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ALLOZYME ANALYSIS OF GIBEL CARP *CARASSIUS GIBELIO* (BLOCH, 1782) POPULATIONS IN BULGARIA

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Abstract

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Genetic diversity among 10 populations of gibel carp *Carassius gibelio* in Bulgaria were analyzed using general muscle proteins and five enzymes (encoded by 16 loci) as markers. All of the six systems investigated indicated polymorphism and nine polymorphic loci (*PROT-3*, EST-1*, EST-2*, sMDH-2*, sMDH-3*, mMEP*, sMEP*, LDH-B*1* and *SOD**) were detected. These loci were encoded by codominant alleles and their inheritance patterns were analyzed. The gene frequencies of polymorphic loci can be used as genetic markers for distinguishing of gibel carp populations in Bulgaria. The analyses of genetic distances between the populations of *C. gibelio* indicated a natural dispersal and/ or deliberate introduction of this invasive species from the Danube River basin to the other inland water bodies in Bulgaria.

Key words: *Carassius gibelio*, Danube River basin, Black Sea, Aegean Sea, electrophoresis, enzymes, general muscle proteins, genetic distance

Introduction

Gibel carp *Carassius gibelio* (Bloch, 1782) was first introduced into Europe from Asia in the 17th century and has since become widely distributed throughout Europe (Lever, 1996). The species is well known as hazardous fish species for native fish communities. The gibel carp easily becomes one of the dominant species in stagnant and slow running waters and may change the flow of nutrients in the whole ecosystem (Paulovits et al., 1998).

In Bulgaria, the species was first reported in the late 1940s in the Danube River and the Black Sea coastal lakes (Drensky, 1948 and Stojanov, 1949). Later, its occurrence was confirmed in the Danube (Marinov, 1966, 1978; Karapetkova et al., 1998; Sivkov, 1998 - 1999 and Polacik et al., 2008), most of the Danube tributaries (Karapetkova, 1994; Trichkova et al., 2004 and Trichkova et al., 2009) and some reservoirs (Trichkova et al. 2001 and Trichkova and Zivkov, 2007). The species appeared in most of the Black Sea lakes and rivers (Manolov-Gheorghiev, 1967 and Karapetkova, 1976). It was first recorded in the Kamchiya River in the period 1967-1970 (Karapetkova, 1974), and in the Veleka River in the period 1963-1971 (Karapetkova, 1975). In the reservoirs of the Aegean Sea drainage basin (Batak, Dospat, Ovcharitsa), the species was found in the 1960s (Zivkov and Stoyanova, 1976; Zivkov, 1987 and Zivkov and Grupcheva, 1987). In the Mesta River basin, the species introduction was first reported for the Greek part of the river by Economidis (1974), and later for the Bulgarian part by Zivkov (1987), Apostolou (2005), Stefanov and Trichkova (2006). Recently, it was found in Dospat Reservoir, and in the slow-flowing sections of the river and adjacent backwaters (Trichkova and Economidis, 2009). Dikov et al. (1994) reported finding of gibel carp in comparatively low abundance and biomass in the Struma River basin.

The gibel carp populations are characterized as unisexual composed by triploid females, or bisexual, including diploid females and males (Golovinskaya et al., 1965 and Toth et al., 2005). A very high percentage of unisexual populations are reproducing gynogenetically by using the sperm of other species, mainly cyprinids, to activate their eggs (Economidis, 1974 and Penaz et al., 1979).

In Bulgaria, in the 1950-1980s, populations in the reservoirs and fish-ponds consisted mainly of gynogenetic females,

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while males occurred occasionally in an average varying from 0 to 1.5% (Paspalev and Pechev,1955 and Zivkov, 1980). Recently, bisexual populations are found with males ranging from 30 to 50% in the catches (Trichkova and Zivkov, 2007 and Trichkova et al., 2008). Mezhzherin and Kokogiy (2010) showed evidence that *C. gibelio* is hybrid polyphyletic form, which diversity in Europe could be considerable. In this respect genetic structure of the colonies of this "species" attract special interest.

Electrophoretic variants of enzymes are useful as genetic markers for population studies, if the data reliably reflects genetic variation (Yamamoto et al., 1998). Valenta (1977) established polymorphism in *sMDH* in erytocites, which could be used as a marker for fish selection. Ikeda et al. (1997) found that the polymorphism at the *LDH-B1** locus was utilized for genetic characteristics of several races in the goldfish. By applying different biochemical-genetic markers, such as transferrin and isozyme, the individuals and the populations of *Carassius auratus gibelio*, could be well characterized genetically (Gsizmadia et al., 1995 and Yang et al., 2001).

