

Genetic differentiation of two diploid-polyploid complexes of spined loach, genus *Cobitis* (Cobitidae), in the Czech Republic, involving *C. taenia*, *C. elongatoides*, and *C. sp.*: Allozyme interpopulation and interspecific differences

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Abstract. Altogether 375 individuals, sampled at eight localities from the Elbe R. and Danube R. basins in the Czech Republic were studied electrophoretically to detect protein variability at population and specific levels in order to identify the spined loach species occurring on the territory. A further 64 individuals of *C. taenia* sensu Vasičev & Vasičeva (1994) from three localities in northern Europe were analysed as comparative material. The products of 11 loci out of 18 analysed were found to be polymorphic. Analyses of the electrophoretic patterns in individual loci and intensity ratios of respective bands showed that at several localities, both in the Elbe R. and the Danube R. basins, the diploid, triploid and tetraploid forms occur sympatrically suggesting the presence of two different hybrid diploid-polyploid complexes.

In Pšovka (Elbe R. basin), the hypothesis of the hybrid origin of such polyploid individuals was confirmed by the present electrophoretic data using analyses of loaches from diploid populations of *C. taenia* (north-western and northern Germany, north-eastern Poland) and *C. elongatoides* (upper Elbe R. and upper Dyje R. basins). The species-specific alleles in several loci that distinguished the genomes of the species involved were identified, and diploid contribution of *C. elongatoides* and haploid contribution of *C. taenia* was confirmed.

In the Dyje R. (Danube R. basin), one species involved was again *C. elongatoides* constituting always the diploid part of triploid and tetraploid individuals, together with the haploid or diploid contribution of another species still unidentified (designated as *Cobitis* sp.). Contribution of a third species to the genome of some tetraploid individuals is supposed but it could not be identified surely because of the very complicated electrophoretic patterns in most loci of polyploid genomes, which did not allow the individual variants to the respective species to be assigned.

Two species involved in the origin of hybrid / polyploid individuals in both complexes, viz. *C. taenia* and *C. sp.*, showed nearly identical protein markers in nearly all loci, irrespective of the fact that the karyotype of the latter apparently does not correspond to that of *C. taenia*. The results confirmed the previous hypothesis on the presence of two different hybrid diploid-polyploid complexes: *C. elongatoides* and *C. taenia* in the Pšovka Creek (Elbe R. basin), and *C. elongatoides* and *C. sp.* in the Dyje R. at its confluence with the Morava R. (Danube R. basin).

Key words: electrophoresis, protein variability, isozymes, interspecific hybrids, species-diagnostic alleles

Introduction

This study presents the second part of the study by Ráb et al. (1999) where general introductory remarks to the hybrid diploid-polyploid complexes among loaches of the genus *Cobitis* are given.

Biochemical genetic studies of *Cobitis* loaches demonstrated the usefulness of genetic markers to address many problems of their systematics, taxonomy, distribution and also their interrelationships. In the first study dealing with the description of European cobitid fishes using biochemical genetic markers, Grossu et al. (1971) compared their serum protein patterns analysed by starch gel electrophoresis. Sivkov & Dobrovolov (1984) differentiated new species of *Cobitis* on the basis of biochemical genetic markers (soluble muscle proteins). Similarly, biochemical genetic characterisations of the North African and Iberian *Cobitis* populations and species led to the discovery of a new species (Perdices et al. 1995, Doadrio & Perdices 1997).

A series of extensive studies regarding species composition of the hybrid diploid-polyploid complex of *Cobitis* in Russia has been carried out by Vasileva and her co-workers (Vasileva et al. 1989, Osinov et al. 1990, Vasilev et al. 1990a,b) using different enzymatic systems as markers. Similar intraspecific variability, interspecific differences and hybrid complexes of cobitids using such markers have been described by Sezaki et al. (1994) in Japan. The biochemical genetic analyses documented the interspecific hybridisation zone between two species of the genus *Cobitis* also in Southern Korea (Kim 1980, Kim & Yang 1993).

The main goal of this study was the biochemical genetic characterisation of *Cobitis* populations found in the territory of the Czech Republic using the electrophoretic methods describing protein polymorphism, its intrapopulation variability, interpopulation differences and possible detection of hybrids in diploid-polyploid complexes.

