

## Genetic structure and distribution of *Oxynoemacheilus bureschi* (Balitoridae, Teleostei), and its phylogenetic relationships with other European stone loaches

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**A b s t r a c t.** The genetic differentiation of *Oxynoemacheilus bureschi* (Drensky, 1928) from all three drainages (Struma=Strymon, Mesta=Nestos, Vardar=Axios) where this species occurs, as well as its phylogenetic relationships with other European stone loaches, was assessed using the mitochondrial cytochrome *b* gene. The phylogenetic reconstruction revealed two distinct clades within the European stone loaches with high bootstrap values. Very low genetic variability with no internal haplotypic structure has been found between and within all examined *O. bureschi* populations, indicated by low polymorphism and similar haplotypes. According to the nesting design and demographic patterns, the range of *O. bureschi* was not constant, but underwent expansion in the recent past. Lack of variation, a rather unusual phenomenon for fishes from the southern Balkans, is ascribed to facilitation of dispersal due to seashore regression, confluence events and river capture during the Pleistocene.

**Key words:** range expansion, Pleistocene, mitochondrial DNA, Aegean Sea, Balkans, *Barbatula*

### Introduction

*Oxynoemacheilus bureschi* (Drensky, 1928) is one of the few species of stone loaches of the family Balitoridae currently recognized in Europe (also includes *O. pindus* and species of the genus *Barbatula*). According to present knowledge, the entire species range is restricted to the relatively small area of three river systems of the eastern Balkans on the Aegean Sea slope: Struma (=Strymon), Mesta (=Nestos) and Vardar (=Axios) rivers. The species was first reported from the Struma River in Bulgaria (D r e n s k y 1928). Later, C h i c h k o f f (1939) also found *O. bureschi* in the Mesta River (Bulgaria), K a r a m a n (1955) in the Strumica River (a tributary of the Struma River, Macedonia), and E c o n o m i d i s (1974) in the Strymon, Nestos, and Nevrokopi River valley drainages in Greece. Recently, Š o r i ć (1999) reported this species also in the Vardar River system, whose ichthyofauna has been well studied in the past. It also represents a type locality for several new species. The Vardar

R. is the only known river system where *O. bureschi* occurs sympatrically with *Barbatula* species. Therefore, there is a possible hypothesis of the recent introduction of *O. bureschi* to the Vardar R. system.

The terminology of *O. bureschi* has a rich history. The species was originally described as *Nemacheilus bureschi*, and later recognized as a subspecies of *Nemacheilus angorae* (Chichkoff 1939), and later still as a subspecies of *Orthrias brandti* (Bănărescu et al. 1978) included in the genus *Orthrias* Jordan et Fowler, 1903. Bănărescu et al. (1978) placed 10 nominal species of the Balitorids from Turkey, Bulgaria and Greece into the genus *Orthrias*. Kottelat (1990) showed that *Barbatula* Linck, 1790 is the earliest name available for this genus. Bănărescu & Nalbant (1966) accepted *Oxyonemacheilus* as a synonym of *Barbatula*. However, Kottelat (in Stomboudi et al. 2006) validated both generic names to be available for two distinct, morphologically recognized lineages within the European stone loaches. Despite this controversy, the phylogenetic relationships of *O. bureschi* and other taxa of stone loaches from Europe and western Anatolia remain unclear, because there is no phylogenetic information on the genus, nor is there data on genetic structure of the species.

In the present study, the genetic differentiation of *O. bureschi* populations from three different river systems and its phylogenetic relationships with other European stone loaches was examined using the mitochondrial cytochrome *b* gene. Based on genetic data, the evolutionary history and distribution of the species was proposed. The results should outline the phylogenetic relationships of the European stone loaches, and contribute to our knowledge on the role of recent geological events in the evolutionary history of Balkan freshwater fish fauna.

