol/µL), 1 µL $MgCl_2$ 2.5mM, 5µL 10X PCR buffer, 25µL 10% trehalose, 0.2 µL 1U Kawsar Company *Taq* DNA polymeras, with sterilized water added to make up the final volume to 50µL. PCR setting for amplification consists of initial denaturing of 1min at 94ºC, five cycles of 40s at 94ºC, 40s at 45ºC, 1min at 72ºC, then 35 cycles of 40s at 94ºC, 40s at 51ºC, 1min at 72ºC and final 5min extension at 72ºC (Witt *et al.*, 2006 with some modifications). PCR products were purified using Gel Extraction kit (Fermentase) and then were sent to Macrogen Inc. in South Korea for sequencing. Finally, at least there were five sequencing data for each species.

Molecular analysis

All of the obtained sequences were verified as being derived from Amphipod DNA using the GenBank Blast algorithm. BioEdit software version 3.2 was used for editing sequences. Then, all of them were aligned using Clustal W (Thompson *et al.*, 1997). Finally, they were scanned by eye for conserved, variable and parsimony informative sites. Thirteen taxa plus one out-group were included in the phylogenetic analysis in MEGA4 software ((Kumar *et al.*, 2004) with different methods of phylogeny inference like parsimony, neighbor-joining and maximum-evolution as well as UPGMA analysis. Divergence time was estimated using Tajima's test (Tajima, 1993) as well as the average net distance between groups (Tajima and Nei, 1984) beside molecular clock approximation for COI of 2.4% nucleotide sequence divergence per million years (Knowlton *et al.*, 1993). This rate was derived from the study of several malacostracan crustaceans whose divergence resulted from a distinct geological event, the formation of Isthmus of Panama (Knowlton *et al.*, 1998).

Figure 2: Map of Iranian provinces with demonstrating Chamahal-Va-Bakhteyari ■ and kohgyloye-Va-BoyerAhmad □ provinces (central Zagros area).

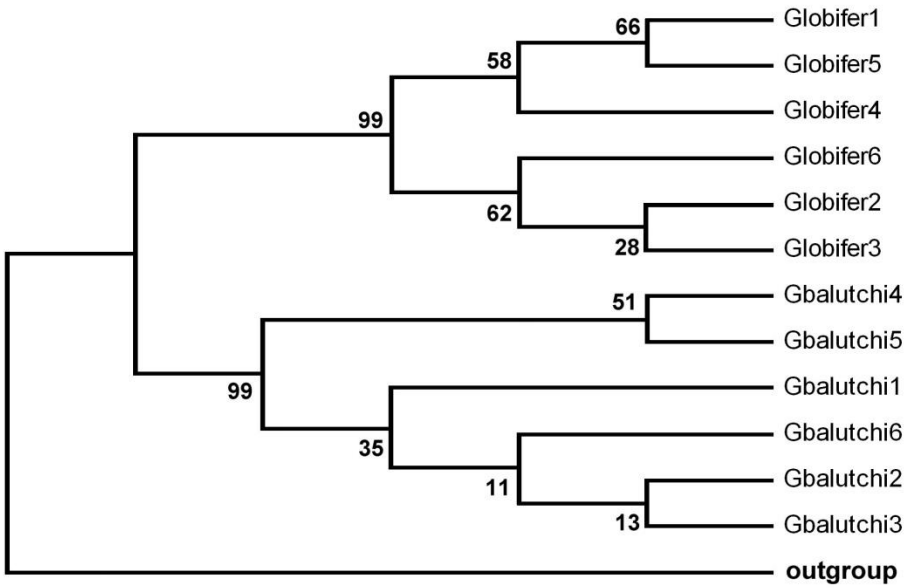

Figure 3: Maximum-Parsimony tree based on the COI data. Bootstrap values>50% are shown based on 500 replicates. Ingroup is rooted by the outgroup taxa *G. lacustric*.

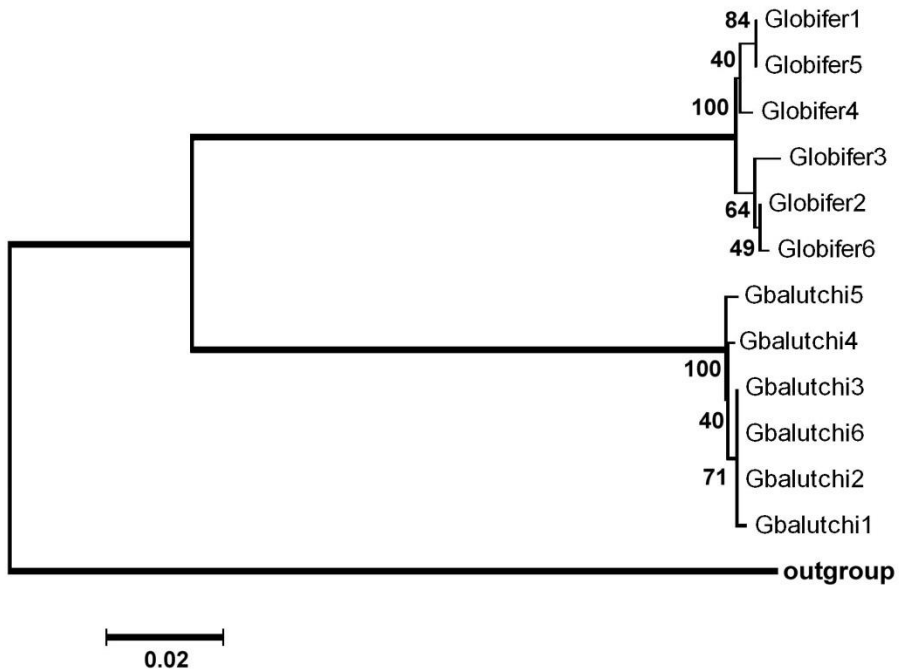

Figure 4: Neighbor-Joining tree based on the COI data. Bootstrap values>50% are shown based on 500 replicates. Ingroup is rooted by the outgroup taxa *G. lacustric*.