Material and Methods

Fishes were sampled at seven localities in both North and Black Sea basins of the territory of the Czech Republic (Table 1, Fig. 1). The accession codes and deposition of material examined were the same as in Ráb et al. (1999). The concise description of locations follows.

1. River Dyje

a) Nová Říše reservoir (N = 49.09.11,6 lat. / E = 15.32.46,4 long.) (Locality No. 1.)

The dam was constructed and its reservoir filled in 1985 on a small brook in the headspring area of the river. The original population living in the brook was not

Table 1. List of localities sampled for *Cobitis*.

No.	Sample size	River	Localitv	Sea basin
	74	Dyje	Nová Říše	Black
-	14	Pšovka	upper part – Pšovka I.	North
3	39	Pšovka	upstream Lhotka – Pšovka II.	North
4	33	Dyje	cutoff – Košárské louky	Black
5	66	Dyje	confluence with Morava R.	Black
-	91	Lužnice	pool	North
-	42	Pšovka	downstream Lhotka – Pšovka III.	North
8	17	Klawój Lake	near Olsztyn	Baltic
9	27	Hunte / Weser	Oldenburg	North
10	20	Kleiner Plöner See	Plön	Baltic
11	16	Jevišovka	Lechovice	Black
Total	439			

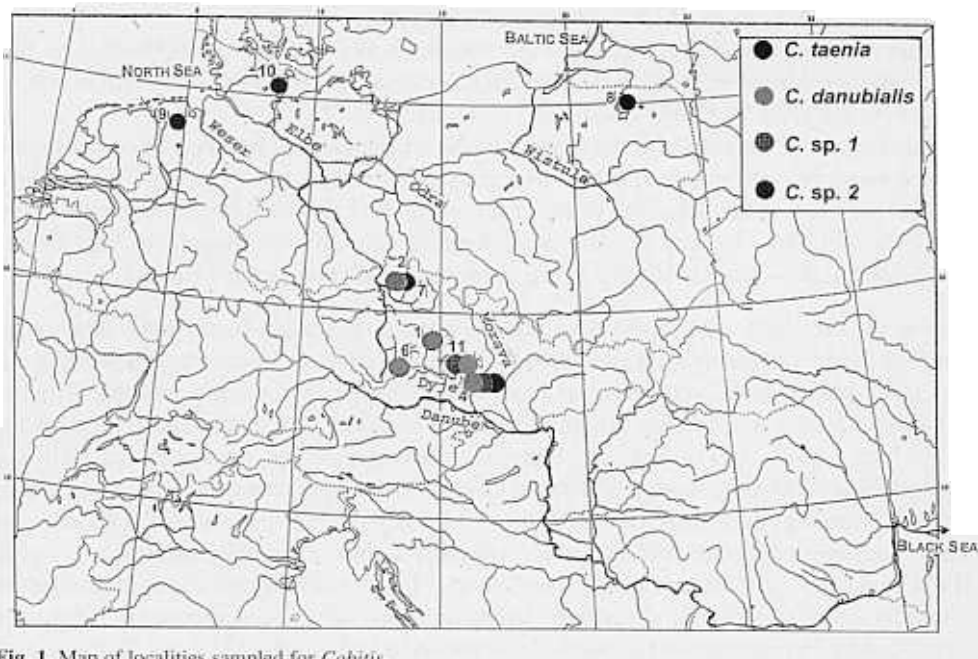


Fig. 1. Map of localities sampled for *Cobitis*.

