Bulgarian Journal of Agricultural Science, 21 (Supplement 1) 2015, 100–105
Agricultural Academy

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

F. TSERKOVA[1,2], I. KIRILOVA[2], T. TCHOLAKOVA[4], M. GEVEZOVA-KAZAKOVA[3], D. KLISAROVA[1], J. JOHANNESEN[4] and I. DENEV[2,*]

[1] *Institute of Fish Resources (Agricultural Academy), BG – 9000 Varna, Bulgaria*
[2] *University of Plovdiv, Department of Plant Physiology and Molecular Biology, BG – 4000 Plovdiv, Bulgaria*
[3] *Institute of Fisheries and Aquaculture, BG – 4000 Plovdiv, Bulgaria*
[4] *Johannes Gutenberg University of Mainz, Institute of Zoology, Dept. Ecology, J.-J. Becher-Weg 7, 55128 Mainz, Germany*

Abstract

TSERKOVA, F., I. KIRILOVA, T. TCHOLAKOVA, M. GEVEZOVA-KAZAKOVA, D. KLISAROVA, J. JOHANNESEN and I. DENEV, 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. Agric. Sci.,* Supplement 1, 21: 100–105

The round goby (*Neogobius melanostomus*) is a euryhalinebottom-residing fish, native to central Eurasia. It is widespread in the Sea of Marmara, Black Sea, Caspian Sea and Sea of Azov where it has been reported along all coasts and in fresh water bodies, including coastal lakes and lagoons. The species was accidentally introduced via ballast water of cargo ships in North West Europe and North America as well as the basins of several major West European rivers like the Rhine, Mosel, Elba etc. In the present study, we used variability of cytochrome b gene to compare 30 samples from the West European Rivers Rhine and Mosel with 41 samples representing Black Sea natural populations in order to study phylogenetic relations between these regions. Five haplotypes were identified. While four of them were found at isolated locations in Black Sea region, the haplotype H1 was found throughout the Black Sea and exclusively in the rivers Rhine and Mosel. H1 is identical to the dominant haplotype reported in North America and other North European locations. These results support the concept that the invasive gobies originated from the Black Sea. Further phylogenetic analyzes are under way to analyze the exact mechanisms of introduction and whether there is a gene flow among subpopulations of invasive gobies.

Key words: Cytochrome b, Gobiidae, molecular taxonomy, *Neogobius melanostomus*

Introduction

The family Gobiidae comprises 6 subfamilies (Amblyopinae, Benthophilinae, Gobiidae, Gobionellinae, Oxudercinae, Sicydaiinae) and 230 genera, including more than 1900 species (Vassilev et al., 2012; Nelson, 1994).Gobies are distributed worldwide in marine, estuarine and freshwater habitats. They are usually benthic, but may occupy different ecological niches e.g. lagoons, costal lakes and rivers. Gobies attain a small body size (often less than 50 mm) and most species have pelvic fins wholly or partially joined ventrally into a disc (Thacker and Roje, 2011).Two allopatric groups of gobiides fishes exist in European waters: the first group inhabits the Atlantic–Mediterranean and Ponto-Caspian (or Sarmatian) regions(Miller, 1986 and Smirnov, 1986), while the second group inhabits the Black, Azov and Caspian Seas

**E-mail: iliden@uni-plovdiv.bg*

(Simonovic, 1999).Most of the introduced (invasive) species in freshwater bodies of Western/Central Europe and North America originate from the Ponto-Caspian region.

Two different introduction mechanisms of Ponto-Caspian species have been described: "corridor expansion" via natural European rivers and/or human–made canals and "jump dispersal" via ballast water from international shipping (Mills et al., 1993; Grigorovich et al., 2002; Stepien et al., 2005 and Neilson and Stepien, 2011).

Upon corridor expansion via rivers and canals the genetic structure of the new population usually resembles that of nearby "source" population (isolation by distance), or is a mixture of those that the "corridor" links. Moreover, other associated biota (e.g. parasites, prey) typically move with the "invader" through the corridor, upon jump dispersal a founder effect coupled with geographic isolation of the new, small propagule from its origin may result in strong genetic drift. Given benign environmental conditions, the new population may rapidly multiply because of lack of natural enemies in the new habitat.

