

Genetic diversity of *Cobitis* spp. (Cypriniformes: Cobitidae) from different drainage areas

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Abstract. Genetic diversity of three pure populations of *Cobitis elongatoides* from hydrologically and geographically different drainage areas, the Lužnice River (48.49.60 N, 14.55.60 E, Labe River drainage area, North Sea basin), the Nová Říše reservoir (49.09.11 N, 15.32.464 E, Morava River drainage area, Black Sea basin), and the Ida River (48.32.284 N, 21.03.421 E, Tisza River drainage area, Black Sea basin) was evaluated by sequencing of mitochondrial cytochrome *b* gene. Species identification of unknown *Cobitis* samples from Vipava River drainage area in Slovenia was also performed based on cytochrome *b* sequence. All forms of the genus *Cobitis* are protected by national legislatives and, in accordance with the Council Directive No. 925/43/EEC, they enjoy protection in selected localities of European importance. In total, 11 haplotypes of *Cobitis elongatoides* were found in Czech and Slovak waters, manifesting low overall genetic diversity. Samples from Vipava River drainage area were identified as *Cobitis bilineata*.

Key words: Danubian spined loach, population diversity

Introduction

Intensive research of members of Cobitidae family in the last 20 years brought many crucial findings, which radically changed original insights of both species and intraspecies structure (Vasileva 2000, Bohlen & Ráb 2001, Boroń et al. 2006). As was shown by researches presented during 3rd conference “Loaches of the genus *Cobitis* and related genera”, held in September 2006 in Šibenik, Croatia, the research potential of this family has not run out yet.

Territories of the Czech Republic and Slovakia territories lie on a water-divide of three or two basins, respectively (North S., Baltic S. and Black S.). Previous studies have proved that in contrary to original assumptions, which classified populations in Czech and Slovakian waters as *Cobitis taenia* Linnaeus, 1758 (Holčík 1995), in fact it is *Cobitis elongatoides* Bacescu et Maier, 1969 and populations of hybrids with other species (Lusk et al. 2000, Ráb et al. 2000, Šlechtová et al. 2000). In a territory of the Czech Republic in Lužnice R. drainage area (basin Vltava-Labe, North Sea) and in the upper part of Dyje R. drainage area (basin Morava-Danube, Black Sea) occur populations of pure species of *C. elongatoides*. In middle and lower parts of the Dyje R. and in the Morava R. drainage areas occur populations of hybrid diploid-polyploid complex *C. elongatoides* x *C. tanaitica*. In

the Labe basin occur populations of diploid-polyploid complex *C. elongatoides* x *C. taenia* (L u s k et al. 2000, R á b et al. 2000, Š l e c h t o v á et al. 2000). In Slovakia, populations of pure *C. elongatoides* were found only in the Slaná R. drainage area, while in the Bodrog and Danube drainage areas exist populations of a hybrid diploid-polyploid complex *C. elongatoides* x *C. tanaitica* (L u s k et al. 2003, L u s k o v á et al. 2004).

Members of genus *Cobitis*, both in pure and hybrid form, are protected by national legislature both in Czech Republic and Slovakia, under the previously broadly-used name *Cobitis taenia*. Within European Union, spined loach is (under name *C. taenia*) listed in Supplement II of Council Directive No. 92/43/EEC. Member states are obliged to define so called special areas of conservation, in which relevant populations are protected.

The aim of this study was to evaluate genetic diversity of tested pure *C. elongatoides* populations, which occur in separated basins in territories of the Czech Republic and Slovakia. Secondary aim was to determine species pertinence of several spined loach samples from the Vipava River drainage area in Slovenia. Previously, the occurrence of *C. bilineata* was reported in the Vipava River drainage area (P o v ž & Š ũ m e r 2000), but no genetic analyses proving this have been performed so far.

Material and Methods

In this study were used samples from pure populations of *C. elongatoides*, which were collected in 2006: 7 individuals from Lužnice drainage area population, locality Halámky (48.49.60 N, 14.55.60 E), 35 individuals from Nová Říše reservoir in upper Dyje drainage area (49.09.11 N, 15.32.464 E), and 8 individuals from Ida River – Slaná drainage area ((48.32.284 N, 21.03.421 E). Furthermore, 4 individuals suspected to be *C. bilineata* from Vipava River drainage area were analyzed. Total genomic DNA was isolated from fin clippings stored in ethanol by standard phenol-chlorophorm-isoamylalcohol extraction (S a m b r o o k et al. 1989) with minor modifications. Mitochondrial cytochrome b gene was amplified in full length (1140 bp) with primers L15267 (B r i o l a y et al. 1998) and ThrR (D o a d r i o et al. 2002) according to protocol in Janko et al. (2003). PCR products were purified and sequenced in automatic sequencer.

