RESEARCH ARTICLE

Feriha Tserkova [1, 2]
Daniela Klisarova [1]
Iliya Denev [2]

Molecular taxonomy study of representatives of the genus *Gobius* inhabiting coastal waters of Black Sea region

Authors' addresses:
[1] Institute of Fish Resources (Agricultural Academy), 9000 Varna, Bulgaria.
[2] Department of Plant Physiology and Molecular Biology, University of Plovdiv Paisii Hilendarski, 4000 Plovdiv, Bulgaria.

Correspondence:
Feriha Tserkova
Institute of Fish Resources, Primorski 4 Bulv., 9000 Varna, Bulgaria.
Tel./fax: +359 52 632066
e-mail: feya1980@gmail.com
Iliya Denev
Department of Plant Physiology and Molecular Biology, University of Plovdiv Paisii Hilendarski, 24 Tzar Asen Str., 4000 Plovdiv, Bulgaria.
Tel.: +359 32 261501
e-mail: iliya.denev@gmail.com

Article info:
Received: 2 November 2016
Accepted: 9 December 2016

ABSTRACT
According to their origin the Black Sea gobies can be divided to Ponto-Caspian relicts and Mediterranean immigrants. The increase of species diversity in the Black Sea is a consequence of Mediterranean immigrants. The present study investigated the variability in Cytochrome b sequences isolated from two former Mediterranean immigrant species: *Gobius niger* and *Zosterisessor ophiocephalus.* The annotated at NCBI Cyt b sequences of the species were also used to extend the reach of the study. The analyses demonstrated that Black Sea inhabited by four haplotypes of *G. niger.* Four other haplotypes were found in sequences of Mediterranean black gobies. In the Black Sea haplotype, 6 is predominant and 83.5% of all *G. niger* representatives belong to it. Three other haplotypes (H_1; H_7 and H_8) were found to form isolated populations. The Tajima D-test indicated that in the Black Sea *G. niger* in a stage of expansion and significant evolutionary pressure according to data from Maximum Composite Likelihood model of Tamura-Nei, which can explain the accumulation of mutation and appearance of new haplotypes. Unlike *G. niger*, *Z. ophiocephalus* populations are shrinking according to Tajima D-test and only one haplotype is still surviving in isolated locations in the Black Sea. These data are in agreement with previous reports of other authors which alarmed that the grass goby is a critically endangered species close to extinction.

Key words: Cytochrome b, fishes, Gobiidae, Mediterranean immigrants, *Gobius*, *Zosterisessor*

Introduction

The family Gobiidae counts probably more than 1900 species. They are classified into 6 subfamilies: Amblyopinae, Benthophilinae, Gobiinae, Gobionellinae, Oxudercinae, Sicydiinae and approximately 230 genera (Nelson, 1994; Hoese & Larson, 2006). Various authors assume that in the Black Sea live between 23 and 35 gobiid species (Svetovidov, 1964; Gheorghiev, 1966; Smirnov, 1986; Miller 1986; Rass, 1993; Engin & Bektas, 2010). According to their origin, the Black Sea gobies can be divided to Ponto-Caspian relicts and Mediterranean immigrants. The increasing species diversity in some Black Sea regions recently is due to Mediterranean immigrants (Vassilev et al., 2012).

One such species considered to be migrant from the Mediterranean Sea is *Gobius niger* (Linn.). The native range of the black goby includes Eastern Atlantic from Scandinavian Peninsula to north-western Africa (Mauritania), Baltic Sea, Mediterranean, Aegean, Adriatic, Marmara Sea, and the Suez Canal. Nowadays it is widespread in the Black Sea. In Bulgarian waters, the black goby can be found everywhere along the Black Sea coast and it is one of the most common gobies. *G. niger* (Linn.) inhabits estuaries, lagoons and coastal sea waters, in depths from 0.5 down to 50 – 75 m, over muddy or sandy bottoms, among shells and algae (Vassilev et al., 2012).

Another similar migrant is grass goby (*Zosterisessor ophiocephalus* Pallas), whose distribution includes Mediterranean Sea and the adjacent parts of the Atlantics to the Canary Islands and Marmara Sea. After its entry through the Bosphorus it has spread in the Black and Azov seas. In the Black Sea it generally can be found near areas with developed macrophyte cover, especially sea grass *Zostera spp*. In the Bulgarian part of Black Sea it used to be one of the most abundant species. Due to overfishing during last century and continuing losses of habitats it became an endangered species (Vassilev et al., 2012).