## Materials and Methods

To reveal the genetic variability and population structure of *O. bureschi*, 10 individuals from each river system (Struma R. – Blagoevgrad, Mesta R. – Gotze Deltzev, Lepenac/Vardar



**Fig. 1.** Sampling sites for *O. bureschi*, indicated by asterisks.

R. – Skopje) were examined (Fig. 1). To place our data into a phylogenetic framework and to determine the phylogenetic relationships of the species with other European stone loaches, data on the genetic variability of the genus *Barbatula* were used from our next manuscript. Sequences of two specimens of *O. pindus* from the Devoll R. (Albania; GenBank Accession No. EF562773), three specimens of *O. sp.* from western Turkey (EU015983-85), 16 specimens of *Barbatula barbatula* from the Danube basin (EF562634-35, EF562637-38, EF562644-45, EF562719-20, EF562682-83), Ebro (EF562755-56), Oder (EF562733-34) and Elbe R. (EF562745-46), two specimens of *Barbatula sturanyi* from the Drim R. (Macedonia; EF562766-67), two specimens of *Barbatula zetensis* from the Morača R. (Montenegro; EF562770-71), and two specimens of *Barbatula vardarensis* from the Vardar R. (EF562764-65) were included into analyses. *Sabanejewia balcanica* (GenBank Accession No. EF447289) was used as an outgroup.

DNA was extracted from fin or muscle tissue using the DNeasy Tissue Kit (QIAGEN). All laboratory procedures, including primer selection, PCR amplification and sequencing reaction, followed the methods given in Šlechťo v á et al. (2006) for cytochrome *b* (cyt *b*). The sequencing was performed on ABI Prism 3130 GA. Sequences were assembled manually, and aligned to each other and to the published cyt *b* sequences of *B. barbatula* (GenBank accession number DQ105254-55) with the SeqMan II module of the DNASTar software package. The alignment was manually revised in BioEdit (Biological sequence alignment editor v5.0.9), and the sequences were deposited in GenBank under Accession Nos. DQ996591-600 and EU015983-85.

Statistical information and estimates of haplotype (*h*) and nucleotide ( $\pi$ ) diversities (Nei 1987) were obtained with DNAsp 4.10.3. (Rozas et al. 2005). To investigate the phylogenetic relationships of *O. bureschi* with other European stone loaches, neighbour joining (NJ) algorithm and Bayesian inference (BI) were performed. Prior to the analyses, the best fitting model of nucleotide substitution was assigned using Modeltest 3.06 (Posada & Crandall 1998). Under Akaike information criterion (AIC), the GTR+I+G model was selected. The NJ tree was constructed in PAUP\* version 4.0b10 (Swofford 2002) with parameter settings as estimated by Modeltest. Statistical support for branching patterns was estimated by 1000 bootstrap replications. For Bayesian analysis, six Monte Carlo Markov Chains were run simultaneously in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) for 1,000,000 generations with a sampling frequency of 100. From the resulting trees, 500 were discarded and the remaining trees were used to construct a 50% majority-rule consensus tree.

To estimate the relationships among haplotypes of *O. bureschi*, a haplotype network was constructed employing the statistical parsimony implemented in the TCS v.1.21 program (Clement et al. 2000).

Three approaches were employed to detect the traces of historical demographic changes in the studied *O. bureschi* populations. DnaSP software was used to assess the distribution of the number of pairwise mutation differences between sequences (the mismatch distribution), which is supposed to be unimodal in recently expanded populations, whereas in stationary populations it is expected to be irregular in shape (Rogers & Harpending 1992). The time of putative population expansions (*t*) is based on the parameter  $\tau$ , and mutation rate of 0.84 sequence divergence per My (Perdices & Doadrio 2001). The relation between *t* and  $\tau$  is:  $t = \tau/2\mu$  (where  $\mu$  equals the substitution rate per locus). In the same software, the Tajima test of neutrality was applied, which is expected to result in significantly negative values