Resulting sequences were aligned in MEGA 3.1 software (K u m a r et al. 2004). Sequences were translated to verify accuracy of alignment. No termination codons were found within sequences. Chi-square homogeneity test of base frequencies was performed in PAUP ver 4.0b10 (S w o f f o r d 2003). Phylogenetic trees were constructed in MEGA 3.1 using neighbour-joining (NJ) (S a i t o u & N e i 1987) an maximum parsimony (MP) algorithms. Previously published sequence of *Misgurnus fossilis* (GenBank accession number AF263097, P e r d i c e s & D o a d r i o 2001) was used as taxonomic outgroup for phylogenetic analyses. FindModel web site (<http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html>) was employed to determine best model of evolution that fits to our data. Consequently, genetic distances were computed, and NJ phylogenetic trees constructed using TN93 model with homogenous pattern among lineages and without gamma correction (T a m u r a & N e i 1993). In MP analysis, only minimal trees were retained. Bootstrap analysis with 1000 replicates was used to evaluate robustness of topology.

In order to identify ancestral haplotype and genealogical relationships among haplotypes, haplotype network was constructed by using Network 4.2.0.0 program (B a n d e l t et al. 1999), using median joining algorithm.

Results

Nucleotide sequences of the mitochondrial cytochrome b gene (1140 bp) were determined in 50 *C. elongatoides* individuals from three populations in the Czech Republic and Slovakia. Base frequencies were homogenous across variable sites (chi square = 4.02, df = 165, P = 1.0). These sequences showed 21 variable sites, including 10 parsimony informative, which constituted 11 different haplotypes (Table 1). Sequence variability was mainly due to transitions (Ti/Tv ratio = 3.8) and third-position substitutions (18 out of 21). Genetic distances between haplotypes ranged from 0.0009 to 0.0106 (0.0059 on average).

Table 1. Haplotypes and sequence variations of cytochrome B in examined *C. elongatoides* samples. Values show number of tested individuals. Dots indicate identical nucleotides. All haplotypes reported here will appear in the GenBank database with accession numbers EF012705-EF012715).

Haplotype	Lužnice	Nová Říše	Ida	Total	Variable site
					1 1 1 1 2 2 2 2 3 3 3 4 6 7 8 9 9 9 9 9 0 7 0 8 9 1 3 3 7 3 4 4 8 3 1 5 0 0 2 3 9 8 2 8 3 5 3 2 4 6 3 5 7 6 6 7 2 0 6 1 0 9 4
CE01	6	10		16	CAATGTCTGTGCGTTGCTGT
CE02			1	1	. . G . T
CE03		18	1	19 T
CE04		7		7	. G . . T
CE05	1			1 T C T C T A A T C
CE06			1	1 T C T T A T .
CE07			1	1 T C T A A T A T .
CE08			1	1	T . . C T C T T A C . . . A T .
CE09			1	1	T . . C T C T T A A T .
CE10			1	1	T . . C T C T . A . T . T A . C . . A T .
CE11			1	1 T C T . . C A T A T .
TOTAL	7	35	8	50	

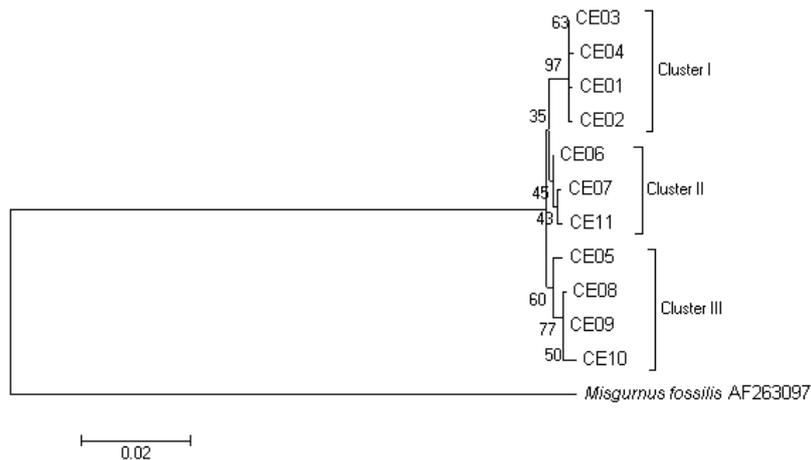


Fig. 1. Phylogenetic tree for 11 cytochrome B haplotypes of *Cobitis elongatoides* in the Czech Republic and Slovakia computed by neighbour-joining algorithm. Values represent estimated bootstrap support.