Results

Analysis of nucleotide sequences

The alignment of COI sequences was conserved in length and no alignment gaps or insertion/deletion events were found. There were 125 parsimony informative sites, 550 conserved sites, 12 singleton sites and also 138 variable sites in these ~ 690bp sequences. The nucleotide sequences of *G. lobifer* and *G. balutchi* are available in GenBank under the accession numbers HQ198587 and HQ198586. The maximum parsimony analysis as well as neighbor-joining analysis in MEGA4 resulted in nearly similar trees (Figs. 3 and 4). With high bootstrap supports, these analyses suggested that we absolutely have two separate monophyletic species. Also other methods of analyzing like UPGMA and minimum-evolution revealed the same results (not shown). It should be mentioned that a sequence of known *Gammarus* species (*G. lacustric*) has been used as out-group.

Analysis of amino acid sequences

Amino acid sequences for these two species were deduced using the genetic code of *Drosophila* mtDNA. They reveal 213 residues in length and just residue 115 was different. Gly for *G. lobifer* and Ala for *G. balutchi.*

G. lobifer

SVVGTSLSVIIRSELSAPGNLIGDDQ
LYNVMVTAHAFVMIFFMVMPIMIG
GFGNWLVPLMLGSPDMAFPRMNN
MSFWLLPPSLTLLLMSGLVESGVG

TGWTVYPPLAGATAHSGGAVDLAI
FSLHLAGASSILGAINFISTVLNMRS
PGMPMDQMPLFVWSVFITAILLLL
SLPVLAGAITMLLTDRNLNTSFFDP
SGGGDPILYQHLFWFFG

G. balutchi

SVVGTSLSVIIRSELSAPGNLIGDDQ
LYNVMVTAHAFVMIFFMVMPIMIG
GFGNWLVPLMLGSPDMAFPRMNN
MSFWLLPPSLTLLLMSGLVESGVG
TGWTVYPPLAGATAHSGAAVDLAI
FSLHLAGASSILGAINFISTVLNMRS
PGMPMDQMPLFVWSVFITAILLLL
SLPVLAGAITMLLTDRNLNTSFFDP
SGGGDPILYQHLFWFFG.

Time of divergence

The net distance between these two species were 18%. So we can estimate that these species diverged nearly 7.5 million years ago from the same ancestor as we can see in Fig.4.

Discussion

Allopatric divergence

Sometimes unexpected species diversity or cryptic species (phenotypically similar but genomically different) are found during molecular studies (Taylor *et al*., 1998; Klautan *et al*., 1999; Witt and Hebert, 2000; Müller, 2000; Collin, 2002; Müller *et al*., 2002). The phenotypic similarities that persist in the face of extensive molecular evolution present a dilemma (Taylor *et al*., 1998; Jarman and Elliott, 2000; Mayer and Van Helversen, 2001; Wellporn, 2004). The role of allopatry in the diversification of taxa is well studied. Natural geographical barriers resulting in a sudden interruption of gene flow between diverging lineages that would result in allopatric speciation. This study has demonstrated that *G. lobifer* and *G. balutchi* are distinct species based on molecular studies. The close phenotypic similarity of these species arose from the same ancestor. The molecular divergence suggests that speciation is most likely due to geographical isolation after the Zagros orogeny. This pattern has been previously reported among freshwater and marine amphipoda (Meyran *et al.*, 1997; Müller, 2000; Witt and Hebert, 2000).

Divergences times in relation to historical events

Molecular clocks have profoundly influenced modern views on the timing of important events in evolutionary history. Among several "universal" molecular clocks, the most prominent has been the "mtDNA clock" (Brown *et al*., 1979; 1982), which holds that animal mtDNA, evolves at a rate of ~2% sequence divergence per million years. Based on the molecular rate of 2.4% divergence per million years that is generally used for crustaceans and also amphipods, it appears that the species diverged approximately 7.5 million years ago during the late Miocene. The "Zagros Orogenesis" took place in the Miocene era in Iran producing a series of parallel ridges interspersed with plains that bisect the country from northwest to southeast (Alavi, 1994). These mountains are located in western Iran at the eastern

edge of the Persian Gulf and are a part of the Alpine-Himalayan mountain chain that stretches from the northwest to the southeast, dividing the region into two distinct geographical regions. The tectonic evolution of the Zagros Mountains was entirely due to plate tectonics and the converging of the Arabian and Eurasian continents. The timing of the collision of the Arabian and Eurasian plates is generally known to be in the Miocene (Takin, 1972; Agard *et al.*, 2005). The tectonic processes were begun during Permian to Triassic in northwest and later in south at the late Triassic (Alavi, 1994).

Late Cretaceous to Eocene rocks represents deposits of the foreland basin prior to the Zagros orogeny and subsequent incorporation into the colliding rock packages (Alavi, 2004). The uplifting of the Zagros Mountains was complete in the Pleistocene and it is likely that allopatric speciation took place as a result. . The studied species are located on either side of the Zagros Mountains with no natural path for migration (Fig. 2). Based on the estimation of the divergence time, the scenario of allopatric speciation because of Zagros uplifting is corroborated. Similar stories were also found in Amphipoda (Thomas *et al.*, 1994; Takhteev, 2000).

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