- numerous; the conditions in the reservoir (48 ha of flooded area) allowed the population to increase so that now its size has reached more than ten thousand individuals.
- b) Jevišovka rivulet – tributary (N = 48.52.35 / E = 16.32.29) (Locality No. 11.)
The left-side tributary enters the river Dyje at its river km 83.1. The occurrence of loach is insular in the middle and lower part of the stream between river km 10.0 and 26.0. Material was sampled at the beginning of the mentioned part.
 - c) lower part (N = 48.37.28 / E = 16.55.53 to N = 48.45.57 / E = 16.53.10) (Locality No. 5.)
This locality covers the stream from its confluence with the river Morava (river km 0.0) upstream to the weir in the town Břeclav (river km 26.7), left-side floodplain between the river and levee. During high water, this whole area is flooded. Loaches occurred both in the river itself and its branches of the parapotamon type and also in some artificial ponds. Fishes from the cutoffs were analysed as a separate group (Locality No. 4.).
2. River Lužnice - upper part, floodplain, pool “U třešně” (N = 48.49.60 / E = 14.55.60) (Locality No. 6.)
The pond – the rest of the former river branch – has 380 square metres and is situated in an active alluvial area 150 m away from the main river bed. The biotope is stable with a constant water level; during the spring flood in some years, the floodplain is overflowed. The abundance of individuals of an age above one year was estimated to be 95 fish per 100 m² (Hartvich et al. 1998).
 3. brook Pšovka
Left-side tributary of the river Elbe with a length of 33.6 km. Mean year discharge in Mělník (estuary) $Q_a = 0.86 \text{ m}^3 \cdot \text{s}^{-1}$. The sampling sites were organised into three major groups.
 - a) Pšovka I. (N = 50.27.53 / E = 14.36.56) (Locality No. 2.)
Upper part of the watercourse upstream of the village Ráj; isolated from the lower parts by swampy meadows and pond.

b) Pšovka II. (N = 50.25.48 / E = 13.32.43) (Locality No. 3.)

Part of the watercourse upstream of the village Lhotka limited from both sides by two ponds; penetration of individuals from the downstream parts is practically impossible.

c) Pšovka III. (N = 50.22.04 / E = 14.33.31) (Locality No. 7.)

Middle part of the stream in the vicinity of the village Lhotka, between a pond upstream and swampy meadows downstream that prevent free migration.

Fish from three other locations in adjacent regions were also used for reference: two from Germany (Plön – locality No. 10, and Oldenburg – locality No. 9) and one from Poland (Klawój Lake – locality No. 8), where occurrence of *C. taenia* was assumed.

For the electrophoretic analyses of protein variability, altogether 439 individuals were sampled. After overdosing with anaesthetics (2-phenoxyethanol), tissue samples were taken, i. e. trunk muscle and liver. Tissue extracts were prepared by the homogenising of tissue aliquots with the same amount (weight / volume) of buffer (0.1 mol.l⁻¹ Tris-HCl pH 8.5 – Valenta et al. 1971) and clarifying the homogenate by centrifugation. All the manipulations with tissues were performed on ice; clear supernatant as well as stored tissue samples were kept deep frozen at -70°C up to the analyses. Refrigerated electrophoresis on starch gel was performed and proteins / enzymes stained essentially after Harris & Hopkinson (1976) and Pasteur et al. (1987) with modifications described in Šlechtová et al. 1998; altogether 15 protein / enzyme systems representing products controlled by 26 presumptive loci were electrophoresed and stained (see Table 2). Phenotype readings from the evaluated electrophoregrams were statistically processed using BIOSYS-1 software package (Swofford & Selander 1981) to obtain basic genetic population data (mean number of alleles per locus; percentage of polymorphic loci; mean observed and expected heterozygosity). The nomenclature of loci and alleles essentially followed the recommendations of Shalee et al. 1990.

Results and Discussion

Among the loci analysed, products of nine of them – i. e. *sAat**, *Adh**, *Gpi-A** and *-B**, *mIdhP**, *Ldh-B**, *sMdh-A**, *Pgm-2**, and *Sod** – were found to be polymorphic at different level; products of eight another loci (*Ak**, *Ck**, *G3pdh-1** and *-2**, *sIdhP-1** and *-2**, *Ldh-A**, and *mMdh**) were monomorphic both within individual samples and among them. Results of electrophoretic separation and / or isozyme detection in the remaining nine loci (*6Pgdh**, *mAat**, *Ldh-C**, *sMdh-B**, *Me-1**, and *-2**, *Pgm-1**, *Est** and *Myo**) were not satisfactory, or not all population samples were analysed for these loci. They were therefore not considered in processing of results notwithstanding the occurrence of variability in many of them and also of species-specific alleles present in some of them (*Est**, *Me-1**, *Myo**).

Among the most variable loci we can include *Adh**, *Gpi-A**, *sMdh-A**, *Pgm-2**, and *Sod** that possessed polymorphism in nearly all samples; on the other hand, *Gpi-B** and *Ldh-B** each showed variability in two population samples only. In *sAat** and *Gpi-A** five alleles were found altogether in all samples analysed; other polymorphic loci showed two to three alleles.