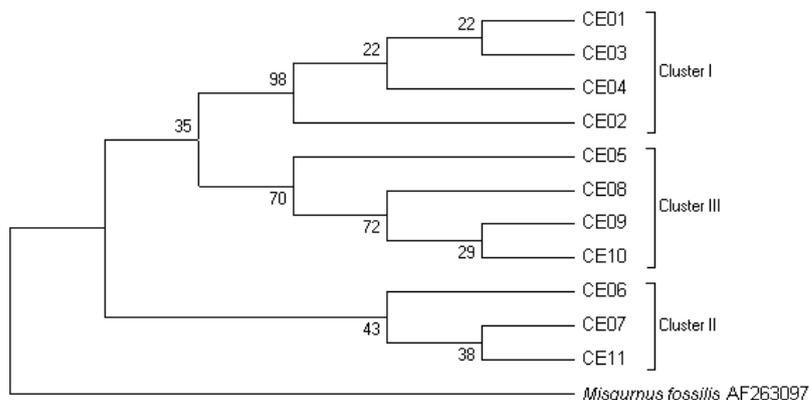


Fig. 2. Phylogenetic tree for 11 cytochrome B haplotypes of *Cobitis elongatoides* in the Czech Republic and Slovakia computed by maximum parsimony algorithm. Values represent estimated bootstrap support.

Out of 11 haplotypes, eight were found in a single individual only. Only two haplotypes were found on more than one locality (CE01 – Lužnice, Nová Říše; CE03 – Nová Říše, Ida). In Lužnice, all samples were identical, except for one individual. In Ida, all individuals differed from each other. In Nová Říše, three haplotypes were found. Phylogenetic tree for 11 haplotypes constructed by the neighbour-joining and maximum parsimony method showed existence of three clusters (Figs 1 and 2). First cluster (I) comprised four haplotypes and had strong bootstrap support (97/98 %). Second cluster was weakly supported (45/43 %) and contained three haplotypes. Third cluster had moderate support (60/70 %) and comprised four haplotypes. However, trees constructed by NJ and MP differed in topology – in NJ, clusters I and II formed superior cluster, whereas in NP, clusters I and III grouped together. Haplotype network (Fig. 3) indicates that CE03 is the most ancestral haplotype. Along with three other haplotypes, it forms a clade equal to Cluster I from phylogenetic analysis. The rest of network also corresponds well with phylogenetic trees.

Sequencing of the whole cytochrome B gene in samples of fishes from the Vipava River drainage area (Slovenia) and comparison with sequences of *Cobitis* spp. obtained from

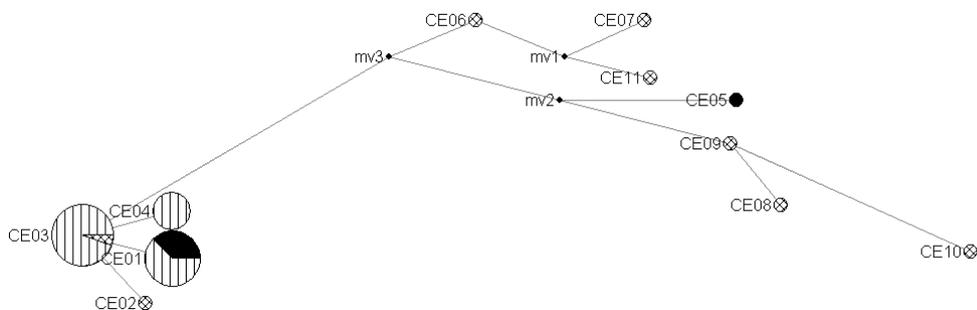


Fig. 3. Haplotype network and geographic distribution of 11 cytochrome B haplotypes of *Cobitis elongatoides* in the Czech Republic and Slovakia. Closed circle – Lužnice R., vertical hatch – Nová Říše, cross hatch – Ida R. Size of the circle is in proportion with number of individuals carrying respective haplotype. Mv1, mv2, mv3 – hypothetical intermediate haplotypes, which were not actually observed. Branch lengths are not in proportion to number of nucleotide changes.