So far, genetic-biochemical parameters of gibel carp were studied in some Bulgarian and Greek water basins (Apostolou et al., 2007) and in the Kamchiya River (Trichkova et al., 2010).

The main goal of the present study was to make comparative electrophoretic analysis and to find genetic markers for distinguishing different gibel carp populations in Bulgaria.

Materials and Methods

Specimens of *Carassius gibelio* were collected by electrofishing, gill nets and hand nets in the period 2003-2009. Experimental material was represented by 205 individuals of *Carassius gibelio* from 10 localities in the three river drainage basins in Bulgaria (Figure 1): Danube River basin - 1. Poletkovtsi Reservoir (19.04.2006); 2. Drenovets Reservoir (05.11.2003); 3. Ogosta Reservoir (15.04.2009); 4. Danube River at Silistra (28.07.2004); Black Sea river basin - 5. Kamchiya River, lowest reaches (14-15.06.2009); 6. Veleka River, lowest reaches (27.07.2005); Aegean Sea river basin - 7. Struma River, Arkata (19.10.2009), 8. Mesta River, Ormanski Gjol (13-16.05.2008), 9. Koprinka Reservoir (19.10.2009), 10. Ovcharitsa Reservoir (14-16.02.2007).

Fig. 1. Sampling localities of Carassius gibelio in Bulgaria

The samples were analyzed by electrophoresis. The electrophoretic procedures were carried out following Smithies (1955) and modification of Dobrovolov (1973). Homogenates obtained from muscle were run in horizontal starch gel electrophoresis for five enzymatic systems, such as: esterase (EC 3.1.1.1 - EST), lactate dehydrogenase (EC 1.1.1.27 - LDH), malate dehydrogenase (MDH), malic enzyme (MEP) and superoxide dismutase (EC 1.15.1.1 – SOD), as well as general muscle proteins (PROT). Staining of different enzymes was performed according to Shaw, Prasad (1970). The buffer systems of Clayton and Gee (1969) and Dobrovolov (1976) were used for the electrophoresis. The nomenclature of loci and alleles followed essentially the recommendations of Shaklee et al. (1990). Presumptive alleles were designed alphabetically by their relative mobility, with the allele variant migrating close to the start position - marked with "a". Calculation of indices of genetic similarity and genetic distance was performed after Nei (1972). The loci were named from the most cathodal to the most anodal position.

Results and Discussion

Analyses of allozymic variations

The assessment of genetic variability in *C. gibelio* populations was carried out on 16 allozyme loci. Allele frequencies at all loci are given in Table 1. Intrapopulation χ^2 analysis indicated that the allelic distributions did not depart from equilibrium expectations.

General muscle proteins (***PROT***) – On the electrophoregrams on starch gel, five fractions probably coded from five loci were visualized (Figure 2), while on isoelectric focusing the number of bands increased to 37 (Figure 4). Polymorphism was registered only in one locus -*PROT-3**.

All *C. gibelio* specimens studied had two allelic polymorphism in the *PROT-3** locus. The frequencies of *PROT-3*a* allele in the Kamchiya and Poletkovtsi populations were the highest (0.736 and 0.611). In all other populations analyzed the frequencies of *PROT-3*a* were in the range of 0.389-0.563 (Figure 2, Table 1). The third (*PROT-3*c*) rare allele was represented only in the specimens from the Struma River and Koprinka Reservoir (Figure 2, Table 1). The *PROT-3*c* frequency in specimens from Koprinka Reservoir

was not included in Table 1, because only two heterosygotes with rare allele were registered.

It was reported that diploid *C. gibelio* could form hybrids with cyprinids (common carp, crucian carp and goldfish) (Tóth et al., 2005). Tsekov and Dobrovolov (1999) showed genetic-biochemical evidence for the existence of hybrids between *C. gibelio* and *Cyprinus carpio.* In Drenovets Reservoir, a typical hybrid spectrum between *C. gibelio* and other

Table 1
Estimated allele frequencies of polymorphic loci on general muscle proteins (*PROT*), esterases (*EST*), malate dehydrogenase (*MDH*), malic enzyme (*MEP*), lactate dehydrogenase (*LDH*) and superoxide dismutase (*SOD*) in *C. gibelio* from 1 - Poletkovtsi Reservoir, 2 - Drenovets Reservoir, 3 - Ogosta Reservoir, 4 – Danube River, 5 - Kamtchiya River, 6 - Veleka River, 7 - Struma River - Arkata, 8 - Mesta River – Ormanski Gjol, 9 – Koprinka Reservoir and 10 – Ovcharitsa Reservoir

