MAKÜ FEBED
ISSN Online: 1309-2243
http://dergipark.ulakbim.gov.tr/makufebed

Mehmet Akif Ersoy Üniversitesi Fen Bilimleri Enstitüsü Dergisi 7(Ek Sayı 1): 248-253 (2016)
The Journal of Graduate School of Natural and Applied Sciences of Mehmet Akif Ersoy University 7(Supplementary Issue 1): 248-253 (2016)

Araştırma Makalesi / Research Paper

Comparing the Efficancy of mtDNA COI-based Taxonomy in the Freshwater Gastropod Genus *Graecoanatolica in* West Anatolia (Gastropoda: Hydrobiidae)

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Geliş Tarihi (Received): 27.07.2016, Kabul Tarihi (Accepted): 12.10.2016
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ABSTRACT

Taxonomic identification among species for biological discipline is vital. Morphological characteristics of the genus *Graecoanatolica*, which it spreads West Anatolia in Turkey, have been comprehensively studied but molecular Systematics of the *Graecoanatolica* species have not been investigated. In this research, phylogenetic analysis of cytochrome oxidase subunit I (620bp) sequences for all living species in Anatolia of *Graecoanatolica* were performed and phylogenetic trees were created by using data of COI sequencing. Mitochondrial DNA (mtDNA) COI gene sequences of different genus were achieved from GenBank and used as outgroup for molecular systematic analysis. mtDNA COI sequence data analysis indicate that species determined as *G. lacustristurca* in Burdur is sister of *G. tenuis* in Denizli but it is still diffirent species. Also molecular data indicates that *G. pamphylica* is different molecularly from genus *Graecoanatolica,* which has been described into this genus and presenced in Antalya Kirkgöz spring.

Keywords: Hydrobiidae, *Graecoanatolica*, COI, phylogeny, taxonomy

Batı Anadolu'da Bulunan Tatlı Su Gastropodu *Graecoanatolica*'nın mtDNA COI Temelli Taksonomisinin Kıyaslanması

ÖZ

Biyolojik disiplinler için türler arası taksonomik tanımlamalar hayati öneme sahiptir. Türkiye'nin Batı Anadolu bölgesinde yayılış gösteren *Graecoanatolica* cinsinin morfolojik özellikleri çok iyi şekilde çalışılmış olmasına rağmen bu cinsin moleküler sistematiği önceden hiç çalışılmamıştır. Bu çalışmada Anadoluda yayılış gösteren *Graecoanatolica* cinsine ait nesli devam eden türlerin, sitokrom oksidaz I genine ait sekanslar kullanılarak filogenetik analizi yapıldı ve filogenetik ağaçlar oluşturuldu. Moleküler sistematik analizi için farklı türlere ait mitokondrial DNA (mtDNA) sitokrom oksidaz I (COI) genine ait sekans verileri gen banktan alındı ve dış grup olarak kullanıldı. Mitokondrial COI genine ait sekans analizi karşılaştırmaları, Burdur'da yayılış gösteren *G. lacustristurca* türünün Denizli'de bulunan *G. tenuis* türüne kardeş ve bu türden farklı bir tür olduğunu göstermetedir. Bunun yanısıra moleküler veriler *Graecoanatolica* cinsi içinde tanımlanan ve Antalya Kırkgöz kaynağında bulunan *G. pamphylica* türünün moleküler olarak *Graecoanatolica* cinsinden farklı olduğunu işaret etmektedir.

Anahtar Kelimeler: Hydrobiidae, *Graecoanatolica*, COI, filogeni, taksonomi

INTRODUCTION

The family Hydrobiidae (Gastropoda: Risooidae) has cosmopolitan spread (Kabat and Hershler, 1993) and large taxonomically diversity compared to many group of prosobranch gastropods (Arconada and Ramos, 2003). The family originated in Laurasia during the transition period between Carboniferous and Permian which was around 280 million years ago (Knight et al., 1960). Hydrobiidae family has been included in some superfamilies: Rissooidea, Hydrobioidea and Truncatelloidea (Arconada and Ramos, 2003). The latest estimates suggest that the family has more than 1000 described living species and almost 400 recent and fossil genera (Georgiev and Gloer, 2011). Hydrobiid populations show little morphological differences, even species living in adjacent drainage (Ponder et al., 1999). The hydrobiids are mostly very small Gastropod and their shell height rarely exceed 8 mm so anatomical study of these animals is too difficult (Kabat and Hershler, 1993).