Molecular phylogenetic analysis of sequence variations in marker genes can provide information about the status, origin and gene exchange with populations of different organisms. For vertebrates the mitochondrial DNA including protein-coding genes are preferred as marker genes because they evolve than the nuclear genome. Therefore, mtDNA sequences have been used to examine various phylogenetic relationships. They are powerful markers for inferring evolution history in lower taxonomic categorical such as genera, species and their populations (Wan et al., 2004).

One of the most widely used molecular markers is the mitochondrial gene encoding cytochrome b (Cyt b) (Zhang & Jiang, 2006). The variability of Cyt b were used by Stepien et al. (2005) to study the genetic structure of *N. melanostomus* populations in the North American – Great Lakes. Comparative analyses of NCBI sequences from Cyt b of *N. melanostomus* showed that the invasive species originates from the Ponto-Caspian group of gobies, characteristic to the Black Sea (Stepien & Tumeo, 2006; Tserkova et al., 2015).

The present study aimed to investigate the variability of Cyt b sequences isolated from *Gobius niger* and *Zosterisessor ophiocephalus* and to apply this information to determine the number of haplotypes of the mitochondrial gene and their geographic distribution. The annotated at NCBI Cyt b sequences were also used to extend the reach of the study.

Materials and Methods

Materials analyzed

In the study we use 17 samples of *Gobius niger* from different habitats – Varna Bay, Golden Sands, cape Kaliakra, Turkey (Fatsa and Trabzon) and Romania. Additionally annotated at NCBI by other authors sequences were incorporated in the analyses (FJ526782, KR811061, KR811060, KF415583, AY884591). As critically endangered and included in the Red Book of the Black Sea, only two samples of *Zosterisessor ophiocephalus*, were caught, but the data was combined with these annotated at NCBI sequences by other authors (FJ526748, FJ526747, EU444670, AY884592, KR811067 и KF415684).

The collected samples were stored in 95% ethanol until processing.

DNA isolation

The total DNA (containing also mitochondrial DNA) was extracted from the caudal fins of the samples using QiagenDNeasy Blood & Tissue Kit Cat No./ID: 69506. The original manufacturer's protocol was followed (https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/dneasy-blood-and-tissue-kit/#resources). The amount of isolated DNA was determined by its absorbance at 260 nm and the quality was controlled by electrophoresis in a 0.8% agarose gel.

Design of primers for isolation of the cyt b mitochondrial gene cluster of the genus Gobius

In order to increase versatility for designs of primers, we used combinations of all known sequences of the Cyt b gene in the genus Gobius. This was done to allow us to use the same primer combinations in the future studies of the genus. Initially, all annotated in the NCBI nucleotide sequences of Cyt b from the Gobius were downloaded (*G. niger* – **FJ526782; KR811061; KR811060; KF415583.1; AY884591;** *Z. ophiocephalus* – **FJ526748**; **FJ526747**; **EU444670**; **AY884592; KR811067**; **KF415684**; *G. bucchichi* – **KR811037; KR811036; KR811029; KR811035; KR811034; KR811032; KR811033; KR811031; KR811030**).

Sequence alignments were done using the Vector NTI 10.1 software. The resulting consensus sequence was transferred to the on-line PRIMER 3 Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) for the preparation of primers.

The following primers were designed to isolate the Cyt b:

Gobi Cyt b Fw 1 5'-CMCTVCTTAAAATYGCAAACCA-3'
Gobi Cyt b Fw 2 5'-GCCCCCTCTAACATTTCTGC-3'
Gobi Cyt b Rev1 5'-AGGGCRAGBACTCCKCCWAGTTT-3'
Gobi Cyt b Rev2 5'-GCAAAHAGHAAGTAYCACTCTGG-3'

Isolation of the Cyt b sequences by PCR

For the PCR reactions, all 4 combinations of right and reverse primers were tested: Gobi Fw1, Gobi Fw2, Gobi Rev1 and Gobi Rev2. The combination of the outer primers Gobi Fw1 and Gobi Rev1 gave a sufficient amount of product with the expected size and because they flank a longer region of the gene were used to isolate it.