In several of those loci – *sAat**, *Gpi-A**, *Sod**, species-specific alleles were observed; moreover, some other loci – *sMdh-A**, *mIdhP**, *Ldh-B** possessed, in addition to the alleles common for all populations studied, alleles occurring only in one of the supposed species (see Table 6). These variants allowed us to distinguish species included in the study and to detect possible hybrid individuals.

Table 2. Electrophoretic analysis of *Cobitis* – enzymes, loci, conditions.

Protein / enzyme	Locus	Tissue [Ⓛ]	Buffer [Ⓛ]	Status [Ⓛ]
6-phosphogluconate dehydrogenase	<i>6Pgdh*</i>	L	V	(P)
aspartate amino transferase	<i>sAat*</i>	L, M	V, MC2	P, D
	<i>mAat*</i>	L, M	V, MC2	(P)
alcohol dehydrogenase	<i>Adh*</i>	L	V, MC2	
adenylate kinase	<i>Ak*</i>	M	V	M
creatine kinase	<i>Ck*</i>	M	V	M
glucosephosphate isomerase	<i>Gpi-A*</i>	L, M	V, F	P, D
	<i>Gpi-B*</i>	M	V, F	P
glycerol-3-phosphate dehydrogenase	<i>G3pdh-1*</i>	M	V	P
	<i>G3pdh-2*</i>	M	v	D
isocitrate dehydrogenase	<i>sldhP-1*</i>	L	MC2	M
	<i>sldhP-2*</i>	L	MC2	M
	<i>ml dhP*</i>	M	Ph	P
lactate dehydrogenase	<i>Ldh-A*</i>	M	V	M
	<i>Ldh-B*</i>	M	V	P, d
	<i>Ldh-C*</i>	L	V, Po	(D)
malate dehydrogenase	<i>sMdh-A*</i>	L	V, MC2	P, d
	<i>sMdh-B*</i>	M	V, MC2	(P)
	<i>mMdh*</i>	M	V, MC2	M
malate dehydrogenase decarboxylating	<i>Me-1*</i>	L	MC2	(P,D?)
	<i>Me-2*</i>	M	Ph	(D)
myoglobin	<i>Myo*</i>	M	Ph	(P,D?)
phosphoglucomutase	<i>Pgm-1*</i>	L	V, Po	(P)
	<i>Pgm-2*</i>	L, M	V, Po	P
superoxide dismutase	<i>Sod*</i>	L	F, Po	M, D
esterase	<i>Est*</i>	L	F, V	(P, D)

[Ⓛ] L = liver; M = trunk muscle

[Ⓛ] V = Valenta et al. 1971; F = Ferguson & Wallace 1968; Ph = Philipp et al. 1979; Po = Poulík 1957; MC2 = Clayton & Tretiak 1972

[Ⓛ] M = monomorphic; P = polymorphic; D = species discriminating, d = auxiliary; in brackets = separation not satisfactory (i. e. not included into analyses)

All individuals from Klawój Lake (locality No. 8), Oldenburg (locality No. 9) and Plön (locality No. 10) were diploids and assigned to *C. taenia*. This conclusion is supported by the analysis of their karyotypes (Ráb et al. 2000) and also by the comparison with the results of Osinov et al. (1990). The alleles found in these populations were therefore determined to be characteristic for this species.

At the locations Nová Říše (locality No. 1) and Lužnice (locality No. 6), all individuals examined were also of diploid constitution and they represented bisexual diploid populations.

Table 3. List of polymorphic loci and allelic frequencies found in diploid *Cobitis*.

Locus	Allele	Population							
		1	2	3	4	5	6	7	8
<i>sAatI</i> *	100	1.000	1.000	1.000	0.894	1.000			
	085				0.106				
	071						1.000	1.000	1.000
<i>ADH</i> *	100	0.830	0.750	0.917	0.949	0.950	1.000	0.680	0.882
	240	0.170	0.250	0.083	0.051	0.050		0.320	0.118
<i>GpiA</i> *	100	0.826	0.464	0.375	0.472	0.393			
	M	113	0.174	0.536	0.625	0.528			0.025
	C	074							0.975
<i>GpiB</i> *	100	1.000	1.000	1.000	1.000	0.988	1.000	1.000	1.000
	200					0.012			
<i>mLdhP</i> *	100	1.000	1.000	1.000	1.000	0.969	1.000	1.000	0.700
	110					0.031			0.300
<i>LdhB</i> *	100	1.000	1.000	1.000	1.000	1.000	1.000	0.870	0.955
	117							0.130	0.045
<i>sMdhA</i> *	100	1.000	0.654	0.700	1.000	0.560			
	070		0.154	0.100		0.155	1.000	1.000	1.000
	040		0.192	0.200		0.286			
						1.000	0.969	0.407	0.158
							0.031	0.593	0.842
						1.000			
	000	0.005			0.002		1.000	1.000	1.000