Gastropod have very different shell form, sculptures and color. Although shell characters are used widely for discrimination among species (Schander and Sundberg, 2001), shell characters may have been affected by environmental factors (e.g. competition, parasitism, availability of food, substratum, calcium availability, salinity) and cannot be correspond to anatomical characters. Therefore, shells are not reliable character for species discriminating in many Mollusca groups (Wilke and Falniowski, 2001) and so the species discriminating have been used lately molecular, morphological and anatomical characters together (Bieler, 1992). Phylogenetic analyses obtained by comparing DNA sequencing that provides information about evolutionary relationships between species and similarities or differences of organisms. These informations help to create phylogenetic trees (Librado and Rozas, 2009).

Turkey has wide species diversity and endemism for hydrobiid snails and its known there are 46 hydrobiids species. Most of these species are endemic to Anatolia up to %50 (Sahin et al., 2012). The *Graecoanatolica* genus is small freshwater snails, aquatic gastropod molluscs in the family Hydrobiidae and has been represented 4 living species in Turkey. Those ones are *G. lacustristurca* Radoman (1973)*, G. tenuis* Radoman (1973)*, G. kocapınarica* Radoman (1973)*, G. pamphylica* Schütt (1964). There were also two species, which they have been named as *G. conica* and *G. brevis,* and they extincted in time. All of these species are endemic to Anatolia (Yildirim, 1996). There is also Balkan species described as *G. macedonica* by Radoman and Stankovic (1979) from Lake Dojran, Macedonia and *G. vegorriticola* was initially described as *Hydrobia vegorriticola* by Schütt (1962) from the Petron and Vegorritis lakes in Greece. Then Radoman (1983) revised this species as *G. vegorriticola*. Both of these species are presumed extinct in time (Ryan and Griffith, 2001; Griffith et al., 2002; Albrecht et al., 2006) and all extant *Graecoanatolica* species are restricted to Turkey (Kebapci et al*.,* 2012). Also, *G. lacustristurca* was reported from Eğirdir Lake and Kovada Channel by Zeybek et al. (2013).

Koca (2007) reported that *G. lacustristurca* from Isparta Egirdir Lake and Burdur-Karamanli Village Kocapınar spring, *G. tenuis* from Denizli-Gemiş Town Esref Otuzbir Park, *G. kocapınarica* from Egirdir Yukarıgökdere Village Kocapınar spring, *G. pamphylica* from Antalya Kirkgöz spring. Kebapci et al. (2012) also described a new *Graecoanatolica* species named *G. dinarica* from Çapalı Lake. Also, *G. lacustristurca* was reported from Eğirdir Lake and Kovada Channel by Zeybek et al. (2013).

The purpose of this study is comparison of the *Graecoanatolica* populations using mtDNA COI sequencing data. More particularly, on basis of the available *Graecoanatolica sp.,* our objective is to test whether molecular data are available with morphological data or not.

MATERIAL AND METHODS

Materials for this study were collected either by hand, by washing submerged stones, or by sweeping vegetation with a hand sieve from type localities (Table 1) stated by Koca (2007). *G. lacustristurca* species was collected as being stuck in rocks from Egirdir Lake mutual shores and also Burdur-Karamanli Village Kocapinar Spring. *G. tenuis* was collected 2 different localities. All samples were put into 80% ethyl alcohol and kept at -20ºC until DNA isolation. DNA was isolated for four sample for each group. Sequencing data belong to some species of Hydrobiidae family were used for phylogenetic analyses as outgroup. These sequencing data were supplied with GenBank accession number (Supplementary Figure 1). Samples of this study were collected from different localities which have been defined for *Graecoanatolica sp.* Samples are named as A, B, C, D, E, K and L, and GPS coordinates are shown

Table 1. Locality data for samples of *Graecoanatolica*. All localities are in West Anatolia.