Neogobius (Apollonia) melanostomus (Neogobiinae: Gobiidae: Teleostei) is bottom-dwelling, euryhaline, very adaptable and invasive species. In the sea it inhabits coastal zones with few stones, shells and sand bottom, at depths up to 20 m (50–60 m in winter). It also lives in lower and middle river streams as well as in coastal lakes, where it successfully survives and breeds. The species can tolerate temperatures from – 1°C to + 30°C and a salinity range between 0 and 40.5‰ (Vassilev et al., 2012). Although it's original habitats are the Black, Azov, and Caspian Seas the round goby has colonized the great rivers of the Ponto-Caspian region and Eastern Europe by corridor expansion.

It was also accidentally introduced by ballast water of cargo ships in North West Europe and North American. In North America, rapidly expanding populations were reported in the Great Lakes and the St. Clair River. In Northern Europe, the invasion started from the Gulf of Gdańsk and recently it is invading different parts of the Baltic Sea, North-Sea basin and basins of several major West European rivers (Rhine, Elba etc). Being free of native predators and parasites, the round goby has out-competed native species for food, shelter and nesting sites, substantially reducing their numbers (Stepien et al., 2005; Stepien and Tumeo, 2006; Brown and Stepien, 2008, 2009 and Neilson and Stepien, 2011). The new, invasive populations have remained relatively predator– and parasite-freefor20 years (Kvach and Stepien, 2008).

Mitochondrial DNA is present in most cells in high copy number and is relatively easy, rapid, and inexpensive to sequence. If a rapidly evolving locus is chosen, researchers can analyze the frequency of haplotypes and to draw conclusions about the genetic structure of studied species. The genealogy of mtDNA haplotypes, rooted with a closely related taxon, additionally reveals the phylogenetic ancestry of each haplotype. The combination of haplotype frequency distributions and their phylogentic ancestry can be used to assess whether the genetic structure of the population is viscous or characterized by substantial gene flow (Avise et al., 1987 and Zink and Barrowclough 2008).

The cytochrome *b (Cyt b)* gene has been used in numerous studies of phylogenetic relationships within species and it is a gene about which a lot of sequence information from different species is available (Irwin et al., 1991; Meyer, 1994; Johns and Avise, 1998; Stepien and Brown, 2008, 2009). The results obtained by different phylogenetic studies, in which this gene has been used, led to the proposition of new classification schemes that better reflected the phylogenetic relationships among the species studied (Arnason et al., 1995; Lara et al., 1996; LeDuc et al., 1999 and Matthee and Robinson, 1999). The sequence variability of *Cytb* makes it useful for the comparison of closely related species and the populations within species.

The aim of this work was to use variability of *Cyt b* sequences to study phylogenetic relations between populations *Neogobius melanostomus* inhabiting Black Sea and North-West European water basins.

Materials and Methods

Samples studied

Ninety-one round goby samples collected from South and North Bulgarian, the Turkish Black Sea coast and from invasive populations of West European freshwater rivers Rhine and Mosel, Germany, were studied (Table 1). The fish were caught during spring and summer of 2014. Caudal fins of round goby samples were taken and placed directly in 95% ethanol and stored in refrigerator in labeled vials (4°C) until DNA extraction. Before extraction, samples were washed for 24 h in ddH_2O to remove the ethanol.

DNA isolation

One genomic DNA was isolated from different samples by caudal fins by DNeasy Blood and Tissue kit (Cat № 69504) applying the standard protocol. The amount of the isolated DNA was determined by its absorbance at 260 nm and the quality by electrophoresis in 0.8% agarose gel.

Primers

All nucleotide sequences of Cyt B annotated in the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) for the genus Neogobius were used to design primes suitable for studying repre-

sentative of this genus. The following GenBank Accession were used:*EU 331156 to EU 331236, EU 564119 to EU 564125, AY 884582 to AY 884583, HQ 452491 to HQ 452492, KC 800809, KC 814168 to 814174, KC 886276 to KC 886278 and NMU 53673 to NMU 53677*.

The multiple alignments of sequences were performed using Vector NTI 10.1 software (http://www.lifetechnologies.com/bg/en/home/life-science/cloning/vector-nti-software.html). The consensus sequence was used for design of set of two forward (Fw) and reverse (Rev) primers by Primer 3 plus software:

http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/
NeogobiFw 1 5' – TACRAAAAACRCACCCACTGC -3'
NeogobiFw 2 5' – GCAAAYCAYGCACTRGTAGACC -3'
Neogobi Rev 1 5' – GKAGRATGGCGTAWGCAAAT -3'
Neogobi Rev 2 5' – AGAAGTAYCAYTCRGGYTTRATGTGG-3'

The primers were provided by "Metabion International AG", Martinsried, Germany. The primers were dissolved in DNase-free water to 100 mmol.L^{-1} stocks. The external set of Fw1/Rev1primers were used for isolation of Cyt b fragment by PCR amplification of the flanked region, while the internal set Fw2/Rev2 was used to bi-directional for sequencing of the products.