The karyotype analysis (Ráb et al. 2000) showed that they pertain to another species quite different from *C. taenia* that was identified as *C. elongatoides*. Also fishes from upper and lower parts of Pšovka creek (Pšovka I. and Pšovka III. – localities No. 2 and 7, respectively) and some individuals from Pšovka II. (locality No. 3) were of the same constitution. The same biochemical markers as in mentioned fishes were found in loaches from the vicinity of Bucharest, Romania (results not presented here) and further support this conclusion.

The samples taken from these diploid, specifically pure populations were analysed and results were processed to obtain the basic genetic parameters. The list of all alleles found in polymorphic loci under consideration together with their frequencies in respective diploid populations is presented in Table 3. As to the population genetic parameters calculated, the genetic variability differed in individual population samples (Table 4). The mean number of alleles per locus was between 1.1 and 1.4; the percentage of polymorphic loci varied from 5.9 to 29.4; the mean observed heterozygosity was found between 0.004 and 0.099, and the expected heterozygosity was 0.004 to 0.091. Populations of *C. taenia* from Poland (locality No. 8) and of *C. elongatoides* from river Dyje (locality No. 1 – Nová Říše) showed the lowest values in these parameters.

Some individuals sampled at the Pšovka Creek (Pšovka II. – locality No. 3) and nearly all fishes from the river Dyje (Košárské louky – locality No. 4, confluence with Morava R. –

Table 4. Genetic parameters of diploid *Cobitis* populations analyzed.

Population	No	Mean sample size / locus	Mean no. of alleles / locus		% of loci polymorphic*	Mean heterozygosity			
						H_o	H-W H_e^{**}		
Dyje-Nová říše		67.3 (2.0)	1.2 (0.1)		17.6	0.031 (0.019)	0.041	(0.024)	
Pšovka I.	2	11.5 (1.1)	1.2 (0.1)		17.6	0.099 (0.054)	0.091	(0.049)	
Pšovka II.	3	17.7 (1.1)	1.2 (0.1)		17.6	0.072 (0.043)	0.066	(0.039)	
Lužnice	6	64.8 (1.7)	1.2 (0.1)		23.5	0.050 (0.034)	0.049	(0.031)	
Pšovka III.	7	39.6 (1.2)	1.4 (0.1)		29.4	0.066 (0.038)	0.074	(0.043)	
Poland-Klawój	8	11.3 (1.9)		(0.1)	5.9	0.004 (0.004)	0.004	(0.004)	
Oldenburg	9	25.9 (0.7)	.2 (0.1)		17.6	0.065 (0.036)	0.069	(0.039)	
Plön	10	17.6 (1.1)	1.3 (0.1)		29.4	0.076 (0.040)	0.064	(0.032)	