Samples	Named	Coordinates	Localities
Graecoanatolica tenuis	A	37°46,351'N 029°50,593'E	Gemiş Town, Denizli, Turkey
G. lacustristurca[1]	B	37°18,376'N 029°55,936'E	Kocapınar Spring, Burdur, Turkey
G. tenuis[1]	C		Gemiş Town, Denizli, Turkey
G. pamphylica	D	36°56,52'N 30°36,30'E	Kirkgöz spring, Antalya, Turkey
Graecoanatolica lacustristurca	E	37° 52,58' N 030° 51,30' E	Isparta Eğirdir Lake
G. kocapınarica	K	37°41,45'N 030°50,78'E	Eğirdir Yukarıgökdere Village, Isparta, Turkey
G. lacustristurca	L	37°52,58'N 030°51,30'E	Eğirdir Lake, Isparta, Turkey

DNA Isolation and Sequencing

Genomic DNA from all samples was harvested dissected snail standard methods described by Wilke et al. (2006). mtDNA COI gene (620 base pairs excluding primer sequence) was used in determining genetic differences between species and form phylogenetic trees. COI gene fragments were amplified with LCO1490 and HCO2198 primers described by Folmer et al. (1994). The PCR conditions for COI were optimized as first step 30 sec. at 94°C to denaturation and followed by 25 cycles of 15 sec. at 95°C, 45 sec. at 52°C, 1 min. at 72°C, and after all cycles an additional elongation step of 10 min. at 72 ºC was performed. The protein-coding COI sequences data were aligned clearly by using BioEdit v7.0.9 (Hall, 1999).

Phylogenetic analyses

The results of the mtDNA COI sequences were translated into different formats by NaSP5 program (Librado and Rozas, 2009). K2 parameter (Kimura, 1980) is used for phylogenetic analysis and phylogenetic trees were formed according to Neighbor-Joining (NJ) (Saitou and Nei, 1987) and Unweighted Pair Group Method of Arithmetic Averages (UPGMA) (Davis et al., 2004) through MEGA4 program (Kumar et al., 2008).

RESULTS

COI Sequence Polymorphism between *Graecoanatolica sp.*

From 620 sites in 28 aligned sequences, number of variable sites was 112 and total number of mutations was 115. Also 112 sites (18%) were polymorphic and 108 sites (17.4%) were parsimony informative. All sequences G+C content was 0.361. The average overall K2P-distance was 0.216 and the lowest distance is 0.003 between *G. kocapınarica* and *G. lacustristurca* from Egirdir Lake, and the highest distance is 0.195 between *G. lacustristurca* (from Burdur) and *G. pamphylica* (from Antalya).

Table 2. The average genetic distance between groups of *Graecoanatolica sp* distributed in different regions

Groups	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1. A (*G. tenuis*)							
2. B (*G. lacustristurca*)	0.012						
3. C (*G. tenuis*)	0.008	0.014					
4. D (*G. pamphylica*)	0.192	0.195	0.188				
5. E (*G. lacustristurca*)	0.024	0.026	0.016	0.180			
6. K (*G. kocapınarica*)	0.026	0.027	0.020	0.181	0.006		
7. L (*G. lacustristurca*)	0.026	0.027	0.020	0.179	0.003	0.003	

COI Phylogeny

The phylogenetic trees were created using four different outgroups (*Belgrandia thermalis, Horatia klecakiana, Sadleriana fluminensis, Orientalia callosa*) and *Graecoanatolica* species consensus sequences. The sequence information of external groups was reached with GenBank accession numbers (Wilke et al., 2001)

Once analyzed the phylogenetic trees, we found out that *Belgrandia thermalis* was the nearest genus to *Graecoanatolica* genus in all outgroups. *B. thermalis which* distributes in the southern part of Italy is freshwater snail (Bodon et al., 1999).