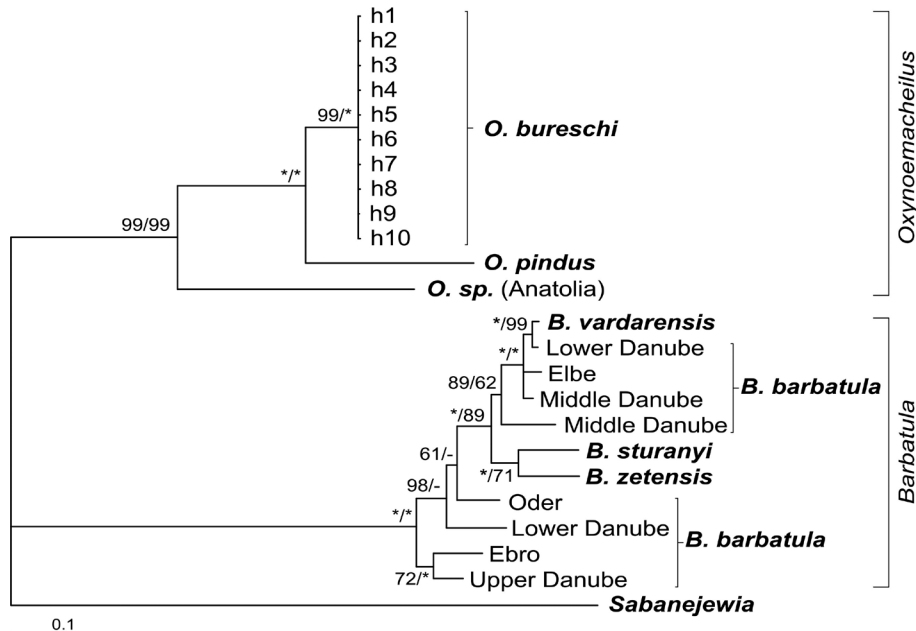
under selective sweep or population growth and/or bottleneck (Ramos-Onsins & Rozas 2002). Maximum Likelihood was computed based on estimators of theta ( $\theta_{ML}$ ) and exponential growth rate ( $g$ ) using the software FLUCTUATE v. 1.4 (Kühner et al. 1998). In this analysis, we were not interested in exact values of the  $g$  parameters, but rather in the question of whether the scenario of constant population size may or may not be excluded for our data. The above analyses were performed on the whole dataset, as well as separately for samples from each river system.

To detect whether there is any signal of geographical structure in the genetic variability, the Nested clade analysis (NCA) was applied (Templeton et al. 1995, Templeton 1998). Calculations were performed in GEODIS software, version 2.01, with 1000 permutations of the clades against the sampling sites to generate the null distribution (Posada et al. 2000). The dichotomous inference key provided in Templeton (2004) was used to identify plausible processes compatible with the observed patterns.

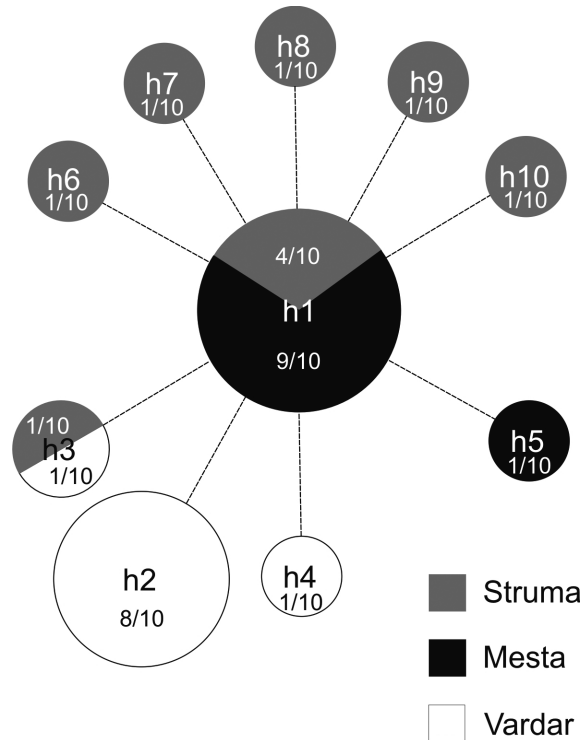
## Results

Among 30 specimens of *O. bureschi*, we identified 10 different haplotypes defined by nine (0.81%) variable sites, including two (0.18%) parsimony-informative sites in the 1106 bp long alignment.