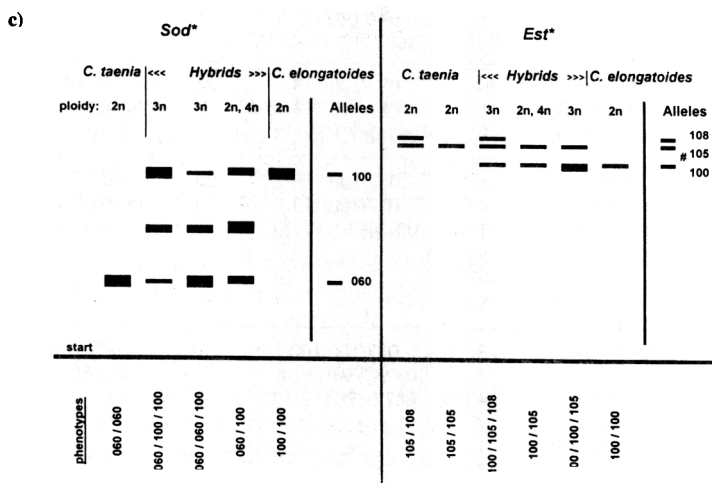
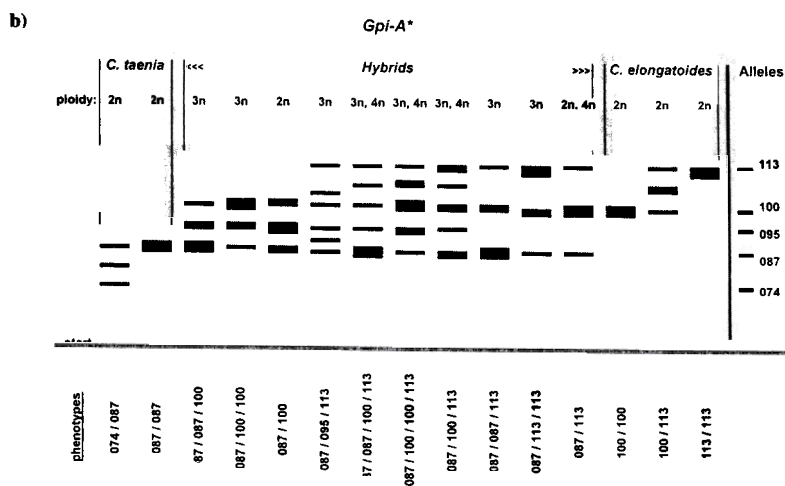
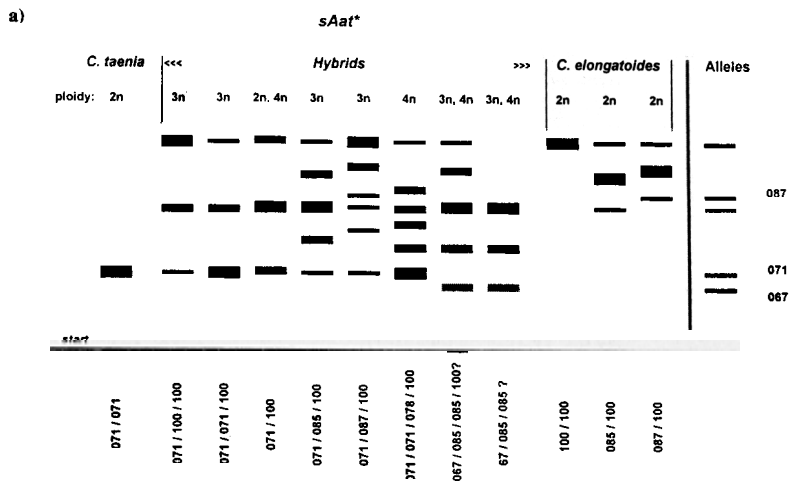
* A locus is considered polymorphic if more than one allele was detected

** Unbiased estimate (see Nei 1978)

locality No. 5, Jevišovka – locality No. 11) showed an asymmetrical intensity distribution of individual isozymes on the electrophoreograms of particular enzyme systems (see Fig. 2). Such fish were assumed to be polyploids – triploids in most cases. Several fishes also showed isozyme patterns that were interpretable only through the presence of more than two separate alleles in the respective locus; such individuals were then assumed to be triploids or

Table 5. Occurrence of phenotypes in polyploid *Cobitis* individuals at selected enzyme loci.

Locality	Locus						
	[loc. No.] (No. of fish)	<i>s-Aat-I</i> * phenotype	N	<i>Gpi-A</i> * phenotype	N	<i>Sod</i> * phenotype	N
Pšovka II. [3] (N = 20)		071/100/100	19	087/100/100	9		20
		071/085/100		087/100/113	11		
Dyje - cutoff [4] (N = 33)		071/100/100	24	087/100/113	24	060/100/100	24
		071/071/085/100		087/113/113	1	060/060/100/100	
		071/071/100/100		087/087/100/113	8		
Dyje-confluence [5] (N = 69)		071/085/085	1	087/100/100	26	060/100/100	56
		071/087/087		087/100/113	35	060/060/100/100	8
		071/085/100		087/087/100/113	8	not tested	6
		071/087/100					
		071/100/100		46			
Lechovice [11] (N = 15)		071/071/100/100	8				
		067/085/085	3	087/100/100	10	060/100/100	9
		067/085/100		087/087/100/100	1	060/060/100/100	6
		071/085/085		087/087/100/113	4		
		071/085/100		1			
	071/071/085/085	-					
	071/071/100/100	5					