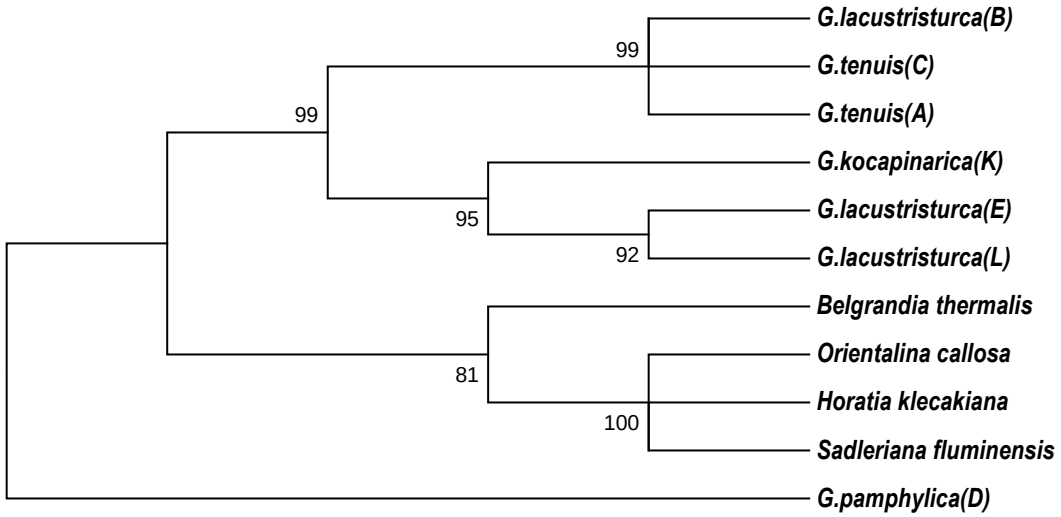

Figure 1. The Phylogenetic tree of *Graecoanatolica sp*. is obtained with the Neighbor Joining test (NJ)

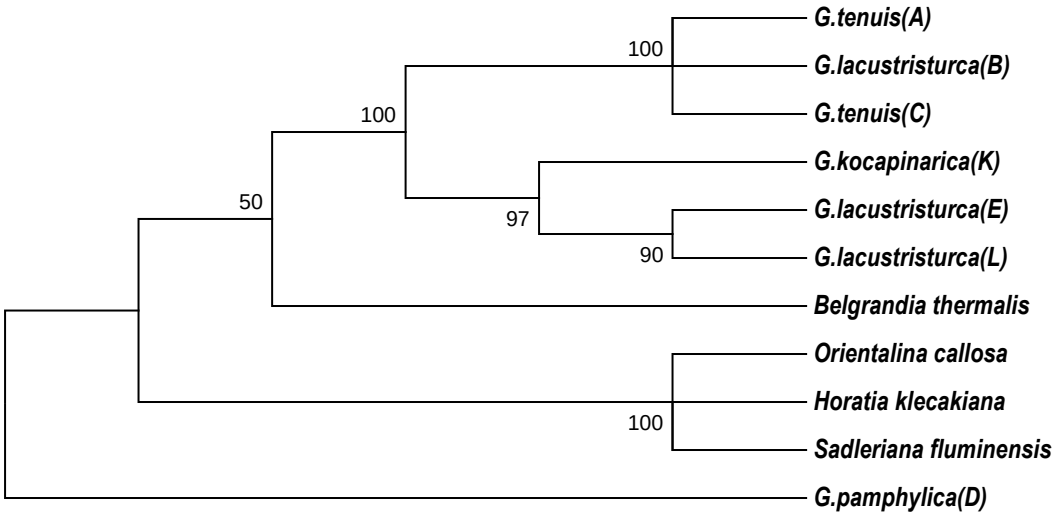

Figure 2. The phylogenetic tree of *Graecoanatolica sp*. is created with UPGMA

DISCUSSION

This research aimed that identification molecular relationship between species belong to *Graecoanatolica* genus, which have been spreading in Western Anatolia of Turkey (Schütt, 1980; Schütt 1990). *Graecoanatolica,* which is a genus of aquatic snails, exists in clean freshwater springs. Six species of *Graecoanatolica* were identified in Western Anatolia however four species of them can be found nowadays. These are *G. lacustristurca*, *G. tenuis, G. kocapınarica, G. pamphylica* (Yıldırım, 1996). Lately a new species described by Kebapci et al. (2012) as *G. dinarica*.