Locus	Allele	1	2	3	4	5	6	7	8	9	10
PROT-3*	a*	0.611	0.471	0.556	0.469	0.736	0.389	0.500	0.563	0.450	0.500
	b*	0.389	0.529	0.444	0.531	0.264	0.611	0.484	0.437	0.550	0.500
	c*	0	0	0	0	0	0	0.016	0	0	0
EST-1*	a*	0.445	-	0.438	0.429	0.467	0.364	0.463	0.458	0.393	0.333
	b*	0.555		0.562	0.571	0.533	0.636	0.537	0.542	0.607	0.667
EST-2*	a*	0.423	0.572	0.444	0.444	0.486	0.091	0.470	0.400	0.457	0
	b*	0	0.428	0.250	0.556	0	0	0.116	0.167	0.543	0.537
	c*	0	0	0	0	0	0	0	0	0	0
	o*	0.577	0	0.305	0	0.514	0.909	0.414	0.433	0	0.463
EST-3*	a*	1	1	1	1	1	1	1	1	1	1
	b*	0	0	0	0	0	0	0	0	0	0
mMDH-1*	a*	1	1	1	1	1	1	1	1	1	1
	b*	0	0	0	0	0	0	0	0	0	0
mMDH-2*	a*	1	1	1	1	1	1	1	1	1	1
	b*	0	0	0	0	0	0	0	0	0	0
sMDH-1*	a*	1	1	1	1	1	1	1	1	1	1
	b*	0	0	0	0	0	0	0	0	0	0
sMDH-2*	a*	0.556	1	1	0.567	0.603	0.458	1	1	0.591	1
	b*	0.444	0	0	0.433	0.397	0.542	0	0	0.409	0
sMDH-3*	a*	0.300	0	0	0	0.382	0	0	0	0	0
	b*	0.700	1	1	1	0.618	1	1	1	1	1
mMEP*	a*	0.111	0	0.346	0	0	0.267	0.250	0.444	0	0.111
	b*	0.889	1	0.654	1	1	0.733	0.750	0.556	1	0.889
sMEP*	a*	0.889	1	1	1	1	0.700	0.750	1	0.696	1
	b*	0.111	0	0	0	0	0.300	0.250	0	0.304	0
LDH-A*1		1	1	1	1	1	1	1	1	1	1
		0	0	0	0	0	0	0	0	0	0
LDH-A*2		1	1	1	1	1	1	1	1	1	1
		0	0	0	0	0	0	0	0	0	0
LDH-B*1	a*	0.583	1	0.786	1	0.574	0.500	0.667	0.679	0.696	1
	b*	0.417	0	0.214	0	0.426	0.500	0.333	0.321	0.304	0
LDH-B*2		1	1	1	1	1	1	1	1	1	1
		0	0	0	0	0	0	0	0	0	0
sSOD*	a*	1	1	0.912	0.941	1	1	0.925	0.906	1	0.912
	b*	0	0	0	0.030	0	0	0	0	0	0.029
	c*	0	0	0.088	0.029	0	0	0.075	0.094	0	0.059

species was found on the basis of general muscle proteins (PROT) using both methods (starch gel and isoelectric focusing) (Figures 3 and 4, N2).

Esterases (*EST*) - Three zones with esterase activity were visualized on the esterase electrophoregrams in the studied *C. gibelio* populations; they were probably coded from three loci (*EST-1*, EST-2** and *EST-3**) (Figure 5). The *EST-1** locus was polymorphic in all populations analyzed with the exception of Drenovets Reservoir, where no data was obtained. Koprinka, Ovcharitsa and Veleka populations had allele frequencies in the range of 0.333-0393. The other populations had close values of allelic frequencies (Table 1). The *EST-2** locus had two allele system of inheriting in the populations in the reservoirs Poletkovtsi, Drenovets, Koprinka and Ovcharitsa, and in the rivers Danube, Kamchiya and Veleka. The *EST-2** locus had three allele system of inheriting with null allele in the populations in the rivers Struma and Mesta, as well as in the Ogosta Reservoir (Table 1). *EST-3** locus was monomorphic in all populations. The detection of null alleles in a single locus is visible when there is an absence of activity in the homozygote. The existence of null allele polymorphism was also of interest and importance in the diploidization process of duplicate gene loci in the salmonid fish, which were thought to have been derived from an autotetraploid