The PCR reactions were carried out by mixing 1 µl of genomic DNA in each sample, 1 µl of the each Fw and Rev primers; 12.5 µl of PCR master mix and 9.5 µl of ddH_2O. The PCR products were separated by gel electrophoresis on 1% agarose gel applying 150 volts. The products are visualized with UV light and sliced out of the gel with a pure surgical blade. The products were further purified from the agarose by QIAquick Gel Extraction kit following the original Qiagen protocol. Isolated and purified PCR products were sequenced bilaterally at GATC Ltd. Germany. For sequencing, the gene primers Gobi Fw1 and Gobi Rev1 were used.

Bioinformatics analyses

Initially, the sequences were subjected to online analyses in NCBI database to confirm that the isolated sequences are indeed those of interest using the nblast algorithm of Altschul et al. (1997).

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20163241

RESEARCH ARTICLE

Table 1. *Nucleotide substitutions and frequencies of the round goby Cyt B haplotypes. The numbers indicate positions of substitutions in Cyt b sequences. Dots indicate identical bases.*

Haplotypes	Positions												
	59	61	62	91	124	127	151	154	163	165	259	268	301
H_1	T	A	G	C	A	C	G	A	T	C	T	G	G
H_2	.	G	T	.	.	.	.	.	C	.	.	.	.
H_3	.	.	T	.	G	.	.	.	.	.	.	.	A
H_4	.	.	T	.	.	.	A	G	C	.	.	A	.
H_5	C	.	T	G	.	.	.	.	G	T	C	.	.
H_6	.	.	T	.	.	.	.	.	.	.	.	.	.
H_7	.	.	T	.	.	.	A	.	.	.	.	.	.
H_8	.	.	T	.	.	T	.	.	.	.	.	.	.

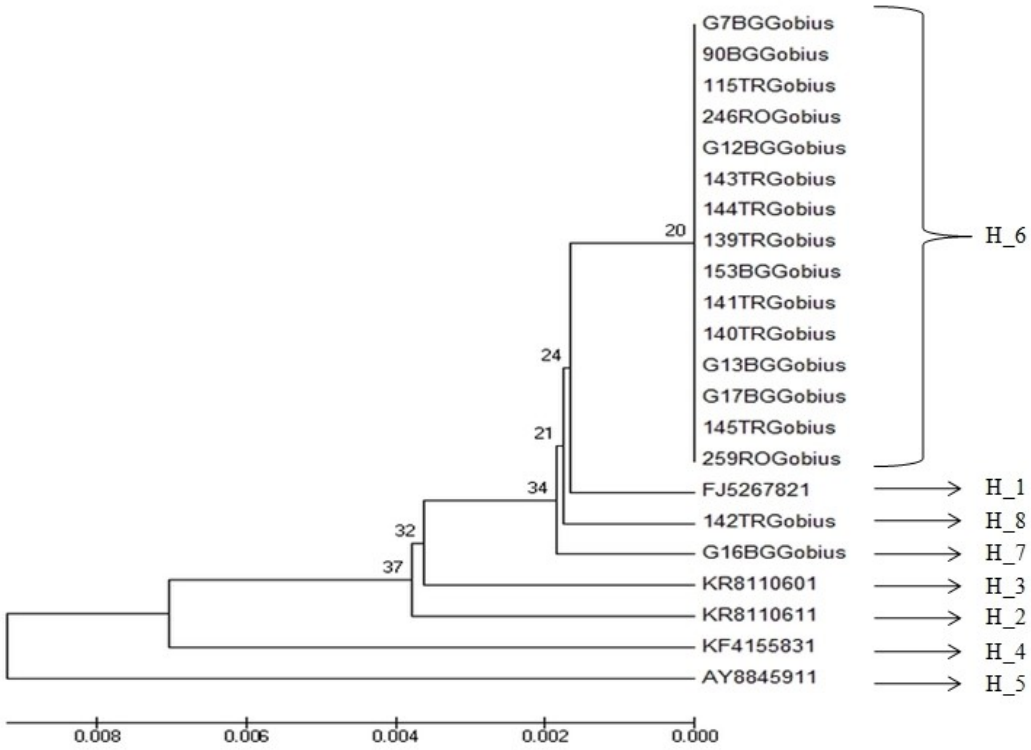

Figure 1. *Phylogenic tree build by experimental and annotated in NCBI sequences of Cyt b gene isolated from G. niger. The tree was build by applying Neighbor joining algorithm under general time reversal model. Phylogeny Test – Bootstrap method by 500 replications.*

Phylogenetic and molecular evolutionary analyzes were performed using MEGA 6 software (Tamura et al., 2013). The number of haplotypes, haploid diversity and nucleotide diversity were calculated with DNA SP 5.10.01 software (Librado & Rozas, 2009). The same program was used to construct a haplotype network.