In previous studies, the samples which were picked up from Burdur Karamanli Village has been identified as *G. lacustristurca* (Radoman 1973) (group B) however according to our results did not place same branch with *G. lacustristurca* (Figure1 and 2). Analysis of phylogenetic tree and genetic distance show that samples of *G. lacustristurca* which are from Burdur Karamanli Village were found closer to *G. tenuis* (Denizli Gemis Town, Side of Esref Otuzbir Park and a source of Acigol Lake) but still different from this species. Molecularly, any difference was not observed between populations of *G. tenuis* which are from 2 different places. The samples of *Graecoanatolica* which are from Burdur are seemed to be a new and different species from *G. lacustristurca* according to molecular data.

Radoman (1985) suggested that inland water basins of the Aegean and Anatolia divided from Tethys widely by

the rising of mountains formation. Therefore, some of marine fauna disappeared. Then, evolution process started from sea enviroment to fresh and brackish water. Radoman (1973) explained this evolution as giving example *Graecoanatolica* genus, which belongs to Hydrobioidea superfamily and represented by him in Turkey. According to this study, ancestor species of *Graecoanatolica* emerged in one of these basins in ancient times and it spread from this basin to the other ones. Subsequently, he indicated that, as a result of proceeding to move up of mountains, previous water systems were divided to numerous secondary systems. The complexes, which belong to Kirkgöz such as Beysehir, Egirdir, Burdur and Acigol are some examples for those kind of systems. He also declared that some species of *Graecoanatolica* were identified from sources, which are connected with Egirdir, Burdur and Acigol in Turkey. As a result, it was indicated that there was only a uniform lake in this area. This big lake was divided by tectonic movements, and small isolated lakes and their connected sources emerged. Then, among these sources, pure source population was formed by geographical isolation. (Radoman, 1973; 1985; Koca, 2007).

The samples (group L) which were picked up from opposite of Egirdir Lake used to be identified as *G. lacustristurca*. According to our molecular data, this population is not *G. lacustristurca*, it is seemed that it belongs to *G. kocapinarica*.

Egirdir Lake has quite long distance between its two opposite coasts and the water flow is limited. Also, it is known that there is a connection on the opposite coast of Egirdir Lake with Kocapinar. Therefore, its not surprising that population on the opposite coast of Egirdir Lake is different from harbor side population of lake and to be similar with Kocapinar.

The generally accepted view of speciation that geographic isolation is very significant in this process. Gene flow will proceed normally in the system and speciation will not occur, as long as populations of a species is interacted directly or indirectly. if a stable population system cleavage with some geographical formation that blocks spread of species, separated population system will not be exchanged genes and evolution of these species will continue independently so new species will appear. Given enough time, they will get more different from each other because each one of those different population systems will evolve by their own way. At the beginning, reproductive isolation for these species is only geographic and they will keep reproduced between each other. According to modern type definition, in this kind of conditions, these population is inclusive of the same species. However, they will turn to be so different genetically in time so much after that, there will not be any effective gen flow even if an interconnection occurs. When differentiation reaches up to this point, two different population systems are going to compose two different species (Keeton et al., 1999).

Antalya Kirkgöz source isolated from other areas, which *Graecoanatolica sp* placed, once regarding geographical position of this area. Very long time ago occurred this isolation might be good enough time for speciation, even enough time forming a new and different genus. More studies should be performed about the Kirkgöz population.

Antalya Kirkgöz source isolated from other areas placed by other *Graecoanatolica sp*. when geographical position is regarded. Very long time ago occurred this isolation might be good enough time for speciation, even enough forming a new and different genus. More studies should perform about the Kirkgöz population.

According to our data, *Graecoanatolica* population which is on the opposite side of harbor of Egirdir Lake may be removed from *G. lacustristurca* population and incorporate to *G. kocapınarica.* Also, the localized population in Burdur - Karamanli may be renamed as a new species.

ACKNOWLEDGEMENTS:

This study was supported by Mehmet Akif Ersoy University Scientific Research Projects Unit (0083-YL-09).

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Suplementary Figure 1. Accession numbers belong to species which used as outgroups sequencing data

Accession Number	**Species**	**Bp**
AF367648	Belgrandia thermalis	638
AF367637	Horatia klecakiana	638
AF367649	Orientalina callosa	638
AF367647	Sadleriana fluminensis	638