Table 2. Comparison of cyt B sequences of Vipava River samples with GenBank sequences. Dots indicate identical nucleotides.

Sample identification	Variable site
	1 2 3 6 7 7 8 9
	0 8 4 2 3 9 4 9
	8 2 5 1 5 8 3 0
GenBank AY191558.1	GTCCTCTC
GenBank AY191559.1	A . . . C . C .
GenBank AY191560.1	. . T . . T . .
Vipava 1 C . . T
Vipava 2	. A . T
Vipava 3	. . T T
Vipava 4 C . . T

GenBank confirmed these samples to be *Cobitis bilineata*; analyzed haplotypes differed from sequences in GenBank by maximum of 2 nucleotides (Table 2).

Discussion

In order to assess genetic diversity of *Cobitis elongatoides* in Czech Republic and Slovakia, we sequenced whole cytochrome B gene. 11 haplotypes were divided into three relatively weak-supported clusters, which probably correlates with low diversity among analyzed haplotypes. Out of these 11 haplotypes, six (CE01-CE06) were previously found in various *Cobitis* samples by J a n k o et al. (2005) in shorter, 1088 bp long variants (Table 3). However, haplotypes CE03 (E1 in J a n k o et al. 2005) and CE05 (E30 in J a n k o et al. 2005) were so far described only in individuals with character of diploid-polyploid complex. Five haplotypes from Ida River (CE07-CE11) are novel.

Presence of individual *C. elongatoides* cytB haplotypes, which we describe in Czech Republic and Slovakia territories, is not exclusively limited to particular examined localities.

Table 3. Comparison of geographical origin of haplotypes found in both Janko et al., 2005 and our study. CZE – Czech Republic, SVK – Slovakia, HUN – Hungary, ROM – Romania, GER – Germany, UKR – Ukraine.

Haplotype	Haplotype (J a n k o et al. 2005)	Water body (basin) (J a n k o et al. 2005)	Country (J a n k o et al. 2005)	Water body (basin)	Country
CE01	E2	Lužnice R.(Elbe)	CZE	Lužnice R. (Elbe) Nová Říše (Danube)	CZE CZE
CE02	E7	Dyje R. (Danube) Čierna voda (Danube)	CZE SVK	Ida R. (Danube)	SVK
CE03	E1	Szodrakosz creek (Danube) Comana R. (Danube) Mures R. (Danube)	HUN ROM ROM	Nová Říše (Danube) Ida R. (Danube)	CZE SVK
CE04	E4	Lužnice R. (Elbe)	CZE	Nová Říše (Danube)	CZE
CE05	E30	various localities	GER, SVK, HUN, ROM, UKR	Lužnice R. (Elbe)	CZE
CE06	E10	Čierna voda (Danube)	SVK	Ida River (Danube)	SVK

Haplotypes from a single cluster were found in geographically rather distant localities from different basins. Therefore, overall genetic diversity of three tested *C. elongatoides* populations is very low. However, intrapopulation diversity significantly differs – while in Ida we found eight different haplotypes belonging to all three detected clusters, in Nová Říše reservoir only three haplotypes were found (all belonging to cluster I) and in Lužnice only two haplotypes were detected (one of them in a single individual only). These conclusions are necessarily influenced by the relatively low number of available samples from Lužnice and Ida. Nonetheless, they correspond with Šlechťová et al. (2000) who, while evaluating heterozygosity based on allozyme analysis, found very low variability in Nová Říše and Lužnice populations even within larger number of tested samples.

While in lower parts of Czech and Slovakian basins populations with character of diploid-polyploid complex are found, examined pure populations of *C. elongatoides* occur mostly in upper parts of respective river drainage areas (Lusk et al. 2003, Lusková et al. 2004). Abundance of the Nová Říše reservoir population is estimated up to 15 000 individuals. This numerous population developed after 1985, based on less than 150 individuals, which had occurred in 2 km stretch of a creek flooded when the reservoir was formed. According to our findings, this isolated population sustains high abundance firmly. In the Lužnice River there are several metapopulations, which can connect both by river itself and in times of floods, when the river is connected with neighbouring floodplain and particular biotopes. A similar situation arises in the Ida River, where no migration barriers exist.

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