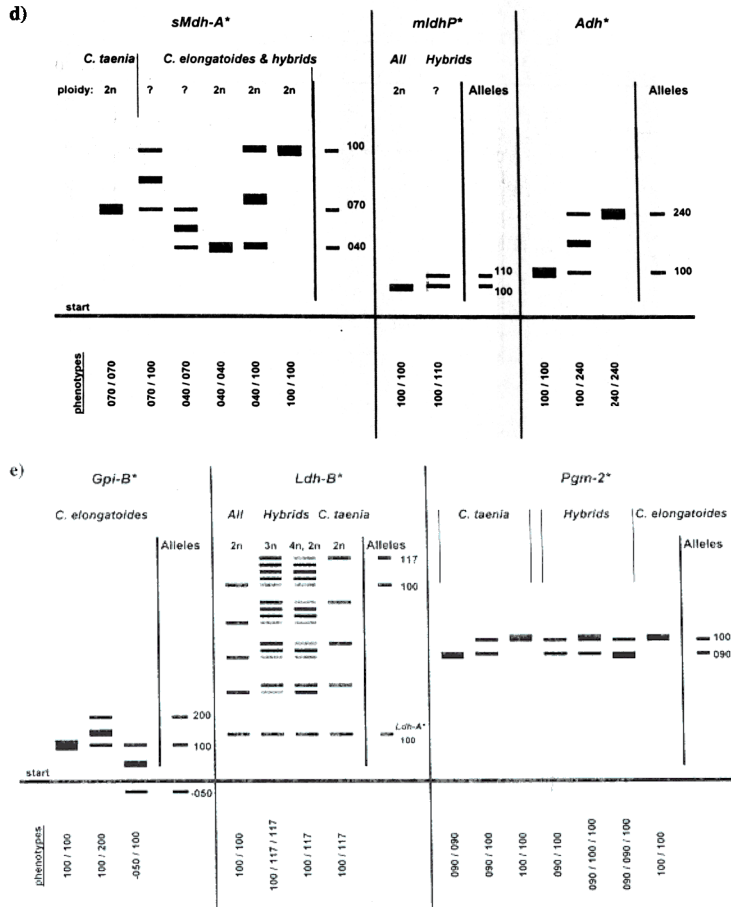


Fig. 2. Schematic drawings of different electrophoretic phenotypes found in selected polymorphic loci of *Cobitis*: a) *sAat**; b) *Gpi-A**; c) *Sod**, *Est**; d) *sMdh-A**, *mldhP**, *Adh**; e) *Gpi-B**, *Ldh**, *Pgm-2**.

tetraploids (three or four different alleles, respectively). In most cases these expectations were confirmed by simultaneous cytogenetic analyses.

Polyploid fish from these locations (Pšovka II. – locality No. 3, Košárské louky – locality No. 4) possessed, in all concerned loci, alleles that were found in different *Cobitis* species (Table 5) and were believed to be characteristic for them (Table 6). We therefore considered such individuals to be of hybrid origin. In this respect, differences exist among the mentioned localities as to species composition of hybrid fish:

- In Pšovka II. (locality No. 3) we assumed the genomes of all polyploid fish analysed to be composed of two sets of *C. elongatoides* and one or two sets of *C. taenia* (in triploids and tetraploids, respectively).
- In the Dyje R. – at its confluence with the Morava R. and neighbouring regions (localities No. 4 – Košárské louky, 5 – confluence itself, and 11 – Jevišovka) – the composition of polyploid fishes included two sets of *C. elongatoides* and one to two sets of other genome. This genome pertains to the other, presently unidentified species different from both *C. elongatoides* and *C. taenia* as confirmed by cytogenetic analysis (Ráb et al. 2000).