PCR reaction conditions

Approximately 250 ng DNA template was taken from each sample and mixed in 200 μL PCR tube with 1 μL of each primer (10 mmol.L^{-1} concentration), 25 μL PCR master mix (Fermentas, Cat N K0171) and 21 μLDNase – free water (supplied with the master mix kit). PCR amplification was carried-out in a TC-512 THERMAL CYCLER (Techne) using the following program: initial DNA melting at 94°C – 5 min; next 35 cycles of 94°C – 45 s; 58°C – 45 s; 72°C – 2 min 30 s and final extension at 72°C for 1 min.

Purification of the PCR products

Initially PCR products were separated by agarose gel electrophoresis. For this purpose each sample was mixed with 5 mL of loading dye (Fermentas, Cat № R0611), loaded onto 1% agarose gel containing 0.5 mg/mL ethidium bromide (final concentration) covered with 0,5 X TBE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined with 1 kB DNA ladder (Fermentas Gene Ruler Cat № SM0311). The PCR products were visualized by UV light and documented with BIO-VISION+3026.WL system (Vilber Lourmat).

PCR product isolation and sequencing

The PCR amplification products of interest were sliced out of the gels by clear surgical blades and isolated from the agarose with the QIAquick Gel extraction kit (Qiagen, Cat № 28704) according to the original protocol. Purified PCR products were sent for sequencing with the internal set Fw2/Rev2 primers to GATC – Biotech AG, Cologne, Germany.

Data Analysis

The online blas (Altschul et al., 1997) analyses were used initially to confirm that the isolated sequences belong to *cytb.* Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 software (Tamura et al., 2013). The number of haplotypes, haplotype diversity and nucleotide diversity were calculated using DNA SP 5.10.01 software (Librado and Rozas, 2009). The same program was used to construct haplotype parsimony network.

Results and Discussion

The mitochondrial DNA is a small fraction of the total DNA isolated. Therefore initially we tested several different PCR conditions in order to achieve optimal amplification. We varied the amounts of DNA template from 50 to 400 ng and found optimal product amplification when 250 ng of total DNA was used. In addition the suitability of the primers was tested using annealing temperatures from 53°C to 65°C. The optimal amplification was achieved with annealing temperature 58°C. The amplified PCR products were with the expected size – about 850 bp (Figure 1). They were isolated from the gel and sent for direct sequencing as described in materials and methods.

When the sequences were received from GATC Biothech AG, first an online blastn algorithm was used to com-

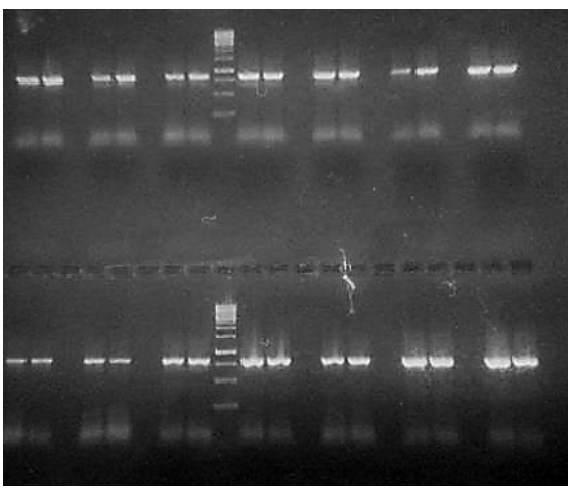

Fig. 1. The *cyt b* products amplified by primers Fw1 and Rev1, separated on 1% agarose gel containing 0.5 mg/mL ethidium bromide and visualized by UV light. The PCR products size was determined using 1 Kb FermentasGeneRuler (Cat. № SM0311)

Table 1

Samples locations and frequencies of haplotype variation in the cytochrome b gene in the round goby (*N. melanostomus*)