Results

The Cyt b sequences were successfully isolated from all 17 *Gobius niger* samples. Initially, we made a comparison through nblast between them and those annotated in NCBI (FJ526782, KR811061, KR811060, KF415583, AY884591). This allowed us first to confirm the quality of isolated by us sequences and second independently of morphological determination to confirm that our samples belong to *G. niger*.

The DNA SP 5.10.01 (2009) software was used to determine the frequency and position of nucleotide substitutions in both our and annotated in NCBI sequences. The results are shown in Table 1.

According to them, there are eight haplotypes, which differ with 13 transitions and 9 transversions. Four of the haplotypes are present only in Mediterranean region. Within

the Black Sea haplotype 6 is predominant and 83.5% of all *G. niger* representatives belong to it. Three other haplotypes (H_1, H_7 and H_8) were found to form isolated populations, which together encompass 16.5% of remaining Black Sea gobies.

These data are also supported by phylogenic tree shown on Figure 1.

Similarly, we isolated and compared by nblast sequences of Cyt b from *Zosterisessor ophiocephalus*. Thus, we confirmed by molecular markers that our samples belong to *Z. ophiocephalus.*

For the need of molecular-phylogenic assays, our data were combined with those from NCBI (FJ526748, FJ526747, EU444670, AY884592, KR811067 and KF415684). The DNA SP 5.10.01 software revealed 3 haplotypes (Table 2 and Figure 2). While H_1 and H_2 are widely presented in the Mediterranean Sea, none of them was reported in the Black Sea. Instead H_3 is represented only in the Black Sea.

The number of nucleotide substitutions in the Cyt b of *Z. ophiocephalus* is insignificant in number. There are 3 transitions and 1 transversion (Table 2).

Table 2. *Nucleotide substitutions and frequencies of the round goby Cyt B haplotypes. The numbers indicate positions of substitutions in Cyt b sequences, while dots indicate identical bases.*

Haplotype	Positions			
	1 0 5	2 0 4	3 5 4	3 6 9
H_1	G	T	A	A
H_2	A	C	C	G
H_3	G	T	C	A

Discussion

As it can be clearly seen from Figure 1, the *G. niger* representatives with haplotype 6 are widely spread in the south of the Black Sea, they were found in the coastal areas of Turkey, Bulgaria, Romania and Russia. The Tajima D-test of Cyt b sequences resulted in D = -2.123065. This is an indication that the former Mediterranean migrants of *G. niger* are well adapted to the conditions the Black Sea offers and their populations are still in a stage of expansion (Tajima, 1989).

Together with *G. niger* H_6, three other haplotypes were found: in Northern parts of the Bulgarian Black Sea coast representatives of H_7 were found. In the area of Trabzon, Turkey *G. niger* haplotype 8 were found, while in the bay of Yalta representatives of H_1 were caught.

To better understand the population dynamics in the Black Sea, frequencies of nucleotide substitutions were calculated, using the Maximum Composite Likelihood model of Tamura-Nei (Tamura et al., 2004).