The tree topologies from both phylogenetic analyses of 10 unique haplotypes of *O. bureschi*, together with the other representatives of the stone loaches of Europe, revealed the presence of two well differentiated clades: one comprising species of the genus *Oxynoemacheilus*, and the second representing the genus *Barbatula* (Fig. 2). Their



**Fig. 2.** Bayesian phylogenetic tree for the cytochrome *b* gene of the European stone loaches. Numbers at the branches indicate statistical support for BI and NJ, respectively. Where the statistical support was 100%, a star replaces the values.



**Fig. 3.** Haplotype network of *O. bureschi* populations. Number of individuals from a particular river system bearing the designated haplotype is indicated below and above the haplotype designations.

relationship, however, cannot be resolved. *O. bureschi* from all three river systems form a monophyletic clade with no intraclade structure. This is in sharp contrast with populations of *B. barbatula*, which do not form a monophyletic clade, and have pronounced internal structures even within a single river system (Danube R.).

The *O. bureschi* mtDNA dendrogram had a star-like structure, characterised by one most-frequent haplotype surrounded by nine haplotypes, mostly singletons (Fig. 3). All the polymorphisms were single nucleotide substitutions. The most common haplotype (h1), with the absolute frequency=13, was identified in individuals from the Mesta R. (9/10) and the Struma R. (4/10). All other haplotypes (h5-h10) from these rivers were singletons, with the exception of one specimen from the Struma R., sharing haplotype (h3) with one specimen from the Vardar R. Eight out of ten individuals from the Vardar R. shared the same haplotype (h2) and single fish from this site carried haplotype h4.

Significantly negative values of Tajima's D were detected in the whole sample set for *O. bureschi*, as well as for the Struma R. population (Table 1). The distribution of pairwise among-sequence differences was unimodal and smooth in the whole sample set, the Struma R. and in the Mesta R. populations. In both types of analyses, the Vardar R. population did not deviate from expectations under neutrality. The growth parameter for the whole sample set was much greater than its three SD, and the  $\theta$  value was excluded based on the shape of the likelihood surface as provided by FLUCTUATE. The same result was obtained for the Mesta R. population. On the other hand, the hypothesis of long-term constant population size in the Vardar R. and Struma R. populations could not be rejected, but in the latter case, this

was caused by huge confidence intervals of  $g$ , probably resulting from the low sample size.

The entire variability of *O. bureschi* was grouped into a single one-step clade and the null hypothesis of no association of the genealogical position of the haplotypes/clades with the geographical distribution was highly significantly rejected ( $p < 10^{-4}$ ; chain of inference 1-2-11-12-13-14-21-NO). Our limited dataset prevented us from discriminating between two plausible hypotheses explaining the observed pattern, i.e. the long-range dispersal and gradual range expansion followed by area fragmentation. In any case, however, our data suggest that the range of *O. bureschi* was not constant, but underwent expansion in the recent past, which is also consistent with the above-mentioned demographic patterns.

**Table 1.** Molecular diversity of *O. bureschi* populations (nucleotide diversity ( $\pi$ ), haplotype diversity (Hd) and Tajima's  $D$  value) and parameters of demographic analyses (time of putative population expansions (t) based on the parameter  $\tau$ , exponential growth rate ( $g$ )). Number of specimens (N), number of polymorphic sites (S) and number of haplotypes (h) for the studied populations.

	N	S	h	Hd	$\pi$	$\tau$ (t)	$g$ (SD)	Tajima's $D$
Struma	10	6	7	0.867	0.00108	1.20 (135 623)	10 000* (8 008)	-1.800*
Mesta	10	1	2	0.200	0.00018	0.28 (31 645)	8 095* (1 703)	-1.112 NS
Vardar	10	3	3	0.378	0.00068	NS	1 610 (1 589)	-1.340 NS
Total	30	9	10	0.754	0.00090	1.00 (113 019)	1 230* (27)	-1.740*

Statistical significance of population expansion: 1. log likelihood of the zero value of  $g$  is more than two units lower than the log likelihood for the best estimate of the  $g$  (marked by symbol \*). 2. values are greater than three standard deviations (SD) of  $g$ .

Statistical significance of Tajima's  $D$  test: NS, departure from neutrality non-significant ( $p > 0.1$ ).