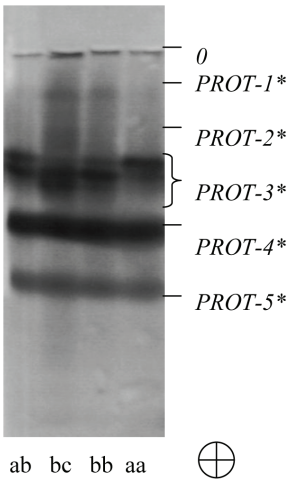

Fig. 2. Starch gel electrophoresis on general muscle proteins (PROT) of C. gibelio (Koprinka Reservoir). Polymorphism on PROT-3* was marked; aa, ab and bb phenotypes; 0 – origin

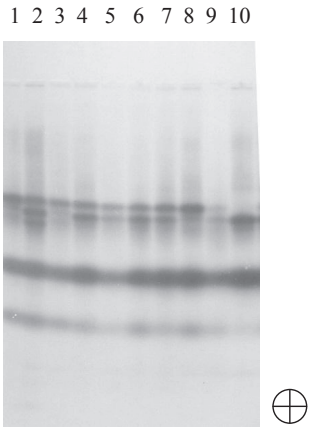

Fig. 3. Starch gel electrophoresis on general muscle proteins (PROT) of C. gibelio in: 1-3- Drenovets Reservoir, 4-10 – Danube River. Hybrid of C. gibellio with other cyprinid species (N2) is presented. 0 – origin

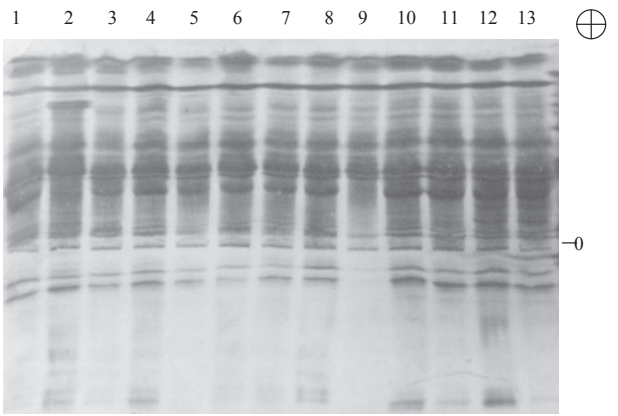

Fig. 4. Isoelectric focusing (IEF) on polyacrylamide gel with pH gradients 3-10 on C. gibelio in: 1-3 –Drenovets Reservoir, 4-9 – Danube River and 10-13 Veleka River. 0- origin

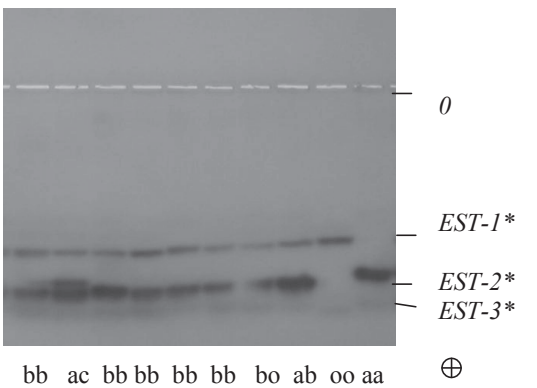

Fig. 5. Esterase zymograms on starch gel of C. gibelio (Ovcharitsa Reservoir). EST-1*, EST-2* and EST-3* - loci with enzyme activity; bb, ac, aa - phenotypes in EST-2* locus; 0- origin

ancestor. Since the cyprinid fish showed a diploid-tetraploid relationship, the existence of null allele polymorphism would be expected in crucian carp (*C. auratus*) population (Yamamoto et al., 1998).

Lactate dehydrogenase (*LDH*) –The enzyme was represented by 9 fractions, which were coded from four loci (*LDH-A*1, LDH-A*2, LDH-B*1* and *LDH-B*2*). In all studied populations the three LDH loci were monomorphic. Only the *LDH-B*1* locus was polymorphic in all populations with exception of Drenovets Reservoir, Danube River and Ovcharitsa Reservoir (Table 1).