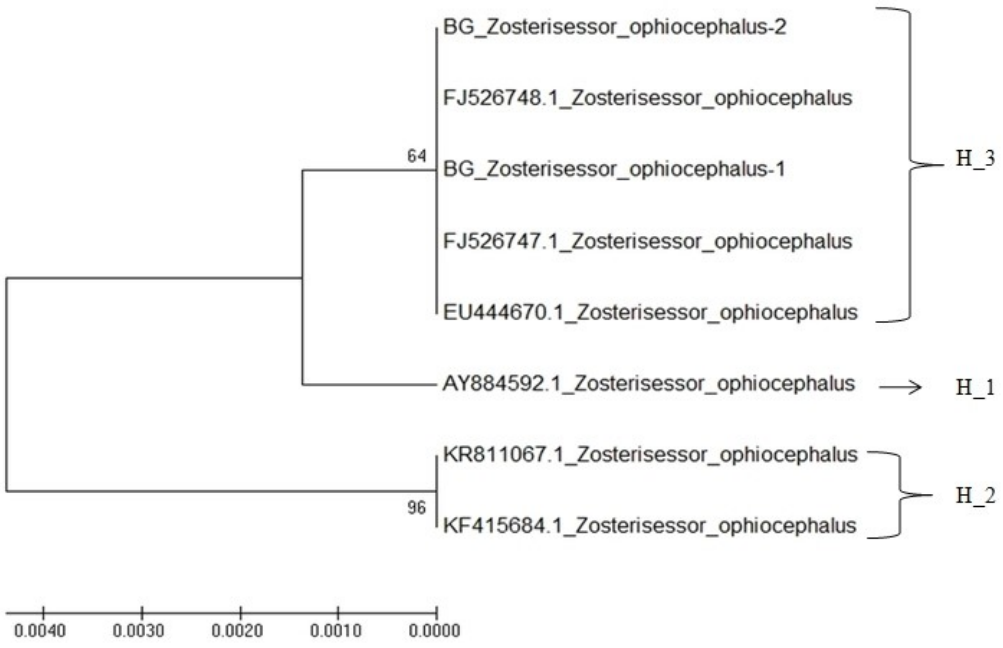

Figure 2. *Phylogenic tree with haplotypes estimated from experimental and annotated in NCBI sequences of Cyt b gene isolated from Z. ophiocephalus. Neighbor joining algorithm was applied to build the tree, applying general time reversal model. Phylogeny Test – Bootstrap method by 500 replications.*

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20163241

Table 3. *The frequencies of different substitutions for G. niger estimated by Maximum Composite Likelihood model of Tamura-Nei. Transitions are shown in bold and transversions in italics.*

	A	T	C	G
A	-	*2.6*	*3.09*	**25.53**
T	*1.87*	-	**15.29**	*1.72*
C	*1.87*	**12.84**	-	*1.72*
G	**27.79**	*2.6*	*3.09*	-

Table 4. *The frequencies of different substitutions for Z. ophiocephalus estimated by Maximum Composite Likelihood model of Tamura-Nei. Transitions are shown in bold and transversions in italics.*

	A	T	C	G
A	-	*2.55*	*2.2*	**31.71**
T	*1.54*	-	**9.48**	*1.51*
C	*1.54*	**11**	-	*1.51*
G	**32.22**	*2.55*	*2.2*	-

The frequencies of different substitutions are presented in Table 3. In general Cyt b sequences of *G. niger* possess 20.15% (A), 27.99% (T / U), 18.51% (C) and 33.35% (G). The transverse / transverse deviations rate (k1) for purines are 14.872, while for pyrimidines (k2) are 4.945. The total transition / transversion ratio (R) is 4.286. The value of R is an indication that *G. niger* representatives are still under significant evolutionary pressure Zvelebil & Baum (2008) and Tamura et al. (2013) with higher level of transitions according to our data. We can hypothesize that Black Sea was colonized by Mediterranean migrants of *G. niger* haplotype 6. Perhaps the evolutionary pressure caused later accumulation of mutations leading to appearance of H_1, H_7 and H_8. These three haplotypes were not reported in Mediterranean Sea.

These events are not exclusive for the Black Sea – in the Mediterranean Sea haplotypes 4 and H_5 are widely spread while H_2 and H_3 were reported so far only along Croatia coastal areas (Kovacic & Sanda, 2016).

Phylogenic analyzes of the available Cyt b sequences of *Z. ophiocephalus*, as presented on Figure 2 showed that only one haplotype – H_3 was found in Black Sea. It forms small isolated populations along the Bulgarian coast and the coastal zones of Russia and Ukraine.

The Tajima D-test showed a value of D = +0.23902. According to Tajima (1989) this is evidence that the habitats of *Z. ophiocephalus* are in process of shrinking. This conclusion is in agreement with Vassilev et al. (2012), according to which the grass gobies were widely spread species in the past. Excessive fishing of the species in the last century and the destruction of its habitats – especially the *Zostera spp.* fields in the Black Sea have brought the species to a state of critical danger of extinction (Vassilev et al., 2012). Perhaps just like *G. niger* H_6 the haplotype 3 of *Z. ophiocephalus* was the most widely spread in the past.

Frequencies of nucleotide substitutions were calculated also for *Z. ophiocephalus* using the Tamura-Nei model (Table 4). The nucleotide frequencies for Cyt b are 19.71% (A), 32.71% (T/U), 28.18% (C) and 19.40% (G). The transverse / transverse deviations are k1=20.985 (purines) and k2=4.317 (pyrimidines). The total transit / transversal ratio is R=5.041, which indicates that both in the Black Sea and the Mediterranean Sea *Z. ophiocephalus* is under evolutionary pressure (Zvelebil & Baum, 2008; Tamura et al., 2013). However, the apparent lack of other haplotypes in the Black Sea gives us reason to hypothesize that there is no new mass migration from the Mediterranean and recolonization of the Black Sea with new *Z. ophiocephalus* migrants. It may be due to the lack of suitable habitats for grass gobies or other factors. Future investigations with non-lethal methods for samples collections are needed in order to be able to better understand the processes of migrations and gene flow between the local and Mediterranean gobies.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. A new generation of protein database search programs. Nucleic Acids Research., 25: 3389-3402.