Species	Body of water	Location	Latitude	Longitude	№ of fish samples (71)	Haplotype(s) and relative frequencies, %
N. (A.) melanostomus (Pallas, 1814) Round goby	w. Black Sea	Bulgaria, Burgas	42º33′N	27º30′E	2	H_1 (50%) H_4 (50%)
		Bulgaria, Varna	43º12′N	27º56′E	3	H_1 (67%) H_3 (33%)
		Bulgaria,Varna lake	43º11′N	27º48′E	4	H_1 (100%)
		Bulgaria, Krapets	43º37′N	28º34′E	3	H_1 (67%) H_4 (33%)
		Bulgaria, Shabla	43º32′N	28º36′E	2	H_1 (100%)
		Bulgaria, Durankulak	43º41′N	28º34′E	7	H_1 (70%) H_2 (15%) H_5 (15%)
		Bulgaria, Ahtopl	42º05′N	27º56′E	10	H_1 (100%)
	s. Black Sea	Turkey, Fatsa	41º02′N	37º29′E	6	H_1 (100%)
		Turkey, Trabzon	40º58′N	39º50′E	4	H_1 (100%)
	River Rhine	Germany	50.04N	7.5E	20	H_1 (100%)
	River, Mosel	Germany, Perl	49.47N	6.42E	10	H_1 (100%)

Table 2

Nucleotide substitutions and their relative position in different haplotypes of the round goby *cyt b* gene

Haplotype No.	Relative position in the sequence*					
	11	12	13	14	84	111
Hap_1	C	T	C	T	G	G
Hap_2	.	.	.	.	A	.
Hap_3	.	C	T	C	.	.
Hap_4	.	.	.	.	.	C
Hap_5	T	.	.	.	.	.

* – Numbers indicate relative positions there substitutions occur; letters – type of substitution; (.) – identical bases.

pare the isolated by us sequences with those annotated in NCBI database. The results demonstrated high similarity between the annotated *Cyt b* sequences (E value 3e-110) for *Neogobius(A.) melanostomus*.

Next we analyzed variability in the *Cyt b* sequences of our 71 individual samples comparing them with *Cyt b* haplotypes of *N.(A.) melanostomus* annotated in NCBI (Accessions EU 331156 – 331236, EU 564119 – 564125, AY 884582 – 884583, HQ 452491 – 452492, KC 800809, KC 814168 -814174, KC 886276 – 886278 and NMU 53673 – 53677). In order to prevent appearance of false SNPs we limited length of analyzed sequences to 352 bp. That enables us to identify with high probability 5 haplotypes (Table 1).

Cyt b haplotypes differ by six single base substitutions – 5 transitions and 1 transversion (Table 2). Numbers of haplotypes per location ranged from 1 to 3, while haplotype diversity ranged from 0.0 to 0.0098 at the different locations.

Maximum Likelihood algorithm was used to estimate the Transition/Transversion Bias (R). It was 1.61. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameters model. The nucleotide frequencies were A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%.

Transition and transversion substitution rates are presented in Table 3.

Table 3

Rates of different transitional and transversion substitutions. Each entry is the probability of substitution (r) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model

From\To	A	T/U	C	G
A	–	5.7254	7.2514	17.1294
T/U	3.6387	–	12.3473	2.8546
C	3.6387	9.7489	–	2.8546
G	21.8340	5.7254	7.2514	–

