Molecular Phylogenetics and Evolution 96 (2016) 187–194

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogenetic evidence for a new species of *Barbus* in the Danube River basin ☆

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ARTICLE INFO

Article history:
Received 30 September 2015
Revised 19 November 2015
Accepted 30 November 2015
Available online 15 December 2015

Keywords:
Cyt *b*
ATPase 6/8
Act-2
Barbus biharicus
Biharian barbel
Cyprinidae

ABSTRACT

Three species of small-sized rheophilic *Barbus* fishes are endemic to and widely distributed throughout the mountain regions in the Danube River basin. In Hungary, barbels referred to as *B. petenyi* occur in streams in the foothills of the Carpathians near the borders with Slovakia, Ukraine and Romania. However, up to now, no genetic investigations were carried out on rheophilic barbels in this region. This study aims to clarify the taxonomic identity and distribution of the rheophilic barbels in the Hungarian plain based on molecular and morphological analyses. Two mitochondrial genes (cytochrome *b*, ATPase 6/8) and one nuclear gene (beta-actin intron 2) were sequenced and several morphometric and meristic characters were recorded. Phylogenetic and morphological analyses revealed that there are four genetically distinct lineages among the rheophilic barbels in the Carpathian Basin. The results demonstrated that North-Hungarian *Barbus* populations belong to *B. carpathicus* and that *B. petenyi* presumably does not occur in Hungary. As expected, *B. balcanicus* was only recorded in samples from the Balkans analyzed for reference. A distinct species, new to science, was discovered to be present in Sebes-Körös River (Crişul Repede) in eastern Hungary and western Romania and is formally described here as *B. biharicus* Antal, László, Kotlík – sp. nov.

1. Introduction

Petenyi's barbel (*Barbus petenyi* Heckel, 1852) is a small-sized rheophilic cyprinid species endemic to mountain regions of the Carpathian Basin (Kotlík et al., 2002; Bănărescu and Bogutskaya, 2003). The species was originally described from Transylvania in the present day Romania and was named after János Salamon Petényi, an eminent Hungarian zoologist (Bănărescu and Bogutskaya, 2003). Since the original description the taxonomic status of the species was controversial, with some authors considering it a subspecies of *B. meridionalis* Risso, 1827, or of *B. peloponnesius* Valenciennes, 1842 (Bănărescu, 1964; Karaman, 1971; Doadrio, 1990). Despite superficial morphological similarity of barbels from the different parts of the Danube River basin, separate evolutionary lineages were described within *B. petenyi* based on the high genetic divergence of mitochondrial DNA (mtDNA) sequences (Tsigenopoulos and Berrebi, 2000; Machordom and Doadrio, 2001a; Kotlík and Berrebi, 2002). Later on, Kotlík et al. (2002) formally described two new species from the Danube River basin, distinct from *B. petenyi*, which they named *B. carpathicus* Kotlík, Tsigenopoulos, Ráb and Berrebi, 2002, and *B. balcanicus* Kotlík, Tsigenopoulos, Ráb and Berrebi, 2002. Further phylogenetic analyses of nuclear and mitochondrial genes confirmed the presence of the three different species, which most likely diverged from each other in the Miocene (Marková et al., 2010; Gante, 2011; Berrebi et al., 2013; Konopiński et al., 2013; Buonerba et al., 2015). The Petenyi's barbel inhabits the Eastern and Southern Carpathians and the Stara Planina Mountains, the Carpathian barbel *B. carpathicus* occurs in the Western and Eastern Carpathians, and the large spot barbel *B. balcanicus* lives in the Dinaric and Western Stara Planina Mountains (Kotlík et al., 2002; Kottelat and Freyhof, 2007) (Fig. 1).

☆ This paper was edited by the Associate Editor G. Orti.

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http://dx.doi.org/10.1016/j.ympev.2015.11.023
1055-7903/