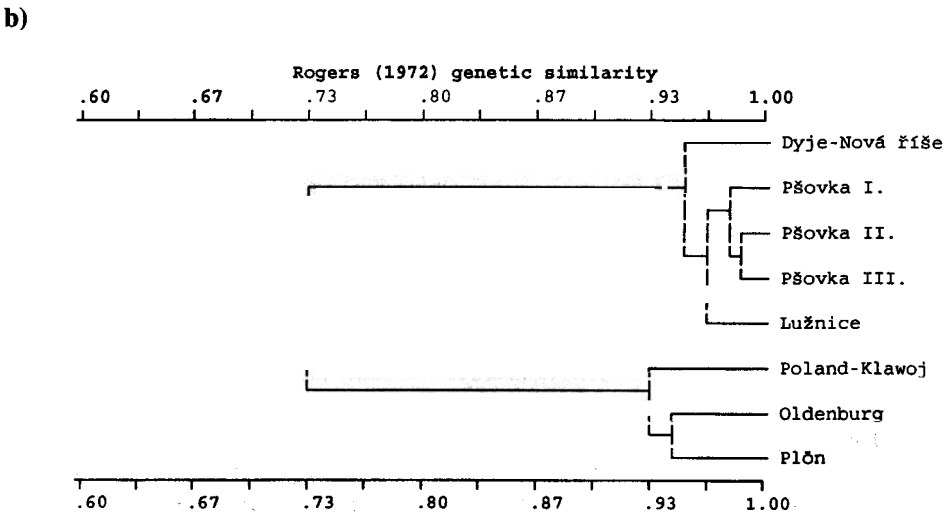
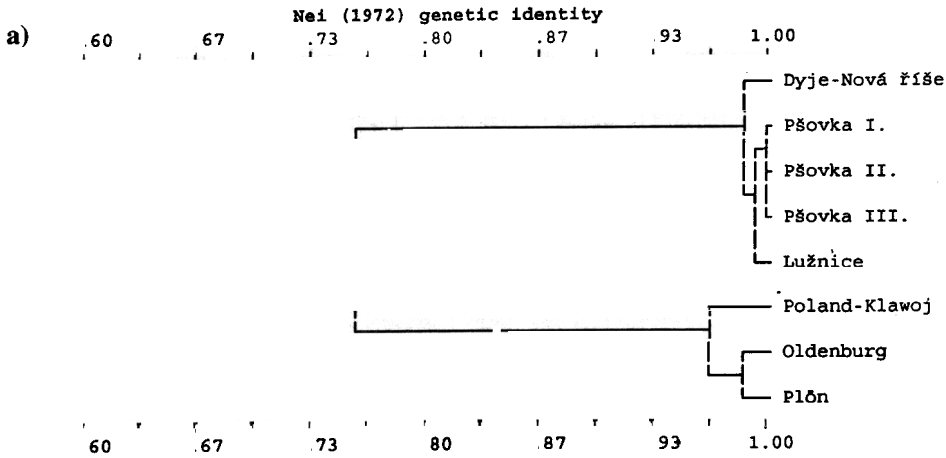


Fig. 3. UPGMA phenotype trees of *Cobitis elongatoides* and *C. taenia* population samples derived from a) Nei (1972) genetic identity and b) Rogers (1972) genetic similarity matrices.

As for the isozymes this unidentified species did not differ from both mentioned species probably apart from the *sAat** locus where the allele *sAat*-067* occurred very rarely. This finding nevertheless has to be confirmed. Anyhow, the presence of three different genomes were already discovered in the hybrid complex of *C. taenia*, *C. melanoleuca* and *C. sp.* in the Moscow R. (Vasiiev et al. 1989).

Table 6. List of species-specific alleles found in *Cobitis*.

Locus	Species		
	<i>C. elongatoides</i>	<i>C. taenia</i>	<i>C. sp.</i>
<i>sAar</i> *		071	071, 067
<i>GPI-A</i> *		087	087
<i>Sod</i> *		060	060
<i>sMdh-A</i> *		070	100
<i>mIldhP</i> *		100, 110	110 (?)
<i>Ldh-B</i> *		100, 117	100
<i>Est</i> * §		105, <u>108</u>	105

bold = species-specific

underlined – auxiliary

§ = not included due to incomplete data

In conclusion, the existence of such complicated hybrid diploid-polyploid species complexes on a rather small territory of the Czech Republic where the presence of only one species was just recently assumed – i. e. *C. taenia* (Baruš & Oliva 1996), documents a much higher specific diversity and complicated evolutionary patterns of *Cobitis* loaches.

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