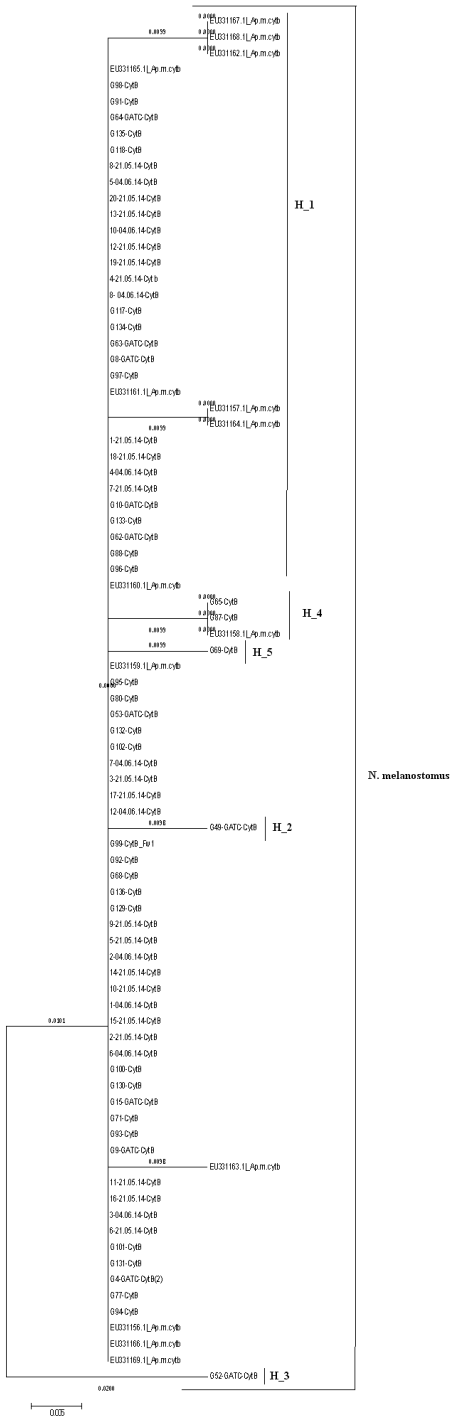

Fig. 2. Phylogenic tree build by experimental and annotated in NCBI sequences of cyt B gene. Neighbor joining algorithm was applied to build the tree, applying general time reversal model and uniform rate of substitution. Phylogeny Test – Bootstrap method by 500 replications

Each entry is the probability of substitution (r) from one base (row) to another base (column). Substitution patterns and rates were estimated under the Tamura-Nei (1993) model. The nucleotide frequencies are 18.69% for Adenine, 29.41% for Thymine (/U), 37.24% for Cytosine and 14.66% for Guanine. The maximum Log likelihood for this computation was –268.561.

Phylogenic relations between studied samples as well as those annotated in NCBI database were analyzed by MEGA6 (Tamura et al., 2013) and are presented on Figure 2.

The grouping of the samples on Figure 2 as well as their distribution by locations clearly demonstrated that H1 is the most widely spread haplotype in all studied Black Sea locations. It is the only one found in samples from rivers Rhine and Mosel. This haplotype is identical with the main haplotype reported in all big European rivers flowing into the Black Sea and annotated in NCBI by other (Stepien and Brown, 2008, 2009). This finding suggests that nonindigenous round gobies in Rhine and Mosel populated these rivers not only by accidental ballast waters (jump dispersal mechanism) but also by corridor expansion from the river Danube by canals, which connect it with other major European rivers. More investigations with additional genetic markers, including both mitochondrial, i.e. maternally inherited, and nuclear, i.e. bilaterally inherited, are needed to clarify which mechanism of invasion prevails and if the invasive subpopulations are connect by ongoing gene flow or evolve independently.

Four other haplotypes indigenous for Bulgarian Black Sea coast were identified that correspond to geographical features, notably basins and tributaries.In the region Durankulak, we found the local haplotypes H2 and H5 (Table 1). Two other haplotypes were found in Varna bay (H3) and H4 near Burgas and Krapets. Additional research is needed to further explore the fine-scale distribution patterns of local and invasive representatives of round goby.

Acknowledgments

This study was performed with the help of PROJECT BG-051PO001-3.3.05-0001 “Science and Business”. The project is implemented with the financial support of Operational Programme “Human Resources Development” financed by the European Social Fund of the European Union. We express our gratitude to Assist. Professor Dr. Yilmaz CIFTCI from Ordu University, Department of Fisheries Technologies Engineering, Faculty of Marine Sciences (FMS), Fatsa, Ordu, Turkey for providing us with goby samples from Turkish Black Sea coast.

References

Altschul, S. F., T. L. Madden, A. A. Scheffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* **25:** 3389–3402

Arnason, U. K., Bodin, A. Gullberg, C. Ledje and S. Mouchaty, 1995.A molecular view of pinniped relationships with particular emphasis on the true seals. *Journal of Molecular Evolution,* ***40*:** 78–85.

Avise, J.C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, A. C. Reeb and N. C. Saunders, 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematic,* **18:** 489–522.

Brown, J. E. and C. A. Stepien, 2008. Ancient divisions, recent expansions: phylogeography and population genetics of the round goby *Apollonia melanostoma. Molecular Ecology*, **17:** 2598–2615.

Brown, J. E. and C. A. Stepien, 2009. Invasion genetics of the Eurasian round goby in North America: tracing sources and spread patterns. *Molecular Ecology*, **18:** 64–79.

Corkum, L. D., M. R. Sapota and K. E. Skora, 2004.The round goby, Neogobiusmelanostomus, a fish invader on both sides of the Atlantic Ocean. *Biological Invasions*, **6:** 173–181.