Malate dehydrogenase (*MDH*) – two mitochondrial loci (*mMDH*-1** and *mMDH-2**) and three *sMDH** loci (*sMDH-1*, sMDH-2** and *sMDH-3**) were detected. The two mitochondrial loci and the first *sMDH* locus were monomorphic in the investigated river and reservoir populations (Figure 6). *s-MDH-2** locus was polymorphic in the reservoirs Politkovtsi and Koprinka, and in the rivers Danube, Kamchiya and Veleka. The locus *sMDH-3** appeared to be polymorphic only in the Poletkovtsi Reservoir and Kamchiya River (Table 1). Interloci hybrid fractions were also presented (Figure 6).

Malic enzyme (*MEP*) - Two zones with enzyme activity, determined from two loci (*mMEP** and *sMEP**) were visualized on the electrophoregrams. Polymorphism was found in the *mMEP** locus in the Poletkovtsi Reservoir, Ogosta Reservoir, Struma and Mesta Rivers, Ovcharitsa Reservoir and Veleka River (Figure 7). *sMEP** locus was polymorphic only in Poletkovtsi Reservoir, Struma River, Koprinka Reservoir and Veleka River (Table 1, Figure 7). All other populations were monomorphic on these loci.

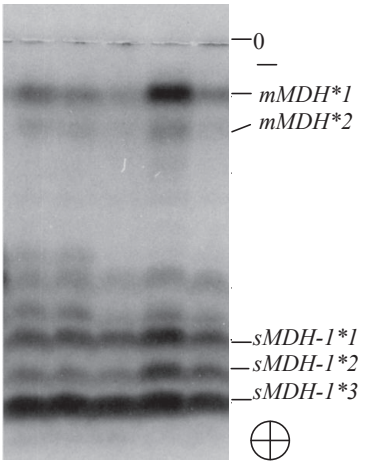

Fig. 6. Malate dehydrogenase zymograms on starch gel of C. gibelio (Kamchiya River). 0- origin

Equal values of allelic frequencies in *mMEP** were found in the populations in the Poletkovtsi Reservoir and Ovcharitsa Reservoir. This is a rarely found phenomenon and so far hardly can be explained in the population genetics. The two reservoirs are quite distant from each other and they are characterized with different environmental conditions. Drenovets Reservoir is located in North-West Bulgaria, Danube River basin, it is mainly used for irrigation, power generation and commercial and recreational fishing. Ovcharitsa Reservoir is located in the Tundzha River basin, Aegean Sea basin. Its main use is as a cooling-reservoir of the Maritsa East 2 TPP. Because of the high average water temperature maintained throughout the year, it is also intensively used for aquaculture and commercial fishing. We suppose that the equal values of allelic frequencies in *mMEP** found can be a result of exchange of stocking material together with gibel carp fingerlings between the two reservoirs.

Superoxide dismutase (*SOD*) - *sSOD* was polymorphic in all populations with exception of reservoirs Poletkovtsi, Drenovets and Koprinka, and rivers Kamchiya and Veleka. In the Danube and Ovcharitsa populations polymorphism with three alleles were found (Figure 8, Table 1).

The polymorphism found and the distribution of allele frequencies of *PROT-3*, EST-1*, EST-2*, mMEP*, sMEP-2*, LDH-B** и *SOD** can be used as genetic markers for the characterization of gibel carp populations in Bulgaria.

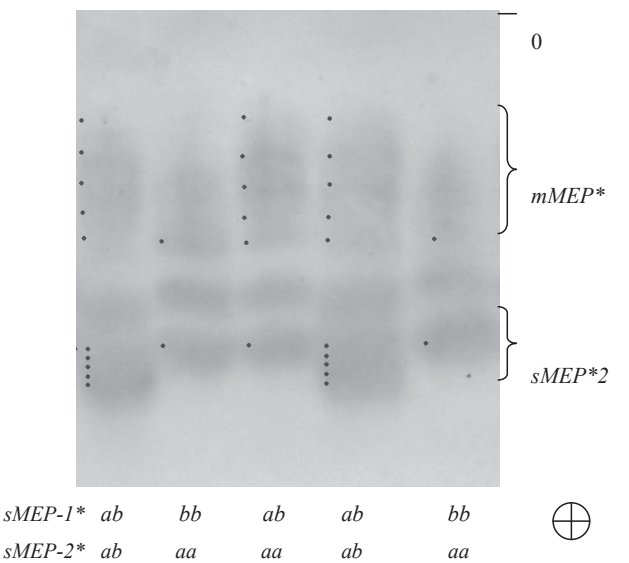

Fig. 7. Malic enzyme (MEP) zymograms on starch gel of C. gibelio (Veleka River). Polymorphism in sMEP-1* and sMEP-2*detected were marked with dots; aa, ab and bb – genotypes; 0- origin