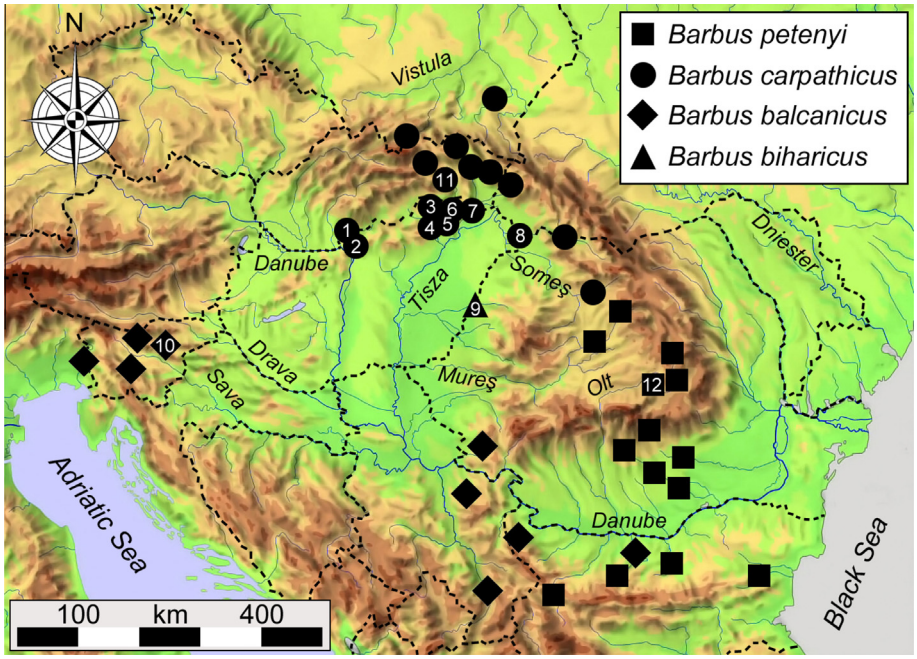

Fig. 1. Map of the Carpathian (Pannonian) Basin showing the geographic location of sampling sites of rheophilic barbels. Symbols without numbers indicate the original records of three *Barbus* species based on Kotlík et al., 2002. Symbols with numbers represent the sampling sites of the present study: 1. Kemence stream (samples HU006-008), GPS: 48.0461°N 18.9185°E; 2. Török stream (HU018-020), 47.8371°N 19.0062°E; 3. Bódva River (HU001-002), 48.3330°N 20.7306°E; 4. Sajó River (HU012-014), 48.1509°N 20.8071°E; 5. Vadász stream (HU021-023), 48.3227°N 20.8863°E; 6. Hernád River (HU003-005), 48.4198°N 21.2130°E; 7. Kemence stream (HU009-011), 48.4652°N 21.4963°E; 8. Tisza River (HU015-016), 48.1072°N 22.8315°E; 9. Sebes-Körös River (HU024-028), 47.0146°N 21.6255°E; 10. Hudinja River (SLO001-004), 46.2323°N 15.2778°E; 11. Torysa River (SK001-004), 48.9775°N 21.2539°E; 12. Aita stream (RO001-004), 45.9838°N 25.6509°E.

Due to their preference for mountain habitats, the rheophilic barbels are largely absent from the Hungarian (Pannonian) plain (Fig. 1). In Hungary, barbels referred to as *B. petenyi* occur in streams in the foothills of the Carpathians near the borders with Slovakia, Ukraine and Romania (Halasi-Kovács and Harka, 2012; Fig. 1). Due to the limited distribution in the country, *B. petenyi* is listed as strictly protected species in Hungary. However, up until now, no genetic investigations were carried out on rheophilic barbels in Hungary and their taxonomic status is therefore an open question. This study aims to clarify the taxonomic identity and distribution of the rheophilic barbels from the Hungarian plain based on molecular and morphological analyses.

2. Materials and methods

2.1. Sampling

Altogether, 28 individuals provisionally identified as *B. petenyi* were collected from nine geographical locations in Hungary, from all regions where the species was assumed to occur. Altogether 12 individuals of *B. petenyi*, *B. carpathicus* and *B. balcanicus* were collected as references from their habitats in Romania, Slovakia and Slovenia, respectively (Fig. 1). Samplings were carried out using electric sampling equipment (HansGrassl, Germany) in Hungary and hand nets in the other countries, between 2010 and 2012. Some individuals and the tissues samples were stored in 96% ethanol at −20 °C until DNA extraction. The collection and storage of the samples were approved by the National Inspectorate for Environment, Nature and Water, Hungary (permission number: 14/4620-3/2010.).

In 2015, additional 27 individuals of the suspected new species were collected from Sebes-Körös River (Crişul Repede) near Bratca, Romania (46.9279°N 22.5934°E), for the morphometric and meristic examination.

2.2. Molecular methods

DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Germany) from tissue samples. Two mitochondrial genes and one nuclear gene were analyzed by polymerase chain reaction (PCR) with high fidelity AccuTaq™ DNA polymerase (Sigma–Aldrich, USA). Partial sequence of mitochondrial cytochrome *b* (Cyt *b*) gene (598 bp) was amplified with primers L15267 (5′AAT GACTTGAAGAACCACCGT3′) and H15891 (5′GTTTGATCCCGTTTCGT GTA3′) designed by Briolay et al. (1998). The same Cyt *b* fragment was used in previous studies (Tsigenopoulos and Berrebi, 2000; Kotlík and Berrebi, 2002; Tsigenopoulos et al., 2002), which allowed direct comparison with published data. The complete mitochondrial ATPase 6 and 8 genes (842 bp) were amplified with primers ATP8.2 (5′AAAGCRTYRGCCTTTTAAGC3′) and Co3.2 (5′GTT AGTGGTCAKGGGCTTGGRTC3′) designed by Machordom and Doadrio (2001b). The second intron of the nuclear-encoded beta-actin (Act-2) gene (496 bp) was amplified with primers Act18U21 (5′GCTCCAGAAAAACCTATAAGT3′) and Act554L21 (5′CTCACTGAA GCTCCTCTTAAC3′) described by Marková et al. (2010). Species from the genus *Barbus* are evolutionarily tetraploid, so that the primers were designed to selectively amplify a single paralogous copy of Act-2 (Marková et al., 2010). Briefly, the two paralogs (corresponding to the two ancestral diploid chromosome sets) were separated by electrophoresis based on the length difference between them, gel-purified and sequenced, and specific primers were designed to match regions differing in sequence, allowing selective amplification and sequencing of only the longer paralog in different species (Marková et al., 2010). The use of locus-specific primers is essential because it allows distinguishing allelic variation segregating at a single locus from variation between paralogous gene duplicates (Marková et al., 2010; Gante et al., 2011). The average uncorrected distance between the Act-2 paralogs (12.0%) largely exceeds average levels within the paralogs (1.6% and 1.1%;