Grigorovich, I. A., H. J. Mac Isaac, N. V. Shadrin and E. L. Mills, 2002.Patterns and mechanisms of aquatic invertebrate introductions in the Ponto-Caspian region.*Canadian Journal of Fisheries and Aquatic Sciences,* **59:** 1189–1208.

Irwin, D. M., T. D. Kocher and A. C. Wilson, 1991.Evolution of the cytochrome *b* gene of mammals.*Journal of Molecular Evolution,* **32**: 128–144.

Johns, G. C. and J. C. Avise, 1998 A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b. Molecular Biology and Evolution,* **15**: 1481–1490.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution,* **16:** 111–120.

Kvach, Y. and C. A. Stepien, 2008. Metazoan parasites of introduced round and tubenose gobies in the Great Lakes: support for the ''enemy release hypothesis''. *Journal of Great Lakes Research,* **34:** 23–35.

Lara, M. C., J. L, Patton and M. N. DaSilva, 1996. The simultaneous diversification of South American echimyid rodents (Hystricognathi) based on complete cytochrome *b* sequences. *Molecular phylogenetics and evolution*, **5:** 403–413.

LeDuc, R. G., W. F. Perrin and A. E. Dizon, 1999. Phylogenetic relationships among the delphinid cetaceans based on full cytochrome *b* sequences. *Marine Mammalian Science,* **15**: 619–648.

Librado, P. and J. Rozas, 2009. DnaSP v5, A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics,* **25:** 1451–1452.

Matthee, C. A. and T. J. Robinson, 1999. Cytochrome *b* phylogeny of the family bovidae: resolution within the alcelaphini, antilopini, neotragini, and tragelaphini. *Molecular phylogenetics and evolution,* **12**: 31–46.

Meyer, A., 1994. Shortcomings of the cytochrome *b* gene as a molecular marker. *Trends in Ecology and Evolution,* **9**: 278–280.

Miller P. J., 1986. Reproductive biology and systematic problems in gobioid fishes. In: T. Uyeno, R. Arai, T. Taniuchi and K. Matsuura, (eds.), *Indo-Pacific Fish Biology*: 640-647. Tokyo: *Tokai University Press.*

Mills, E. L., J. H. Leach, J. T. Carlton and C. L. Secor, 1993. Exotic species in the Great Lakes: a history of biotic crises and anthropogenic introductions. *Journal of Great Lakes Research*, **19:** 1–54.

Neilson, M. E. and C. A. Stepien, 2011. Historic speciation and recent colonization of Eurasian monkey gobies (Neogobiusfluviatilis and N. pallasi) revealed by DNA sequences, microsatellites and morphology.*Diversity and Distributions,* pp. 1–15.

Nelson, J. S., 1994. Fishes of the World. *John Wiley and Sons,* Inc. New York. 3rd edition. 600 pp.

Simonovic, P. D., 1999. Phylogenetic relationships of Ponto-Caspian gobies and their relationship to the Atlantic–Mediterranean *Gobiinae*. *Journal of Fish Biology,* **54:** 533–555.

Smirnov, A. I., 1986. Perciformes (Gobioidei), Scorpaeniformes, Pleuronectiformes, Gobiesociformes, Lophiiformes. – Fauna Ukrainy 8, Ribi (5). *Naukova Dumka,* Kiev. 320 pp. (Ru)

Stepien, C. A., J. E. Brown, M. E. Neilson and M. A. Tumeo, 2005. Genetic diversity of invasive species in the Great Lakes versus their Eurasian source populations: insights for risk analysis. *Risk Analysis,* **25:** 1043–1060.

Stepien, C. A. and M. A. Tumeo, 2006. Invasion genetics of Ponto-Caspian gobies in the Great Lakes: a 'cryptic' species, absence of founder effects, and comparative risk analysis. *Biological Invasions,* **8:** 61–78.

Tamura, K. and M. Nei, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution,* **10:** 512–526.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumara, 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, **30:** 2725–2729.

Thacker, C. E. and D. M. Roje, 2011. Phylogeny of Gobiidae and identification of gobiidlineages. *Systematics and Biodiversity,* **9:** 329–347.

Vassilev M., A. Apostolou, B. Velkov, D. Dobrev and V. Zarev, 2012. Atlas of the Gobies (Gobiidae) in Bulgaria. Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 112 pp (Bg/Eng).

Zink, R. M. and G. Barrowclough, 2008. Mitochondrial DNA under siege in avian phylogeography. *Molecular Ecology,* **17:** 2107–2121.