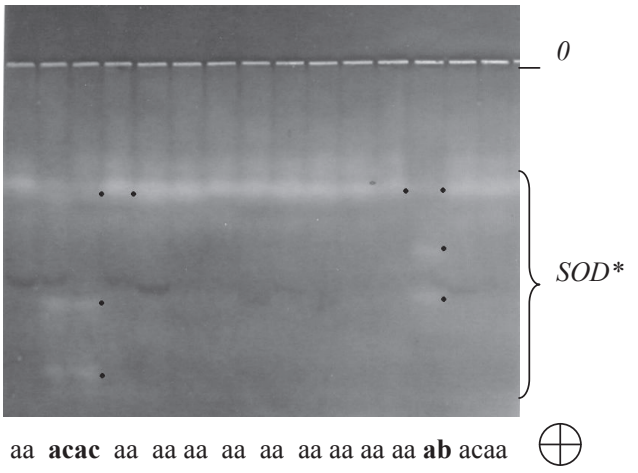

Fig. 8. Superoxide dismutase (SOD) zymograms on starch gel of C. gibelio (Ovcharitsa Reservoir). Polymorphism was detected and marked; variants were marked with dots; ac and ab – polymorphic variants; 0 – origin

The studied polymorphism in all systems (enzymatic and non-enzymatic) was under Hardy-Weinberg equilibrium, which indicates the stability of the studied populations and the insignificant participation of gynogenesis in their formation. The populations consisted of individuals from both sexes.

The results from the analysis of genetic distances between the studied populations of *C. gibelio* are shown on Table 2. All *C. gibelio* populations were well diverged with exception of Danube River and Koprinka Reservoir, which were close to each other. These results are difficult to be explained, because the two populations belong to different river basin systems. The same situation was observed with other Danube populations (Drenovets and Ogosta), which were close to populations from the Aegean Sea basin (Ovcharitsa, Struma and Mesta). Probably the reasons are natural dispersal and/ or deliberate introductions by humans (by stocking, fishery, recreational activities) of specimens from the Danube River system to the water bodies of the Agean Sea basin. Gene flow from the Danube to the Black Sea populations was also illustrated (Table 2). The Poletkovtsi population was most diverged from the other investigated Danube populations (Table 2).

Conclusions

Based on allozyme analyses, nine genetic markers for distinguishing ten gibel carp (*C. gibelio*) populations in Bulgaria were found. Most of the *C. gibelio* populations were well diverged. The analyses of genetic distances between the populations of *C. gibelio* indicated a gene flow from the Danube River basin to the water bodies of the Aegean Sea basin and the Black Sea basin. Probably the reasons are natural dispersal and/ or deliberate introductions by humans (by stocking, fishery, recreational activities).

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Table 2
Genetic distances (D_{Nei}, above asterisks) and genetic identity (I_{Nei}, under asterisks) based on 16 loci in 10 populations of *C. gibelio*

Region	1	2	3	4	5	6	7	8	9	10
1. Poletkovtsi Reservoir	*	0.074	0.038	0.062	0.004	0.026	0.026	0.034	0.052	0.066
2. Drenovets Reservoir	0.929	*	0.021	0.016	0.075	0.122	0.035	0.045	0.025	0.038
3. Ogosta Reservoir	0.963	0.979	*	0.037	0.043	0.068	0.008	0.004	0.039	0.030
4. Danube River	0.940	0.984	0.964	*	0.068	0.093	0.052	0.060	0.011	0.040
5. Kamchiya River	0.974	0.966	0.992	0.949	*	0.050	0.035	0.040	0.063	0.076
6. Veleka River	0.967	0.956	0.996	0.942	0.992	*	0.047	0.054	0.071	0.074
7. Struma River	0.949	0.975	0.962	0.989	0.960	0.945	*	0.008	0.040	0.040
8. Mesta River	0.936	0.963	0.970	0.960	0.960	0.962	0.951	*	0.057	0.039
9. Koprinka Reservoir	0.996	0.928	0.958	0.934	0.966	0.960	0.939	0.927	*	0.050
10. Ovcharitsa Reservoir	0.974	0.885	0.934	0.911	0.954	0.947	0.931	0.929	0.951	*

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