## Discussion

### Phylogenetic relationships of *O. bureschi* with other European stone loaches

The phylogenetic analyses showed that the European stone loaches split into two main clades, whose monophyly is supported by high bootstrap values, and indicate the existence of two different genera in Europe. Their relatedness, however, cannot be assessed, as we were unable to find any recent common ancestor.

Our results further confirm the relationships of *O. bureschi* and *O. pindus* with the Anatolian species *O. sp.* in a monophyletic clade that correspond to the genus *Oxynoemacheilus*, and *B. barbatula*, *B. vardarensis*, *B. sturanyi* and *B. zetensis* in the monophyletic clade *Barbatula*, as previously suggested by Stoumboudi et al. (2006), based on morphology.

### Genetic structure and evolutionary history of *O. bureschi* populations

According to our results, *O. bureschi* from all three river systems was characterized by very low inter- and intrapopulation genetic diversity. No discernible substructuring was detected within the *O. bureschi* clade. Compared to that, there is pronounced substructuring, even within a single river system (Danube basin), in *B. barbatula* populations, though monophyly was not confirmed. This topic will be addressed in detail in our next manuscript. Although the sample

size was limited in this study, the results of demographic analyses suggest that the populations of *O. bureschi* were not constant, but underwent expansion in the recent past, since the tree topology highly significantly deviated from the neutral null-hypothesis. This applies to the whole sample set, as well as to each river system, with the exception of the Vardar River. The beginning of putative population expansion was dated to be around 113,000 years ago (Table 1), which corresponds to the end of the most extensive glaciations (Bianco 1990). The observed pattern therefore may be accounted for by oscillations in population size due to Pleistocene climatic shifts. Lack of polymorphism is rather unexpected for fish populations from southern regions, which are generally supposed to be less directly affected by glaciations (Bernatchez & Wilson 1998, Hewitt 1999, 2000). Nevertheless, considerably low genetic variation was also described for populations of *Barbus barbus* from the eastern Balkans and northern Anatolia (Kotlík & Berrebi 2001). During the glacial maxima, the climate in the Balkans did not provide very suitable conditions for freshwater fishes due to the region's arid, semi-desert character (Adams & Faure 1997). It is therefore likely that the lack of detectable polymorphisms at *cyt b* was due to smaller effective population sizes caused by more restricted habitat availability for *O. bureschi* at that time.

Since samples from just one locality per each river system were available for this study, only preliminary conclusions about the history of *O. bureschi* range may be suggested. It is nonetheless evident that *O. bureschi* populations from all three river systems are closely related. The genetic variability among populations from different river systems was not larger than variability within populations, and it seems likely that all populations recently shared an ancestor or were connected via gene-flow. Such a conclusion is further strengthened by the signal of range expansion suggested by NCA and by inferred population expansions (see above). According to Bianco (1990), freshwater fish dispersion in this area was mostly the result of Late Pleistocene events, when river confluences occurred in epicontinental seas during the more extended marine regressions. Throughout the glacial maxima, the global sea level was more than 100 m below its present level (Bianco 1990, Fairbanks 1989). This would have facilitated dispersal between the rivers in geographical proximity. According to Zagorchev (2002), large lakes existed in the peri-Aegean coastal plain south of the Middle-Mesta R. fault zone with the existing connection of the Struma and Mesta fluviolacustrine system. River capture may have been also an important dispersal mechanism as a complement to confluence events (Economidis & Bănărescu 1991) and this was documented for Struma and Mesta rivers (Psilovikos 1987). In case of the Struma – Vardar R., we assume possible gene flow through the Strumica R. (tributary of the Struma R.), which is in close proximity to the Vardar R. Contact could have been enabled by river capture. Alternatively, the late discovery of *O. bureschi* in the Vardar R. system (Šorić 1999), despite thorough ichthyological surveys of the Vardar drainage over the past 100 years and Karaman's findings (1928, 1955), may result from human-mediated introduction into this river, since this fish is often used as bait by anglers (personal observation). However, our data do not support this hypothesis, since in such a case, one would expect the most common h1 to be present in the Vardar R., which was not the case.

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