Engin S, Bektaş Y. 2010. Morphologic and habitat characteristics of Black Sea's endemic goby *Neogobius platyrostris* (Gobiidae). Tr. J. FAS., 10: 263-269.

Gheorghiev JM. 1966. Composition d'espece et caracteristique des Gobiides (Pisces) en Bulgaria. Izv. Nauchn. Issled. Inst. Rib. Stop. Okeanogr. Varna, 7: 159-228.

Hoese DF, Larson HK. 2006. Gobiidae. Gobies. – In: Hoese DF, Bray DJ, Paxton JR, Allen GR. Fishes. In: Beesley P.L. & Wells A. (eds). Zoological catalogue of Australia. Volume 35. Parts 1-3. ABRS and CSIRO Publishing, Canberra, p. 1612-1697.

Kovacic M, Sanda R. 2016. A new species of *Gobius* (Perciformes: Gobiidae) from the Mediterranean Sea and the redescription of *Gobius bucchichi*, J. Fish Biol., 88 (3): 1104-1124.

Librado P, Rozas J. 2009. DNA SP v. 5, A software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25: 1451-1452.

Miller PJ. 1986. Gobiidae. – In: P. J. P. Whitehead ML., Bauchot JC, Nielsen HJ. & Tortonese E. (eds), Fishes of the North-eastern Atlantic and the Mediterranean, 3: p. 1019-1085.

Nelson JS. 1994. Fishes of the world. Third edition. John Wiley & Sons, Inc., New York. 600 p.

Rass TS. 1993. Ichthyofauna of the Black Sea and some phases of its history. – In: Oven LS. (ed.) Ichthyofauna of the Black Sea bays in conditions of anthropogenic impact. Naukova Dumka, Kiev, p. 6-16 (in Russian).

Smirnov AI. 1986. Perciformes (Gobioidei), Scorpaeniformes, Pleuronectiformes, Gobiesociformes, Lophiiformes. – Fauna Ukrainy 8, Ryby (5). Naukova Dumka, Kiev (in Russian).

Stepien CA. Brown JE. Neilson ME. Tumeo MA. 2005. Genetic diversity of invasive species in the Great Lakes versus their Eurasian Source Populations: Insights for risk analysis. Risk Analysis, Blackwell Sci. 25 (4): 1043-1060.

Stepien CA, Tumeo MA. 2006. Invasion genetics of Ponto-Caspian gobies in the Great Lakes: a 'cryptic' species, absence of founder effects, and comparative risk analysis. Biol. Invasions, 8: 61-78.

Svetovidov AN. 1964. – Fishes of the Black Sea. Nauka, Moskow.

Tajima F. 1989. Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism. Genetics, 123: 585-595.

Tamura K, Nei M, Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. PNAS., 101: 11030-11035.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol., 30: 2725-2729.

Tserkova F, Kirilova I, Tcholakova T, Gevezova-Kazakova M, Klisarova D, Johannesen J, Denev I. 2015. Comparative study of round goby (*Neogobius melanostomus*) populations inhabiting Black Sea and North-West European water basins as revealed by variability in cytochrome b gene. Bulg. J. of Agric. Sci., 21: 100-105.

Vassilev M, Apostolou A, Velkov B, Dobrev D, Zarev V. 2012. Atlas of the Gobies (Gobiidae) in Bulgaria. – Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Bulgaria.

Wan QH, Wu H, Fujihara T, Fang SG. 2004. Which genetic marker for which conservation genetics issue. Electrophoresis, 4 (25): 2165-2176.

Zhang F, Jiang Z. 2006. Mitochondrial phylogeography and genetic diversity of Tibetan gazelle (*Procapra picticaudata*): implications for conservation. Molec. Phylog. & Evol., 41: 313-321.

Zvelebil M, Baum JO. 2008. Understanding Bioinformatics. Garland Science, Taylor & Francis Group, NewYork, London.

http://www.jbb.uni-plovdiv.bg
DOI: 10.69085/jbb20163241